TRANSFORMATION AND CRYOPRESERVATION OF EMBRYOGENIC AVOCADO (*Persea americana* Mill.) CULTURES

By

DARDA EFENDI

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I dedicate this work to my beloved wife, Neneng Nurhasanah,
my late parents, Dahlan Rasyad and Nuraya Rasyad, and my extended family.

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TRANSFORMATION AND CRYOPRESERVATION OF
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By

Darda Efendi

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The avocado fruit is climacteric and ethylene acts as a natural triggering mechanism for the induction of ripening. Genetic transformation of avocado with a gene construct that could block ethylene biosynthesis would extend on-tree storage and shelf life of avocado fruit. The *samK* gene encodes SAM hydrolase, which converts SAM (S-adenosylmethionine) to methylthioadenosine and homoserine so that it is not available for ethylene biosynthesis.

Embryogenic avocado cultures were genetically transformed using an *Agrobacterium tumefaciens*-mediated protocol. Transformed embryogenic avocado cultures harboring AGT01/NPTII::ACP/SamK were selected in MS3:1P medium supplemented with 100 to 300 mg l^{-1} kanamycin sulfate. Further selection was accomplished on somatic embryo development medium supplemented with 200 mg l^{-1} kanamycin sulfate, which completely inhibits development and maturation of nontransformed somatic embryos.

Embryogenic cultures experience major developmental problems over time, which include a low rate of somatic embryo germination and loss of embryogenic competence. The latter can make it difficult to develop elite lines that have been genetically engineered, and cultures of some genotypes cannot be used for medium-term research. Long-term storage of embryogenic cultures is critical to address this problem.

Avocado embryogenic cultures can be cryopreserved by slow cooling and vitrification. The ability to withstand cryopreservation appears to be genotype-dependent. Following cryopreservation, embryogenic cultures can proliferate in liquid medium, and somatic embryo development and germination do not appear to be negatively affected.

This study should have a major impact on biotechnology research involving avocado. This is the first report of regeneration of transgenic avocado with a horticulturally important trait. The cryopreservation protocols developed in this study will have major impact on the management of avocado genetic resources and experimental material in the laboratory.

CHAPTER 1
INTRODUCTION

Importance of Avocado

The avocado is one of the major fruit crops of the world. Average world avocado production has been approximately 2.4 million metric tons per annum for the last six years (1996-2001) (Food and Agriculture Organization of the United Nations Statistical Databases, FAOSTAT, 2002). Mexico is the world's largest avocado producer and exporter, i.e., 37% of the world's avocado production (Table 1-1). Other important producers include USA, Indonesia, Colombia, Dominican Republic, Brazil, Chile, Peru, Israel, South Africa, China and Spain (Table 1-1) (FAOSTAT, 2002). Together, those countries account for 80% of world avocado production. Avocado production ranks 10th after citrus, *Musa* (banana and plantain), grape, apple, mango, pear, peach, plum and papaya (FAOSTAT, 2002). In the USA, avocado production has recently been approximately 192,000 metric tons per annum with a value of $307 million. California contributes approximately 95% of the production and Florida accounts for approximately 5% (Table 1-2) (National Agricultural Statistics Service-The United State Department of Agriculture, NASS-USDA, 1999-2002).

The top ten exporting countries are Mexico, Spain, Israel, South Africa, Chile, Netherlands, USA, France, Belgium-Luxemburg and Dominican Republic (Table 1-3) (FAOSTAT, 2002). Together those countries account for 91% of the word's avocado exports. The Mexican 'Hass' and to a much lesser extent ' Fuerte' have dominated the world's avocado export market. 'Hass' is the most important cultivar grown in the USA

(Bergh, 1976). The West Indian ('Simmons', 'Waldin' etc.) and hybrid Guatemalan x West Indian ('Choquette', 'Monroe', etc.) cultivars predominate in the tropics and south Florida (Crane et al., 1998) but have failed to gain significant market share due to significant post-harvest problems.

Table 1-1. Average yearly production of avocado during 1996 - 2001

No.	Country	Production (Tons)	Production (% of World)
1	Mexico	860,961	36.6
2	USA	177,562	7.5
3	Indonesia	131,422	5.6
4	Colombia	122,778	5.2
5	Dominican Republic	89,825	3.8
6	Brazil	84,928	3.6
7	Chile	80,500	3.4
8	Peru	79,499	3.4
9	Israel	74,028	3.1
10	South Africa	67,981	2.9
11	China	59,750	2.5
12	Spain	57,082	2.4
	World	2,353,265	100.0

(FAOSTAT, 2002)

Table 1-2. Average of yearly production of avocado in USA in 1996-2001

States	Bearing Acreage		Production Quantity		Production Value	
	Acres	%	Tons	%	$1,000	%
California	59,517	90.69	169,167	87.95	292,112.17	95.26
Florida	5,883	8.97	22,917	11.91	14,309.33	4.67
Hawaii	225	0.34	262	0.40	290.50	0.09
Total USA	65,625	100.00	192,345	100.00	306,635.33	100.00

(NASS-USDA, 1998-2002)

The avocado fruit is a major source of antioxidants, a source of fruit protein and fiber, and avocado oil has several culinary and health benefits (Bergh, 1992a; Knight, 2002). Avocado fruit contains ca. 30% fat; however, Bergh (1992b) noted that it is predominantly monounsaturated oleic acid and maintains high levels of beneficial high-

density lipoprotein (HDL). It has been shown to reduce levels of low-density lipoprotein (LDL) in blood. In addition avocado fruit has other potential heart-protective benefits due to its high content of antioxidant vitamins A, C and E, high densities of other nutrients and high soluble fiber content. Hardison et al. (2001) found that 'Anona', 'Fuerte', 'Hass' and 'Orotava' on average contain the following levels (in mg/100g of edible portion) of macro elements: sodium 66.4, potassium 99.4, calcium 7.7, magnesium 52.8, phosphorus 15.6 and microelements: iron 40.7, copper 33.7, zinc 32.8, manganese 40.5 and boron 9.0. Of these four cultivars, 'Hass' has the highest content of macro elements, while 'Fuerte' is the richest in microelements.

Table 1-3. Average yearly export of avocado during 1996 - 2001

No.	Countries	Export Quantity		Export Value	
		Tons	%	X $ 1,000	%
1	Mexico	68,856	22.9	54,390	19.1
2	Spain	42,647	14.2	49,105	17.2
3	Israel	38,285	12.7	38,476	13.5
4	South Africa	36,457	12.1	17,670	6.2
5	Chile	35,092	11.7	15,834	5.6
6	Netherlands	14,002	4.7	19,523	6.9
7	USA	13,963	4.6	15,834	5.6
8	France	13,858	4.6	18,263	6.4
9	Belgium-Luxembourg	10,321	3.4	25,149	8.8
10	Dominican Republic	8,219	2.7	4,119	1.7
	World	300,649	100.0	284,831	100.0

(FAOSTAT, 2002)

Control of Fruit Ripening

An extended shelf life of avocado fruit is a major goal of many avocado producers (Lahav and Lavi, 2002); however, there are no breeding programs that specifically target on-tree storage and extended shelf-life of avocado fruit. This is in part due to the typical problems of conventional breeding of woody perennial species, i.e., a long juvenile

period, seasonal flowering and low fruit set (Pliegro-Alfaro and Bergh, 1992; Lavi et al., 1993b; Lahav and Lavi, 2002).

There are two types of avocado with respect to fruit maturity: cultivars whose fruit cannot ripen on the tree and cultivars whose fruit readily ripen on the tree. The fruit of Mexican and Guatemalan types and their hybrids cannot ripen while they are still attached to the tree and can remain on the trees for 2-4 months (Tingwa and Young, 1975; Sitrit et al., 1986; Whiley, 1992). The ability to remain on the tree can be used to prolong avocado supply by "on-tree-storage." A year-round supply of 'Hass' avocado in California exploits this feature together with different climatic regions that can affect production times. Whiley et al. (1996) indicated, however, that a delay of 2 months for harvesting can initiate an alternate bearing cycle that lowers the production in the subsequent year and reduces the average annual yield by 26%. Extending the shelf life would permit earlier harvesting of avocado of this type and would overcome the problem of alternate bearing.

The fruit of West Indian and West Indian x Guatemalan hybrids cannot be stored on trees because they ripen and drop if they are not harvested at maturity (Whiley, 1992). Consequently, to ensure availability of fruit year-round in tropical production areas, several avocado cultivars, each with a different harvesting season, must be grown. In Florida, approximately 23 major and 38 minor avocado cultivars are commercially grown in order to ensure fruit availability from the end of May through the beginning of March (Crane et al., 1998; Newett et al., 2002). Therefore, there is no uniform standard for appearance and quality of tropical avocado. Extending on-tree-storage and shelf-life of

fruit of this avocado type could result in a uniform fruit standard and overcome marketing problems.

The avocado fruit is climacteric and ethylene acts as a natural triggering mechanism (Adato and Gazit, 1974; Morton, 1987; Kays, 1997). Ethylene also regulates fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including enhancement respiration rate, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars and increased activity of cell wall degrading enzymes (Gray et al., 1992).

Various methods that interfere with ethylene have been utilized in order to prolong avocado fruit shelf-life and prevent senescence; these include controlled atmosphere and/or low-temperature storage, and chemical application, e.g., aminoethoxyvinylglycine (AVG), norbornadiene (NBD), diazocyclopentadiene (DACP) and 1-methylcyclopropane (1-MCP) (Blankenship and Sisler, 1993; Sisler and Serek, 1997; 1999; Feng et al., 2000; Jeong et al., 2002). However, these techniques are relatively expensive and fail to slow fruit senescence satisfactorily. Storage at low temperature is a problem for tropical/subtropical fruits like avocado, because the fruit is subject to chilling injury (Morton, 1987; Kays, 1997). Ethylene hastens the chilling injury process (Pesis et al., 2002).

Lowering the production of endogenous ethylene from avocado fruit could delay avocado fruit ripening and extend fruit shelf-life without extensive use of chemicals and/or highly controlled atmosphere storage. This could have a great impact on the efficiency of avocado production. It would replace reliance of on-tree storage (and prevent alternate bearing) of Mexican, Guatemalan varieties and their hybrids without

affecting continuity of supply. It could also assure the continuity of supply of good quality West Indian and West Indian x Guatemalan hybrids with fewer cultivars.

Endogenous ethylene production can be suppressed by blocking specific gene activity, e.g., the genes encoding ACC synthase and ACC oxidase, or by introducing transgenes encoding SAM hydrolase and ACC deaminase**.** Genetic transformation has been utilized successfully to interfere with ethylene production in tobacco (Bestwick et al., 1991), tomato (Hamilton et al., 1991; Klee et al., 1991; Penarruba et al., 1992; Oeller et al., 1991; Theologies et al., 1992; Good et al., 1994; Kramer et al., 1997), melon (Amor et al., 1998; Clendennen et al., 1999), red raspberry and in strawberry (Mathews et al., 1995a,b). There is almost 99% inhibition of ethylene biosynthesis in tomato fruit after transformation with antisense ACC synthase (Oeller et al., 1991), ca. 90-97% if transformed with ACC deaminase (Klee et al., 1991; Klee, 1993) and ca. 80% if transformed with SAM-ase (Good et al., 1994). Transformed plants have not demonstrated any apparent morphological abnormalities, fruits exhibited significant delays in ripening, and the mature fruits remained firm for at least 6 weeks longer than the control fruit. This approach has not been attempted with any woody tree species.

Genetic Transformation and Cryopreservation

Genetic transformation of avocado with a gene construct that would block ethylene biosynthesis could extend on-the-tree storage of tropical avocado and shelf life of avocado fruit generally. The genetic transformation of avocado is based on the embryogenic avocado system. Embryogenic avocado cultures are easy to manipulate and the protocols have been developed (Mooney and van Staden, 1987; Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999a,b). Embryogenic avocado cultures

have been successfully transformed with selectable nptII and GUS reporter genes (Cruz-Hernández et al., 1998) and with chitinase+glucanase and antifungal (AFP) protein genes (Raharjo et al., unpublished).

Embryogenic avocado cultures, however, lose their embryogenic competence over time and appear to affect some genotypes more than others. Loss of embryogenic capacity is associated with morphological changes and the loss of organization of proembryonic masses (PEMs). This phenomenon can occur as early as 3 months after induction of embryogenic cultures and is genotype dependent (Witjaksono and Litz, 1999a). Loss of morphogenic competence must be addressed for the following reasons: 1) to support medium- to long-term research involving embryogenic avocado cultures, 2) to preserve elite embryogenic lines developed from crop improvement research and 3) potentially to back up ex *situ* germplasm banks.

Objectives

1. Establishment of embryogenic avocado cultures from different cultivars.
2. Transformation of embryogenic avocado lines with the S-adenosylmethionine hydrolase (SAM-ase) gene construct to block ethylene biosynthesis in avocado fruit, recovery of transformed avocado somatic embryos and plantlets, and confirmation of genetic transformation.
3. Study of the growth of transformed embryogenic cultures, somatic embryo development and maturation in selection medium.
4. Development of a cryopreservation procedure for embryogenic avocado cultures and studies the effect of cryopreservation on embryogenic culture growth, somatic embryo development and plant recovery.

CHAPTER 2
LITERATURE REVIEW

Taxonomy

Avocado (*Persea americana* Mill.) is a member of the Lauraceae. It is a diploid species (2n = 2x = 24) with a genome size of 8.83 x 10^8 bp (Arumuganathan and Earle, 1991; Scora et al., 2002). Avocado is the only economically important food species in the Lauraceae. Other economically important species includes spices, *Cinnamomum zeylanicum* Blume and *C. cassia* (Nees) Nees and Eberm. Ex Blume, medicinal plants, *C. camphora* (L.) J. Presl., timber, *Nectandra spp.* Roland ex Rottb, *Ocotea* Aubl and *Phoebe*, and ornamentals, *Persea indica* Spreng (Schroeder, 1995).

The Lauraceae family is considered to be among the oldest flowering plants together with the Annonaceae, Magnoliaceae and Proteaceae (Scora et al., 2002). There are two subgenera of *Persea* (Clus.) Miller: *Eriodaphne* and *Persea*. The former is primarily a South American entity and the latter is meso-American. The commercial avocado belongs to subgenus *Persea* (Kopp, 1966; Scora et al., 2002). Subgenus *Persea* consists of relatively few species that have large fruits and are susceptible to phytophthora root-rot (PRR). Subgenus *Eriodaphne* consists of a large number of species, most of which have small fruit and are resistant to PRR (Kopp, 1966; Zentmeyer, 1980). According to Bergh and Lahav (1996), members of each subgenus are graft and sexually compatible; however, they are incompatible with members of the other subgenus. Subgenus *Persea* consists of three species: *P. schiedeana* Nees, *P. parviflora* William, and *P. americana* Mill. (Scora et al., 2002). *Persea americana* is polymorphic,

consisting of several separate taxa that may be considered as subspecies, botanical varieties or horticultural or geographic races (Scora et al., 2002). The species can be divided into three geographic races: *P. americana* Mill. var. *americana* (West Indian), *P. americana* Mill. var. *guatemalensis* L. Wans. (Guatemalan) and *P. americana* Mill. var. *drymifolia* Blake (Mexican) (Morton, 1987). The three races increase in their tropical adaptation from Mexican (subtropical) to Guatemalan to West Indian (Whiley, 1992). Hybridization readily occurs among the three races (Lahav and Lavi, 2002). The West Indian race is also known as the Antillean race or more correctly as the lowland race (Knight, 2002; Lahav and Lavi, 2002)

Fiedler et al. (1998) analyzed the three avocado races using RAPDs (randomly amplified polymorphic DNA). The average similarity within races of avocado was 75% for the Mexican, 73% for the Guatemalan and 71% for the West Indian, while similarity between these races ranged from 53% to 58% (Fiedler et al., 1998). These results support the present classification of *P. americana* into three subspecies. Based upon RFLP markers (Davis et al., 1998) and mini satellite and SSRs (simple sequence repeats) (Mhameed et al., 1997), 'Guatemalan' and 'West Indian' races are more similar to each other than to the 'Mexican' race. The three races and hybrid commercial avocados are more closely related to each other than to *P. schiedeana* (Davis et al., 1998).

Classification of complex hybrids of commercial avocado is difficult and contradictory in some points. 'Hass' and 'Fuerte' traditionally were assumed to be [(Guatemalan x Mexican) x Guatemalan] or (GxM)xG and (Guatemalan x Mexican) or GxM, respectively (Smith et al., 1992). Mhameed et al. (1997), using DNA finger printing (DFP), and Fiedler et al. (1998), using RAPD markers, found that 'Fuerte' and

'Hass' are closer to the Mexican than to the Guatemalan race. Davis et al. (1998), using RFLP markers, however, considered these cultivars to be more closely related to Guatemalan and West Indian avocados than to the Mexican race.

Sharon et al. (1997) obtained an initial map of the avocado genome using 50 SSRs, 17 RAPDs and 23 DFP markers. They found that SSR markers were better than DFP and RAPD markers for an avocado genetic linkage map because they are polymorphic, abundant and locus-specific. Although DFP markers are highly polymorphic, they are not PCR-based or locus-specific, while RAPD markers are PCR-based but less informative since they are a dominant marker.

Origin and Distribution

Avocado originated in southern Mexico (Chiapas) and Guatemala-Honduras (Whiley, 1992). Avocado has been cultivated in the area from the Rio Grande to central Peru long before the arrival of Europeans (Morton, 1987). The avocado fruit has been an important food in Mexico and Central America since antiquity (Popenoe, 1927). Avocados arrived in Spain as early as 1600 AD and in West Africa before 1750 AD (Smith et al., 1992). The avocado was introduced to Indonesia in the mid-18th century (Morton, 1987) and to Brazil in the early 19th century (Knight, 2002). It was introduced from Mexico to Florida in 1833 and to California in 1848 (Gustafson, 1976). Avocado was introduced in 1908 into Palestine (Knight, 2002).

Breeding

Conventional avocado breeding is difficult for several reasons: 1) there is only one seed per fruit; 2) low fruit set and heavy fruit drop; 3) long juvenile phase; 4) seasonal flowering; and 5) large tree size (Pliegro-Alfaro and Bergh, 1992; Lahav and Lavi, 2002). On the other hand, genetic variation is very broad, and there are no genetic barriers

among the races (Lahav and Lavi, 2002). The juvenile phase of avocado ranges from 3 to 11 years (Lahav and Lavi, 1992). Only ca. 0.1% of avocado flowers will form fruit (Davis et al., 1998).

Breeding objectives for scion avocado cultivars include high yield with high quality fruit, and long shelf life (Bergh, 1976; Lahav and Lavi, 2002; Newett et al., 2002). Rootstock breeding objectives include tolerance of phytophthora root rot (PRR), tolerance of salinity, and dwarf size for easier management (Lahav and Lavi, 2002; Newett et al., 2002). Although 'Hass' and 'Fuerte' still dominate avocado export markets, several new cultivars have been produced from classical breeding. These include 'Gwen', 'Lamb Hass', 'BL 667', 'GEM', etc. from the University of California Riverside avocado breeding program, and 'Iriet', 'Eden', 'Galil' and 'Arad' from the Israeli avocado breeding program (Lahav and Lavi, 2002). Rootstocks with tolerance of PRR include 'Duke', 'Duke7', 'Barr-Duke', 'D9', 'Martin Grande' and 'Thomas' (Lahav and Lavi, 2002; Newett et al., 2002). Approximately 50 West Indian rootstock clones that are tolerant of salinity, including the 'Ashdot' series, 'Degania' series and 'Maoz', have resulted from the Israeli breeding program (Newett et al., 2002).

There has been an assumption that much of the genetic variation in avocado is additive; however, Lavi et al. (1993b) reported significant non-additive genetic variance for nine traits: anise scent, fruit density, flowering intensity, fruit weight, harvest duration, inflorescence length, seed size, softening time and tree size. Most avocado traits, including skin color, flowering group and anise scent, are coded by several loci with several alleles in each locus (Lavi et al., 1993a). This conclusion resulted from crossing experiments, where any combination between skins color (green and purple),

flowering group (A and B), and anise scent (anise and no anise) always resulted in more green color, flowering type B, and no anise scent, respectively (Lavi et al., 1993a).

Marker assisted selection would facilitate avocado breeding by enabling screening for certain traits at the seedling stage. Mhameed et al. (1995) reported several DNA fragments that are associated with harvest duration, skin color, skin thickness and skin texture, i.e., fragments P4, P8, E2 and E5, respectively. The P8 fragment was associated with black-purple skin color, the skin color of 'Hass' (Mhameed et al., 1995).

The Role of Ethylene

Avocado Fruit Ripening

Mexican and Guatemalan avocados cannot ripen while the fruit are still attached to the tree and remain on the trees accumulating oil for 2-4 months after reaching maturity (Tingwa and Young, 1975; Sitrit *et al*., 1986; Whiley, 1992). This has been variously attributed to 1) the presence of an ethylene inhibitor in the fruit stem (Tingwa and Young, 1975; Morton, 1987); 2) translocation of an ethylene inhibitor into the fruit from the tree (Adato and Gazit, 1974; Whiley, 1992; Kays, 1997); and 3) the emission of trace amounts of ethylene from avocado fruit that are attached to the tree (Sitrit *et al.,* 1986). The ability to remain on the tree for 2-4 months can prolong avocado supply by "on-tree-storage"; however this can also cause alternate bearing and lower production in subsequent years.

The fruit of West Indian and West Indian X Guatemalan hybrids mature, ripen and drop if not harvested at maturity. The fruit cannot be stored on the trees (Whiley, 1992). Consequently, to ensure availability of fruit year-round in tropical zones, several avocado cultivars, each with a different harvesting season, must be grown. For example, Crane *et al.* (1998) noted that in Florida, approximately 30 avocado cultivars are grown

commercially in order to ensure fruit availability from the end of May through the beginning of March. There is therefore no uniform standard for appearance and quality for the tropical avocado. Extending the on-tree storage of West Indian and Guatemalan X West Indian avocado types could overcome this problem.

Avocado fruit is strongly climacteric (Adato and Gazit, 1974; Morton, 1987; Kays, 1997). The ripening phase is biphasic; the first phase is a lag phase or preclimateric and the second phase is the climacteric peak (Sitrit *et al*., 1986; Starret and Laties, 1991, 1993; Kays, 1997). Starret and Laties (1991) referred to the lag phase as System I Ethylene, where endogenous ethylene is low, and the second phase as System II Ethylene that causes and accompanies a respiration climax attended by ripening phenomena. Starret and Laties (1993) found that cellulase, polygalacturonase, and ACC oxidase are not involved in the initiation of the climacteric, because none of them is induced during the lag period in intact fruit.

Ethylene is thought to act as a natural triggering mechanism for the induction of the respiration climacteric (Kays, 1997). Ethylene also regulates fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including enhancement of the rate of respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars and increased activity of cell wall degrading enzymes (Gray *et al*., 1992).

