Persea americana Mill. Avocado

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11.1 *Persea americana* Mill. Avocado

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I. Introduction

A. Botany and History

The humid tropical highlands of Central America, including southern Mexico (Chiapas), Guatemala and Honduras is considered to be the centre of origin of *P. americana*, and wild *P. americana* trees occur in the forests of this region (Kopp, 1966). There are eight well-defined subspecies or geographical ecotypes of *P. americana* and they appear to have evolved in different climatic environments and in geographical isolation from each other (Scora *et al*., 2002). These include *P. americana* var. *nubigena* (Williams) Kopp, var. *steyermarkii* Allen, var. *zentmyerii* Schieber and Bergh, var. *floccosa* Mez. and var. *tolimanensis* Zentmyer and Schieber. The remaining three subspecies comprise the common avocado (Bergh and Ellstrand, 1986): 1) the Mexican subspecies *P. americana* var. *drymifolia* (Schlect. and Cham.) Blake, which is thought to have originated in the highlands of south-central Mexico and is adapted to the tropical highlands; 2) the Guatemalan subspecies *P. americana* var. *guatemalensis* Williams, which is adapted to medium elevations in the tropics; and 3) the West Indian subspecies *P. americana* var.

americana Mill., which is adapted to the lowland, humid tropics (Popenoe, 1941). The Guatemalan race may have originated in the interior valleys of the Central American highlands and its ancestry may include var. *nubigena*, var. *steyermarkii*, var. *zentmyerii* and var. *tolimanensis* (Kopp, 1966; Schieber and Bergh, 1987; Furnier *et al*., 1990). Williams (1976; 1977) argued that the West Indian subspecies may have evolved from the Mexican subspecies, and Storey *et al.* (1986) speculated that the West Indian avocado may have originated in the Pacific lowlands.

The avocado tree is evergreen, approx. 20 m at maturity. The tree is supported primarily by a shallow unsuberized secondary root system, although anchorage roots can penetrate to 3-4 m (Whiley, 1992). Flowers are borne on the terminals of twigs as panicles of cymes. Avocado fruit is a large, fleshy pyriform or globose berry with a single seed.

The word avocado is derived from the Nahuatl "ahuacatl", which was transliterated into Spanish as aguacate. The Mexican avocado was apparently selected as early as 7000-8000 BC (Smith, 1966; 1969). According to Gama (1994), domestication of the common avocado most probably occurred over a long period of time among Meso-American cultures.

B. Importance

World production of avocados was estimated to be approx. 2,406,343 Mt in 2000 (FAOSTAT, 2001). The leading producing countries include Mexico (939,118 Mt), USA (164,500 Mt), Indonesia (121,822 Mt), South Africa (104,000 Mt), Chile (100,000 Mt), Brazil (85,000 Mt), Dominican Republic (81,720 Mt), China (78,000 Mt), Colombia and Peru (each 75,000 Mt). Although the avocado is consumed primarily as a fresh fruit, it is

also a rich source of oil. The international trade in fresh avocado fruit is very important; the estimated value of avocado exports in 1999 was $360,991,000 (FAOSTAT, 2001). Leading exporting countries include Chile, Mexico, Israel, USA and South Africa. The European Union and North America are the biggest importers of avocados. The most important export avocado cultivar is 'Hass'.

C. Breeding and Genetics

The avocado tree is a large spreading canopy tree in its original habitat. The species is genetically very heterozygous with a long juvenile period and a very high rate of flower abscision and immature fruit drop. Flowering in avocado is dichogamous, protogynous and synchronous (Whiley, 1992). Each flower opens twice during two consecutive days. Therefore, all open flowers are either functionally male or functionally female. Cultivars are classified as either type A or type B, depending on whether the flowers are female in the morning (type A) or in the afternoon (type B). This flowering behaviour ensures that self-pollination cannot occur. The chromosome number is 2n=2x=24 (Garcia, 1975). Despite the relatively high costs of conventional breeding of avocado with respect to time, labour and land, conventional breeding programmes have been moderately successful, although leading cultivars are still mostly derived from open pollinations. Bergh and Lahav (1996) and Lahav and Lavi (2002) have reviewed the current status of avocado breeding.

There are no genetic barriers among the avocado subspecies/races, and many cultivars are hybrids involving two or more subspecies. The widely grown 'Hass' and 'Fuerte' avocados are considered to be Guatemalan x Mexican hybrids. The earliest California cultivars originated either as selections made in Mexico and Central America

or from imported seeds (Bergh, 1957). 'Benik', 'Itzamna' and 'Nabal' (Guatemalan) were introduced as bud wood, and 'Dickinson' and 'Fuerte' originated from seed. Openly pollinated seedlings in California were selected later, e.g., 'Bacon, 'Hass', 'Pinkerton', 'Reed' and 'Zutano', and these cultivars are grown in most regions having Mediterranean and subtropical climates. The Florida avocado industry is based upon West Indian and Guatemalan x West Indian hybrids, and these selections have been successfully introduced to many tropical areas.

1. rootstocks

a. major breeding objectives

i. Phytophthora root rot (PRR)

This disease, caused by the soil-borne pathogen *Phytophthora cinnamomi* Rands., is one of the most important limiting factors for avocado production worldwide (Coffey, 1987; Ben-Ya'acov and Michelson, 1995; Whiley, 1992). There is no resistance to PRR in avocado and in other species of subgenus *Persea*. Species within subgenus *Eriodaphne*, however, are highly resistant to the disease, but are sexually and graft-incompatible with species in subgenus *Persea* (Lahav and Lavi, 2002; Bergh and Ellstrand, 1986; Zentmyer, 1980).

ii. dwarfing

Dwarfing rootstock could have a significant impact on avocado production for several reasons: 1) control of tree size is a major production cost; 2) harvesting is cheaper from compact trees; 3) plantings can be high density, resulting in greater yields.

iii. salinity stress tolerance

Much avocado production is in areas where water is either saline or in short supply, necessitating the use of brackish water for irrigation, e.g., California USA, Israel and Australia. Consequently, in order to optimize production, clonal rootstocks should be tolerant of saline conditions.

b. breeding accomplishments

i. Phytophthora root rot

'Duke' and its progeny, 'Duke 7', 'Barr-Duke' and 'D9', and 'Thomas' have good tolerance of PRR. The 'G6' selection (Mexican) is also fairly tolerant of PRR. Some of the PRR-tolerant selections have serious limitations. 'Martin Grande' (G755), a cross involving *P. schiedeana* x *P. americana* (Guatemalan race) (Ellstrand *et al*., 1986), produces low-yielding trees. Tsao *et al.* (1992) observed that some rootstocks with tolerance of *P. cinnamomi* have no tolerance for another serious soil-borne pathogen, *Phytophthora citricola*. Guatemalan rootstocks appear to be more sensitive than Mexican rootstocks to *Dothiorella* and *Verticillium* wilt (Zentmyer *et al.*, 1965).

ii. dwarfing

Although Sánchez-Colin and Barrientos-Priego (1987) reported that 'Colin V-33', used either as an interstock or as a rootstock, could confer dwarf habit to the scion, this selection has tested positive for the avocado sunblotch viroid (ASBv).

iii. soil stress

West Indian avocados demonstrate the greatest resistance to salinity and Mexican cultivars are least resistant, although there is significant variability within each race (Kadman and Ben-Ya'acov, 1976) and among seedlings from the same tree (Kadman, 1968). Resistance to Ca^{++}-induced chlorosis is greatest in West Indian cultivars, but there

is also considerable variability for this trait within seedling populations (Ben-Ya'acov, 1972). West Indian rootstocks perform poorly in heavy soils and under waterlogged conditions (Ben-Ya'acov *et al*., 1974).