Various methods for prolonging fruit shelf-life and preventing senescence have been employed, e.g., application of aminoethoxyvinylglycine (AVG) as an ethylene synthesis inhibitor and silver ions (Ag^{+}) and 2,5-norbornadiene (NBD) as ethylene action inhibitors, carbon dioxide, controlled atmosphere, and low-temperature storage. These

techniques are expensive and fail to prevent fruit senescence satisfactorily. Storage at low temperature is a problem for tropical fruits like avocado, because the fruit is subject to chilling injury (Morton, 1987; Kays, 1997). However, low-temperature storage can delay ripening and retard the appearance of mRNAs for cellulase, polygalacturonase, ethylene forming enzyme and other mRNAs of unknown function (Dopico et al., 1993). Lowering the production of endogenous ethylene from avocado fruit should delay avocado fruit ripening.

Ethylene and Fruit Ripening

Fruit ripening is under genetic control, e.g., genes encoding β-1,4-glucanase (avocado), polygalacturonase (tomato) and trypsin inhibitor (tomato) show increased expression during ripening. During ripening of mature avocado fruit, a number of mRNAs increase, i.e., the messages for cellulase (Christoffersen et al., 1984; Dopico et. al., 1993); a cytochrome P-450 oxidase (Bozak et al., 1990); polygalacturonase and ACC oxidase (Dopico et al., 1993). Different cDNAs associated with avocado fruit ripening have been reported: polygalacturonase cDNA, referred to as pAVOpg (Kutsunai et al., 1993), and pAVOe3 (McGarvey et al., 1990; 1992). McGarvey et al. (1991; 1993) demonstrated that pAVOe3 is similar (76%) to pTOM13, an ACC oxidase gene from tomato but is also weakly similar to E8 protein of tomato (31%). A high degree of conservation between pTOM13 and pAVOe3 implies a conservation of function (McGarvey et al., 1992).

Theologis et al. (1992) noted that the cloning of the genes involved in ethylene biosynthesis in tomato, e.g., ACC synthase and ACC oxidase, enabled the regeneration of plants bearing fruit with extended shelf-life using antisense technology. The antisense

phenotype can be reversed by application of ethylene or propylene, an ethylene analog (Theologis et al., 1992). Treated fruits are indistinguishable from naturally ripened fruits with respect to texture, color, aroma and compressibility. They also noted that the use of antisense technology and overexpression of metabolizing enzymes in controlling fruit ripening is only the first step toward controlling fruit senescence. Indeed, they noted that expression of antisense RNA using regulated promoters may eliminate the use of exogenous ethylene for reverting mutant phenotypes.

The ethylene biosynthesis pathway is indicated below:

Methionine ⟶ AdoMet (SAM) ⟶ ACC ⟶ Ethylene

AdoMet (SAM) = S-adenosylmethionine

ACC = 1-aminocyclopropane-1-carboxylic acid

During ethylene biosynthesis, ATP-methionine-S-adenosyltransferase converts methionine to SAM (S-adenosylmethionine). ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase) converts SAM to ACC (1-aminocyclopropane-1-carboxylic acid). ACC is converted to ethylene by ACC oxidase (Kionka and Armhein, 1984; Kende, 1989; Penarruba et al., 1922; McKeon et al., 1995; Kende and Zeevaart, 1997). Kionka and Armhein (1984) and Penarruba et al. (1992) also noted that malonyl transferase can irreversibly conjugate the ACC to N-malonyl-ACC (MACC), thereby removing ACC from ethylene production.

At the molecular level, the production of ethylene during fruit ripening depends on activating some genes that encode enzymes in the ethylene biosynthesis pathway. Conversely, ethylene production can be reduced by blocking specific gene activity, e.g., methionine-S-adenosyltransferase, the genes encoding ACC synthase, ACC oxidase, and

by overexpressing the genes encoding malonyl transferase, SAM hydrolase and ACC deaminase. Suppressing ethylene biosynthesis can be achieved accordingly:

1. Inactivation of the gene encoding ACC synthase. ACC synthase has been cloned by several groups (Sato and Theologis, 1989; Nakajima et al., 1990; Van der Straeten et al., 1990). Oeller et al. (1991) used antisense RNA of ACC synthase to inhibit tomato fruit ripening. Although ACC synthase is encoded by a multigene family whose numbers are differentially expressed in response to developmental, environmental and hormonal factors, Klee et al. (1991) and Kende and Zeevaart (1997) cited that tomato plants transformed with antisense ACC synthase show ca. 99% inhibition of ethylene synthesis compared to normal nontransformed plants.
2. Inactivation of the gene encoding ACC oxidase. The activity of ACC oxidase isolated from melon fruits has been demonstrated *in vitro* (Ververidis and John, 1991). Insertion of a chimeric pTOM13 antisense gene from tomato, part of the ACC oxidase system (Hamilton et al., 1990), can reduce ethylene biosynthesis by 87%. Picton et al. (1993) found that insertion of ACC oxidase in the antisense orientation causes an extreme reduction in ethylene production to 5% of normal in vine-ripened fruit and 10% of normal in detached fruit stored in air. Amor et al. (1998) found that the expression of antisense ACC oxidase in *Cucumis melo* transgenic plants is associated with low ACC oxidase activity and ethylene production, whereas the regeneration capacity of the transformed tissue was greatly enhanced (3.5 fold in leaves and 2.8 fold in cotyledons) compared to the nontransformed control.
3. Metabolism of ACC before it can be converted to ethylene. When a bacterial gene encoding ACC deaminase was introduced into tomato (Klee et al., 1991; Klee, 1993),

it caused ethylene synthesis to be reduced by 90-97% in transgenic plants, and did not cause any apparent morphological abnormalities. Transformed fruits exhibited significant delays in ripening, and the mature fruits remained firm for at least 6 weeks longer than those in the control. Klee et al. (1991) noted that degradation of ACC inhibited ethylene synthesis, but did not interfere with the ability of fruit to perceive ethylene because transgenic fruits exposed to exogenous ethylene ripened normally.

4. Reducing or altering the effect of ethylene by expression of genes that causes specific tissues to be insensitive to ethylene, or by inactivating genes that function in specific aspects of fruit ripening. Smith et al. (1988) and Sheehy et al. (1988) transformed tomato with tomato antisense polygalacturonase cDNA. They found that expression of polygalacturonase in the antisense orientation in transformed plants reduced the level of polygalacturonase mRNA by 90% and 94% (Sheehy et al., 1988; Smith et al., 1988, respectively) and polygalacturonase activity by 69 – 93% and 90% (Sheehy et al., 1988; Smith et al., 1988, respectively).
5. Metabolism of SAM so there is no substrate for ACC synthase to produce ethylene. Agritope (1999), a plant biotechnology corporation, utilizes SAM hydrolase (SAMase) to convert SAM to a non-toxic by-product, 5'-methylthioadenosine and homoserine, that is recycled within the plant cell, so that SAM is not available to be converted into ACC. This strategy is referred to as the 'metabolic shunt'. The gene is used behind a specific fruit-ripening promoter so that SAMase would be expressed in mature green fruit before or just as it would normally start to ripen. This approach has been utilized with tobacco (Bestwick et al., 1991), tomatoes (Good et al., 1994; Kramer et al., 1997), raspberry (*Rubus ideaus* L.) and strawberry (*Fragaria x*

ananassa) (Mathews et al., 1995a, b) and cantaloupe (Clendennen et al., 1999). USDA granted approval to Agritope (now Exelixis) for release of transgenic tomato 35 1N in 1996 and gave a pending status for cantaloupe A and B in 1999 (Agribios, 2002a,b).

In order to prolong fruit shelf-life, ethylene biosynthesis must be reduced by >90% (Klee, 2003). Fruit internal quality cannot be preserved as long as its appearance because fruit continue to use sugar and acids as respiration substrates (Klee, 2003).

Neither blocking nor lowering ethylene biosynthesis has been attempted with woody tree species. In this study, we have attempted to suppress ethylene production in avocado fruit using a genetic engineering strategy. A transgenic approach to the post harvest problem would increase on-the-tree storage of tropical avocados and prolong shelf life. Fruit ripening would be controlled by inhibition of ethylene biosynthesis, and would involve the introduction of a gene encoding SAM hydrolase (SAMase) into the avocado genome via transformation of embryogenic avocado cultures.

SAM Hydrolase

The *samK* transgene is a version of SAM hydrolase modified in the 5' region to contain a consensus eukaryotic translation initiation by altering the SAM ATG start codon and encodes functional SAM hydrolase (Good et al., 1994; Kramer et al., 1997). SAM hydrolase originated from bacteriophage T3 and encodes S-adenosyl-methionine hydrolase (AdoMetase or SAMase, EC 3.3.1.2) (Hughes et al., 1987; Bestwick at al., 1991). SAM hydrolase catalyzes the conversion of SAM to methylthioadenosine (MTA) (Figure 2-1) (Good et al., 1994; Kramer et al., 1997).

SAM is not only a metabolic precursor of ethylene but also plays a central role in numerous biosynthetic reactions including, polyamine biosynthesis, DNA methylation

and phospholipid biosynthesis (Good et al., 1994; Ravanel et al., 1998). In order to express the *samK* gene only in ripening fruit, a tissue specific and developmentally regulated expression system has to be employed. According to this strategy, the only impact of SAM hydrolase would be reduction of ethylene biosynthesis through the reduction of the SAM pool. As the pool of SAM is depleted by the action of SAM hydrolase, neither ACC nor ethylene is produced (Good et al., 1994; Kramer et al., 1997).

Klee (2003) indicated that constitutive blocking ethylene biosynthesis even using antisense ACC synthase or ACC oxidase have also caused negative impacts on plant development. Those impacts include preventing adventious roots formation (Clark et al., 1999) and increasing susceptibility of transgenic plant to pathogens (Knoester et al., 1998). Lund et al. (1998), however, reported that tomato mutants impaired in ethylene perception exhibited reduction in plant susceptibility to pathogens. To avoid these effects, the transgene must be expressed at a specific time and developmental stages using specific transcriptional promoters or by targeting specific members of the biosynthetic gene family (Klee, 2003).

Protein blot analysis of transgenic tomato with the *samK* gene driven by a stage and tissue specific promoter showed that SAM hydrolase was detectable only during the climacteric phase but was not detected in green fruit or ripe stage fruit (Kramer et al., 1997). The total concentration of SAM hydrolase at that stage was ca. 0.0026% of the total protein of ripe tomato. Expression of SAM-ase driven by a constitutive promoter in transgenic tomato at the level necessary to alter fruit ripening is detrimental to plant growth and development (Good et al., 1994).

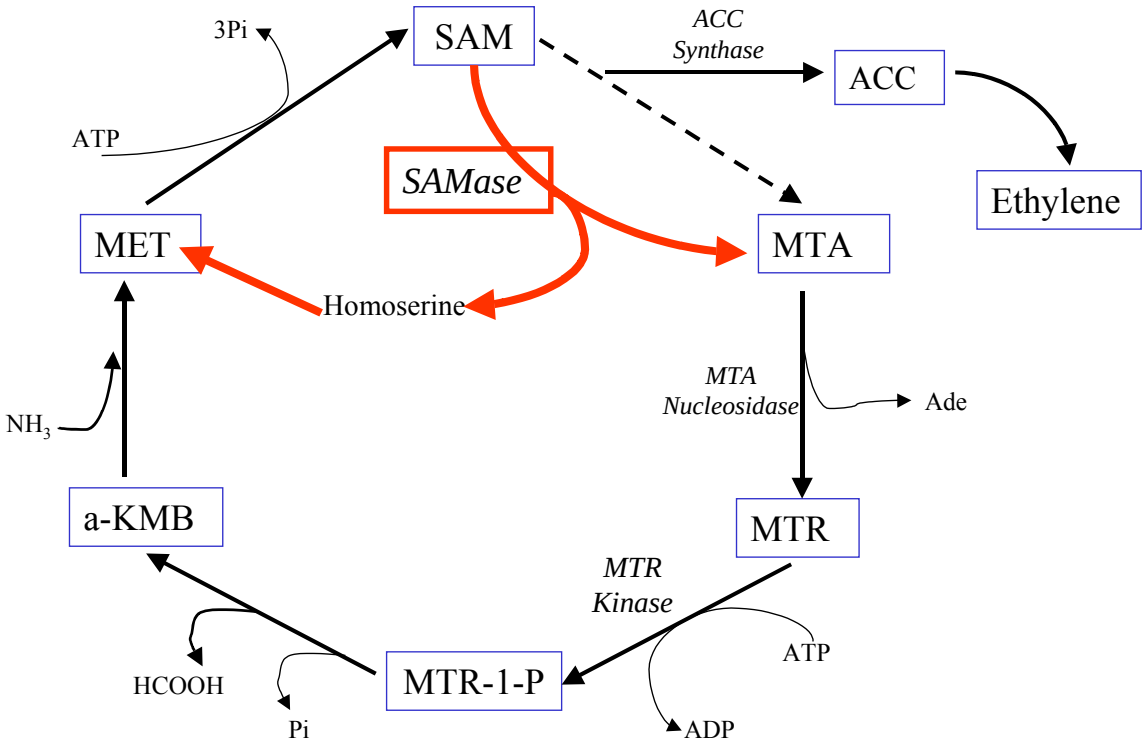


Figure 2-1. The methionine-recycling pathway in plants (Good et al., 1994). Abbreviations: SAM, S-adenosylmethionine; SAMase, SAM hydrolase; MTA, 5'-methylthioadenosine; MTR, 5-methylribose; KMB, α-ketome-thylthiobutyric acid; MET, methionine; ACC,1-aminocyclopropane-1-carboxylic acid.

Stable integration of E8/E4::*samK* gene in the cantaloupe genome resulted in functional SAM hydrolase protein (Clendennen et al., 1999). SAM-ase expression did not alter fruit size and weight, fruit firmness, external and internal color, soluble solids and mold susceptibility. Reduction of ethylene biosynthesis occurred in inbred transgenic as well as in hybrid crosses between the transgenic lines and non-transformed controls. The transgenic fruit matured more uniformly three days later than the control thereby allowing accumulation of more sugar (Clendennen et al., 1999).

Stable integration of the *samK* transgene into the tomato genome slowed the rate of ripening (Good et al., 1994; Kramer et al., 1997). The nutritional composition of

transgenic tomatoes including vitamins, total protein concentration, amino acids and total acids, was not affected (Kramer et al., 1997). The SAM-hydrolase protein is rapidly degraded by heat and gastric conditions and is not toxic or does not cause an allergic reaction (Kramer et al., 1997). Good et al. (1994) reported that reduction of ethylene production from transgenic tomato expressing SAM-ase was ca. 80%. This transgenic tomato required twice as much time to develop to the final ripened state, produced less lycopene and showed increased fruit firmness. Senescence was delayed for as long as three months after harvest.

A variety of integrations of *samK* gene including single, double and multiple insertions was detected in transgenic raspberry and strawberry (Mathews et al., 1995a, b). This is important for the higher expression of SAM hydrolase that is needed to by-pass the ethylene biosynthesis pathway. A high level of SAMase expression is needed to deplete the SAM pool from the methionine-recycling pathway, since substrate affinity of ACC synthase (Km 12-60 μM) is lower than SAM hydrolase (Km 200 μM) (Good et al. (1994).

Somatic Embryogenesis

Somatic embryogenesis refers to the development of plants from somatic cells through embryological stages without fusion of gametes (Williams and Maheshwaran, 1986). It is a natural process, and is identical to adventious embryony within ovules of polyembryonic plant species (Litz and Gray, 1992). Somatic embryos can develop either directly or indirectly from somatic tissues. They develop from small, rapidly dividing meristematic cells, having dense cytoplasm, a large nucleus, and a small vacuole (Williams and Maheshwaran, 1986). Somatic embryos have a single cell origin (Litz and

Gray, 1992) and develop through globular, heart, torpedo and mature (cotyledonary) stages in the same manner as zygotic embryos. A somatic embryo is a bipolar structure with a shoot and a root meristem (Litz and Gray, 1992).

Direct somatic embryogenesis occurs from pre-embryonic determined cells (PEDC's) that require only the presence of an exogenous growth regulator or favorable conditions to develop (Evans et al., 1981; Williams and Maheshwaran, 1986; Wann, 1988). Indirect somatic embryogenesis, however, requires determination of differentiated cells, usually by callus proliferation and development of embryogenically determined cells (IEDC's). Plant growth regulators are essential for determination of the embryogenic state (Williams and Maheshwaran, 1986).

Schroeder (1957) first reported avocado callus initiation and maintenance. The callus did not develop shoots, although a few roots developed from long-term callus (Schroeder et al., 1962; Schroeder, 1973). Pliego-Alfaro and Bergh (1992) indicated that callus cultures can be established from almost any avocado explants, but the callus is usually non morphogenic.

Pliego-Alfaro (1981) first described the induction of embryogenic cultures from avocado zygotic embryos. Zygotic embryos 0.6-0.8 mm long from immature fruit (0.9 mm long) were used as explants (Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988). Zygotic embryos of this size correspond to the early heart stage (Witjaksono, 1997; Witjaksono and Litz, 1999a; Witjaksono et al., 1999). The stage of development of zygotic embryos and the presence of 0.41 μM (0.1 mgl-1) picloram in the induction medium appear to be critical for induction (Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988; Witjaksono, 1997; Witjaksono and Litz, 1999a). Embryogenic cultures

have also been induced in ‘Fuerte’ (Mexican x Guatemalan) and ‘Duke’ (Mexican) with 0.1-0.5 mm long zygotic embryos, which are at the globular to early heart stage of embryo development (Witjaksono et al., 1999; Mooney and van Staden, 1987).

Two types of culture proliferation occur on induction medium; 1) a grayish to tan-colored amorphous callus; and 2) growth of glossy-textured and light creamy colored cultures (Pliego-Alfaro and Murashige, 1988). Upon subculture onto initiation or basal media, the latter develop as proembryos that later enlarge and germinate (Pliego-Alfaro and Murashige, 1988). These embryo-like structures are very similar anatomically to zygotic embryos, with well-defined cotyledons, an embryonic axes and a developing shoot and root meristem (Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988). Mooney and van Staden (1987) indicated that embryogenic avocado cultures consisted of friable globular structures or proembryonic masses (PEMs).

Embryogenic cultures that develop on induction medium can be categorized as two distinct types: SE-type and PEM-type (Witjaksono, 1997; Witjaksono and Litz, 1999a; Witjaksono et al., 1999). SE-type cultures consist of various development stages (globular, heart and torpedo) and PEMs. SE-type embryogenic cultures produce somatic embryos that develop to maturity on induction medium, while PEM-type cultures consist of friable PEMS that must be transferred to embryo development medium to form somatic embryos (Witjaksono, 1997; Witjaksono and Litz, 1999a; Witjaksono et al., 1999). Both PEM- and SE-type embryogenic cultures show some disorganization after several subcultures (Witjaksono and Litz, 1999a). The SE-type response is most common for avocado.

SE-type cultures readily form somatic embryos while PEM-type cultures are associated with low frequency somatic embryo production (Witjaksono and Litz, 1999a; Pliego-Alfaro et al., 2002); however, several problems are associated with this type of embryogenic culture. After several subcultures, SE-type cultures are comprised of PEMs, disorganized PEMs and callus-like masses, representing gradual loss of morphogenic potential. These changes over time cause loss of the ability to form globular, heart and early cotyledonary stages of somatic embryos and the cultures became completely disorganized approximately 6-8 months after induction (Witjaksono and Litz, 1999a). This can be an important limiting factor for somatic embryo-based crop improvement.

Somatic embryogenesis can be divided into four stages: 1) induction of embryogenic cultures, 2) maintenance of embryogenic cultures, 3) somatic embryo development and maturation, and 4) somatic embryo germination and plant conversion.

Induction of Embryogenic Cultures

Embryogenic avocado cultures can be induced from early developmental stages of zygotic embryos: globular (0.1- 0.5 mm) (Mooney and van Staden, 1987), early heart stage (0.6- 0.8 mm) (Pliegro-Alfaro and Murashige 1988) and from globular to torpedo stage (2.7 mm) (Witjaksono 1997; Witjaksono and Litz, 1999a, b). They can also be induced from cotyledon pieces excised from later stages of embryo development (Raviv et al., 1998). It is also possible to induce embryogenic cultures from nucellar tissue, which represents mature phase or elite material (Witjaksono et al., 1999).

Induction medium consists of MS (Murashige and Skoog, 1962) basal medium supplemented with (in mg l-1) sucrose 30,000, thiamine HCl 0.4, i-inositol 100, picloram 0.1, and solidified with 8% agar (Pliego-Alfaro and Murashige, 1988). Witjaksono

(1997) and Witjaksono et al. (1999a) modified the induction media by substituting B5 (Gamborg et al., 1968) major salts, resulting in a greater embryogenic response.

Conditions for induction of embryogenic avocado cultures have been described for several avocado cultivars including: 'Hass' (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999a), 'Duke 7' and 'Fuerte' (Mooney and van Staden, 1987); 'Booth 7', Booth 8', 'Duke', 'Esther', 'Hass', 'Lamb', 'T362', 'Thomas' and 'Waldin' (Witjaksono, 1997; Witjaksono and Litz, 1999a) and 'RCF Purple' (Raviv et al., 1998). Embryogenic cultures are evident 18-40 days after explanting with a frequency that ranges from 0-25% (Witjaksono, 1997; Witjaksono and Litz, 1999a).

Maintenance of Embryogenic Cultures

Embryogenic cultures are not calluses but consist of PEMs (Litz and Gray, 1992). Proembryonic masses proliferate by repetitive embryogenesis that involves continuous cycles of secondary somatic embryogenesis from PEMs that have lost their ability to form single somatic embryos (Williams and Maheshwaran, 1986; Ammirato, 1987; Litz and Gray, 1992), usually in the presence of inductive agent.

Embryogenic avocado cultures can be maintained either on semi solid medium or as suspension cultures. Semi solid maintenance medium consists of MS medium supplemented with 0.41µM picloram and solidified with 8.0 g l^{-1} TC agar in 110 x 15 mm Petri dishes (Witjaksono and Litz, 1999a). Cultures on semi solid medium are maintained in darkness at 25^{o}C, and subcultured at 3-5 week intervals.

The establishment of suspension cultures is important for somatic cell-based crop improvement since growth and proliferation in liquid medium is generally superior to the responses on semi solid medium (Witjaksono et al., 1999). Both PEM- and SE-type cultures can be maintained and retain their characteristic in suspension (Witjaksono and

Litz, 1999a). Suspension cultures can be initiated by inoculating approximately 0.8-1.0 g of the smallest fraction of embryogenic cultures that passes through sterile nylon filtration fabric (1.8 mm or 0.8 mm mesh) into 80 ml liquid medium (Witjaksono and Litz, 1999a).

The optimal liquid medium is either filter-sterilized MS medium supplemented with 30-50 g l^{-1} sucrose, 100 mg l^{-1} inositol, 4 mg l^{-1} thiamine HCl, and 0.41 μM picloram (Witjaksono and Litz, 1999a) or autoclaved MS3:1 medium, which is basically the same formulation but modified to contain 30.3 mg l^{-1} KNO_3 and 12 mg l^{-1} NH_4NO_3 (Witjaksono and Litz, 1999b). Embryogenic suspension cultures are maintained on a rotary shaker at 125 rpm under diffuse light and are subcultured biweekly (Witjaksono and Litz, 1999a).