2. scions

a. major breeding objectives

i. fruit quality

The international market standard for avocados is the black skinned 'Hass', and to a much lesser extent, the green skinned 'Fuerte'. Several cultivars that closely resemble 'Hass' have been released in order to supplement this selection, particularly in its off season, and these include 'Gwen', 'Jim', 'Lamb Hass' and 'Reed'. The optimum fruit size for most markets is about 250-350 g (Lahav and Lavi, 2002). Size is highly variable in each genotype, and is affected by stage of maturity, cultural practices and climatic conditions (Lahav and Kalmar, 1977; Whiley and Schaffer, 1994). The shapes of pyriform 'Hass' and ovate 'Bacon' and 'Gwen' are desirable. Easily removed peel of 'Fuerte' and 'Hass' is preferred. In Mexican and Mexican x Guatemalan types, small seed size is desirable; however, this trait is uncommon in West Indian avocados.

ii. shelf life

Mexican type avocado fruit are strongly climacteric (Adato and Gazit, 1977). According to Eaks (1980), a fixed climacteric phase appears to precede a variable lag phase. Low levels of endogenous ethylene accumulate during the lag phase and trigger the climacteric as sensitivity to ethylene increases (McMurchie *et al*., 1972). Following its initiation, ripening cannot be arrested. According to Whiley (1992), fruit of Guatemalan and Mexican cultivars can be stored on the tree during the lag phase for 2-4

months after reaching maturity, and accumulate oil during this period. Ripening begins to occur only after the fruit are picked. West Indian and West Indian x Guatemalan cultivars, on the other hand, cannot be stored in this manner, and must be picked at maturity. On-tree storage together with different climatic conditions has enabled producers in many areas to concentrate production on a single cultivar, 'Hass' (Griswold, 1945). In order to assure year round production of West Indian and West Indian x Guatemalan fruit in the tropics, several cultivars having different maturity dates must be grown (Crane *et al*., 1996). Consequently, there is no single market standard for tropical avocados.

iii. fruit diseases

In the humid tropics and subtropics, fruit diseases are a serious problem. 'Fuerte' and other Mexican avocados are highly susceptible to anthracnose or black spot (*Colletotrichum gloeosporioides* Penz.) (Ruehle, 1963). 'Collinson', 'Fuchsia' and 'Pollock' are apparently fairly resistant to Cercospora spot or blotch (*Pseudocercospora purpurea* Cooke). 'Fuchsia', 'Pollock', 'Booth 1' and 'Waldin' are moderately resistant to avocado scab (*Sphaceloma perseae* Jenkins).

iv. tree architecture

Compactness and dwarf or semi-dwarf trees are ideal for ease of harvesting, grove management and high density plantings. According to Lahav and Lavi (2002), tree vigour and fruitfulness are inversely related, and robust seedling trees often have few fruit.

b. breeding accomplishments

Classical breeding has provided relatively little genetic information about *Persea*. With respect to Mendelian genetics, the dwarfing character of *P. schiedeana* has been attributed to a single gene (Bergh and Lahav, 1996). Lavi *et al*. (1993a,b) have concluded that morphological traits in avocado are probably coded by several loci with several alleles in each locus. Several cultivars have been released from the California and Israeli avocado breeding programmes.

II. Molecular genetics

Two main avenues of research involving molecular studies have been followed: the generation and application of various markers and the identification of avocado genes and ESTs.

A. Gene Cloning

Cellulase was the first gene to be identified in avocado (Christoffersen *et al*., 1984); pAV5 is a ripening specific cDNA clone identified to be a cellulase gene on the basis of co-migration of the *in vitro* translation product, on SDS-PAGE and immunoprecipitation by antiserum to purified avocado cellulase. This enzyme is regulated at the transcription level since a 50-fold increase in cellulase mRNA occurs during ripening. Tucker *et al*. (1987) used the pAV5 clone to screen a cDNA library prepared from ripe 'Hass' fruit and isolated clone pAV363 containing 2021 transcribed nucleotides. The full length message is approximately 2.2 kb having an ORF of 1482 bp coding for a 54.1 kD polypeptide. Further analysis of this family was carried out by Cass *et al*. (1990), who screened an avocado genomic library with the cellulase cDNA probe and identified two family members: *cel1* and *cel2*; *cel1* is highly homologous to cellulase cDNA and represents the ripening related cellulase gene, whereas, *cel2* although related to *cel1*, is divergent in its

5' end and no cellulase cDNA derived from ripe fruit represents *cel2* transcripts. Thus, the *cel1* gene is responsible for the major portion, if not all of the cellulase transcripts in ripe fruit. Only *cel1* transcripts have been detected in ripe mesocarp and its accumulation has been detected in the fruit abscission zone (Tonutti *et al.,* 1995). It is has been suggested that the *cel1* expression in the mesocarp during fruit ripening is associated with abscission of mature avocado fruits. Several control upstream motifs have been identified in the cellulase and other ethylene-regulated genes.

A cytochrome P-450 has been purified from the mesocarp of ripe avocado fruit by O'Keefe *et al.* (1992). The enzyme is functional as *p*-chloro-*N*-methylaniline demethylase. Bozak *et al*. (1990) isolated two overlapping cDNAs that accumulate during ripening and which have been were identified as P-450 (Bozak *et al*., 1992). The N-terminus of the predicted polypeptide derived from the cDNA is identical to the N-terminus of the purified avocado enzyme. The gene was designated as CYP71A1 and the mRNA was determined to be 1800 bp. The predicted protein has less than 40% homology with other genes of the P-450 family and thus could represent a separate family. During ripening there is accumulation of the CYP71A1 gene product which correlates with increased total P-450 and enzyme activities. The functional role of the CYP71A1 gene remains obscure. Substrate studies (Christoffersen *et al.*, 1995) showed that various mono-terpenes, e.g., nerol and geraniol, are either hyroxylated or epoxylated by the CYP71A1 enzyme, although these have not been detected in ripening fruit.

Avocado polygalaturonase (PG) cDNA has been isolated from a cDNA library by heterologous hybridization with a tomato cDNA probe. The full length mRNA is 1900 bp, coding for 453 amino acids, which is similar in size to the reported avocado PG

protein (Kanellis *et al.*, 1991). Avocado PG is similar to the tomato and corn enzymes and contains the conserved octa-peptide which characterizes PGs from plants, fungi and bacteria (Kutsunai *et al.*, 1993; Dopico *et al.*, 1993). Wakabayashi and Huber (2001) have isolated an endo-PG from cell walls of avocado mesocarp by sequential ion exchange and gel permeation chromatography. They recovered two isoforms of approximately 46 and 48 kD, while the latter has slightly higher catalytic activity. The purified enzymes catalyzed significant molecular mass downshifts in the polyuronides of preripened fruits; however, they had limited capacity to solubilize polyuronides from cell walls of these fruits.

The AVOe3 mRNA has been identified as a ripening-related gene and is detected after 12 h of propylene treatment. The clone encodes a soluble, monomer polypeptide of 34kD. Because of its pattern of induction and its relationship to an ethylene-related tomato gene, it might be involved in ethylene biosynthesis (McGarvey *et al.*, 1992).

Twenty three cDNA clones have been identified by differential screening of a cDNA library made from 7^{o}C stored fruits (Dopico *et al.*, 1993). These clones were grouped as 10 families, six of which had increased expression during cold storage and normal ripening. These sequences had homologies to PG, endochitinase, a cysteine proteinase inhibitor and several stress-related proteins. In clones from six families, no homology was detected.

The ethylene forming enzyme (EFE) has been cloned and over expressed in *E. coli*. This Fe (II) and ascorbate requiring oxidase is responsible for the last step of ethylene biosynthesis in which 1-aminocyclopropane-1-carboxylic acid (ACC), is converted to ethylene. The kinetic mechanism of ACC oxidase is currently being

investigated. (R. Cristoffersen, University of California – Santa Barbara, personal communication).

At the time of writing, there have been 140 avocado sequences, including sequences of the avocado sunblotch viroid, in the NCBI data base.

B. Genetic markers and Marker Assisted Selection

Molecular tools for marker identification and mapping are increasingly being exploited in avocado breeding and genetic analysis. Isozymes have been used as geentic markers in avocado (Torres and Bergh, 1980); however, current research has focused on the application of various DNA markers, e.g., Restriction Fragment Length Polymorphism (RFLP) (Furnier et al., 1990; Bufler and Ben-Ya'acov, 1992), Random Amplified Polymorphic DNA (RAPD) (Bufler and Ben-Ya'acov, 1992) and Variable Number of Tandem Repeats (VNTR), including minisatellites (Lavi *et al*., 1991) and microsatellites or Simple Sequence Repeats (SSRs (Sharon *et al*., 1997).

1. assessment of heterozygosity

Genetic variation and the level of heterozygosity in avocado have been studied. The level of heterozygosity was found to be 100% in five crosses and 90-94% in the analysis of self-pollinated progeny using mini- and microsatellites (Lavi *et al*., 1994). Typing of 59 loci with microsatellites in five avocado cultivars revealed ans average heterozygosity of 0.58 (Nei and Roychoudhury *et al*., 1974). Gene diversity (Rongwen *et al*., 1995) was 0.42 to 0.66. Analysis of 11 cultivars with 17 microsatellites revealed an average of 6.1 alleles per locus, a heterozygosity level of 0.79 and an average Gene Diversity of 0.78. A relatively low level of self-pollination could explain the high level of heterozygosity in avocado.

2. genetic relationships

Davis *et al*. (1998) attempted to determine the paternal origins of important avocado cultivars and the frequency of outcrossing in populations of avocados using RFLP and microsatellite markers. Furnier *et al.,* (1990) used RFLP markers and suggested that *P. nubigena* and *P. steyermarkii* is ancestral to *P. americana* var. *guatemalensis* as suggested by Kopp (1966) and Scora and Bergh (1990). In addition, they suggested that *P. americana* consists of *P. schiedeana* and a large taxon containing the other species and varieties. Thus, *P. flococcosa* may be a variety of *P. americana* and the root rot-tolerant rootstock G755A probably resulted from a cross between *P. americana* and *P. schiedeana.* Buffler and Ben-Ya'acov (1992), using ribosomal DNA probes, were able to identify var. *drymifolia* while *guatemalensis* and *americana* could not be separated. This is in agreement with Kopp (1996), but is in contrast with Scora and Bergh (1990) and Pliego-Alfaro and Bergh (1992), who suggested that the three races are equally distinct from each other. These authors have also used RAPDs to distinctly identify each of the avocado races and various avocado accessions.

The relationship among avocado cultivars and among *Persea* species using mini- and microsatellite markers was explored by Mhameed *et al*. (1997), who assigned 19 avocado cultivars (out of 24 tested), to each of the three avocado races. Other cultivars of unknown origin were compared with DNA patterns of DNA mixes and were found to be hybrids between the various races. In addition, the Guatemalan and the West Indian races were found to be more closely related to each other than to the Mexican race. Mhameed *et al*. (1997) observed that the *P. americana* races and three accessions of *P. schiedeana* are quite distinct from each other. It is our opinion that neither the morphological nor the

DNA markers are superior to each other in phylogeny studies but that the two tools should complement each other. Thus, the relatively high levels of genetic variation observed in selfing progeny using both tools, pose some questions regarding the validity of races and species in avocado (Mhameed *et al*., 1997).

3. genetic mapping and marker assisted selection

Fifty progeny of the cross 'Ettinger' x 'Pinkerton' were genotyped by 93 microsatellites (of which 51 were found to be polymorphic in this family), 17 polymorphic RAPD markers and 23 minisatellite markers (Sharon *et al.,* 1997). The resulting preliminary genetic map consists of 12 linkage groups having 2-5 markers in each group (a total of 35 markers) and covering about 357 cM. Mhameed *et al*. (1995) identified DFP fragments associated with skin colour, harvest duration, skin thickness, skin surface, fruit weight, seed size and peeling ability. The fragment P8 was associated with black-purple skin colour based upon two half-sib families. Sharon *et al.* (1998) also used microsatellites to identify specific bands that are associated with genes controlling skin gloss and seed size. The SSR marker AVAO4 (mapped to linkage group 3), is linked to a gene controlling the amount of fibre in the flesh (p= 0.00001). Correlations between morphological and DNA markers together with a linkage map should eventually enable marker assisted selection for avocado improvement.

III. Micropropagation

The main goal for micropropagating avocados is for clonal rootstocks with tolerance of soil-borne diseases caused by *Phytophthora cinnamomi* and *Rosellinia necatrix* and of saline or calcareous soil limestone conditions. Ontogeny of the explants, juvenile or adult, is critical for *in vitro* development of shoots.

A. Explanting and shoot proliferation

1. juvenile material

Nodal sections with lateral buds have been used successfully for *in vitro* establishment of 1- to 4-year-old seedlings of 'Hass' and 'Hopkins' (Cooper, 1987) and of 1- to 3-year-old seedlings of 'Fuerte' (Schall, 1987) and 'Duke' (Capote *et al.*, 2000). *In vitro* germinated seedlings of 'Gvar-am 13' have been used as a source for explants (Barceló-Muñoz *et al.*, 1990), while Barringer *et al.* (1996) used embryonic axes as explants for several West Indian cultivars.