Growth of embryogenic cultures in suspension with respect to settled cell volume follows a short lag phase (1 day), followed by exponential growth (5 days), a linear phase (9 days), progressively decelerating (5 days) and declining growth during 25 days of culture (Witjaksono and Litz, 1999a). The lag phase is 1 day longer and the exponential phase is 1 day shorter with respect to fresh weight (Witjaksono and Litz, 1999a). They indicated that there was no lag phase in dry weight accumulation, but the exponential and linear phases are 1 day shorter and the decelerating phase is 1 day longer (Witjaksono and Litz, 1999a). The dry weight, fresh weight and settled cell volume peaked at 18, 20 and 21 days while increasing 7.9, 6.4 and 14-fold, respectively (Witjaksono and Litz, 1999a). They found that picloram has no effect on volume and dry weight of settled cells.

Witjaksono (1997) and Witjaksono et al. (1998) described the isolation and culture of morphogenic protoplasts from embryogenic cultures, and have been able to regenerate plants. Protoplast isolation, culture and regeneration are genotype and culture age dependent (Witjaksono and Litz, 2002). PEM-type cultures release protoplast more readily and produce fewer protoplasts that do not divide than SE-type embyogenic cultures (Witjaksono and Litz, 2002). Older cultures that consist of disorganized and less embryogenic PEMs result in a high number of protoplasts that only form microcalluses without somatic embryo recovery (Witjaksono and Litz, 2002).

Avocado protoplasts can be cultured either in liquid (Witjaksono et al., 1998) or on medium solidified with 20 g l^{-1} agarose type VII (Witjaksono et al., 1999). The number of PEMs that develop from protoplast culture in liquid is affected by medium osmolarity, source of nitrogen and their interaction, and plating density (Witjaksono et al., 1998, 1999). Somatic embryo development from protoplasts derived from morphogenic PEMs occurs on SED medium. The conversion rate of somatic embryos was ≤ 1% (Witjaksono et al., 1998).

Somatic Embryo Development

Somatic embryos develop on hormone-free medium (Pliego-Alfaro and Murashige, 1988), but the absence of picloram is not a perquisite for somatic embryo development, especially for SE-type cultures (Witjaksono and Litz, 1999a). Raviv et al. (1998) reported that cotyledonary-stage somatic embryos can develop on semi solid proliferation medium with 9.04 µM 2,4-D and 2.22 µM Benzyladenine (BA). Absicic acid (ABA) does not improve somatic embryo development (Pliego-Alfaro and Murashige, 1988).

The presence of picloram in induction medium arrests somatic embryo development and maturation (Witjaksono and Litz, 1999b; Witjaksono et al., 1999). Therefore, removal of picloram would improve somatic embryo development.

Semisolid medium is better than liquid medium for somatic embryo development and maturation because somatic embryos that develop in suspension are usually hyperhydric and fail to develop normally upon transfer to semi solid medium (Witjaksono and Litz, 1999b; Witjaksono et al., 1999). The optimum medium for somatic embryo development consists of MS basal medium supplemented with (in mg l^{-1}) 30,000 sucrose, 4 thiamine HCl and 100 myo-inositol, 6,000 gellan gum in 110 x 20 mm Petri dishes (Witjaksono and Litz, 1999b). Cultures are maintained at 25^{o}C in darkness.

Germination of Somatic Embryo

Somatic embryo germination can occur on hormone-free medium (Pliego-Alfaro and Murashige, 1988), or somatic embryo development (SED) medium (Witjaksono and Litz, 1999b). Somatic embryos turn green upon transfer to light (Witjaksono and Litz, 1999b). Well-developed opaque and mature somatic embryos (0.8 cm length) are transferred individually to semi solid germination medium (Witjaksono and Litz, 1999b). Somatic embryo maturation and germination medium is similar to induction medium but without picloram and supplemented with 4.44 µM BA and 2.89 µM Gibberelic acid (GA_3). The medium is solidified with 8 g l^{-1} TC agar and aliquots of 25 ml medium are dispensed in 150 x 25 mm glass test tubes, closed with polypropylene Kaputs and autoclaved for 15 min at 121^{o}C and 1.1 kg cm^{-2} (Witjaksono and Litz, 1999b). After transferring one somatic embryo into each tube, the tubes are closed with vented transparent film Sun Caps® and secured with rubber bands. Somatic embryos are

subcultured onto fresh medium of the same composition at 2-3 month intervals (Witjaksono and Litz, 1999b).

The frequency of somatic embryo germination is low, approximately 0 to 5%, depending on genotype (Mooney and van Staden, 1987; Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999b; Raviv et al., 1998). Application of gibberellic acid (GA_3), benzyladenine (BA) and low temperature (12^{o}C for 3 weeks) do not increase germination frequency (Pliego-Alfaro and Murashige, 1988). Low germination and plant conversion are related to the presence of underdeveloped root and shoot meristem and lack of bipolarity in somatic embryos (Mooney and van Staden, 1987; Pliego-Alfaro and Murashige, 1988; Witjaksono et al., 1999).

To increase the efficiency of plant recovery, emerging shoots can be micropropagated on MS medium (without NH_4NO_3) supplemented with 4.44 µM BA for several subcultures and then transferred onto the same composition of MS medium supplemented with 800 mg l^{-1} NH_4NO_3, and 2200 mg l^{-1} KNO_3 (Witjaksono and Litz, 1999a; Witjaksono et al., 1999). Rooting is induced by pulsing 1.5-2.0 cm nodal cuttings in MS medium (without NH_4NO_3) and supplemented with 122.6 µM IBA for 3 days, and followed by transfer onto medium of the same formulation supplemented with 1 g l^{-1} activated charcoal (Witjaksono, 1997; Witjaksono et al., 1999). Raharjo et al. (unpublished data) have micrografted the somatic embryo shoots onto avocado seedlings under aseptic condition in order to optimize plant conversion from avocado somatic embryos.

Genetic Transformation

General

Genetic transformation is the process whereby foreign DNA is integrated into cells. Genetic transformation is now a core research tool in plant biology and a practical tool for crop improvement (Birch, 1997), especially for trees that have a long juvenile period (Witjaksono and Litz, 2002). Birch (1997) indicated that there are several potential uses for genetic transformation: e.g., 1) generation of plants with useful traits unachievable by conventional breeding; 2) correction of faults in a cultivar more efficiently than conventional breeding; and 3) protection of germplasm under intellectual property rights.

Genetic transformation was first reported with tobacco using an organogenic regeneration pathway (Horsch, et al., 1984; De Block et al., 1984). Several genetic transformation techniques now are available: *Agrobacterium*-mediated transformation, bombardment with DNA-coated microprojectiles, electroporation or PEG-treatment of protoplasts, microinjection of DNA into zygotic proembryos, in planta transformation, silicone carbide whiskers and laser microbeams (Pontrykus, 1991; Birch, 1997; Hansen and Wright, 1999). The *Agrobacterium* system is attractive because it is relatively easy, involving minimal cost and resulting in transgenic plants with simple copy insertion (Hansen and Wright, 1999).

Agrobacterium species are aerobic, gram-negative, soil bacteria that are capable of saprophytic or parasitic growth and are the cause of crown gall and hairy root diseases of dicotyledonous plants (De Cleene and De Ley, 1976). *Agrobacterium tumefaciens* genetically transforms plants by transferring T-DNA (transferred DNA), a portion of the resident tumor-inducing plasmid (Ti-plasmid) to the plant (Gelvin, 2000). Ti-plasmids

include T-DNA that is bordered by 25 bp imperfect repeats, known as left and right border and 35 kb virulence genes (Sheng and Citovsky, 1996; Hellens et al., 2000). T-DNA transfer is accompanied by several virulence (Vir) proteins that assist in T-DNA transfer, nuclear targeting and integration into the plant genome (Gelvin, 2000). There are three genetic components of *Agrobacterium* that are required for plant cell transformation (Sheng and Citovsky, 1996). The two main components for successful *Agrobacterium*-mediated gene transfer are the T-DNA and the vir region located on Ti-plasmid (Hellens et al., 2000). The third component is the suite of *chromosomal virulence (chv)* genes located on the *Agrobacterium* chromosome that are involved in bacterial chemotaxis toward and attachment to the wounded plant cell (Sheng and Citovsky, 1996). Both T-DNA and the vir genes can reside on separate plasmids, where the *vir* gene function is provided by the disarmed Ti-plasmids resident in *Agrobacterium* and the T-DNA is provided on the vector plasmid. (Hellens et al., 2000). The T-DNA can be engineered to contain a selectable marker and/or gene or genes of interest to be transferred into the plant genome (Hansen and Wright, 1999; Hellens et al., 2000).

Agrobacterium tumefaciens has the capacity for gene transfer to many plant species and to many regenerable plant cell types (Birch, 1997). Embryogenic cultures offer several advantages for genetic transformation because they are highly competent to respond to hormonal stimuli for *in vitro* manipulation, they are able to express foreign DNA, and regeneration from single cells results in solid transformed plants (Ellis, 1995). Embryogenic avocado cultures are amenable to *Agrobacterium*-mediated transformation (Witjaksono and Litz, 2002) and have been transformed successfully (Cruz-Hernández, 1998; Raharjo et al., unpublished).

The efficiency of transformation is dependent upon: 1) the ability of *Agrobacterium* to efficiently transform cells; 2) efficient selection of transformed cells; 3) efficient regeneration from transformed cells; and 4) the stability of the incorporated DNA in the plant genome (Dandekar, 1992).

The low frequency of transformation and high frequency of undesired changes or unpredictable transgene expression, due to the untargeted integration (Hellens et al., 2000) limit the practical transformation of many plant species (Birch, 1997).

Potrykus (1991) proposed that proof of integrative transformation requires combination of genetic, phenotypic and physical data, including molecular and genetic analysis of offspring populations. Proving the latter is problematic in trees that are slow to reproduce sexually (Birch, 1997). Birch (1997) proposed that southern DNA hybridization analysis and phenotype data are adequate for proving gene integration. Phenotype data, however, require negative results from all untransformed controls (Birch, 1997). Furthermore Birch (1997) noted that survival of lines on 'escape-free' selection medium is not sufficient, because of the possibility of selection of mutants that are resistant to the selective agent, and cross-protection by secreted product of contaminating microbes or transgenic tissue.

Embryogenic Avocado Culture Transformation

Avocado embryogenic cultures were first transformed with a construct containing a kanamycin resistance gene as a selectable marker (*nptII*) and GUS (β-glucuronidase) as a reporter gene (Cruz-Hernández et al., 1998). Proembryonic masses were transformed by co-cultivation with *Agrobacterium tumefaciens*. Disarmed, acetosyringone-activated *Agrobacterium tumefaciens* strain A208 was used, which harbored a co-integrative vector

pTiT37-ASE::pMON9749. Genetic transformation of embryogenic cultures and somatic embryos was confirmed by the X-gluc reaction. The integration of *nptII* and GUS gene into the avocado genome was confirmed by PCR and Southern hybridization. Transgenic plants were not regenerated (Cruz-Hernandez et al., 1998). Raharjo et al. (unpublished) have integrated chitinase, β-1,4-glucanase and the antifungal (AFP) protein genes into embryogenic avocado cultures and have regenerated transgenic plants that are undergoing nursery evaluation. Plant conversion was increased by micrografting transformed shoots onto avocado seedlings. Constructs were pGPTV-BAR-CG (chitinase, glucanase), and pGPTV-BAR-AFP (antifungal protein). Cultures were selected for resistance to Basta and GUS was the reporter gene. The transgenes were delivered by *Agrobacterium tumefaciens* strain EHA 105.

In this research plasmid pAG-4092 (Figure 2-2) was used. This plasmid has a transgene construct that contains the *samK* gene, a version of the SAMase or SAM hydrolase gene and the selectable marker gene, neomycin phosphotransferase II (*nptII*) (Figure 2-2). The *samK* gene encodes the S-adenosylmethionine hydrolase (SAMase or AdoMetase, EC 3.3.1.2) (Good *et al*., 1994). The *samK* gene is driven by an avocado cellulase promoter from avocado fruit, an organ-specific (fruit) and a stage- and temporally-regulated (climacteric phase) promoter (Agritope, 2000). The *nptII* gene confers for kanamycin resistance, and is used for selection of transformed embryogenic cultures and somatic embryos. *Agrobacterium tumefaciens* strain EHA101 was used to introduce pAG-4092 harboring the *samK* and *nptII* genes into embryogenic avocado cultures.

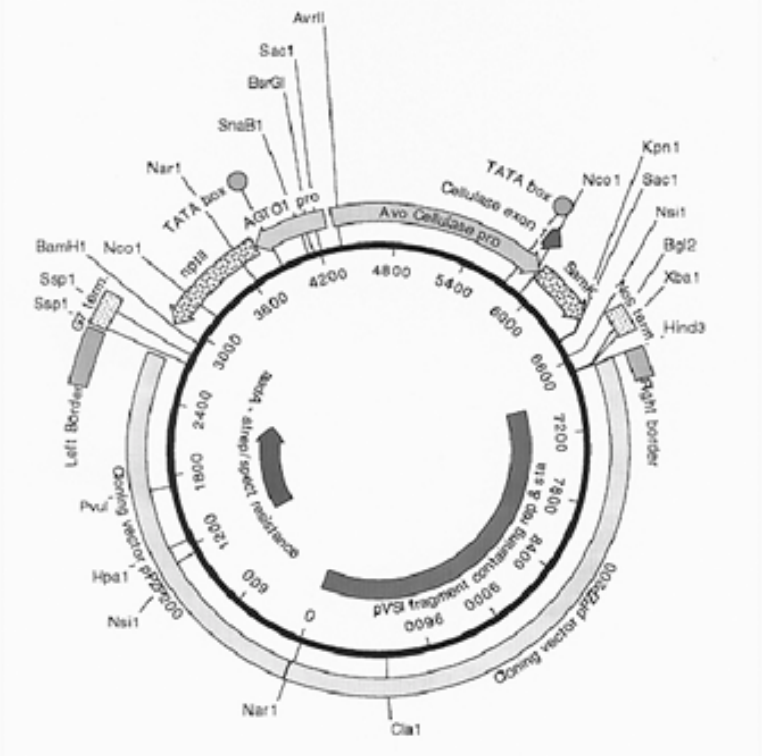


Figure 2-2. The plasmid pAG-4092 harboring *samK* with Avocado Cellulase promoter and selectable marker *nptII* under control of AGT01 promoter (Agritope, 2000)

Cryopreservation

Importance of Cryopreservation

Tissue culture techniques are useful tools for propagation and can be used for short-term and medium term preservation of genotypes. However, problems associated with maintenance and repeated subculture expose plant material to danger of contamination and epigenetic changes that can cause loss of morphogenic competence. Loss of embryogenic competence can occur as early as 3 months after induction with some genotypes (Witjaksono and Litz, 1999a). As a result, it is difficult to use embryogenic cultures of some genotypes for medium and long-term research, and embryogenic cultures must be induced annually. The loss of embryogenic competence also

contributes to problems associated with developing elite lines of embryogenic cultures that have been genetically engineered. Long-term storage is critical to overcome these problems.

Cryopreservation is considered to be an ideal method for long-term storage of germplasm since it offers long-term storage capability with stability of genotypic and phenotypic characteristics of stored germplasm, and only minimal space and maintenance are required (Engelmann, 1997). Cryopreservation can be used as an alternative to back up *ex situ* field plantings and to support availability of embryogenic cultures for long-term in vitro research such as genetic transformation. Cryopreservation also can be utilized for eradication of several microorganisms that cause diseases in the plants, e.g., Helliot et al. (2002) reported that cryopreservation can eliminate cucumber mosaic virus and banana streak virus from *Musa spp*., where the frequency of virus eradication was 30% and 90%, respectively. Withers (1992) indicated that clonal genotypes of highly heterozygous plants tend to be the target of cryopreservation since their seed are not true to type and the stock plants for mass clonal propagation system often do not have a natural storage form. Many tropical woody species come into this category (Withers, 1992).

Cryopreservation involves storage at ultra-low temperature (-196^{o}C) with liquid nitrogen being the most widely used cryogen (Grout, 1995). There is cessation of biological activities at this temperature and material can be stored for extremely long periods (Grout, 1995; Benson, 1997). Cryopreservation can be achieved by either slow or fast cooling, which differ in the pre-cooling protection, and cooling rate (Grout, 1995; Benson, 1997). Other approaches to cryopreservation involve the use of desiccation and

encapsulation either by slow cooling or vitrification (Engelmann, 1991; Bajai, 1995; Benson, 1997).

Cryopreservation of Tropical Tree Species

Cryopreservation protocols have been developed for a few tropical trees, e.g. cacao, citrus, coffee, rubber, jackfruit and oil palm (Table 2-1). Some non-woody tropical species have also been cryopreserved, i.e., banana, cassava, rice, and sugarcane (Table 2-5). Plant material used for cryopreservation includes somatic embryos, embryogenic cells, protoplasts, embryo axes, seeds, shoot tips and apices (Table 2-5). Small cells that are highly cytoplasmic, thin-walled and nonvacuolate are able to withstand freezing much better than large, thick-walled, vacuolate cells (Engelmann, 1991; Bajai, 1995). The late lag phase or materials undergoing the exponential growth stage have the highest cryotolerance (Withers, 1985; Reinhoud et al., 1995). Somatic embryos have less survival potential compared to proembryonic masses, since the former have a large size and are thick-walled and more vacuolate.

Several cryopreservation protocols are commonly used, i.e., 1) cryoprotection followed by slow cooling; 2) vitrification using a plant vitrification solution, 3) desiccation followed by either slow cooling or fast cooling; and 4) encapsulation-dehydration followed by slow cooling.

Slow Cooling Cryopreservation

Slow cooling cryopreservation is based on vitrification of intracellular solutes during freezing and thawing process to avoid cell damages. In order to be vitrified, intracellular solutes must be highly concentrated. In slow cooling cryopreservation, this is reached by cryodehydration (a process whereby water is lost due to ice crystal growth)

(Muldrew, 2003). During slow cooling, intracellular water moves out and is frozen extracellularly and increases the concentration of intracellular solutes.

Slow cooling techniques involve pretreatment and incubation of materials with cryoprotectants, slow cooling (0.5-1.0 C^o min^{-1}) to –30 to –40°C, immersion into liquid nitrogen, thawing to 35-40°C, removal of cryoprotectant and restoration of osmolarity of cultures to physiological level before transfer to recovery medium (Towill, 1995; Benson, 1997). The slow cooling process can be attained using a programmable freezer. It also can be achieved with a simple freezing device consisting of a plastic box containing 250ml isopropyl alcohol with cooling rate very close to 1°C min^{-1} ("Mr. Frosty' Nalgene). The container is placed in a –80°C freezer for 120 min to achieve –40°C (Simione, 1998). Engelmann et al. (1994) suggested that this permits sufficient cell dehydration to obtain high survival rates after a cryogenic treatment.

Cryoprotectants are chemicals that reduce the injury of cells during freezing and thawing. There are two kinds of cryoprotectant, penetrating and non-penetrating, based on their ability to cross cell membranes (Pérez, 2000, Muldrew, 2002). Dimethyl-sulfoxide (DMSO), propylene glycol, ethylene glycol and glycerol are all penetrating cryoprotectants. Hydroxyethyl starch, polyvinylpyrrolidone and polyethylene oxide are non-permeating cryoprotectants (Muldrew, 2002). Glycerol is unique as a cryopro-tectant, since it is a penetrating protectant if added at physiological temperatures, but is non-permeating if used at 0°C (Muldrew, 2002).

The mechanism of action of a penetrating cryoprotectant is due to their role in cryodehydration (Grout, 1995) and also due to their colligative properties to buffer salt concentrations extra- and intracellularly at low temperatures within the physiological

range. Non-permeating protectants act by dehydrating cells at subfreezing temperatures, thereby reducing water activity to a much greater extent (Muldrew, 2002).

Table 2-1. Tropical plant species that have been cryopreserved; arranged by plant material used for cryopreservation (somatic embryos, embryogenic cultures, cell cultures, embryo axes, seed and zygotic embryos, shoot tips and apices, and meristematic clumps)

Botanical Name	Cryoprotectants/ Techniques[1)]	Cooling[2)]	Thawing[3)]	References
Somatic Embryos				
Citrus sinensis	10% DMSO	Slow	Slow	Marin and Duran-Villa (1988)
Citrus sinensis	10% DMSO	Slow	Fast	Marin et al. (1993) Duran-Villa (1995)
Elais guineensis	Preculture, Dehydration	Fast	Fast	Dumet et al. (1993)
Camellia japonica	Vary	vary		Janeiro et al. (1996)
Manihot esculenta	10% DMSO+ 10% sucrose	Fast	Fast	Stewart et al. (2001)
Embryogenic cultures				
Citrus sinensis	PVS2, Vitrification	Fast	Fast	Sakai et al. (1990)
Citrus sinensis	5% DMSO+1.2M sucrose	Slow	Fast	Kobayashi et al. (1990)
Citrus sinensis	2M glycerol	Slow	Fast	Sakai et al. (1991)
C. sinensis, C. aurantium C. aurantifolia, C. limon C. paradisi, C. hybrid	10% DMSO	Slow	Fast	Duran-Villa (1995) Perez et al. (1997, 1999)
Hevea brasiliensis	10% DMSO+1.M sucrose	Slow	Fast	Engelmann and Etienne (2000)
Oryza sativa	1M DMSO+ 1M glycerol+ 2M sucrose+0.09M L-proline	Slow	Fast	Jain et al. (1996)
Oryza sativa	Vitrification	Fast	Fast	Wang et al. (1998)
Euphoria longan	Vitrification	Fast	-	Sudarmonowati (1996)
Cell cultures				
Doritaenopsis sp.	Vitrification	Fast	Fast	Tsukazaki et al. (2000)
Oryza sativa	5% DMSO+ 10% D-glucose	Slow	Fast	Watanabe et al. (1995;1999)
Embryo axes				
Camellia japonica	Vary	vary		Janeiro et al. (1996)
Artocarpus heterophylus	Vitrification	Fast	Fast	Thammasiri (1999)
	Cryoprotectant	Slow	-	Normah and Marzalina (1996)
Camellia sinensis	Desiccation	Fast	Fast	Chaudhury et al. (1991)
Citrus madurensiss	Vitrification	Fast	Fast	Cho et al. (2001)
Poncirus trifoliata	Desiccation	Fast	Fast	Radhamani and Chandel (1992)

Citrus sinensis	Encapsulation-dehydration; Vitrification	-	-	Sudarmonowati (1999)
Citrus halimii, C. mitis, C. aurantifolia,	Desiccation	-	-	Normah and Marzalina (1996)
Bacaurea polyneura	Desiccation	-	-	Normah and Marzalina (1996)
Nephelium lappaceum	Desiccation	-	-	Normah and Marzalina (1996)
Seeds and zygotic embryos				
Theobroma cacao	10% DMSO+ 0.5M sucrose	Slow	Slow, Fast	Pence (1991)
Coffea arabica	desiccation	Slow	Fast	Dussert et al. (2000)
Nephelium lappaceum	Two-step freezing	-	-	Sudarmonowati (1996);
Litchi sinensis	Vitrification	Fast	-	Sudarmonowati (1996)
Bacaurea polyneura	Desiccation	-	-	Normah and Marzalina (1996)
Carica papaya	Desiccation	-	-	Normah and Marzalina (1996)
Manilcara zapota	Desiccation	-	-	Normah and Marzalina (1996)
Shoot tips and apices				
Manihot esculenta	10% DMSO+ 1M sorbitol	Slow	Fast	Escobar et al. (1997)
Colocasia esculenta	Vitrification	Fast	Fast	Takagi et al. (1997)
P. trifoliate x C. sinensis	Encapsulation-dehydration	Fast	Fast	Wang et al. (2002)
Poncirus trifoliata	Encapsulation-dehydration	Slow; Fast	Slow	Gonzales-Arnao (1998)
Meristematic clumps				
Banana spp.	Vitrification	Fast	Fast	Helliot et al. (2002)

Note: 1) Cryoprotectants = cryoprotectant used on slow cooling cryopreservation, techniques = methods used other than slow cooling.
2) Slow cooling including cooling 0.5-1.0C^{o} min^{-1}, either by programmable freezer or by 'Mr. Frosty'; Fast cooling means direct immersion into liquid nitrogen
3) Slow thawing means thawing at room temperature; Fast thawing means thawing by immersion in water bath with temperature 37-60^{o}C, average ca. 40^{o}C.
- No information available.

Fast Cooling Cryopreservation or Vitrification

Vitrification is a simplified cryopreservation procedure that involves rapid freezing by direct immersion into liquid nitrogen (Sakai et al., 1990; Grout, 1995; Towill, 1995; Benson, 1997). Vitrification refers to the phase transition of water from liquid directly into a vitreous, non-crystalline or amorphous phase by increasing the viscosity during

rapid cooling without ice formation (Fahy et al., 1984; Engelmann et al., 1991; Grout 1995; Towill, 1995; Benson, 1997). Some advantages of vitrification over slow cooling or two-step-cooling include simplicity, less cell damage due to ice formation, and applicability to larger pieces of tissue (Towill, 1995).

As with slow cooling cryopreservation, the key to successful vitrification is the increase of intracellular solutes, achieved by removal of intracellular water by vitrification solutions. The protocol consists of two cell protection steps: loading with a loading solution and dehydration using a vitrification solution. Cultures are directly immersed into liquid nitrogen, and after thawing, the cryopreserved cultures are osmoconditioned with a high concentration of sucrose. The loading step enhances permeation of cryoprotectant through cell membranes and prevents cell damage as a result of exposure to vitrification solution (Towill, 1995). The most common loading solution is a 25% strength (Wang et al., 1998) plant vitrification solution no. 2 (PVS2) consisting of 30% glycerol, 15% ethylene glycol, 15% DMSO, and 0.15 M sucrose (Sakai et al., 1991). A simpler solution also can be used, e.g., a solution of 0.3-0.4 M sucrose + 2.0 M glycerol (Ishikawa et al., 1997; Takagi et al., 1997; Thammasiri, 1999; Pennycooke and Towill, 2000; Tsukazaki et al., 2000).

Dehydration using a vitrification solution deprives intracellular water from cells and permits an intracellular solution to solidify, forming an amorphous glass state upon direct immersion into liquid nitrogen (Grout 1995; Towill, 1995; Wang et al., 1998). The Plant Vitrification Solution number 2 (PVS2) is the most common vitrification solution used for vitrification of plant material. This solution consists of 30% glycerol, 15% ethylene glycol, 15% DMSO with 0.15 M sucrose (Sakai et al., 1990), or with 0.4 M

sucrose (Ishikawa et al., 1997; Takagi et al., 1997; Wang et al., 1998; Thammasiri, 1999; Pennycooke and Towill, 2000; Tsukazaki et al., 2000). Modified vitrification solution for apple consists of 7% (w/v) DMSO, 15% (w/v) ethylene glycol, 15% (w/v) propylene glycol, and 15% (w/v) glycerol in 0.5 M sorbitol (Seufferheld et al., 1991).

Thawing

Bajai (1995) suggested that fast thawing at 37-40^{o}C yields better results than other methods. Rapid thawing prevents fusion of ice microcrystals, which would thereby form larger crystals, and damage the integrity of the cells (Engelmann, 1991). It also prevents formation of ice crystals from amorphous vitrified intracellular solutions (Grout, 1995; Muldrew, 2002). Thawing of cryopreserved citrus at 37^{o}C for 5 min ensures a high rate of survival (Pérez et al., 1997; Pérez, et al., 1998; Marin et al., 1993). The thawing procedure for vitrified material is similar to that of slow cooling cryopreservation. However, immediately after thawing the material must be unloaded to reach the concentration of physiological level of intracellular solution (Towill, 1995; Wang et al., 1998).

Viability

The fluorescein diacetate (FDA) staining of cryopreserved material performed immediately after thawing has been shown to overestimate cell survival (Perez et al., 1997). Staining with 2,3, 5-triphenyltetrazolium (TTC), however, has resulted in a good correlation between viability and the recovery of cryopreserved materials (Watanabe *et al*.,1999; Ishikawa *et al*., 1997). Bajai (1995) suggested that the staining method alone may not provide accurate data about survival since some cells that stain positively later die in culture. Growth of cryopreserved materials on recovery medium is the only reliable test for viability (Engelmann, 1991; Duran-Vila, 1995; Perez et al., 1997). Perez

et al. (1997) also noted the importance of evaluation of the embryogenic potential of cryopreserved embryogenic cultures.

Growth generally occurs three days (Sakai, et al., 1991) or 2-6 weeks (Duran-Vila, 1995) after plating embryogenic cultures on recovery medium. Viability is 5% with slow thawing and 30% with fast thawing in 37^{o}C for somatic embryos of citrus (Marín et al., 1993) and 100% for proembryonic masses of citrus (Pérez, et al., 1997).

The reports of growth and embryogenic capacity of cryopreserved cultures of different plant species are somewhat contradictory. Sakai et al. (1991) reported that growth of cryopreserved embryogenic cultures of naval orange is lower than the unfrozen control until 12 days of cultures. Perez et al. (1997) reported that in sweet orange, lemon, Cleopatra Mandarin and Mexican lime the appearance and growth rate are the same as the nonfrozen controls. In rice, cryopreservation does not apparently affect morphogenic competence (Carnejo et al., 1995; Moukadiri, 1999).

Genetic Integrity

White spruce regenerated from embryogenic cultures that have been cryopreserved for 4 years are genetically stable (De Verno et al., 1999). Cryopreservation does not affect phenotypic and genetic stability of *Citrus* (Duran-Vila, 1995), *Picea glauca* (Cyr et al., 1994; De Verno et al., 1999) and *P. abies* (Häggman et al., 1998). Cryopreservation does not apparently affect ploidy level, and there is no change in pattern and number of DNA fragments in *Malus fumila* (Hao et al., 2001). Genetic fidelity of *Anigozanthos viridis* is maintained following tissue culture, cold storage and cryopreservation (Turner et al., 2001). Elleuch et al. (1998) reported that cryopreservation of transgenic *Papaver somniferum* does not affect the integrity and transcription of the transgene, and the enzymatic activity of its product. Aronen et al.

(1999), however, reported that cryoprotectants may cause some genetic alteration, e.g., approximately 17% alteration of RAPD bands occurs in DMSO treated but nonfrozen samples.

There have been some beneficial effects of cryogenic storage on embryogenic capacity, i.e., non-embryogenic cells appear to be eliminated from cultures (Gupta et al., 1995) and there appears to be increased synchrony of development from cryopreserved somatic embryos (Häggmann et al., 1998). However, Perez et al. (1997) reported that cryopreserved Succari sweet orange, Red Marsh grapefruit and Mexican lime cannot produce somatic embryos if the cultures have lost embryogenic competence prior to cryopreservation.

CHAPTER 3
TRANSFORMATION OF EMBRYOGENIC AVOCADO CULTURES

Introduction

Although most avocados are consumed locally, they are also a major export commodity. Average world avocado production and exports for 1996-2001were approximately 2.4 and 0.3 million metric tons per annum, respectively (FAOSTAT, 2002). An extended shelf life of avocado fruit is one of the major goals of many avocado producers (Lahav and Lavi, 2002). Despite of its importance, however, there are no breeding programs that specifically target extended shelf life of avocado fruit. This could be due to the typical problems of conventional breeding of woody perennial species, i.e., a long juvenile period, seasonal flowering and low fruit set (Pliegro-Alfaro and Bergh, 1992; Lavi et al., 1993; Lahav and Lavi, 2002).

The hybrid cultivars 'Hass' and 'Fuerte', both Mexican x Guatemalan hybrids, have dominated the world's avocado export market (Bergh, 1976; Lahav and Lavi, 2002; Newett et al., 2002). Although 'Hass' is the most important avocado grown in the USA, the West Indian ('Simmonds', 'Waldin', etc.), and Guatemalan x West Indian ('Choquette', 'Monroe', etc.), cultivars predominate in south Florida and also in the tropics (Crane et al., 1996; Newett et al., 2002). The Mexican, Guatemalan, and Mexican x Guatemalan type avocados do not ripen on the tree and remain attached to the trees 2-4 months after reaching maturity (Tingwa and Young, 1975; Sitrit et al., 1986; Whiley, 1992). This characteristic is used to extend avocado supply by "on-tree-storage". However, Whiley et al. (1996) reported that delaying of harvesting reduces

average annual yield and initiates an alternate bearing cycle. Extending the shelf life would permit earlier harvesting of these types of avocados and would overcome the problem of alternate bearing.

The fruit of tropical avocados cannot be stored on the tree because fruit mature and ripen on the tree and drop if not harvested (Whiley, 1992). In order to ensure year-round availability of avocado fruit, several avocado cultivars with different harvesting windows must therefore be grown, e.g., Florida's avocado growers grow approximately 23 major and 38 minor avocado cultivars for this purpose (Crane et al., 1996; Newett et al., 2002). Consequently, a uniform standard for appearance and quality for the tropical avocado is difficult. Extending on-tree-storage of fruit of these avocado types could result in continuous supply of more uniform fruits.

The avocado fruit is climacteric and ethylene plays a central role in ripening (Adato and Gazit, 1974; Morton, 1987; Kays, 1997). Ethylene acts as a natural triggering mechanism for the induction of climacteric respiration and also regulates fruit ripening, including autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars and increased activity of cell wall degrading enzymes (Gray *et al*., 1992; Kays, 1997). Lowering ethylene concentration during fruit storage can prolong fruit shelf life.

Chemical compounds that interfere with ethylene with or without controlled atmosphere and low-temperature storage can be used to prolong avocado fruit shelf-life: aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor (Kays, 1997); NBD (2,5-norbornadiene) and diazocyclopentadiene (DACP), both ethylene action inhibitors

(Blankenship, 1993; Sisler and Serek, 1997; 1999); and 1-methylcyclopropane (1-MCP), an ethylene perception blocker (Feng et al., 2000; Jeong et al., 2002).

Lowering the production of endogenous ethylene from avocado fruit could delay avocado fruit ripening. Ethylene production can be suppressed by blocking specific gene activity, e.g., the genes encode methionine-S-adenosyltransferase, ACC synthase and ACC oxidase, and by overexpressing the genes encoding malonyl transferase, SAM hydrolase and ACC deaminase**.** Genetic transformation has been utilized to interfere with ethylene production in tobacco (Bestwick et al., 1991), tomato (Hamilton et al., 1991; Klee et al., 1991; Penarruba et al., 1992; Oeller et al., 1991; Theologies et al., 1992; Good et al., 1994; Kramer et al., 1997), in melon (Amor et al., 1998; Clendennen et al., 1999), and red raspberry (Mathews et al., 1995). These approaches have not been attempted with any woody tree species. Genetic transformation of avocado with a gene construct that could block ethylene biosynthesis may extend on-the-tree storage and shelf life of avocado fruit.

Avocado embryogenic cultures are not recalcitrant to *Agrobacterium tumefaciens*-mediated transformation. Avocado embryogenic cultures were first transformed with a construct containing a kanamycin resistance gene neomycin phosphotransferase (*nptII*) as a selectable marker and *GUS* (β-glucuronidase) as a reporter gene (Cruz-Hernández et al., 1998). Proembryonic masses were transformed by co-cultivation with disarmed, acetosyringone-activated *Agrobacterium tumefaciens* strain A208, which harbored a co-integrative vector pTiT37-ASE::pMON9749. Transformed embryogenic cultures and somatic embryos were confirmed by the X-gluc reaction, and integration of *npt*II and GUS into the avocado genome was confirmed by PCR and Southern hybridization (Cruz-

Hernández et al., 1998) but transgenic plants were not regenerated. Raharjo et al. (unpublished) have integrated chitinase+β-1,4-glucanase and the antifungal (AFP) protein genes into embryogenic avocado cultures and have regenerated transgenic plants that are undergoing nursery evaluation. Plant conversion was increased by micrografting transformed shoots onto avocado seedlings. Raharjo et al. (unpublished) utilized constructs consisting of pGPTV-BAR-CG (chitinase, glucanase), and pGPTV-BAR-AFP (antifungal protein). The transgenes were delivered by *Agrobacterium tumefaciens* strain EHA 105. Transformed embryogenic cultures and somatic embryos were selected for resistance to Basta. The GUS gene was used as a reporter gene and transgene insertion was confirmed by the X-gluc reaction.

The aim of this study has been to transform embryogenic avocado cultures with the S-adenosylmethionine hydrolase (*sam-ase*) gene construct, to recover transformed avocado somatic embryos and plantlets, and confirm genetic transformation. The ultimate goal is to block ethylene biosynthesis in avocado fruit.

Material and Methods

Induction of Embryogenic Cultures

Embryogenic cultures were induced from immature zygotic embryos of avocado (*Persea americana* Mill.). Avocado fruits ⩽1.0 cm in length representing different cultivars of different races were collected from the USDA-ARS Subtropical Horticultural Research Station (Miami, FL) National Avocado Germplasm Repository and the germplasm collection of the University of California (Riverside, CA) (Table 3-1). The immature fruits were surface-disinfested in a 20% (V/V) solution of commercial bleach supplemented with 10–20 drops of Tween 20® per liter for 20 min and were rinsed with two changes of sterile, deionized water in a laminar flow hood. The fruits were bisected

longitudinally under sterile conditions, and the immature seed was removed from each fruit. Six seed halves were cultured on semi solid induction medium in a 65 x 15 mm Petri dish containing 10–15 ml $B5^+$ medium. Petri dishes were sealed with Parafilm® and the cultures were maintained in darkness at room temperature (25°C). The $B5^+$ medium consisted of B5 (Gamborg et al., 1968) major salts, MS (Murashige and Skoog, 1962) minor salts, 0.41 μM picloram and (in mg l^{-1}) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and TC agar (8,000) (Sigma) (Witjaksono, 1997; Witjaksono and Litz, 1999a).

Table 3-1. Avocado genotypes used for the experiments, their botanical description and source.

Genotypes	Races[1)]	Source	Embryogenic lines
'Suardia'	WI?	USDA-ARS, Miami	SA1.1, SC3.1
'T362'	G	UC Riverside	T2.11.1
'Nabal'	G	USDA-ARS, Miami	NC2.2

Note: 1) Smith et al. (1992). WI = West Indian, G = Guatemalan

Maintenance of Embryogenic Cultures

Embryogenic cultures consisting of proembryonic masses (PEMs) and early cotyledonary somatic embryos that developed on induction media were transferred onto fresh semi solid MSP medium (30-35 ml in each 100 x 20 mm Petri dish) 2-4 week after explanting. This medium consists of MS basal medium, supplemented with 0.41 μM picloram and (in mg l^{-1}) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and TC agar (8,000) (Witjaksono, 1997). The pH of the medium was adjusted to 5.7 with either KOH or HCl prior to addition of agar. Medium was sterilized by autoclaving at 1.1 kg cm^{-2} and 120°C for 20 min.

Tissues were subcultured onto semi solid MSP at 3-5 week intervals. Approximately 200-300 mg PEMs that passed through 1.8 mm mesh sterile nylon filtration fabric were subdivided to form 7 inocula of 0.3-0.5 mm in diameter. Inocula were flattened on the surface of semi solid MSP medium in Petri dishes, one in the center and 6 around it. Petri dishes were closed, sealed with Parafilm®, and maintained in darkness at room temperature (25^{o}C).

After 3 to 6 subcultures, embryogenic cultures were transferred into liquid MS3:1P media. This medium is a modification of MS basal medium, containing 60 mM inorganic nitrogen in which 75% of nitrogen is NO_3^- and 25% is NH_4^+, and supplemented with induction medium addenda but without solidifying agent (Witjaksono and Litz, 1999b). The pH was adjusted to 5.7 prior to autoclaving. Approximately 0.5-1.0 g of embryogenic culture from semisolid medium was inoculated into 40 ml medium in 125 ml Erlenmeyer flasks, which were capped with aluminum foil and sealed with Parafilm®. The cultures were maintained on a rotary shaker at 125 rpm and 25°C with diffuse light, and were subcultured at 2-3 week intervals.

Genetic Transformation

***SamK* gene**

The construct used in this experiment was a binary vector pAG4092 (10.7 kb) in *Agrobacterium tumefaciens* strain EHA 101 (Agritope, 2000). This plasmid was constructed using the backbone of the pPZP200 binary vector (Agritope, 2000). The pAG4092 has the *nptII* gene that encodes resistance to the antibiotic kanamycin under the AGT01 promoter located near the left border and the *samK* gene driven by a fruit-specific avocado cellulase promoter located near the right border (Figure 3-1) (Agritope, 2000). The *samK* gene is a modified *sam-ase* and encodes for SAM hydrolase that

catalyzes the conversion of SAM to methylthioadenosine (MTA) (Good et al., 1994). Since SAM is the metabolic precursor of ACC, the proximal precursor of ethylene, the depleted SAM pool will inhibit ethylene biosynthesis (Good et al., 1994; Kramer et al., 1997).

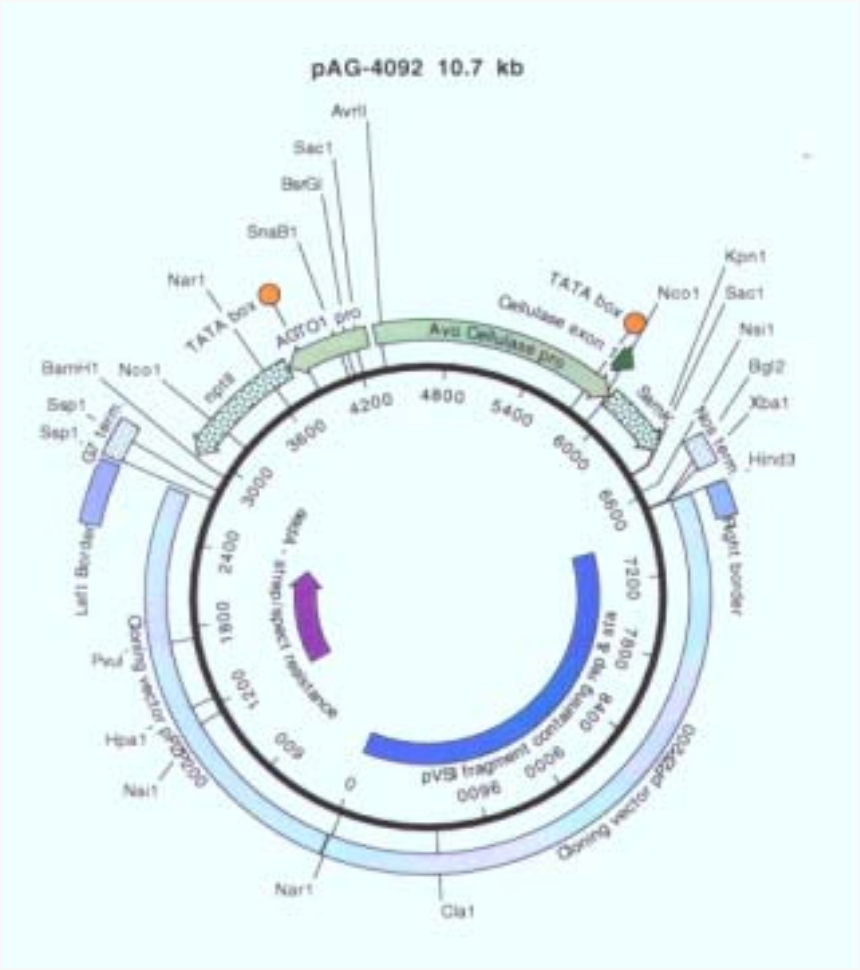


Figure 3-1. Plasmid pAG-4092 harboring *samK* gene with Avocado Cellulase promoter and selectable marker *nptII* under control of AGT01 promoter (Agritope, 2000)

Preparation of bacterial cultures

A single colony of EHA101/pAG4092 from YM medium was plated on fresh medium of the same composition containing 50 mg l^{-1} kanamycin sulfate and 100 mg l^{-1}

spectinomycin. The cultures were incubated at 35^{o}C in the dark for two days. Three colonies of this culture were inoculated into 5 ml liquid medium with the same antibiotic concentrations. Cultures were maintained at 125 rpm on a rotary shaker at room temperature in darkness for 15h. The cultures were transferred to 20 ml medium of the same composition, but supplemented with 100 µl of 3 µM acetosyringone, and were maintained at 125 rpm for 6h at room temperature.

Cocultivation

A modification of the transformation protocol developed by Cruz-Hernández et al. (1998) was used in this study. A 15-day-old embryogenic suspension culture was sieved through sterile nylon filtration fabric (mesh size 1.8 mm), and the smaller fraction was used. Four avocado genotypes were used, i.e., 'T362' line 'T2.11.1', 'Suardia' line 'SA1.1' and line 'SC3.1', and 'Nabal' line 'NC2.2'. One gram of PEMs that passed through 1.8 mm mesh sterile nylon filtration fabric was subcultured into 80 ml fresh MS3:1 medium in 250 ml Erlenmeyer flasks. A 1.0 ml aliquot of 6 h-old acetosyringone-activated *A. tumefaciens* was added. Kanamycin sulfate and spectinomycin were added for a final concentration of 50 and 100 mg l^{-1}, respectively. Flasks were maintained on a rotary shaker at 125 rpm in darkness at room temperature.

After three days, the PEMs were transferred into fresh MSP3:1 medium supplemented with 200 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin in order to kill the *A. tumefaciens*. After two days, the medium was decanted and the PEMs were washed 5x with sterile deionized-water. The PEMs were transferred into 80 ml fresh MS3:1P medium supplemented with antibiotics. After six days of culture, the PEMs were washed three times with sterile deionized water and subcultured into medium of the same composition with antibiotics.

Selection and maintenance

After eight days the embryogenic cultures were transferred into fresh MS3:1P medium without cefotaxime and carbenicillin, but supplemented with 50 mg l^{-1} kanamycin sulfate. Four days later, the PEMs were transferred into fresh medium of the same composition and supplemented with 100 mg l^{-1} kanamycin. For routine maintenance the cultures was kept in MS3:1 medium supplemented with 100 mg l^{-1} kanamycin and subcultured at 2 to 3-week intervals. Cultures also were maintained on semisolid MSP medium supplemented with the same strength of kanamycin. The cultures on semi solid medium were subcultured at 4- to 6-week intervals.