Mineral salts and hormonal balance are important for culture initiation and subsequent shoot proliferation. Cooper (1987) recommended WPM formulation (Lloyd and McCown, 1981) with 4.44 μM benzyladenine (BA). To initiate shoot elongation, medium containing 1.3 μM BA and 0.5 μM indolebutyric acid (IBA) were used. Under these conditions, a 3-fold multiplication rate per month could be obtained over a two-year period. Barceló-Muñoz *et al*. (1990) utilized $N_{45}K$ macroelements formulation (Margara, 1984) with 45 mM total N content (in a 4:1 NO_3^-/NH_4^+ ratio) and 4.44 μM BA. After several subcultures, shoots became miniaturized and could be multiplied for several years. For shoot elongation, 0.3-0.5 cm shoots are cultured in liquid medium for two weeks in the presence of 1.3 μM BA or kept in multiplication medium without subculturing for 8-10 weeks. Similarly, Witjaksono *et al.* (1999b) obtained optimum growth on a modified MS formulation (Murashige and Skoog, 1962) with 67% NO_3^- and 33% NH_4^+ and 40 mM total N content.

The effects of irradiance level and medium (semi solid or double phase) on shoot proliferation have been studied by de la Viña *et al.* (2001), using shoot cultures derived

from 2-year-old seedlings. Cultures on semi solid medium with 2.89 μM BA resulted in reduced shoot length and proliferation than those growing under double phase conditions (same composition of semi solid medium but with 3 ml of liquid medium supplemented with 0.44 μM BA overlying the semi solid phase). In 30% of cultures growing on semi solid medium, the main shoot demonstrates symptoms of apical necrosis after 3 subcultures, while axillary shoots growing under double phase conditions are hyperhydric. Chlorophyll a and carotenoid concentrations generally decrease when irradiance is increased, e.g., shoots growing under 60-85 μmol m^{-2} s^{-1} are yellowish while those at 35 μmol m^{-2} s^{-1} are green. Scanning electron microscopy studies have shown that leaf stomata from double phase medium remain open, deformed and asymmetric, while those from semi solid medium are half open and below the level of epidermal cells. Double phase conditions negatively affect rooting of cuttings. Barceló-Muñoz (1995) observed that spongy parenchyma cells of hyperhydric shoots are larger and with larger intercellular spaces than those of control leaves; moreover, starch accumulates in the chloroplasts, probably due to their inability to export sugars. Length of hyperhydric shoots is greater and they have a slightly lignified vascular system.

2. mature phase material

Schall (1987), working with axillary buds of adult phase 'Fuerte', initiated axillary bud proliferation on semi solid, half strength MS medium with 22.2 μM BA. Shoot development occurred on semi solid medium with 4.44 μM BA; however, shoots could only be maintained on proliferation medium for 3-4 subcultures. Similar results have been obtained with 'Duke 7' (Harty, 1985; Cooper, 1987). Harty (1985) used semi solid DF medium (Dixon and Fuller, 1976) with 46 μM kinetin and Cooper (1987) used WPM

semi solid medium with 0.44 μM BA. Using etiolated axillary buds of 'Duke', Capote *et al.* (2000) obtained bud sprouting and shoot growth on DF medium with 8.88 μM BA and 5.77 μM gibberellic acid (GA_3); however, growth decreased with subculturing.

Using nodal sections with axillary buds from severely pruned IV-8 rootstocks, Pliego-Alfaro *et al.* (1987) obtained proliferating shoots on semi solid, half strength MS medium with 1.3 μM BA. To stimulate proliferation, shoots are maintained in double phase medium with 2.8 and 0.4 μM BA in the solid and liquid phases, respectively; however, hyperhydricity increases with subculturing. This method has also been used to multiply adult material of 'Duke', 'Fuerte', 'Hass' and 'Topa-Topa' (Zirari and Lionakis, 1994), although data on shoot development has not been presented. To control hyperhydricity, shoots of IV-8 must be cultured for 2 weeks in liquid B5 medium (Gamborg *et al.*, 1968) containing 1.3 μM BA, followed by 6 weeks in double phase B5 medium with 2.8 and 0.4 μM BA in the semi solid and liquid phases, respectively. Shoots have been maintained under these conditions for several years (Barceló-Muñoz *et al.*, 1999)

Adult shoots of 'Gvar-am 13' have been maintained as proliferating cultures for >2 years in $N_{45}K$ macroelement formulation with 4.44 μM BA, although weekly subculture is needed to avoid apical necrosis (Pliego-Alfaro *et al.*, 1987). Hyperhydricity increases with subculturing under these conditions.

For avocado cultivars that are difficult-to-establish *in vitro*, grafting of axillary buds onto *in vitro* germinated seedlings (Pliego-Alfaro and Murashige, 1987; Barceló-Muñoz, 1995) can be used to overcome the problem. Shoots of *in vitro*-grown 4-week-old seedlings are decapitated, their axillary buds removed and lateral buds are inserted

into an incision in the rootstock. Approximately 70% of the micrografts develop successfully, providing shoots after 6-8 weeks. Shoots generally show rapid decline in growth rate after separation from the rootstock (Barceló-Muñoz, 1995)

B. Rooting

Rooting of juvenile shoots can be obtained as long as material of adequate size (>1.5 cm with several leaves) is used. Auxin and nutrients are not essential for induction, although their presence favours rooting. In the absence of auxin, Pliego-Alfaro (1988) obtained 60% rooting without basal medium, 100% with 0.3X MS salts and 10% with full strength MS medium. Rooting also occurs in the absence of sucrose. Pliego-Alfaro (1988) found no difference in rooting rate of juvenile shoots in 0-6% sucrose, although callus production increased with sucrose concentration and some yellowing of leaves was observed at the 6% level. No differences in root number were found in 3-6% sucrose, while in the absence of sucrose, root number is reduced by half. Premkumar *et al*. (2002b) associated decreased rooting with yellowing of the leaves at 5% sucrose.

Continuous exposure to auxin does not significantly affect root induction; however, short exposures (3 days) to 123-492 μM IBA significantly increase rooting (Pliego-Alfaro, 1988). Barceló-Muñoz *et al.* (1990) showed that 4.92 μM IBA is equally effective. Cooper (1987) dipped shoots for 1 sec in 1000-3000 mg litre^{-1} IBA or naphthaleneacetic acid (NAA), before transfer to basal medium. Indolebutyric acid induces slightly higher rooting percentages, but NAA promotes faster rooting. The physiological stage of the shoots affects rooting, e.g., etiolated shoots generally root poorly, and auxin reduces their rooting potential (Pliego-Alfaro, 1988). De la Viña (1996)

recommends incubation of light-grown shoots under dark conditions for 3 days with exposure to auxin in order to increase rooting.

Due to the inhibitory role of auxin in root elongation, Pliego-Alfaro (1988) used activated charcoal in the second phase of rooting, while Schall (1987) has recommended 24.6 μM IBA throughout the rooting process, and increased rooting from 26% to 87% after supplementing the medium with activated charcoal.

Auxin metabolism during rooting has been studied by García-Gómez *et al.* (1994). Concentrations of indoleacetic acid (IAA) and the conjugate IAA-aspartate were measured in juvenile shoots that rooted in the absence of auxin and in cuttings treated with IBA or with the auxin transport inhibitor TIBA. They demonstrated the importance of endogenous auxin for rooting; e.g., no rhizogenesis was observed in the presence of TIBA, while activation and development of cambial cells was not inhibited. García-Gómez *et al.* (1994) suggested that auxin is necessary at the early stages of rooting, e.g., activation of cambial cells (days 0-3) and division of cambial cell derivates (days 3-6). García-Gómez *et al.* (1995) observed increased peroxidase activity between days 3 and 6 of the rooting process. Histological studies indicated that there is a close association between peroxidase activity and cambial cell division and differentation.

Ex vitro rooting has been demonstrated with juvenile avocado. Cooper (1987) dipped shoots for 1 sec in 3000 mg litre^{-1} NAA and obtained 100% rooting. Under these conditions shoots formed excellent root systems.

Rooting potential of adult shoots is low and does not increase with subculture (Pliego-Alfaro *et al.*, 1987). Generally, shoot growth ceases, the leaves are shed and the shoots die following transfer to rooting medium (Pliego-Alfaro and Murashige, 1988;

Zirari and Leonakis, 1994). Rooting can be improved following grafting of adult buds onto *in vitro* germinated seedlings. Pliego-Alfaro and Murashige (1988) obtained 50% rooting of adult 'Duke-7' shoots after one graft, although regrafting (as many as 3x) did not improve rooting or shoot vigour. Up to 90% rooting of adult 'Gvar-am 13' shoots occurs following successive *in vitro* grafting (as many as 15x) (Barceló-Muñoz, 1995). The restored rooting competence is stable after 9 subcultures but proliferation is poor.

Approximately 30% rooting has been obtained with shoots derived from severely pruned IV-8 plants (Pliego-Alfaro *et al.*, 1987). Rooting increases up to 72% if the root induction phase (with auxin) is in liquid medium, and up to 77% if root induction and elongation phases occur in liquid medium. Unfortunately, in the latter case, virtually all shoots are hyperhydric, and a liquid-solid medium alteration is preferable. Up to 90% rooting can be obtained with B5 medium, demonstrating the important role of medium on rooting (Barceló-Muñoz *et al*., 1999)

C. Acclimatization

The physiology of juvenile avocado plantlets growing under *in vitro* conditions has been studied. For example, de la Viña *et al*. (1999) investigated the effects of sucrose concentration and carbon dioxide levels on photosynthesis and determined the amount of ribulose 1,5 biphosphate carboxylase-oxygenase (Rubisco protein) in rooted avocado shoots. At high sucrose levels (87.6 mM), Rubisco content is low in leaves, particularly in the presence of high CO_2 (1000 μmol.mol $^{-1}$). Under these conditions, the maximum photosynthetic rate (Pmax) is low.

In a similar study, Witjaksono *et al.* (1999b) grew avocado plantlets in a CO_2-enriched environment with 30 g litre^{-1} sucrose, and observed that the net CO_2 assimilation

rate was reduced by 39%, although these mixotrophic plantlets grew better than those cultured under atmospheric CO_2 conditions. De la Viña *et al.* (1999) also obtained higher leaf area and leaf fresh weight/(stem+root) fresh weight ratio in plants grown under enhanced CO_2, although survival after transplanting was less for plants coming from low sucrose/high CO_2 conditions in comparison to those grown under high sucrose/ high CO_2.

The effect of sucrose in the culture medium prior to acclimatization of juvenile avocado plantlets has been studied by Premkumar *et al.* (2002a,b). No significant differences in total leaf chlorophylls, N mass, flavonoids and total soluble proteins were found in plants under *in vitro* or *ex vitro* conditions. However, *ex vitro* transfer strongly affects foliar ratios of chl a to chl b, and total chlorophylls to carotenoids, indicating that transfer *ex vitro* implies a modification of the light absortion by the leaves. The C:N ratio is affected by changes in sucrose concentration as well as by transfer *ex vitro* suggesting increased structural carbon that is related to hardening of cell walls and increased xylem tissue (Premkumar *et al*. 2002a). Rubisco content decreases with increasing sucrose content, while it greatly increases *ex vitro*, suggesting a possible role as storage pool of reduced nitrogen for growth *ex vitro* (Premkumar *et al.* 2002a). Increased sucrose does not alter the endogenous levels of monosacharides, sucrose and starch in leaves *in vitro*. In general, endogenous concentrations of sucrose and starch are higher in leaves than roots, irrespective of the sucrose treatment, while the starch content increases in leaves of *ex vitro* acclimatized plants and sucrose level is lower. These changes indicate improved sucrose utilization efficiency and starch synthesis *ex vitro* (Premkumar *et al.,* 2002b).

Acclimatization of rooted juvenile shoots under high relative humidity can be routinely accomplished (Barceló-Muñoz, 1990; Cooper, 1987; Schall, 1987). However,

growth and development is improved after inoculation with vesicular-arbuscular mycorrhizal fungi. Vidal *et al.* (1992) obtained improved rooting and shoot growth following inoculation with *Glomus fasciculatum* at the time of transplanting, together with improved survival of inoculated plants. De la Viña *et al.* (1996) obtained similar results with RR-86 rootstock, a two year-old seedling showing tolerance of *P. cinnamomi*, after inoculating rooted shoots with *Glomus deserticola*.

Adult plantlets show slow growth *ex vitro* resulting in a low survival rate (Barceló-Muñoz, 1995). Barceló-Muñoz *et al.* (1999) obtained 70% survival after maintaining IV-8 plants for >4 weeks in polyethylene tunnels with 100% RH, 110-120 μmol m^{-2} s^{-1} irradiance level at 15-30°C. Plants were exposed to increasing periods (5 min the first day, 5 h the last day) of ambient environmental conditions for four weeks. After 4 weeks, plants could be transferred to open tunnels.

IV. Micrografting for elimination of pathogens

Avocado sunblotch viroid (ASBVd), a member of the avsunviroidae family of viroids, causes sunblotch disease of avocado. ASBVd cause symptoms on fruits and leaves and levels of infection in the plant vary according to the tissue origin and environmental conditions (da Graca and Moon, 1983; Schnell et al., 1997). Asymptomatic trees can occur (Semancik and Szychowsky, 1994) and infected plants have appeared to be healthy after further indexing, which is interpreted as unequal distribution of the viroid, lack of efficiency of the indexing technique and silencing mechanism of the host on the viroid (Olano et al., 2003). Transmission of ASBVd can occur via seeds, vegetative material, pollen and contaminated tools (Desjardines, et al., 1979; Wallace and Drake, 1962;

Desjardines et al., 1987; Parker et al., 1932); and the only effective control is eradication of affected plants.

Shoot tips, consisting of the meristem plus 2-3 leaf primordial, from *in vitro* germinated avocado seedlings of ASBVd-infected cultivars were micrografted onto decapitated seedlings of 2 ASBVd-free cultivars, and plants were recovered (Suarez, 2003; Suarez *et al*., in press). Regenerated plants were indexed by RT-PCR for ASBVd infection and amplified products were cloned and sequenced. RT-PCR indicated that ASBVd replicated in the micrografts, while no ASBVd was detected in micrografts from plants that tested negative. In a parallel study, Suarez (2003) screened embryogenic cultures and plants derived from avocado nucellus, and demonstrated with RT-PCR that ASBVd was present in all cultures. ASBVd, therefore, cannot be eliminated either by *in vitro* micrografting or nucellar culture (Suarez, 2003).