Polymerase chain reaction (PCR)

DNA was isolated from proembryonic masses and from somatic embryos using the CTAB protocol (Doyle and Doyle, 1990) and modified at the NCSU Forest Biotechnology laboratory (Ghislain et al., 2002). PEMs and slices of somatic embryos were air-dried in a laminar flow hood for approx. 1 h. Approximately 400 mg air-dried PEMs were ground in a pre-chilled mortar in liquid nitrogen to obtain a fine powder. The powder was transferred into four Eppendorf tubes. To each tube was added 700 µl 2X CTAB buffer and 2 µl ß-mercaptoethanol and vortexed. The tubes were incubated in a 65^{o}C water bath for 45 min, and agitated at 15 min intervals and cooled to room temperature for 2 min. To each tube was added 700 µl chloroform: isoamyl alcohol (24:1) and the mixtures were vortexed briefly and inverted several times. The tubes were centrifuged for 5 min at 14,000 rpm in a microcentrifuge. The aqueous top layer was removed and transferred to a new Eppendorf tube and 50 µl 10% CTAB (in 0.7 M NaCl) was added and vortexed gently. To each tube was added 500 µl cold isopropanol and the tubes were inverted several times and kept at 4^{o}C for 30 min. The tubes were

centrifuged for 20 min at 14,000 rpm. Supernatants decanted carefully to avoid losing the DNA pellet. The tubes were air-dried for 2 min and the DNA pellet was then washed with 1ml of 70% ethanol for 3 min and centrifuged for 5 min at 14,000 rpm. The ethanol was discarded and the pellet was air-dried overnight. DNA was dissolved with 150 µl rehydration solution (Promega).

The presence of *nptII* and s*amK* genes in transformed PEMs and somatic embryos were demonstrated by PCR (Polymerase chain reaction). Two specific oligonucleotides derived from the *nptII* gene were used as primers for *nptII*. The oligonucleotide primers were 5'-GGT GCC CTG AAT GAA CTG-3' and 5'-TAG CCA ACG CTA TGT CCT-3' (Llamoca-Zarate et al., 2002). This pair of primers would be expected to produce a 700 bp fragment. A pair of primers derived from the *samK* gene (Agritope, 2000) was used as primers for *samK* PCR amplification: sammp3 5' – CGC TTT CCG TTC TAA CCT CT - 3' and sammp5 5'- GGC GAC CGA ACT CAT CAA TA- 3'. The expected fragment size of the PCR product is 395 bp (Helena Mathews, Exelixis 2003, personal communication). PCR amplification occurred in 50-µl reactions containing 5 µl DNA sample, 5 µl of each primer, 25 µl PCR master mix (Promega, catalog # M7501) and 10 µl nuclease-free water. The PCR cycle for *nptII* amplification was 95^{o}C for 3 min followed by 35 cycles of 94^{o}C for 1 min, 54^{o}C for 1.5 min and 72^{o}C for 1.5 min followed by a final extension of 7 min at 72^{o}C. PCR conditions for *samK* amplification was 3 min at 94^{o}C, and 35 cycles of 30 sec at 94^{o}C, 30 sec at 60^{o}C and 60 sec at 72^{o}C followed by a final extension of 5 min at 72^{o}C (Helena Mathews, Exelixis 2003, personal communication).

In order to confirm that the PCR products from the *samK* and *nptII* gene in PEMs and somatic embryos was not due to *A. tumefaciens* contamination, PCR analysis was also done with the primers for amplification of a 650-bp fragment of the chromosomal virulence gene *chvA* of the bacterium. The primers were 5'-ATG CGC ATG AGG CTC GTC TTC TTC GAG-3' (*chvA* F1) and 5'-GAC GCA ACG CAT CCT CGA TCA GCT-3' (*chvA* R1) (Bond and Roose, 1998). The PCR cycle of *nptII* was used to amplify the *chvA* fragments. Genomic *A. tumefaciens* DNA was used as a positive control.

The PTC-100™ Programmable Thermal Controller (MJ Research) was used for PCR. The PCR products were electrophoresed on a 1% agarose gel supplemented with 1 µl of 10 mg ml^{-1} ethidiumbromide for 20 ml l^{-1} agar. The bands of the PCR amplified fragment were visualized using UV light and photographed with Nikon coolpix 995 digital camera with a red filter.

Results

General considerations

Antibiotic concentrations of 200 mg l^{-1}cefotaxime and 500 mg l^{-1} carbenicillin during six subcultures can eliminate symptoms of growth of *A. tumefaciens* from avocado embryogenic cultures. Washing of PEMs with sterile deionized water before transfer to fresh medium increased the effectiveness of the antibiotics. After *A. tumefaciens* was eliminated from the cultures, all of the embryogenic cultures were black. Transformed 'Suardia' SA1.1 cultures showed proliferation of new PEMs, while the three other genotypes failed to recover (Figure 3-2). These three lines i.e., 'T362' line 'T2.11.1', 'Suardia' line 'SC3.1', and 'Nabal' line 'NC2.2' may be more sensitive to *A. tumefaciens* and/or to prolonged exposure to cefotaxime and carbenicillin. Elimination of *A. tumefaciens* from embryonic cultures was determined by observation of *A.*

tumefaciens contamination symptoms and by PCR analysis with the primers for amplification of a 650-bp fragment of the chromosomal virulence gene *chvA* of the *A. tumefaciens*.

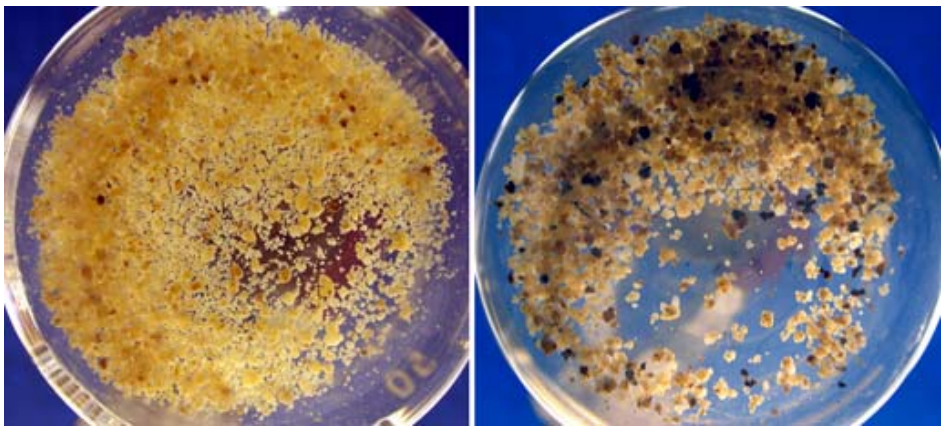


Figure 3-2. Embryogenic suspension cultures of non-transformed 'Suardia' SA1.1 in MS3:1P without kanamycin (left) and transformed 'Suardia' SA1.1 in MS3:1P with 100 mg l^{-1} kanamycin (right).

Polymerase Chain Reaction

The incorporation of the transgene into embryogenic avocado cultures was determined by agarose electrophoresis of PCR-amplified DNA fragments from PEMs and somatic embryos (SEs). There was a 700 bp amplification product of the *nptII* gene generated with a primer pair 5'-GGT GCC CTG AAT GAA CTG-3' and 5'-TAG CCA ACG CTA TGT CCT-3' (Figure 3-3). No amplification could be detected with DNA from the non-transformed control.

The *samK* gene also has been incorporated into the embryogenic avocado culture genome. A 395-bp fragment was generated from transformed PEMs and SEs but not from nontransformed cultures (Figure 3-4). The pair of primers derived from the *samK* gene (Agritope, 2000) were used for *samK* PCR amplification: sammp5 and sammp3.

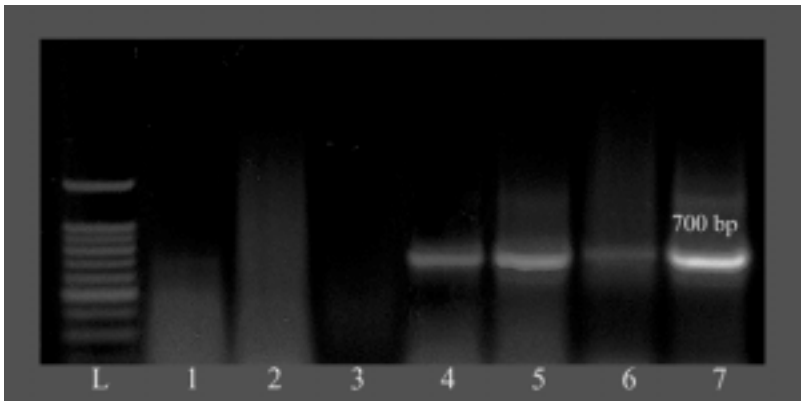


Figure 3-3. PCR amplification of *nptII* gene from 'SA1.1' DNA. Number 1, 2, 4 and 5 are from PEMs and number 3 and 6 from SEs. Numbers 1, 2 and 3 are the non-transformed control, number 4, 5 and 6 are transformed DNA, and L is 100 bp DNA ladder (Promega catalog # G2101). Line 4, 5, and 6 show a 700 bp fragment generated by *nptII* primer. Line 7 is a positive control using the plasmid pAG4092 as a template. PEMs = proembryonic masses; SE = somatic embryos.

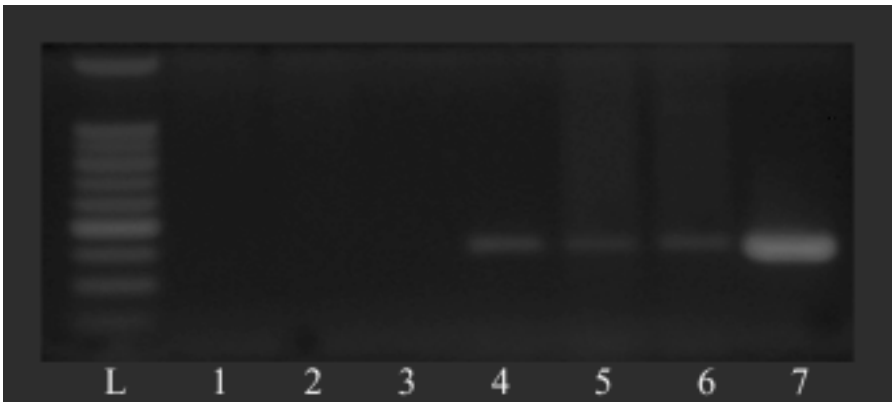


Figure 3-4. PCR amplification of *samK* gene of 'SA1.1' DNA. Number 1, 2, 4 and 5 are from PEMs and number 3 and 6 from SEs. Numbers 1, 2 and 3 are the non-transformed control, number 4, 5 and 6 are transformed DNA, and L is 100 bp DNA ladder (Promega catalog # G2101). Line 4, 5, 6 and 7 show a 400 bp fragment generated by sammp primer. Line 7 is a positive control using the plasmid pAG4092 as a template. PEMs = Proembryonic masses; SE = somatic embryos.

The chromosomal virulence gene A (*chvA*) specific primers were used to demonstrate that none of the PEMs and SEs was contaminated with residual *A.*

tumefaciens. Figure 3-5 shows that there is no amplification of a 650-bp *chvA* fragment from either transformed cultures or from nontransformed cultures.

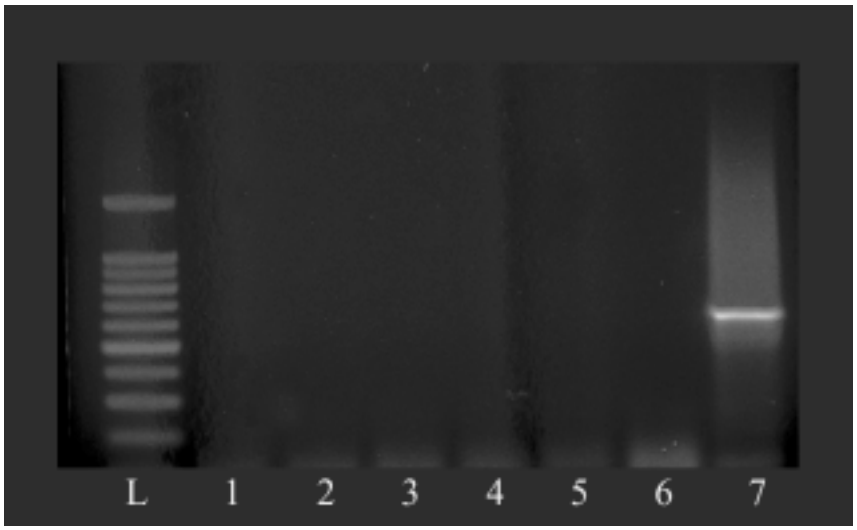


Figure 3-5. PCR amplification of *chvA* gene of 'SA1.1' DNA. Number 1, 2, 4 and 5 are from PEMs and number 3 and 6 from SEs. Number 1, 2 and 3 are the non-transformed control, number 4, 5 and 6 are transformed DNA, and L is 100 bp DNA ladder (Promega catalog # G2101). Line 7 is a positive control using the genomic DNA of *Agrobacterium tumefaciens* EHA101:: pAG4092 as a template.

Transgene Expression

The data for *nptII* gene expression in PEMs and somatic embryos of 'Suardia' SA1.1 are presented in Chapter 4. The transformed 'Suardia' SA1.1 PEMs were able to grow in medium supplemented with 100 to 300 mg l^{-1} kanamycin sulfate, and somatic embryos were able to develop on SED medium supplemented with 100 – 400 mg l^{-1} kanamycin sulfate. The growth of nontransformed cultures was inhibited by 100 mg l^{-1} kanamycin sulfate and somatic embryo development and maturation was completely suppressed by 200 mg l^{-1} kanamycin sulfate (Chapter 4).

Phenotypic expression of *samK* gene could not be determined in PEMs, somatic embryos or plantlet stage since the gene is driven by an avocado fruit-specific promoter that is turned on only at the climacteric phase of fruit ripening.

Discussion

Embryogenic avocado cultures have been successfully genetically transformed by an *Agrobacterium*-based protocol. Suspension cultures consisting of PEMs that proliferated by the production of secondary proembryos (Witjaksono, 1997; Witjaksono et al., 1999a) were co-cultivated with *A. tumefaciens* harboring the binary vector pAG4092.

Transformation of embryogenic cultures of other hardwood species have also been reported, i.e., Poncan citrus (*Citrus reticulata*) (Li et al., 2002) and tangelo (*C. reticulata x C. paradisi*) (Yao et al., 1996), coffee (*Coffea canephora*) (Hatanaka et al., 1999), mango (*Mangifera indica*) (Mathews et al., 1992), cherry (*Prunus Subhirtella autumno rosa*) (Machado et al., 1995), pecan (*Carya illinoensis*) (McGranahan et al., 1993), sandalwood (*Santalum album*) (Shiri and Rao, 1998), tea (*Camellia sinensis*) (Mondal et al., 2001), and walnut (*Juglans regia*) (McGranahan et al., 1990; Tang et al., 2000).

The concentration of 100 mg l^{-1} kanamycin sulfate used for selecting transformed embryogenic avocado cultures is comparable to that used with mango (Mathews et al., 1992), citrus (Yao et al., 1996), walnut (McGranahan et al., 1990; Tang et al., 2000) and grape (Herbert et al., 1993). This concentration, however, is higher than the level used for other species, e.g., transformed embryogenic cultures have been selected on semi solid medium with kanamycin sulfate concentrations ranging from 25 mg l^{-1} with sandalwood (Shiri and Rao, 1998), 20-40 mg l^{-1} with grape (Scorza et al., 1995),

50 mg l^{-1} with pecan (McGranahan et al., 1993), grape (Nakano et al., 1994) and tea (Mondal et al., 2001), and 75 mg l^{-1} with cherry (Machado et al., 1995).

The PCR-amplified fragment of 700 bp agrees with the expected *nptII* gene amplification with the pair of primers used (Llamoca-Zárate et al., 1999). The PCR-amplified fragment of ca. 400 bp is also agrees with the expected *samK* gene amplification with the pair of sammp5' and sammp3' primers (Helena Mathews, 2003 personal communication).

The *nptII* and *samK* PCR-generated fragments must have originated from the transgene that was integrated into the embryogenic avocado culture genome since transformed cultures were not contaminated with residual *A. tumefaciens*. The chromosomal virulence A (*chvA*) gene fragment was generated by PCR from genomic DNA of *A. tumefaciens* with the specific primers as a positive control. The *chvA* fragment length is 650 bp and is also agrees with the expected fragment (Bond and Roose, 1998). Neither the transformed PEMs and SEs nor the nontransformed cultures show the *chvA* PCR-amplified fragment.

Conclusion

Embryonic avocado cultures were genetically transformed by cocultivation with *A. tumefaciens*. Sensitivity of PEMs to *A. tumefaciens* and antibiotics were genotype dependent. The integration of the transgene into the avocado genome was confirmed by the presence of *nptII* and *samK* PCR-amplified fragments of transformed PEMs and somatic embryos DNA. The absence of residual *A. A. tumefaciens* in transformed cultures was confirmed by the negative results of PCR amplification of the chromosomal virulence A (*chvA*) gene.

CHAPTER 4
GROWTH OF TRANSFORMED EMBRYOGENIC AVOCADO CULTURE

Introduction

The gene construct carried by plasmid pAG-4092 was introduced into embryogenic avocado 'Suardia' SA1.1 cultures. This construct contains the s*amK* gene, a version of the *sam-ase* or SAM hydrolase gene and the selection marker neomycin phosphotransferase II (*nptII*) gene (Figure 4-1). The s*amK* gene encodes a functional enzyme S-adenosylmethionine hydrolase (SAMase or AdoMetase, EC 3.3.1.2) (Good et al., 1994). This enzyme can prevent ethylene biosynthesis by altering the methionine-recycling pathway in plants. The ethylene biosynthesis pathway is bypassed and methionine is converted to methylthioadenosin (MTA) and homeoserin (Good et al., 1994). The *samK* gene is driven by an avocado cellulase promoter from avocado fruit, an organ-specific and a temporally regulated (climacteric) promoter (Agritope, 2001). The *nptII* gene encodes for kanamycin resistance, and is used for selection of transformed embryogenic cultures and somatic embryos. *Agrobacterium tumefaciens* strain EHA101 was used to introduce pAG-4092 harboring the *samK* and *nptII* genes into the embryogenic avocado cultures.

The goal of this study was to compare transformed and nontransformed embryogenic 'Suardia' SA1.1 suspension culture growth and somatic embryo development in the presence of kanamycin sulfate. Germination of transformed SA1.1 somatic embryos was also observed.

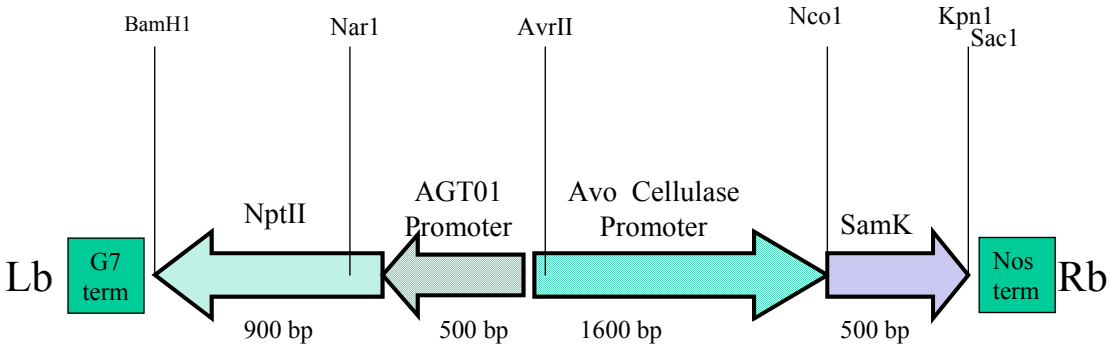


Figure 4-1. Restriction map of the binary vector pAG4092 used in this study.

Material and Methods

Embryogenic Suspension Cultures of Transformed Suardia SA1.1

The growth of transformed and nontransformed embryogenic 'Suardia' SA1.1 cultures in liquid MS3:1P medium containing 0, 100, 200 and 300 mg l^{-1} kanamycin sulfate were compared. Embryogenic cultures were derived from suspension cultures (Chapter 3). Nontransformed 'Suardia' SA1.1 cultures were maintained in liquid MS3:1P medium without kanamycin sulfate. Embryogenic 'Suardia' SA1.1 cultures transformed with *samK* and *nptII* genes were maintained in liquid MS3:1P medium supplemented with 100 mg l^{-1} kanamycin sulfate.

The inocula consisted of 14-day-old cultures from 5 flasks (80 ml MS3:1P media in 250 ml Erlenmeyer flasks) for nontransformed and transformed 'Suardia' SA1.1. Three concentrations of kanamycin sulfate were tested, i.e., 0, 100, 200, and 300 mg l^{-1}. There were 8 treatments, with 4 replications, i.e., total 32 flasks. Kanamycin sulfate stock solution (20 mg l^{-1}) was added to 40 ml MS3:1P medium in 125 ml Erlenmeyer flasks at 0, 400, 800 and 1200 μl to produce final concentrations of 0, 100, 200 and 300 mg l^{-1} kanamycin sulfate. One (1.0 ml) of PEMs settled volume was inoculated into 40 ml MS3:1P liquid medium in 125 ml Erlenmeyer flasks. The flasks were capped with

aluminum foil and sealed with Parafilm®. Cultures were maintained on a rotary shaker at 125 rpm at 25°C under diffuse light.

Settled Cell Volume (SCV) of PEMs was measured at weekly intervals during a 5-week period. The cultures were decanted into sterile graduated plastic centrifuge tubes and the PEM volume was measured after approximately 1 min. The cultures were recultured in the original Erlenmeyer flasks, capped with aluminum foil and sealed with Parafilm® and maintained at 125 rpm and 25°C under diffuse light. Data for settled PEM volume (SCV) were analyzed using ANOVA (SAS, 2002) and were plotted [Sigma Plot (Sigma, 2002)].

Somatic Embryo Development

Embryogenic suspension cultures maintained in MS3:1P without kanamycin sulfate for nontransformed SA1.1 and with 100 mg l^{-1} kanamycin sulfate for transformed SA1.1 were used for these experiments. Both nontransformed and transformed cultures were obtained from 10-day-old cultures (days after last subculture). Cultures were sieved through sterile 1.8 mm nylon filtration fabric and the smaller fraction was used. Embryogenic cultures were air-dried on 16-20 layers of sterile Kimwipes® in open plastic Petri dishes in a laminar flow hood for 1h.

Air-dried embryogenic cultures were plated on somatic embryo development (SED) medium supplemented with different concentrations of kanamycin sulfate. SED medium consisted of MS3:1 medium supplemented with 20% (v/v) filter-sterilize fresh coconut water and solidified with 6 g $liter^{-1}$ Gel-Gro gellan gum (Witjaksono 1997; Witjaksono and Litz, 1999b). Filter-sterilized kanamycin sulfate solution (50 ml) was added together with coconut water (200 ml) to sterile medium. Medium was poured into 100x20 cm sterile plastic Petri dishes, with approximately 50 ml per dish.

Approximately 40-50 mg of air-dried embryogenic cultures was plated by spreading the PEMs evenly over the surface of the medium. The dishes were closed but not sealed and were maintained in darkness at room temperature (25^{o}C) for two months.