See micrografting for rejuvenating mature phase avocados above.

V. Somatic Cell Genetics

A. Regeneration

The chief prerequisite for applying somatic cell genetic approaches to avocado, including protoplast-based technologies, *in vitro* mutagenesis and selection and genetic transformation is a highly efficient regeneration protocol from single cells of elite selections. There were several early reports of callus initiation from various plant partsof avocado; however, the callus was non morphogenic (Blumenfeld and Gazit, 1971; Schroeder, 1956; 1961; 1971; 1980).

1. somatic embryogenesis

a. induction

Conditions for induction of embryogenic cultures from explanted avocado zygotic embryos, using openly pollinated 'Hass' (Mexican x Guatemalan) as a model were described by Pliego-Alfaro (1981) and Pliego-Alfaro and Murashige (1988). These reports have been confirmed by Mooney and van Staden (1987) with openly pollinated 'Fuerte' (Mexican x Guatemalan) and 'Duke 7' (Mexican) later by Witjaksono and Litz (1999a, b) and (Raviv *et al.*, 1998). Various sizes and developmental stages of zygotic embryos have been utilized as explants. Mooney and van Staden (1987) used 0.1–0.5 mm embryos from 3-4 mm fruits, Pliego Alfaro and Murashige (1988) used 0.6–0.8 mm embryos from 9 mm fruits and Raviv *et al.* (1998) used 7–10 mm embryos. After surface-sterilization, the fruit are bisected longitudinally. The immature seed halves can be plated on induction medium so that the zygotic embryo is in contact with medium or the zygotic embryo can be excised and used as the explant. Witjaksono *et al.* (1999a) demonstrated that embryogenic cultures could also be induced from the nucellus (maternal) of immature avocado seeds of the same stage of development on the same induction medium. Cultures are induced on semi solid induction medium in 60x15 mm plastic disposable petri dishes in darkness at 25^{o}C.

Induction of embryogenic cultures from explanted nucellus (Witjaksono *et al*., 1999a) and zygotic embryos (Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988; Mooney and van Staden, 1987) has been reported on semi solid MS medium supplemented with 0.1 mg litre^{-1} thiamine HCl, 100 mg litre^{-1} myo inositol, 30 g litre^{-1} sucrose and 0.41 μM picloram. Witjaksono and Litz (1999a) observed that an induction basal medium consisting of B5 major salts (without NH_4NO_3) with MS minor salts and organic components was superior to MS medium. Embryogenic cultures are induced

approx. 8-25 days after explanting. Cultures consist of proembryonic masses (PEMs) and hyperhydric somatic embryos.

b. maintenance

Medium-term maintenance of embryogenic cultures is essential for many types of *in vitro* manipulation. Witjaksono and Litz (1999a) optimized the growth of embryogenic cultures on semi solid MS induction medium formulation supplemented with agar, but observed that more somatic embryos developed on modified B5 medium (see above) supplemented with gellan gum. Subculture of the smallest PEM fraction at 3–5 week intervals is necessary for cultures on semi solid medium. Maintenance of embryogenic cultures in suspension, however, provides optimum conditions for proliferation of PEMs (Witjaksono and Litz, 1999a). MS medium that has been modified to contain 12 mg litre^{-1} NH_4NO_3 and 30.3 mg litre^{-1} KNO_3 results in the highest PEM FW increase compared to other medium formulations (Witjaksono and Litz, 1999b). Biweekly subcultures are necessary for embryogenic suspension cultures. Usually, 0.5 or 1 g PEMs are inoculated into 40 ml or 80 ml liquid maintenance medium in 125 ml or 250 ml Erlenmeyer flasks, respectively.

There are two distinct types of embryogenic avocado cultures (Witjaksono and Litz, 1999a), and these responses are genotype-dependent: 1) the PEM-type. A few genotypes proliferate as PEMs with no differentiation of cotyledons in the presence of auxin; 2) the SE-type. Heart and later developmental stages of somatic embryos differentiate and develop in the presence of auxin, and this is the most typical response. Suspension cultures of the SE-type must be sieved prior to subculture, and only the <0.8 mm fraction is inoculated into fresh maintenance medium. The loss of embryogenic

potential under maintenance conditions is cultivar-dependent (Witjaksono and Litz, 1999a) and can vary from 3 months with 'Yon' to >2 years with 'Esther'. Cultures on semi solid maintenance medium are incubated in darkness at 25ºC, whereas suspension cultures are incubated in semi darkness.

c. maturation

Although Pliego-Alfaro and Murashige (1988) and Mooney and Van Staden (1987) reported that somatic embryo development followed the transfer of embryogenic cultures onto semi solid medium without picloram, the absence of auxin is not essential for somatic embryo development of SE-type cultures (Witjaksono and Litz, 1999a) (Figure 1). This has been confirmed by Raviv *et al.* (1998), who observed that cotyledonary somatic embryos develop on semi solid medium supplemented with 9.04 μM 2,4-D and 2.22 μM BA. Somatic embryo development following transfer from maintenance to maturation media is genotype-dependent, and the development from SE-type cultures is more efficient (Witjaksono and Litz, 1999a, b).

Development of somatic embryos is also mediated by a few physical parameters (Witjaksono and Litz, 1999b), e.g., gelling agent and sucrose concentration, which affect both the size and number of good quality somatic embryos. Although the largest number of somatic embryos develops on medium supplemented with 6-7 g 1^{-1} gellan gum and 90 g $litre^{-1}$ sucrose, their size is reduced at this concentration of sucrose. Hyperhydricity is invariably a consequence of optimizing maintenance in suspension culture, and somatic embryo development on medium with high gellan gum and sucrose concentrations can reverse or prevent this physiological disorder. The optimum growth conditions for somatic embryo maturation include MS medium which has been supplemented with 30 g

litre^{-1} sucrose, 4 mg litre^{-1} thiamine HCl, 100 mg litre^{-1} myo inositol and 6.0 g litre^{-1}gellan gum in darkness at 25^{o}C. Addition of 20% (v/v) filter-sterilized coconut water increases the frequency of recovery of high quality somatic embryos (Witjaksono and Litz, 2002).

d. germination

Fully enlarged, opaque mature somatic embryos (0.8-1.0 cm) are transferred to semi solid germination medium, which is MS medium supplemented with 4.44 µM BA and 2.89 µM GA_3 (Witjaksono and Litz, 1999b). After six months, somatic embryos develop shoots only, roots only or are bipolar. Shoot development is generally low due to failure of the apical meristem to become organized (Pliego Alfaro and Murashige, 1988). Maturation medium supplemented with filter-sterilized coconut water can increase the frequency of recovery of somatic embryos with apical meristems (Pliego Alfaro, personal communication; Witjaksono and Litz, 2002).

Witjaksono *et al*. (1998) rescued somatic embryo shoots by micropropagating them according to the protocol described above (Witjaksono *et al.*, 1999b). Another procedure that has been successfully exploited has involved the micrografting of somatic embryo-derived shoots on decapitated seedlings (Raharjo and Litz, 2003).