There were 2 experiments: 1) kanamycin sulfate at 0, 100, and 200 mg l^{-1} and 2) kanamycin sulfate at 200, 300, and 400 mg l^{-1}. All protocols for these experiments were the same except for kanamycin sulfate concentration, the time that the experiments were initiated and culture age. In the first experiment, 10-day-old cultures were used for both nontransformed and transformed SA1.1 embryogenic cultures. There were 17 replications for the nontransformed control and 27 replications for the transformed cultures for each treatment. Nontransformed embryogenic avocado cultures were used as a control, and there were 2 concentrations of kanamycin sulfate (100, and 200 mg l^{-1}) with medium without kanamycin sulfate as control, in 6 treatments with 132 plates. In the second experiment, 12-day-old cultures of nontransformed and transformed cultures were used to measure somatic embryo development on SED medium containing 200, 300, or 400 mg l^{-1} kanamycin sulfate. There were 6 treatments with 12 replications each for nontransformed cultures and 26 replications for transformed cultures.

The number of opaque somatic embryos in three categories (<0.5, 0.5-1.0, and >1.0 cm in length) for first experiment and two categories (<0.5 and >0.5 cm) for the second experiment were counted. The number of hyperhydric somatic embryos was also counted. Data were collected at one and two months after plating. One Petri dish was a single experimental unit.

Somatic Embryo Germination

Somatic embryos that developed on SED medium were harvested two months after culturing. Large (>1.0 cm in length) opaque somatic embryos were plated on

germination medium. Germination medium consisted of MS basal medium supplemented with 4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30, 000 mg l^{-1} sucrose, 1 µg l^{-1} benzyladenine (BA), 1 µg l^{-1} gibberellic acid (GA_3) and solidified with 3 g l^{-1} Gel-Gro (Witjaksono, 1997; Witjaksono and Litz, 1999a). Medium was autoclaved for 20 min at 121^{o}C and 1.1 kg cm^{-2}. Aliquots of 63 ml medium were dispensed into 100 x 25 mm sterile plastic Petri dish.

Seven somatic embryos were plated in each Petri dish. The treatments in this experiment included origin of the somatic embryos, i.e., somatic embryos that developed on SED medium containing kanamycin sulfate at 200, 300 and 400 mg l^{-1}. One Petri dish was a single experimental unit. There were 33, 17 and 18 replications for somatic embryos that originated from SED supplemented with 200, 300 and 400 mg l^{-1} kanamycin sulfate, respectively.

Results

Growth of Suspension Cultures

There was no significant difference in the PEM settled volume (SCV) of nontransformed and transformed cultures one week after culturing in liquid MS3:1P medium containing different concentrations of kanamycin sulfate (Table 4-1 and Figure 4-2). A significant difference in settled volume between nontransformed and transformed cultures became obvious at the second week. There was a significant interaction between transformation treatment and kanamycin sulfate concentration, and individually, transformation treatment and kanamycin sulfate concentration also had significant effects (Table 4-1 and Figure 4-2).

Table 4-1. ANOVA of effect of transformation and kanamycin sulfate concentration on settled cell volume (SCV) of 'Suardia' SA1.1 grown in liquid MS3:1P medium.

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
SCV Week 1					
Model	7	0.74	0.10	1.33	0.2805
Error	24	1.90	0.08		
Corrected Total	31	2.63			
Lines	1	0.00	0.00	0.00	0.9504
Kanamycin	3	0.50	0.17	2.10	0.1266
Lines*Kanamycin	3	0.24	0.08	0.99	0.4121
SCV Week 2					
Model	7	89.49	12.78	24.21	<0.0001
Error	24	12.67	0.53		
Corrected Total	31	102.17			
Lines	1	31.80	31.80	60.23	<0.0001
Kanamycin	3	47.94	15.98	30.26	<0.0001
Lines*Kanamycin	3	9.75	3.25	6.16	0.0030
SCV Week 3					
Model	7	193.87	27.70	62.78	<0.0001
Error	24	10.59	0.44		
Corrected Total	31	204.46			
Lines	1	52.79	52.79	119.66	<0.0001
Kanamycin	3	119.18	39.73	90.05	<0.0001
Lines*Kanamycin	3	21.90	7.30	16.55	<0.0001
SCV Week 4					
Model	7	164.59	23.51	42.66	<0.0001
Error	17	9.37			
Corrected Total	24	173.96			
Lines	1	10.69	10.69	19.40	0.0004
Kanamycin	3	124.80	41.60	75.48	<0.0001
Lines*Kanamycin	3	29.10	9.70	17.60	<0.0001
SCV Week 5					
Model	7	145.30	20.75	36.20	<0.0001
Error	16	9.18	0.57		
Corrected Total	23	154.48			
Lines	1	5.76	5.76	10.04	0.0060
Kanamycin	3	117.62	39.21	68.36	<0.0001
Lines*Kanamycin	3	21.92	7.31	12.74	0.0002

Lines = Nontransformed and Transformed 'Suardia' SA1.1
Kanamycin = 0, 100, 200 and 300 mg l^{-1} kanamycin sulfate
Lines*Kanamycin = Interaction between Lines and Kanamycin

For most treatments, except for nontransformed SA1.1 grown without kanamycin sulfate, the maximum PEM SCV occurred 3 weeks after culturing (Figure 4-2). The maximum SCV of nontransformed cultures in medium without kanamycin sulfate occurred 4 weeks after culturing. For nontransformed 'Suardia' SA1.1 the maximum settled PEM volume occurred 3 weeks after culturing, i.e., 4.0±0.2, 3.1±0.2, 3.0±0.2

ml/flask on media containing kanamycin sulfate at 100, 200 and 300 mg l^{-1}, respectively. In medium without kanamycin sulfate the maximum PEM SCV was 10.3±0.2 ml/flask. After three weeks, kanamycin sulfate suppressed growth of nontransformed cultures by 53, 64 and 65% in medium with kanamycin sulfate at 100, 200 and 300 mg l^{-1}, respectively. Settled cell volume of transformed embryogenic 'Suardia' SA1.1 cultures 3 weeks after culturing, was 9.2±0.6, 9.0±0.2, 6.0±0.6 and 4.9±0.4 ml per flask in liquid medium containing 0, 100, 200 and 300 mg l^{-1} kanamycin sulfate, respectively.

Kanamycin sulfate at 100 mg l^{-1} did not inhibit growth of transformed embryogenic avocado cultures harboring *nptII*. Growth of transformed embryogenic cultures was suppressed 35 and 48% when kanamycin sulfate concentration was 200 and 300 mg l^{-1}, respectively (Figure 4-4).

There was an interaction of kanamycin sulfate and transformation treatment on settled PEM volume 2 weeks after culturing. The SCV of nontransformed culture decreased sharply from 6.0±0.2 ml in media without kanamycin sulfate to 3.8±0.1 ml in medium with 100 mg l^{-1} kanamycin sulfate, then dropped slightly to 3.2±0.1 ml in medium with 200 and 300 mg l^{-1} kanamycin sulfate (Figure 4-4). The suppression of nontransformed SA1.1 PEM growth in medium with kanamycin sulfate at 200 and 300 mg l^{-1} was approximately 47% compared to the control.

A different response occurred with transformed 'Suardia' SA1.1. The settled PEM volume of transformed SA1.1/pAG-4092 was always greater than the nontransformed cultures at every concentration of kanamycin sulfate. The settled PEM volume of transformed cultures was similar in medium without kanamycin sulfate and in medium with 100 mg l^{-1} kanamycin sulfate, i.e., 7.4±0.6 and 7.6±0.4 ml, respectively (Figure 4-4).

Increasing kanamycin sulfate to 200 mg l^{-1} reduced the settled PEM volume to 4.9±0.5 ml; however, this was not significantly different from the volume of 4.2±0.4 ml at 300 mg l^{-1} kanamycin sulfate (Figure 4-4). Transformed culture growth was not inhibited by 100 mg l^{-1} kanamycin sulfate, but decreased approximately 34% and 43% at 200 and 300 mg l^{-1} kanamycin sulfate, respectively, compared to growth at 100 mg l^{-1} kanamycin sulfate.

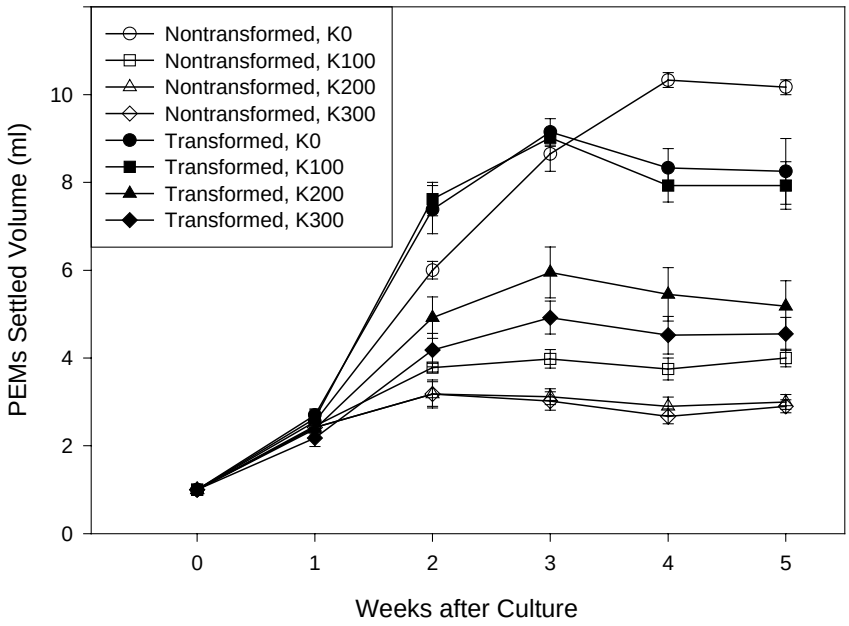


Figure 4-2. Effect of kanamycin sulfate on settled PEM volume of nontransformed and transformed 'Suardia' embryogenic cultures in 40 ml liquid MS3:1P maintenance medium supplemented with 0, 100, 200 and 300 mg l^{-1} kanamycin sulfate (K0, K100, K200 and K300, respectively). Data represent means±standard error of four replications.

Kanamycin sulfate at 100 mg l^{-1} in liquid MS3:1P medium does not affect the settled volume of transformed 'Suardia' SA1.1, but does suppress the growth of nontransformed cultures. Most of the increased volume of nontransformed cultures in kanamycin sulfate-containing media occurred during the first week after culture (Figure 4-2) and little growth occurred between one and two weeks after culture, with growth

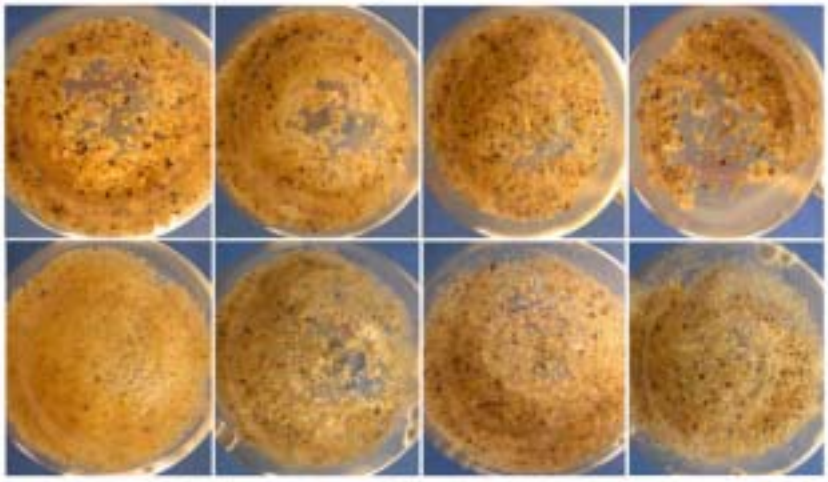


Figure 4-3. Suspension cultures of nontransformed ‘Suardia’ SA1.1 (lower) and transformed ‘Suardia’ SA1.1 with pAG4092 construct (upper) in MSP3:1 maintenance medium supplemented with different concentration of kanamycin sulfate (0, 100, 200 and 300 mg l^{-1} kanamycin sulfate from left to right).

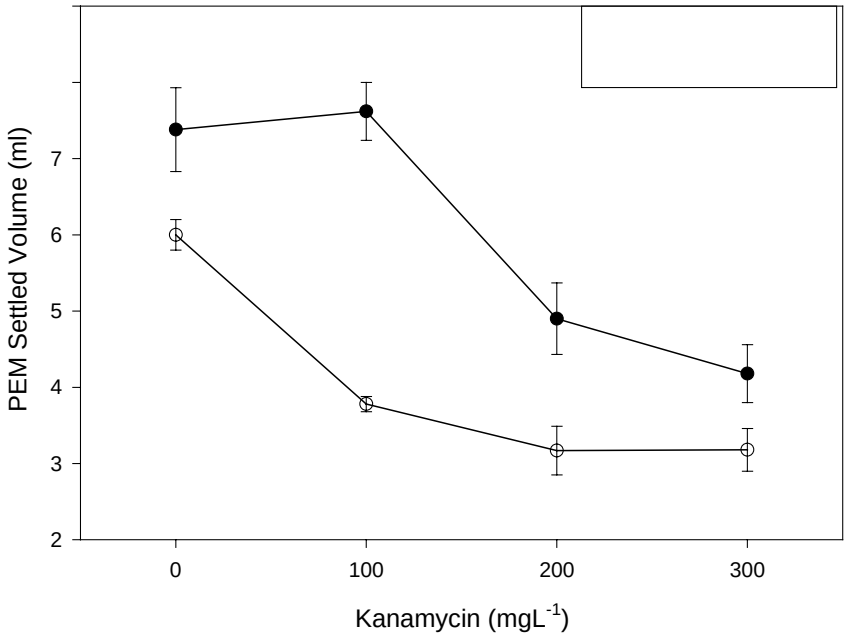


Figure 4-4. Effect of kanamycin sulfate on growth of nontransformed and transformed ‘Suardia’ SA1.1 embryogenic cultures in suspension culture two weeks after culturing. Data represent means±standard error of four replications.

ceasing after two weeks. In contrast, growth of transformed cultures occurred more rapidly between one and two weeks after culturing and this continued for three weeks after culturing (Figure 4-2).

Somatic Embryo Development

White opaque somatic embryos (<0.5 mm diameter) were evident on semisolid SED medium approximately two weeks after plating. Data presented here were from observations two months after plating. The total number of somatic embryos, including opaque and hyperhydric embryos, was very significantly affected by kanamycin sulfate concentration and transformation treatments. There was also a significant interaction between kanamycin sulfate concentration and transformation treatment (Table 4-2 and Figure 4-5).

Table 4-2. ANOVA for the effect of 0, 100 and 200 mg l^{-1} kanamycin sulfate on somatic embryo development of nontransformed and transformed 'Suardia' SA1.1

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Total Somatic Embryo					
Model	5	48832.08	9766.41	67.13	<0.0001
Error	110	16003.47	145.49		
Correction Total	115	68435.54			
Lines	1	39850.07	39850.07	273.91	<0.0001
Kanamycin	2	1647.12	823.56	5.66	0.0046
Lines*Kanamycin	2	7334.89	3667.44	25.21	<0.0001
Number of SE <0.5 cm					
Model	5	6299.64	1259.93	50.81	<0.0001
Error	109	2702.74	24.80		
Correction Total	114	9002.38			
Lines	1	4756.42	4756.42	191.82	<0.0001
Kanamycin	2	940.51	470.25	18.97	<0.0001
Lines*Kanamycin	2	602.71	301.36	12.15	<0.0001
Percent of SE <0.5 cm[*)]					
Model	5	7.19	1.44	14.48	0.0001
Error	109	10.84	0.10		
Correction Total	114	18.04			
Lines	1	1.15	1.15	11.53	0.0010
Kanamycin	2	2.90	1.45	14.59	0.0001
Lines*Kanamycin	2	3.15	1.58	15.84	0.0001
Number of SE 0.5-1.0 cm					
Model	5	5368.73	1073.75	38.88	<0.0001
Error	102	3010.43	27.62		

Correction Total	114	8379.16			
Lines	1	4632.83	4632.83	167.74	<0.0001
Kanamycin	2	83.56	41.78	1.51	0.2249
Lines*Kanamycin	2	652.34	326.18	11.81	<0.0001
Percent of SE 0.5-1.0 cm*)					
Model	5	5.80	1.16	65.54	<0.0001
Error	109	1.93	0.02		
Correction Total	114	7.73			
Lines	1	3.31	3.31	187.14	<0.0001
Kanamycin	2	0.98	0.49	27.69	<0.0001
Lines*Kanamycin	2	1.51	0.75	42.59	<0.0001
Number of SE >1.0 cm					
Model	5	2233.17	446.63	54.44	<0.0001
Error	109	894.27	8.20		
Correction Total	114	3127.44			
Lines	1	1214.51	1214.51	148.03	<0.0001
Kanamycin	2	143.93	71.97	8.77	0.0003
Lines*Kanamycin	2	874.72	437.36	53.31	<0.0001
Percent of SE >1.0 cm*)					
Model	5	3.27	0.65	34.86	<0.0001
Error	109	2.05	0.02		
Correction Total	114	5.32			
Lines	1	1.48	1.48	78.7	<0.0001
Kanamycin	2	0.13	0.06	3.44	0.0356
Lines*Kanamycin	2	1.67	0.83	44.35	<0.0001
Number of Hyperhydric SE					
Model	5	1132.32	226.46	28.67	<0.0001
Error	109	861.07	7.89		
Correction Total	114	1993.39			
Lines	1	966.07	966.07	122.29	<0.0001
Kanamycin	2	70.81	35.41	4.48	0.0135
Lines*Kanamycin	2	95.44	47.72	6.04	0.0033
Percent of hyperhydric SE*)					
Model	5	3.19	0.64	143.03	<0.0001
Error	109	0.49	0.00		
Correction Total	114	3.68			
Lines	1	1.84	1.84	413.06	<0.0001
Kanamycin	2	0.74	0.37	82.81	<0.0001
Lines*Kanamycin	2	0.61	0.30	68.23	0.0001

Lines = Transformation treatments; Nontransformed and Transformed 'Suardia' SA1.1
Kanamycin = 0, 100 and 200 mg l^{-1} kanamycin sulfate
Lines*Kanamycin = Interaction between Lines and Kanamycin
*) Percentage data was transformed by arc sin transformation.

The total number of somatic embryos of nontransformed 'Suardia' SA1.1 was 29.7±2.3, 4.5±0.9 and 0.5±0.2 embryos/plate on somatic embryo development (SED) medium containing 0, 100 and 200 mg l^{-1} kanamycin sulfate, respectively (Table 4-3). The total number of somatic embryos from transformed cultures was 45.6±2.3, 52.7±4.2, and 52.9±3.0 somatic embryos/plate on SED medium supplemented with 0, 100 and 200

mg l^{-1} kanamycin sulfate, respectively (Figure 4-5). Somatic embryo development from transformed PEMs was not inhibited by up to 200 mg l^{-1} kanamycin sulfate. The maximum somatic embryo formation was on 200 mg l^{-1} kanamycin sulfate and the minimum occurred on medium without kanamycin sulfate. Somatic embryo development on medium with 100 mg l^{-1} kanamycin sulfate was similar to that on media with 200 mg l^{-1} or without kanamycin sulfate.

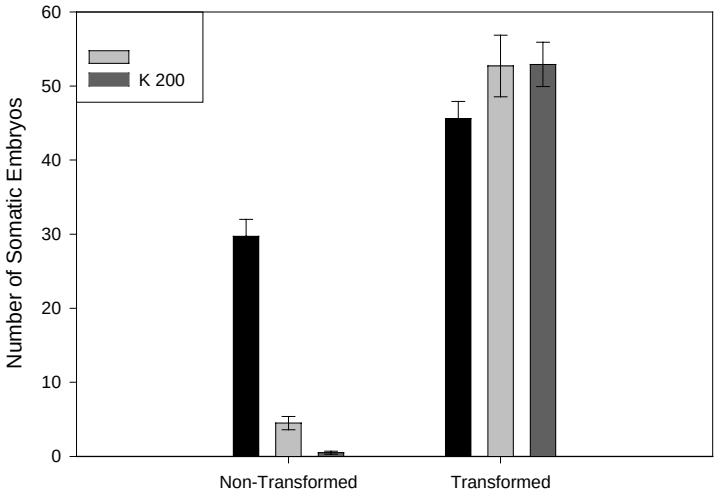


Figure 4-5. Effect of kanamycin sulfate on somatic embryo development of nontransformed and transformed 'Suardia' SA1.1 two months after plating on kanamycin-containing SED medium. K0, K100 and K200 represent kanamycin sulfate concentrations of 0, 100 and 200 mg l^{-1}, respectively. Data represent means± SE of 17 and 27 replicates for nontransformed and transformed culture, respectively.

The number of small (<0.5 cm length) opaque somatic embryos that developed on kanamycin sulfate-containing SED medium was very significantly affected by the interaction between kanamycin sulfate concentrations and transformation treatments as well as by the individual effects of kanamycin sulfate and transformation treatment (Table 4-2). The percentage of small somatic embryo that developed was significantly affected by the interaction between kanamycin sulfate concentrations and transformation

treatments as well as by the individual effects of kanamycin sulfate and transformation treatment.

The number of small (<0.5 cm length) opaque somatic embryos of nontransformed cultures was 13.1±1.1, 3.9±0.8, and 0.4±0.2 embryos per plate on medium supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively (Table 4-3). In terms of percentage of total somatic embryos, the values for small (<0.5 cm length) somatic embryos of nontransformed cultures were 45.3±3.1, 88.8±4.8, and 80.0±11.8% of total somatic embryos on medium supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively. The corresponding values for transformed cultures were 19.8±0.8, 20.0±1.5, and 17.1±1.4 somatic embryo per plate (44.8±1.8, 39.3±2.4, and 33.3±2.8% of total somatic embryos), respectively (Table 4-3). Development of small (<0.5 cm length) somatic embryos of nontransformed 'Suardia' SA1.1 was significantly reduced with respect to increasing kanamycin sulfate concentration. Kanamycin sulfate at 100 and 200 mg l^{-1} suppressed somatic embryo development of nontransformed cultures by 70 and 97% compared to the development of somatic embryos on medium without kanamycin sulfate. For transformed cultures small SEs were increase by 1 and 14% at 100 and 200 mg l^{-1} kanamycin sulfate, respectively. The average number of small embryos that developed from nontransformed cultures in the presence of kanamycin sulfate was only 2.2 compared to 18.6 somatic embryo per plate for transformed cultures, i.e. 8.4-fold difference.

The number of medium sized (0.5-1.0 cm length) somatic embryos was significantly affected by the interaction between kanamycin sulfate concentration and transformation treatment (Table 4-2). Individually, transformation treatment had a very

significant effect on development of medium sized somatic embryos while kanamycin sulfate did not have a significant effect. In terms of the percentage of the medium sized embryos that developed relative to the total number of embryos, there was an interaction between kanamycin sulfate concentration and transformation treatment, and individual treatments also had a significant effect (Table 4-2).

Table 4-3. Effect of kanamycin sulfate on somatic embryo development of nontransformed and transformed 'Suardia' SA1.1. K0, K100 and K200 represent kanamycin sulfate concentrations of 0, 100 and 200 mg l^{-1}, respectively. Data represent means± SE of 17 and 27 replicates for nontransformed and transformed culture, respectively.

Somatic Embryo		Nontransformed			Transformed		
		K0	K100	K200	K0	K100	K200
<0.5 cm	Number	13.1±1.1	3.9±0.8	0.4±0.2	19.8±0.8	20.0±1.5	17.1±1.4
	%	45.3±3.1	88.8±4.8	80.0±11.8	44.8±1.8	39.3±2.4	33.3±2.8
0.5-1.0 cm	Number	7.8±0.8	0.4±0.2	0.0±0.0	14.1±1.2	16.2±1.7	16.9±1.4
	%	25.9±1.4	8.9±4.8	0.0±0.0	30.0±1.8	29.4±1.7	31.2±1.5
>1.0 cm	Number	5.3±0.8	0.2±0.1	0.1±0.1	4.6±0.5	8.6±0.6	12.5±1.0
	%	16.8±2.0	2.3±1.6	20.0±3.1	9.8±1.0	16.8±1.2	24.1±2.24
Hyperhydric	Number	3.6±0.6	0.0±0.0	0.0±0.0	7.1±0.6	7.9±0.8	6.4±0.8
	%	12.0±1.6	0.0±0.0	0.0±0.0	15.4±1.1	14.5±1.1	11.4±1.0

Nontransformed cultures produced opaque, medium sized (0.5-1.0 cm) somatic embryos that ranged from 7.8±0.8, 0.4±0.2, and 0.0±0.0 embryos/plate on medium supplemented with kanamycin sulfate at 0, 100, and 200 mg l^{-1}, respectively (Table 4-3). In terms of the percent of the total number of embryos, the corresponding values were 25.9±1.4, 8.9±4.8, and 0.0±0.0, respectively. Kanamycin sulfate at 100 mg l^{-1} inhibited growth and maturation of nontransformed somatic embryos, where the number of the medium sized somatic embryos was reduced by approximately 95% compared to that of somatic embryos that developed on control medium. Kanamycin sulfate at 200 mg l^{-1} totally arrested nontransformed somatic embryo growth and maturation.

Transformed ‘Suardia’ SA1.1 cultures produced more medium size (0.5-1.0 cm) opaque somatic embryos than nontransformed cultures. The total number of SEs produced was 14.1±1.2, 16.2±1.7, and 16.9±1.4 on medium supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively. In terms of the percentage of the total number of embryos, the corresponding values were 30.0±1.8, 29.4±1.7, and 31.2±1.5%, respectively (Table 4-3).

The number of large opaque white somatic embryos (>1.0 cm in length) was very significantly affected by the interaction between kanamycin sulfate concentration and transformation treatments, as well as by kanamycin sulfate and transformation treatment individually. The percentage of large somatic embryos was also significantly affected by interaction between kanamycin sulfate concentrations and transformation treatments as well as by the individual effect of kanamycin sulfate concentrations and transformation treatments(Table 4-2).

The total number of large somatic embryos that developed from nontransformed cultures was 5.3±0.8, 0.2±0.1, and 0.1±0.1 on media supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively (Table 4-3). In terms of the percentage of the total number of somatic embryos, the corresponding values were 16.8±2.0, 2.3±1.6, and 20.0±3.1, respectively. Kanamycin sulfate reduced the growth and maturation of large nontransformed somatic embryos by 98 to 100% at 100 and 200 mg l^{-1}, respectively. Production of large somatic embryos from transformed embryogenic cultures was 4.6±0.5, 8.6±0.6, and 12.5±1.0 on medium supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively. In terms of percentage, these values correspond to 9.8±1.0, 16.8±1.2, and 24±2.24% of the total somatic embryo production, respectively

(Table 4-3). Kanamycin sulfate at 100 and 200 mg l^{-1} increased the production of large opaque transformed somatic embryos by approximately 87 and 170%.

Both the total number and percentage of hyperhydric somatic embryos were significantly affected by the interaction between kanamycin sulfate concentration and transformation treatment (Table 4-2). The individual effect of the treatments also was highly significant. Nontransformed cultures produced hyperhydric somatic embryos only on medium without kanamycin sulfate, e.g., 3.6±0.6 embryos per plate (12.0±1.6% of total) (Table 4-3). Transformed 'Suardia' SA1.1 cultures produced more hyperhydric somatic embryos than the nontransformed cultures. The total numbers of hyperhydric somatic embryos were 7.1±0.6, 7.9±0.8, and 6.4±0.8, on medium supplemented with 0, 100, and 200 mg l^{-1} kanamycin sulfate, respectively. In terms, of percentage, the corresponding values were 15.4±1.1, 14.5±1.1, and 11.4±1.0, respectively.

Effect of 200-400 mg l^{-1} kanamycin sulfate on development

The total number of 'Suardia' SA1.1 somatic embryos that developed tended (Pr>F=0.0922) to significantly affect by interaction of kanamycin sulfate concentrations and transformation treatments (Table 4-4). Development of somatic embryos from PEMs on SED differed significantly for nontransformed and transformed cultures (Table 4-2 and Table 4-4). Kanamycin sulfate itself did not significantly affect the total number of somatic embryos that developed; however, transformation treatment significantly affected the total number of somatic embryos.

Production of somatic embryos from nontransformed cultures was suppressed by increasing kanamycin sulfate concentration. The total number of somatic embryos from nontransformed cultures two months after plating on SED medium was 9.1±1.4, 3.4±0.3, and 2.1±0.4 on medium with 200, 300 and 400 mg l^{-1} kanamycin sulfate, respectively

(Figure 4-6). Production of somatic embryos on SED medium containing different concentrations of kanamycin sulfate is shown in Figure 4-7.

Table 4-4. ANOVA of the effect of 200, 300 and 400 mg l^{-1} kanamycin sulfate on somatic embryo development of nontransformed and transformed 'Suardia' SA1.1

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Total Somatic Embryo					
Model	5	64882.65	12976.53	89.97	<0.0001
Error	107	15433	144.23		
Correction Total	112	80315.88			
Lines	1	64002.67	64002.67	443.74	<0.0001
Kanamycin	2	176.77	88.39	0.61	0.5437
Lines*Kanamycin	2	703.20	351.60	2.44	0.0922
Number of SE <0.5 cm					
Model	5	31072.26	6214.45	98.22	<0.0001
Error	107	6770.23	63.28		
Correction Total	112	37842.50			
Lines	1	30656.34	30656.34	484.51	<0.0001
Kanamycin	2	169.45	84.73	1.34	0.2664
Lines*Kanamycin	2	246,47	123.24	1.95	0.1476
Percent of SE <0.5 cm*)					
Model	5	0.76	0.15	2.01	0.0829
Error	107	8.10	0.08		
Correction Total	112	8.86			
Lines	1	0.05	0.05	0.69	0.4073
Kanamycin	2	0.45	0.22	2.98	0.0548
Lines*Kanamycin	1	0.26	0.13	1.70	0.1883
Number of SE >0.5 cm					
Model	5	1360.61	272.12	58.09	<0.0001
Error	107	501.24	4.68		
Correction Total	112	1861.86			
Lines	1	1285.64	1285.64	274.44	<0.0001
Kanamycin	2	51.28	25.64	5.47	0.0055
Lines*Kanamycin	2	23.69	11.85	2.53	0.0845
Percent of SE >0.5 cm*)					
Model	5	3.29	0.66	265.04	<0.0001
Error	107	0.26	0.00		
Correction Total	112	3.55			
Lines	1	3.23	3.23	1319.68	<0.0001
Kanamycin	2	0.04	0.02	8.85	0.0003
Lines*Kanamycin	2	0.02	0.02	3.92	0.0228
Number of Hyperhydric SE					
Model	5	2015.08	403.01	16.79	<0.0001
Error	107	2567.93	24.00		
Correction Total	112	4583.01			
Lines	1	1767.54	1767.54	73.65	<0.0001
Kanamycin	2	157.72	78.86	3.29	0.0412
Lines*Kanamycin	2	89.82	44.91	1.87	0.1589
Percent of hyperhydric SE*)					
Model	5	0.46	0.09	1.24	0.2951
Error	107	7.89	0.07		
Correction Total	112	8.35			
Lines	1	0.13	0.13	1.83	0.1792
Kanamycin	2	0.26	0.13	1.76	0.1773
Lines*Kanamycin	2	0.06	0.03	0.43	0.6512

Lines = Transformation treatments; Nontransformed and Transformed 'Suardia' SA1.1; Kanamycin = 200, 300 and 400 mg l^{-1} kanamycin sulfate; Lines*Kanamycin = Interaction between Lines and Kanamycin;
*) Percentage data was transformed by arc sin transformation

Kanamycin sulfate concentrations of 200 – 400 mg l^{-1} did not significantly suppress somatic embryo development from transformed 'Suardia' SA1.1. The total production of somatic embryos was 53.0±3.3, 59.4±0.3, and 56.6±2.2 embryos per dish on medium supplemented with 200, 300 and 400 mg l^{-1} kanamycin sulfate. Nontransformed 'Suardia' SA1.1 produced <10 somatic embryos per plate (mean = 4.9), while transformed SA1.1 produced >50 (mean = 56) somatic embryos per plate (Figure 4-6).

Somatic embryos that developed were categorized as being small (<0.5 cm), large (>0.5 cm) opaque-white, and hyperhydric. Kanamycin sulfate at 200 mg l^{-1} or greater inhibited somatic embryo development/maturation from nontransformed cultures, so there were no somatic embryos that were >1.0 cm. The ANOVA result can be seen in Table 4-4. The number of small opaque somatic embryos was significantly affected by transformation treatment but not by kanamycin sulfate concentration. There was no interaction between kanamycin sulfate concentration and transformation treatment (Table 4-4). In terms of the percentage of small somatic embryos with respect to total somatic embryos, transformation treatments and their interaction with kanamycin sulfate concentrations did not have a significant effect while kanamycin sulfate concentrations alone had a significant effect (Table 4-4).

The number of small (<0.5 cm) embryos from nontransformed SA1.1 2 months after culturing was 7.5±1.3, 2.2±0.3, and 1.2±1.4 embryos per plate on medium containing 200, 300 and 400 mg l^{-1} kanamycin sulfate, respectively (Table 4-5). The percentage of small opaque somatic embryo was 81.5±5.0, 67.4±9.8, and 60.0±11.8%, respectively. The production of somatic embryos <0.5 cm in length from transformed

SA1.1 embryogenic cultures was 38.8±2.3, 41.1±1.6, and 37.9±1.5 somatic embryos per plate on SED media supplemented with 200, 300 and 400 mg l^{-1} kanamycin sulfate, respectively. In terms of percentage, these values corresponded to 74.0±1.5, 70.5±2.0 and 67.4±1.3%.

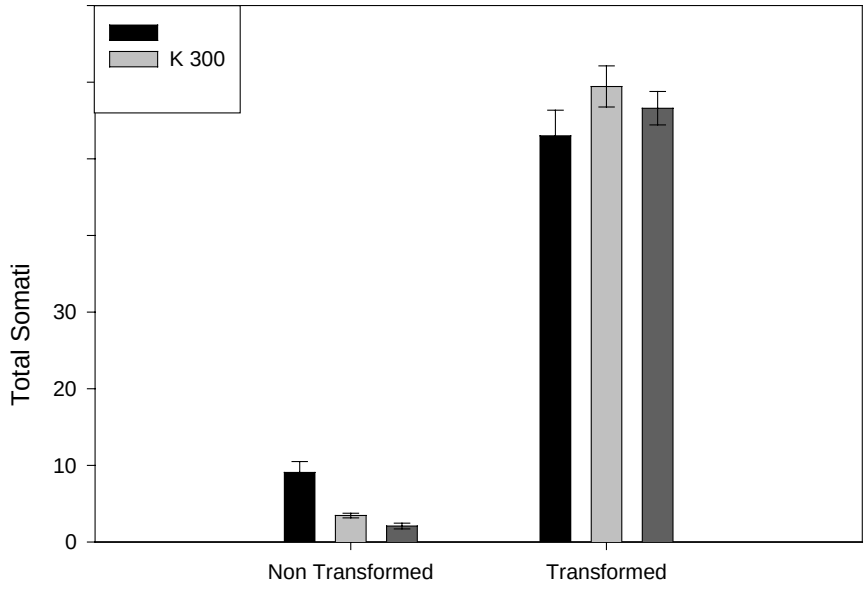


Figure 4-6. Effect of kanamycin sulfate on somatic embryo development from non-transformed and transformed 'Suardia' SA1.1 cultures two months after plating on somatic embryo development medium. K 200, K 300 and K 400 represent kanamycin sulfate concentrations of 200, 300 and 400 mg l^{-1}, respectively. Data represent means±SE of 12 and 26 replications for nontransformed and transformed 'Suardia' SA1.1.

There was a slight interaction (Pr>F=0.0845) between kanamycin sulfate concentration and transformation treatment with respect to number of somatic embryos >0.5 cm in length (Table 4-4). Kanamycin sulfate concentrations and transformation treatments individually had a very significant effect on number of somatic embryo >0.5 cm. In terms of percentage with respect to total somatic embryo development, there was an interaction between kanamycin sulfate concentration and transformation treatment,

and individually, kanamycin sulfate and transformation treatments had a significant effect.

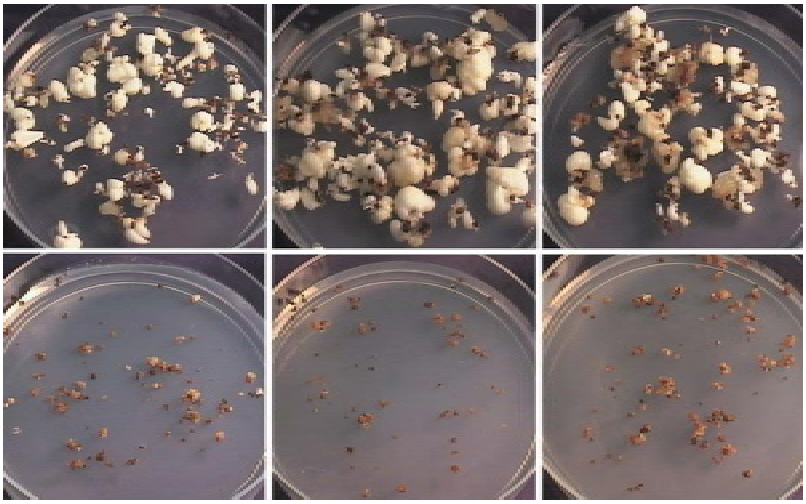


Figure 4-7. Effect of kanamycin sulfate on somatic embryo development from non-transformed (lower row) and transformed (upper row) 'Suardia' SA1.1 two months after culturing on SED medium containing kanamycin sulfate. Column 1, 2, and 3 (from left) represent kanamycin sulfate concentrations of 200, 300 and 400 mg l^{-1}, respectively.

Somatic embryos >0.5 cm in length did not develop from nontransformed embryogenic cultures on any concentration of kanamycin sulfate. Development of opaque somatic embryos >0.5 cm in length from transformed embryogenic cultures of 'Suardia' SA1.1 was significantly affected by kanamycin sulfate concentration. Total number of somatic embryos >0.5 cm that develop from transformed SA1.1 cultures were 6.7±0.4, 6.7±0.5, and 8.7±0.6 on SED containing 200, 300 and 400 mg l^{-1} kanamycin sulfate, respectively. These values corresponded to 13.0±0.7, 10.9±0.7, and 15.5±1.0% of total embryo production (Table 4-5). The maximum production of somatic embryos occurred on 400 mg l^{-1} kanamycin sulfate.

There was no interaction between kanamycin sulfate concentration and transformation treatment with respect to number and percentage of hyperhydric somatic

embryos. The production of hyperhydric somatic embryos was affected by kanamycin sulfate concentrations and embryogenic lines (nontransformed and transformed lines) (Table 4-4). In terms of the percentage of the total number of somatic embryo, production of hyperhydric somatic embryo was not significantly affected by kanamycin sulfate concentration and transformation treatment (Table 4-4).

Table 4-5. Effect of kanamycin sulfate on somatic embryo development from nontransformed and transformed 'Suardia' SA1.1 cultures. K200, K300 and K400 represent kanamycin sulfate concentrations of 200, 300 and 400 mg l^1, respectively. Data represent the total number and percentage of somatic embryo means±SE of 12 replications of nontransformed and 26 replicationsof transformed cultures.

Somatic Embryo		Nontransformed			Transformed		
		K200	K300	K400	K200	K300	K400
<0.5 cm	Number	7.5±1.3	2.2±0.3	1.2±0.4	38.8±2.3	41.1±1.6	37.9±1.5
	%	81.5±5.0	67.4±9.8	60.0±11.8	74.0±1.5	70.5±2.0	67.4±1.3
>0.5 cm	Number	0.0±0.0	0.0±0.0	0.0±0.0	6.7±0.4	6.7±0.5	8.7±0.6
	%	0.0±0.0	0.0±0.0	0.0±0.0	13.0±0.7	10.9±0.7	15.5±1.0
Hyperhydric	Number	1.6±0.4	1.3±0.4	0.8±0.3	7.5±1.1	11.8±1.3	10.0±0.9
	%	18.5±5.0	32.6±9.8	40.0±11.0	13.0±1.4	18.6±1.7	17.1±1.2

There were fewer hyperhydric somatic embryos from nontransformed 'Suardia' SA1.1 cultures, i.e., 1.6±0.4, 1.3±0.4, and 0.8±0.3 per plate on SED containing 200, 300, and 400 mg l^{-1} kanamycin sulfate, respectively (Table 4-5). In terms of percentage of hyperhydric somatic embryos with respect to the total number of somatic embryos, the corresponding value was 18.5±5.0, 32.6±9.8, and 35.4±11.0%. There were 7.5±1.1, 11.8±1.3, and 10.0±0.9 hyperhydric transformed somatic embryos per plate on medium supplemented with 200, 300, and 400 mg l^{-1} kanamycin sulfate, respectively (Table 4-5). In terms of percentage with respect to the total number of somatic embryos, the corresponding value was 13.0±1.4, 18.6±1.7, and 17.1±1.2%.

Somatic Embryo Germination

The percentage of germinating somatic embryos that originated from transformed cultures on kanamycin sulfate-containing SED medium three months after culturing can be seen in Figure 4-8. Transformed somatic embryos that formed roots without shoots and a plantlet are demonstrated in Figure 4-9. The percentage of shoot formation from 7 large opaque somatic embryos per plate was significantly affected by the origin of the somatic embryos. The percentage of somatic embryos forming shoots was 7.4±1.9, 16.8±3.7, and 11.2±3.0 for somatic embryos that developed on SED medium containing 200, 300, and 400 mg l^{-1} kanamycin sulfate, respectively (Figure 4-8). The highest percentage of shoot formation occurred from somatic embryos that originated from SED with 300 mg l^{-1} kanamycin sulfate, but this was not statistically different from the response with 400 mg l^{-1} kanamycin sulfate.

The frequency of somatic embryos that formed roots was lower than the frequency of somatic embryos that formed shoots. Root formation occurred from only 5.2±1.5% of somatic embryos that developed on SED medium with 200 mg l^{-1} kanamycin sulfate, and was 2.4±1.3% from somatic embryos that developed on SED medium supplemented with 400 mg l^{-1} kanamycin sulfate. Somatic embryos that developed on medium with 200 mg l^{-1} kanamycin sulfate did not form roots.

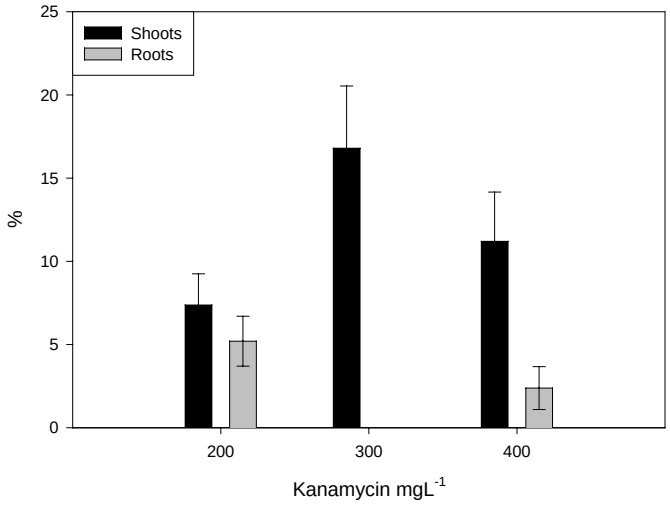


Figure 4-8. Percentage (7 embryos per plate) of opaque transformed somatic embryos that germinated on germination medium without kanamycin sulfate. Transformed somatic embryos were harvested from SED medium supplemented with 200, 300 and 400 mg l^{-1} kanamycin sulfate. Data represent means±SE from 33, 17, 18 replications for somatic embryos that originated from SED medium supplemented with kanamycin sulfate at 200, 300, and 400 mg l^{-1}, respectively.

Figure 4-9. Somatic embryo with a root but no shoot (left) and plantlet from germinated somatic embryo (right) from 'Suardia' SA1.1 transformed with *samK* and *nptII*.

Discussion

Growth in Suspension

Many angiosperm woody species have been successfully transformed by *Agrobacterium tumefaciens*, *A. rhizogenes*, or particle bombardment-based protocols, using either the organogenic or embryogenic pathway. *Citrus* species are probably the most studied among woody tree fruit species, and most citrus transformation has been achieved through organogenesis, i.e., sweet orange (*Citrus sinensis*) (Bond and Roose, 1998; Yu et. al., 2002), grapefruit (*Citrus paradisi*) (Luth and Moore, 1999; Yang et al., 2000; Costa et al., 2002), trifoliate orange (*Poncirus trifoliate*) (Kaneyoshi et al., 1999); Citrange (*C. sinensis x Poncirus trifoliate*) (Peña et. al., 1995) and Mexican lime (*Citrus aurantifolia*) (Peña et al., 1997; Dominguez et al., 2000). Epicotyl or internodal stem segments of citrus are co-cultivated on medium supplemented with 50 – 200 mg l^{-1} kanamycin sulfate in order to select for transformed organogenic shoots (Kaneyoshi et al., 1994; Peña et al., 1995; 1997; Bond and Roose, 1998; Luth and Moore, 1999; Dominguez et al., 2000; Yang et. al., 2002; Yu et al., 2002;).

Transformation of embryogenic cultures of citrus has also been reported, i.e., Poncan citrus (*Citrus reticulata*) (Li et al., 2002) and tangelo (*C. reticulata x C. paradisi*) (Yao et al., 1996). Transformed embryogenic citrus cultures are selected on semi solid medium supplemented with 100 mg l^{-1} kanamycin for 2-3 subcultures and then on medium with kanamycin 200 mg l^{-1} (Yao et al., 1996). Somatic embryos develop on medium without selection pressure.

Several other woody species also have been transformed using embryogenic cultures, i.e., mango (*Mangifera indica*) (Mathews et al., 1992); tea (*Camellia sinensis*) (Mondal et al., 2001), walnut (*Juglans regia*) (McGranahan et al., 1990; Tang et al.,

2000), pecan (*Carya illinoensis*) (McGranahan et al., 1993), sandalwood (*Santalum album*) (Shiri and Rao, 1998), cherry (*Prunus Subhirtella autumno rosa*) (Machado et al., 1995) and grape (*Vitis vinifera*) (Herbert et al., 1993; Nakano et al., 1994; Scorza et al., 1995).

Embryogenic cultures grow more efficiently in liquid medium than on semi solid medium (Witjaksono et al., 1999; Litz et al., 1993; Viana and Mantell 1999). Suspension cultures can be synchronized and single cell and small cell aggregates can be easily separated by sieving (von Arnold et al., 2002). This is difficult to achieve with cultures that grow on semi solid medium.