Individual micropropagated shoots derived from somatic embryos can be rooted according to the induction and development procedure of Pliego Alfaro (1988) and Witjaksono *et al*. (1999b). Individual shoots (1.5–2 cm length with 1–3 leaf primordia and non-expanded leaves) that develop from proliferating shoot cultures are cultured for 3 days on MS medium supplemented with 122.6 µM IBA. Culture conditions include a 16h photoperiod (100-120 µmol m^{-2}s^{-1} provided by cool white fluorescent bulbs) at 25^{o}C.

2. protoplast isolation and culture

The first reports of isolation and culture of avocado protoplasts involved studies of avocado sunblotch viroid using callus-derived protoplasts (Blickel *et al.*, 1986) and fruit ripening studies using protoplasts from fruit mesocarp (Percival *et al.*, 1991). These studies utilized non morphogenic cultures. Witjaksono and Litz (2000) recovered protoplasts from non morphogenic callus suspensions of two *Persea* spp. in the subgenus *Eriodaphne*, i.e., *P. cinnerascens* and *P. pachypoda*. Witjaksono *et al*. (1998) described the isolation and culture of protoplasts from embryogenic avocado cultures, and regenerated plants from somatic embryos. Avocado protoplasts have been successfully cultured in liquid (Witjaksono *et al.*, 1998) and in agarose-solidified (Witjaksono *et al.*, 1999a) media, although the former procedure is more efficient. Yields of $>3x10^6$ protoplast g^{-1} are obtainable from embryogenic cultures maintained in liquid medium. The isolation and culture of protoplasts and their regeneration are genotype dependent, and the procedure is more efficient with PEM-type cultures than SE-type cultures. Development of PEMs from protoplasts in liquid medium is dependent on medium osmolarity, nitrogen source and plating density. PEMs develop in medium with 0.4 M osmolarity, while microcalluses develop in medium with 0.6 M osmolarity. The optimum medium and conditions for recovery of PEMs from protoplasts consists of 0.4 M MS$^-$8P with a 0.8×10^5 ml^{-1} protoplast density.

Protoplasts are cultured in 2-3 ml liquid medium in sealed 60 x 15 mm sterile plastic dishes, and maintained in darkness at 25^{o}C. Approx. 5% of protoplasts divide after 5 days of culture, and the plating efficiency after 12 days is approx. 25%. Protoplast-derived PEMs are able to develop on maturation medium (see *somatic*

embryogenesis maturation above), and mature somatic embryos have germinated, albeit with a low conversion frequency.

B. Genetic Manipulation

1. mutation induction and somaclonal variation

a. breeding objectives

Somatic mutations of avocado that affect tree architecture, leaf size, leaf shape and colour, fruit size and shape, and skin texture have been recognized. Several off types of 'Fuerte', including ‘Weisel’, ‘Newman’ and ‘de Bard’ have been recovered (Hodgson, 1945). The rootstock selection ‘D9’ originated from irradiated ‘Duke’, and has good resistance to PRR together with some dwarfing effect on the scion. Scions grafted on ‘D9’ are more productive than on ‘Martin Grande’ but less so than ‘Borchard’ and ‘Duke 7’ (Arpaia *et al*., 1992). 'Hass' that was irradiated with ^{60}Co showed reduced vegetative growth, and greater flowering and fruit set were observed from 4-year-old plants exposed to 13 Gy (de la Cruz-Torres *et al*., 1995a). Following irradiation at 15 Gy, there was variability in height, rootstock and graft diameter, stomatal density and internode length (de la Cruz-Torres *et al*., 1995b).

Witjaksono and Litz (2004) reported the radiation sensitivity of embryogenic cultures of ''Fuerte' and 'T362'. The effects of irradiation on embryogenic suspensions and on somatic embryo development from irradiated cultures were described. The approximate PD_{50} as determined by linear regression was 35 Gy two weeks after irradiation for 'Fuerte' and four weeks after irradiation for 'T362'. Irradiation did not significantly affect the number of early stage 'Fuerte' 2.11.1 somatic embryos that developed from irradiated cultures; however, 10-50 Gy inhibited somatic embryo

development. Irradiation of 'T362' embryogenic cultures at 25-50 Gy inhibited the number of intermediate and mature stages of somatic embryos that developed from irradiated cultures, and 50 Gy inhibited somatic embryo maturation. Irradiation up to 10 Gy significantly increased the number of mature 'Fuerte' somatic embryos that developed from suspension cultures. Irradiation with doses up to 25 Gy stimulated development of heart stage 'T362' somatic embryos; however, mature somatic embryo development was suppressed at dosages of 10 Gy and greater. The aim of this study is to select *in vitro* for resistance to the culture filtrate of *Phytophthora cinnamomi* and to regenerate plants that may have resistance to PRR (Witjaksono, 2001).

2. somatic hybridization

a. breeding objectives

The immunity to PRR that is associated with species in subgenus *Eriodaphne* is inaccessible to plant breeders due to graft and sexual incompatibility barriers between *Persea* spp. in this group and with species in subgenus *Persea* (Frohlich *et al*., 1958). Pliego-Alfaro and Bergh (1992) suggested that somatic hybridization might be the appropriate way to achieve hybridization between species in the two subgenera.

i. protocol

Witjaksono (1997) reported the attempted somatic hybridization of avocado by means of protoplast fusion involving protoplasts from embryogenic avocado cultures with leaf protoplasts of PRR-resistant species. In order to assure a constant supply of protoplasts, a procedure for micropropagating *Persea* species (subgenus *Eriodaphne*) from *in vitro*-germinated seedlings was developed (Witjaksono, 1997). The results of this study were inconclusive; however, somatic hybrids were recovered as a result of the

fusion of embryogenic avocado protoplasts with non morphogenic protoplasts of Persea spp in subgenus *Eriodaphne* (Witjaksono and Litz, 2000); however, plants were not regenerated from the hybrid somatic embryos.

2. genetic transformation

a. breeding objectives

Genetic transformation could be used to address some important rootstock and scion breeding objectives. A primary breeding objective has been to develop improved avocado rootstock cultivars with greater resistance to PRR and scion cultivars with enhanced resistance to foliar and fruit diseases, using genes that encode for disease resistance or pathogenesis-related proteins. The control of fruit ripening is another important breeding objective that would have two important ramifications: 1) enable the on-tree storage of West Indian and West Indian x Guatemalan avocados; and 2) extend the post-harvest storage time of all types of avocados.

i. protocol

The genetic transformation of avocado has been based upon highly embryogenic suspension cultures described above. Growth of PEM-type embryogenic suspensions can be suppressed by 50% with 50 mg litre^{-1} kanamycin sulfate; whereas, 50% growth suppression on semi solid medium requires 100 mg litre^{-1} kanamycin sulfate (Cruz-Hernandez *et al.*, 1998). Complete suppression of growth of embryogenic cultures occurs on semi solid medium containing 200 mg litre^{-1} kanamycin sulfate.

Cruz-Hernandez *et al.* (1998), utilizing PEM type cultures, described a 2-step selection procedure for recovery of genetic transformants. PEM-type embryogenic cultures growing on semi solid maintenance medium were gently abraded with a soft

camel hairbrush. The abraded embryogenic cultures were then incubated with acetosyringone-activated *Agrobacterium tumefaciens* [strain 9749 ASE2 containing a co-integrate vector pMON9749 with a selectable kanamycin resistant marker (*nptII*) and *GUS* (β-glucuronidase)] in liquid maintenance medium, and co cultured for 3 days at 100 rpm. *Agrobacterium tumefaciens* was then eliminated by incubating the cultures in maintenance medium supplemented with 50 mg litre^{-1} kanamycin sulfate and 200 mg litre^{-1} cefotaxime. After an initial selection for antibiotic resistance in liquid maintenance medium containing 50 mg litre^{-1} kanamycin sulfate for 2-4 months, there was a second, more intensive selection in the presence of 100 mg litre^{-1} kanamycin sulfate for 2 months. Finally, the cultures were cultured in 200 mg litre^{-1} kanamycin sulfate to eliminate chimaeras. Somatic embryo development was initiated by subculture of transformed PEMs onto maturation medium without kanamycin sulfate, followed by subculture onto maturation medium containing kanamycin sulfate. Transformed somatic embryos stained positively for *GUS* (Jefferson, 1987), and the integration of *nptII* and *GUS* into the avocado genome was confirmed by PCR and southern hybridization (Doyle and Doyle, 1990; Miller, 1972). Transgenic plants were not regenerated.

ii.accomplishments

Using a modification of the protocol described above, it has been possible to genetically transform avocado with genes that could affect different horticultural traits. The strategy that has been adopted for controlling avocado fruit ripening is based upon blocking ethylene production with the SAM hydrolase gene (SAMase), which converts SAM to a non-toxic by-product that it is not available to be converted into ACC (Good *et al*., 1994). In this manner, the biosynthesis of ethylene is blocked. The SAMase gene is

in pAG4092 under the control of an avocado fruit-specific cellulase promoter with *nptII* as a selectable marker. Efendi (2003) has described the recovery of transgenic plants that contain the SAMase gene.

Embryogenic avocado cultures have also been genetically transformed with PR-related genes, including β-1,6-glucanase, chitinase and the antifungal protein (AFP) gene in order to address the problem of PRR of avocado rootstocks (S. Raharjo and Witjaksono, Homestead, USA, personal communication) (Figures 2-4). The AFP gene is in pHGAFP together with *uidA*, the gene for resistance to hygromycin and the CaMV 35s promoter. Glucanase and chitinase have been cloned in pGPTV, together with *uidA*, *bar* and the CaMV 35S promoter. The initial greenhouse and field trials commenced in 2003 (Raharjo *et al*., 2003).

C. Cryopreservation

Long term conservation of avocado genetic resources is confounded by the high costs of land, labour, grove management including the control of pests and diseases and inclement weather. Genetic diversity within the genus *Persea*, within the subgenera *Persea* and *Eriodaphne* and within *Persea amsericana* is large, so that meaningful collections of species, subspecies and cultivars for breeding and genetic studies must be extensive. Manipulation of avocado at the somatic cell level requires a constant supply of embryogenic material. There is a strong genotype-dependent loss of embryogenic competence *in vitro* (Witjaksono *et al*., 1999a). Therefore, the annual renewal of embryogenic lines has been essential, resulting in the expenditure of considerable time ande xpense. Cryopreservation could resolve some of the problems associated with ensuring a continuous supply of embryogenic cultures. In addition, cryopreservation

could impact the long-term maintenance of clonal *Persea* genetic resources by providing a back up for existing collections.

Efendi *et al*. (2001), Efendi and Litz (2002; 2003) and Efendi (2003) have described the recovery of embryogenic avocado cultures from cryogenic storage, and successfully regenerated somatic embryos from these cryopreserved cultures. Two procedures were described: 1) stepwise cooling (-1^{o}C min^{-1} from room temperature to -75^{o}C followed by rapid cooling to -196^{o}C; and 2) rapid cooling (vitrification) from room temperature to -196^{o}C. For stepwise cooling, embryogenic cultures were treated with different cryoprotectant combinations consisting of DMSO and glycerol [5% + 5%, 10%+10% and 15%+15% (v/v)], and cooled at the rate of -1^{o}C min^{-1} to –75^{o}C in "Mr. Frosty" containers. The cryovials were removed and were plunged into Dewar flasks containing liquid nitrogen. For cryopreservation by vitrification, embryogenic cultures were suspended in PVS2 vitrification formula (Sakai *et al*., 1991), consisting of glycerol (30%), ethylene glycol (15%) and DMSO (15%) for 15 min prior to rapid cooling by plunging into liquid nitrogen. Following the removal of vials from liquid nitrogen and rapid warming to room temperature, cultures were thoroughly washed with maintenance medium and plated on semi solid maintenance medium formulation. Somatic embryo development was initiated by subculturing on somatic embryo maturation medium.

VI. Conclusions

A few groups in major avocado-producing countries (USA, Spain, Israel, Mexico and Australia) have made an early and significant impact on avocado improvement using modern genetics and cell culture techniques. Perhaps because avocado has been a good model for study of the basic physiology of fruit ripening, there have been a number of

published and unconfirmed reports of gene cloning of the important genes implicated in this process. Moreover, two of the major breeding goals for avocado are fairly well defined: control of fruit ripening and control of Phytophthora root rot. These objectives are attainable using current genetic transformation procedures. It is probable that the avocado PRR problem can also be addressed using *in vitro* mutation induction and selection. There appears to be a strong readiness among groups to collaborate, which should enable steady and rapid progress.

Biotechnology can address other important issues associated with this crop. The use of either shoot tip and nodal culture or somatic embryogenesis as propagation procedures could resolve some of the serious limitations of existing methods for vegetatively propagating PRR-resistant rootstocks by the etiolation method (Frolich and Platt, 1972). Control of avocado sunblotch disease caused by a viroid could possibly be addressed by genetic transformation with the *pac1* ribonuclease gene, which has been demonstrated to be highly effective against potato spindle tuber viroid (Sano *et al*., 1997).

It is expected that important genes will be identified, studied and eventually be applied to the avocado. Major discoveries will occur in the area of genomics research, which is a new platform of technologies aiming at understanding whole genomes, e.g., large scale sequencing, bioinformatics, DNA chips, etc. The function of various genes as well as the interactions between them will be disclosed. Specifically for avocado, it is our expectation that more avocado genes will be identified directly by these tools and by establishing homologies of with genes from other plants in which application of these technologies is more advanced (*Arabidopsis*, corn, rice and tomato). Identification of the genes coding for important traits, including yield, fruit size and shape, will on one hand

improve classical breeding by MAS, and on the other hand provide suitable genes for generation of transgenic plants with the desired characters.

Cryopreservation of embryogenic avocado lines can have great utility not only to ensure a supply of morphogenically competent cells, but also could be used as a back up for avocado germplasm collections and to facilitate the international movement of important genetic resources, particularly from the great genetic repositories in USA, Israel and Mexico and Australia.

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Figures