Selection of transformed cultures in liquid medium is more precise than on semi solid selection medium. Mathews and Litz (1990) reported that growth of nontransformed mango proembryos was arrested in liquid medium with only 12.5 mg l^{-1} kanamycin sulfate, while growth on semi solid medium was inhibited by 200 mg l^{-1} kanamycin. Furthermore, in order to recover purely transformed mango cultures, transformed cultures that were selected on semi solid medium with 400 mg l^{-1} kanamycin must be transferred to liquid medium with 100 mg l^{-1} kanamycin in order to eliminate "escapes" (Mathews et al., 1992). In liquid medium, the proembryonic masses are bathed with the selection agent, which prevents the occurrence of escaped nontransformed cultures. However, cultures that are maintained on semi solid medium are needed to back up the suspension cultures.

In this experiment, a single step selection of transformed cultures in liquid medium was successfully used, where transformed cultures were directly selected in liquid medium supplemented with 100 mg l^{-1} kanamycin sulfate. Cruz-Hernández et al.

(1998) and Mathews et al. (1992) used a two-step selection protocol to recover transformed embryogenic avocado and mango cultures. According to Cruz-Hernandez et al. (1998) transformed cultures were first selected in liquid medium containing 50 mg l^{-1} kanamycin sulfate for 2-4 months, and then selected in medium with kanamycin sulfate 100 mg l^{-1} for 2 months. A similar stepwise selection strategy was used to select transformed embryogenic mango cultures since transformed mango somatic proembryos carrying *nptII* gene and nontransformed cells could grow in the presence of 200 mg l^{-1} kanamycin sulfate resulting in chimeral PEMs (Mathews et al., 1992). Stepwise selection was therefore used to eliminate chimeras and cultures were selected first in medium with 200 mg l^{-1} kanamycin sulfate, and later in medium with 400 mg l^{-1} kanamycin sulfate.

The kanamycin sulfate concentration used for selecting transformed embryogenic avocado cultures in this study was higher than the level used for many species. For example, transformed embryogenic cultures have been selected on semi solid medium supplemented with kanamycin concentrations ranging from 25 mg l^{-1} with sandalwood (Shiri and Rao, 1998), 20-40 mg l^{-1} with grape (Scorza et al., 1995), 50 mg l^{-1} with pecan (McGranahan et al., 1993), grape (Nakano et al., 1994) and tea (Mondal et al., 2001) and 75 mg l^{-1} with cherry (Machado et al., 1995). Comparable concentrations of 100 mg l^{-1} kanamycin have been reported with walnut (McGranahan et al., 1990; Tang et al., 2000) and grape (Herbert et al., 1993). For selection of transformed embryogenic cassava (*Manihot esculenta*), the kanamycin concentration ranged from 10 mg l^{-1} (Schreuder et al., 2001) to 175 mg l^{-1} (Schopke et al., 1996).

After four weeks, kanamycin sulfate at 100 mg l^{-1} suppressed growth of nontransformed embryogenic avocado suspension cultures by >60% . This suppression

was greater than the effect of 50 mg l^{-1} kanamycin, i.e., approx. 50% (Cruz-Hernandez et al., 1998). Growth of transformed cultures after 4 weeks was not affected by 100 mg l^{-1} kanamycin sulfate, relative to the control. Increasing kanamycin sulfate concentration to 200 and 300 mg l^{-1} suppressed growth of transformed cultures by 34 and 46% relative to growth on medium without kanamycin sulfate. These results are comparable to previous research (Cruz-Hernandez et al., 1998); 400 mg l^{-1} kanamycin was toxic to the cultures (Cruz-Hernandez et al., 1998).

Transformed embryogenic avocado culture growth rate in medium without and with 100 mg l^{-1} kanamycin sulfate was similar over a period of 5 weeks of culture. Cruz-Hernández et al. (1998) reported similar results for 4 weeks of culture. However, growth of nontransformed suspension cultures was not completely arrested by 100 mg l^{-1} kanamycin, but was suppressed at 37, 53, 63 and 60% at 2, 3, 4 and 5 weeks after explanting. This data indicates that some nontransformed or chimeral proembryogenic masses can develop in liquid medium supplemented with 100 mg l^{-1} kanamycin sulfate. Since the gene construct used does not have a reporter gene, the percentage of nontransformed, transformed or chimeral PEMs cannot be demonstrated. Maintenance of transformed embryogenic avocado cultures carrying the *nptII* gene in liquid medium supplemented with 100 mg l^{-1}kanamycin sulfate, however, is sufficient for proliferation and selection of transformed PEMs provided that they are used as materials for further selection during somatic embryo development on kanamycin-containing SED medium. However, if PEMs from maintenance medium are the final product for DNA analysis, they must be cultured in medium with 200 or 300 mg l^{-1} kanamycin sulfate to minimize "escapes" of nontransformed PEMs.

Somatic Embryo Development

Somatic embryo development and maturation from transformed embryogenic cultures can be achieved on a single medium [somatic embryo development (SED) medium] (Witjaksono and Litz 1999b) supplemented with kanamycin sulfate as a selection agent. Cruz-Hernandez et al. (1998) used a two-step process: 1) PEMs were plated on maturation medium without kanamycin; 2) somatic embryos were transferred onto medium with the same formulation supplemented with kanamycin sulfate. This two-step process can result in production of fewer mature somatic embryos since some of the small somatic embryos will die following their transfer onto new medium.

Nontransformed embryogenic cultures of 'Suardia' SA1.1 can produce a few (i.e., 11%) medium- to large-sized opaque somatic embryos on SED medium supplemented with 100 mg l^{-1}kanamycin sulfate. Kanamycin sulfate at 100 mg l^{-1} does not totally inhibit somatic embryo growth/maturation. Therefore, somatic embryo development from transformed PEMs on SED medium supplemented with 100 mg l^{-1} kanamycin sulfate risks the development of nontransformed somatic embryos. This problem can be solved by increasing selection pressure in liquid maintenance medium to 200 – 300 mg l^{-1} kanamycin and/or on somatic embryo development medium with kanamycin up to 400 mg l^{-1}.

The growth and maturation of opaque medium (0.5-1.0 cm) and large (>1.0 cm) somatic embryos from nontransformed cultures were completely arrested on medium containing 200 mg l^{-1} kanamycin sulfate. Similar results were reported, in which 200 mg l^{-1} kanamycin sulfate totally inhibited somatic embryo maturation from embryogenic avocado (Cruz-Hernández et al., 1998) and mango cultures (Mathews and Litz, 1990; Mathews et al., 1992). In the presence of kanamycin in somatic embryo development

medium, nontransformed cultures produced only few small opaque and hyperhydric somatic embryos, which are unlikely to reach maturity, whereas large opaque, mature transformed 'Suardia' SA1.1 somatic embryos were produced on SED medium supplemented with up to 400 mg l^{-1} kanamycin sulfate. To reduce the risk of escaped nontransformed opaque somatic embryos, somatic embryo development should be achieved on SED medium supplemented with 200 mg l^{-1} of greater kanamycin sulfate. An alternative is to use stepwise selection (Mathews et al., 1992), where escape PEMs can be minimized in liquid maintenance medium by increasing the kanamycin sulfate to 300 mg l^{-1}, since 400 mg l^{-1} kanamycin sulfate is toxic to embryogenic avocado cultures (Cruz-Hernández et al. 1998).

Increasing kanamycin sulfate concentrations to >200 mg l^{-1} for somatic embryo selection is less effective since it increases the percentage of hyperhydric somatic embryo. Hyperhydricity of somatic embryos has been a limiting factor for somatic embryogenic systems (Monsalud, et al., 1995) because these embryos are incapable of development to maturity (Lad et al., 1997). Hyperhydricity is a result of optimizing in vitro growth medium composition, plant growth regulator, culture vessel type, solidifying agent and water content (Teasdale, 1997; Ziv, 1991a,b), where the cells take up excess water (Pierik, 1988). Benzyladenine (BA) increases somatic embryo hyperhydricity in mango (Monsalud, et al., 1995). Increasing Gel Gro concentration to 7-10 g l^{-1} decreased somatic embryo hyperhydricity in avocado (Witjaksono, 1997).

On SED medium without selection agent, transformed 'Suardia' SA1.1 produced more somatic embryos than nontransformed cultures. Kanamycin sulfate may have

suppressed growth of the less embryogenic cells and caused the cultures to be more synchronized.

Somatic Embryo Germination

Somatic embryo germination and plantlet conversion is a problem with embryogenic cultures. In this study, approx. 7 to 17% of transformed somatic embryos that originated on SED medium supplemented with kanamycin sulfate formed shoots, and only 0-5% formed roots. This frequency of shoot and root formation is due neither to the transformation process nor to a kanamycin sulfate residual effect. Nontransformed 'Suardia' SA1.1 produced shoots and roots at a similar frequency of 0-7% (Chapter 6). This is in agreement with earlier studies (Pliego-Alfaro and Murashige, 1987; Mooney and Van Staden, 1988; Witjaksono, 1997). Witjaksono and Litz (1999b) reported that after 9-10 months in maintenance medium, 2.5% of 'T362' and 5% of 'Booth 7' somatic embryos are able to produce shoots, while somatic embryos of 'Isham' did not develop any shoots. In this study there were no somatic embryos that developed both a shoot and a root. Most avocado somatic embryos lack bipolarity (Pliego-Alfaro and Murashige, 1987; Witjaksono et al., 1999).

More basic research on medium composition, plant growth regulators, and physical environment of induction and maintenance medium is needed to increase production of bipolar somatic embryos. Low levels of endogenous auxin are required to establish polarity and allow bipolar growth in embryogenic cultures. Moreover cytokinins stimulate meristem organization (Michalczuk et al., 1992). Exogenous auxin added to medium to arrest histodifferentiation of somatic embryos may disturb polarity (Michalczuk et al., 1992). The auxin analog, 2,4-D for example, increases endogenous auxin (IAA) in carrot cells (Michalczuk et al., 1992) and in alfalfa (Pasternak et al.,

2002). Lowering the exogenous auxin analog (picloram) by reducing its concentration in the maintenance medium needs to be studied.

The standard practice for producing plantlets is to root the emerging shoots with several subcultures in rooting medium (Witjaksono et al., 1999). Another procedure involves the rescue of emerging shoot by micrografting them aseptically on in vitro-grown zygotic seedlings of avocado (Raharjo et al., unpublished data). This requires many avocado seeds, which are seasonally available.

Although the avocado seed is recalcitrant and does not become desiccated during development, in vitro induced desiccation may increase the frequency of germination. Viana (1997) and Viana and Mantell (1999) reported that *Ocotea catharinensis* (Lauraceae) germination can be increased by desiccating somatic embryos. Low frequency of plant recovery of tea somatic embryos has been addressed by the temporary immersion system (RITA), and resulted in synchronized multiplication and embryo development with a high frequency of plant recovery (Akula et al., 2000). Manipulation of desiccation and rehydration of somatic embryos by controlling immersion time in the temporary immersion system increased plant recovery up to 6x compared to the traditional method involving semi solid medium (Akula et al., 2000).

Conclusion

Liquid MS3:1P medium supplemented with 100 mg l^{-1} kanamycin sulfate can be used to maintain transformed embryogenic avocado cultures carrying the *nptII* kanamycin resistant gene since it does not reduce the growth of transformed PEMs; however, some cultures that have escaped transformation may also grow in this formulation. To avoid escape PEMs, a higher concentration of kanamycin sulfate up to (300 mg l^{-1}) must be used.

Kanamycin sulfate at 100 to 400 mg l^{-1} inhibited somatic embryo development (expressed as somatic embryo <0.5 cm in length) and maturation of somatic embryos (expressed as somatic embryo ≥0.5 cm in length) of nontransformed 'Suardia' SA1.1. At 200 mg l^{-1} kanamycin sulfate, the growth of somatic embryos of nontransformed cultures was totally suppressed. However, transformed 'Suardia' SA1.1 somatic embryos carrying *samK* and *nptII* genes were not affected by up to 400 mg l^{-1} kanamycin sulfate. SED medium supplemented with 200 mg l^{-1} kanamycin sulfate can be used for development and maturation of somatic embryo from transformed embryogenic culture without escapes.

CHAPTER 5
CRYOPRESERVATION OF EMBRYOGENIC AVOCADO (*Persea americana* MILL.) CULTURES

Introduction

Embryogenic avocado cultures can be initiated from early developmental stages of zygotic embryos (Pliegro-Alfaro and Murashige 1988; Mooney and van Staden, 1987; Raviv et al., 1998; Witjaksono 1997; Witjaksono and Litz, 1999a, 1999b) and the nucellus (Witjaksono et al., 1999).

Embryogenic cultures experience developmental problems such as a low rate of germination of somatic embryos and plantlet conversion, and loss of embryogenic competence during the maintenance phase. These responses appear to be strongly genotype-dependent. Loss of embryogenic competence can occur as early as 3 months after induction (Witjaksono and Litz, 1999a). As a result, it is difficult to use embryogenic cultures of some genotypes for medium and long-term research, and embryogenic cultures must be induced annually. The loss of embryogenic competence also contributes to problems associated with developing elite lines of embryogenic cultures that have been genetically engineered. Long-term storage is critical to overcome these problems.

Cryopreservation involves storage at ultra-low temperature (-196^{o}C) with liquid nitrogen being the most widely used cryogen (Grout, 1995). There is cessation of biological activity at this temperature and material can be stored for extremely long periods (Grout, 1995; Benson, 1997). Cryopreservation can be achieved either by slow

cooling or fast cooling (vitrification) which differ with respect to precooling protection and cooling rate (Grout, 1995; Benson, 1997). Slow cooling involves the following protocol: pretreatment and incubation of materials with cryoprotectants, slow cooling (0.5-1.0 ^{o}C min^{-1}) to –40^{o}C, immersion in liquid nitrogen, thawing in a water bath at 35-40^{o}C, removal of cryoprotectant and restoration of osmolarity of cultures to physiological level (Grout, 1995; Benson, 1997). Vitrification refers to the phase transition of water from liquid directly into a vitreous state, a non-crystalline or amorphous phase, by rapidly increasing viscosity during fast cooling (Fahy et al., 1984; Engelmann, 1991; Grout 1995; Benson, 1997). Cryopreservation by vitrification is a simplified cryopreservation procedure involving rapid cooling by direct immersion into liquid nitrogen (Sakai et al., 1990; Grout, 1995; Benson, 1997). The procedure consists of a two-step cell protection phase: 1) loading phase by a loading solution and 2) dehydration phase using a vitrification solution. Cultures are directly immersed into liquid nitrogen, and after thawing; the cryopreserved cultures are osmoconditioned with high a concentration of sucrose. Other approaches to cryopreservation involve the use of desiccation and encapsulation either by slow cooling or vitrification (Engelmann, 1991; Bajai, 1995; Benson, 1997).

Cryopreservation protocols have been developed for only a few tropical tree species, e.g. cacao (*Theobroma cac*ao) (Pence, 1991), citrus (*Citrus spp*) (Sakai et al., 1990; Kobayashi et al., 1990; Marin and Duran-Vila, 1988; Radhamani and Chandel, 1992; Marin et al., 1993; Sakai et al., 1991; Pérez et al., 1997, 1999; Gonzales-Arnao et al., 1998; Cho et al., 2001), coffee (*Coffea arabica*) (Dussert et al., 2000), rubber (*Hevea brasiliensis*) (Engelmann and Etienne, 1995), jackfruit (*Artocarpus heterophylus*)

(Thammasiri, 1999), oil palm (*Elaeis guineensis*) (Dumet et al., 1993), and tea (*Camellia sinensis*) (Chaudhury et al., 1991), ornamental camellia (*Camellia japonica*) (Janeiro et al, 1996). Some non-woody tropical species have also been cryopreserved, i.e., banana (*Musa spp*.) (Helliot et al., 2002), cassava (*Manihot esculenta*), orchid (*Phalaeonopsis x Doritis*) (Ishikawa et al., 1997), rice (*Oryza sativa*) (Jain et al., 1996; Wang et al., 1998; Watanabe et al., 1995 and 1999), sugarcane (*Saccharum officinalis*) (Martinez-Montero, 1998), sweet potato (*Ipomoea batatas*) (Pennycooke and Towill, 2000, 2001), and taro (*Colocasia esculenta*) (Takagi, 1997).

Plant material used for cryopreservation experiments has included the following: somatic embryos, embryogenic cells, protoplasts, zygotic embryo axes, seeds, shoot tips and apices. Cryopreservation techniques that have been used, i.e., 1) cryoprotectant treatment follow by slow cooling; 2) vitrification using plant vitrification solution; 3) desiccation followed by either slow cooling or fast cooling; and 4) encapsulation-dehydration follow by slow cooling.

The aim of this study has been to develop a cryopreservation procedure for embryogenic avocado cultures.

Materials and Methods

Embryogenic Culture Induction

Embryogenic cultures were induced from immature zygotic embryos of avocado (*Persea americana* Mill.). Avocado fruits ⩽1.0 cm in length representing different cultivars of different races were collected from the University of Florida Tropical Research and Education Center (Homestead, FL), the USDA-ARS Subtropical Horticultural Research Station (Miami, FL) National Avocado Germplasm Repository and the germplasm collection of the University of California (Riverside, CA) (Table 5-1).

Table 5-1. Avocado cultivars used for the experiments, their botanical description, source and time of explanting

Cultivar	Race	Source	Explanting Time
'Booth 7'	G x WI [1]	UF-TREC, Homestead	March, 2000
'Suardia'	WI?	USDA-ARS, Miami	May, 2001
'Hass'	(G x M) x G	UC Riverside	June, 1999
'Fuerte'	G x M[1]	UC Riverside	June, 1999
'T362'	G[1]	UC Riverside	June, 1999

Note: 1) Smith et al., (1992) M=Mexican, G=Guatemalan, WI= West Indian

The immature fruits were surface-disinfested in a 20% (V/V) solution of commercial bleach supplemented with 10–20 drops of Tween 20® per liter for 20 min after the sepals and peduncles were removed. The immature fruits were rinsed with two changes of sterile, deionized water in a laminar flow hood. They were bisected longitudinally under sterile conditions, and the immature seed was removed from each immature fruit. Six seed halves from three immature fruits were cultured on semi solid induction medium so that each zygote embryo was in contact with the medium in a 65 x 15 mm Petri dish containing 10–15 ml medium. Petri dishes were sealed with Parafilm® and the cultures were maintained in darkness at room temperature (25^{o}C).

Induction medium was B5^{+}, which consisted of B5 major salts (Gamborg et al., 1968), MS (Murashige and Skoog, 1962) minor salts, 0.41 µM picloram and (in mg l^{-1}) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and solidified with TC agar (8,000) (Sigma) (Witjaksono, 1997; Witjaksono and Litz, 1999a). The pH was adjusted to 5.7 with 0.1–1.0 N KOH or HCl prior to autoclaving at 1.1 kg cm^{-2} and 120^{o}C for 20 min.

Culture Maintenance

Embryogenic cultures consisting of proembryonic masses (PEMs) and early cotyledonary somatic embryos that developed on induction media were transferred onto

fresh semi solid MSP medium (30-35 ml in each 100 x 20 mm Petri dish). This medium is MS basal medium, supplemented with 0.41 μM picloram and (in mg l^{-1}) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and TC agar (8,000) (Witjaksono, 1997; Witjaksono and Litz, 1999a). The pH was adjusted to 5.7 with either KOH or HCl prior to addition of agar. Medium was sterilized by autoclaving at 1.1 kg cm^{-2} and 120°C for 20 min.

Embryogenic cultures were transferred to fresh MSP medium 2-4 weeks after zygotic embryos were explanted. Tissues were subcultured onto semi solid MSP at 3-5 week intervals thereafter. Proembryonic masses ⩽1.8 mm, which passed through sterile nylon filtration fabric with 1.8 mm mesh, was used as inocula. On each Petri dish were plated 7 inocula, one in the center and 6 around it, and each Petri dish was sealed with Parafilm®. Cultures on semisolid media were maintained in darkness at room temperature (25°C).

After 3 to 6 subcultures on semi solid MSP, embryogenic cultures were transferred into liquid MS3:1P media. This medium is a modification of MS basal medium containing 60 mM inorganic nitrogen in which 75% of nitrogen is NO_3^- and 25% is NH_4^+, and supplemented with induction medium addenda but without solidifying agent. The pH was adjusted to 5.7 prior to autoclaving (Witjaksono and Litz, 1999b). Approximately 0.5-1.0 g of embryogenic culture from semisolid medium was inoculated into 40 ml media in 125 ml Erlenmeyer flasks, which were capped with heavy duty aluminum foil and sealed with Parafilm® . The cultures were maintained on a rotary shaker at 125 rpm and 25°C with diffuse light, and were subcultured at 2-3 week intervals.

One-week to two-week-old embryogenic suspension cultures were used for all cryopreservation experiments. Cultures of 'Booth 7' and 'Suardia' were sieved through 1.8 mm nylon fabric and the small fraction was used, while 'Hass', 'Fuerte' and 'T362', which have smaller PEMs, were not sieved.

Slow Cooling Cryopreservation

Cryoprotectant

Preliminary studies indicated that cryoprotectant solution consisting of DMSO or glycerol alone in MS3:1 (MS3:1P without picloram) medium cannot result in viable cultures. Therefore, a combination of DMSO and glycerol in MS3:1 was utilized.

Different combinations of cryoprotectants were testing using 'Hass' line H3.2 and 'T362' line T1.8. Three combinations of DMSO and glycerol were used as cryoprotectant, i.e., 5% DMSO+5% glycerol, 10% DMSO+10% glycerol, and 15% DMSO+15% glycerol. Filter-sterilized DMSO and autoclave-sterilized glycerol were added to sterile MS 3:1 medium. The pH of MS3:1 medium was adjusted to 5.7 prior to autoclaving.

Cryopreservation

Cryoprotectant solution and vials were placed on ice in a laminar flow hood 30 min before experiments were initiated. Embryogenic suspension cultures were sieved using 1.8 mm nylon fabric. Proembryonic masses ⩽1.8 mm in diameter were collected and air dried on 16-20 layers of sterile Kimwipes® EX-L in Petri dishes in a laminar flow hood for approximately 1h. Approximately 200 mg of air-dried embryogenic culture was mixed with 1.0 ml cryoprotectant in 1.2 ml Corning® cryogenic vials and the vials were maintained on ice for 30 min. Vials were inserted into Nalgene® "Mr. Frosty" containers that contained 250 ml isopropanol. The containers were placed in a low

temperature (–80^{o}C) freezer for 2h for slow cooling (-1^{o}C min^{-1}). The vials were removed and inserted into aluminum cryo-cane holders, then immersed directly into liquid nitrogen (-196^{o}C) in a Taylor-Wharton 10LD Dewar container for three days. In this experiment 1 vial represented one experimental unit, with 10 replications and 3 treatments, i.e., 30 vials.

Thawing

In order to rapidly thaw the vials, they were removed from liquid nitrogen and immersed for 5 min in a 40^{o}C water bath. Cryoprotectant was removed from the vials using an Eppendorf pipette, and the embryogenic cultures were washed once with MS3:1 medium. Five vials of cryopreserved cultures were used for the tetrazolium chloride (TTC) viability test and the other five vials were plated onto semi solid MSP medium for recovery.

Vital staining

The post-thaw viability of cryopreserved embryogenic cultures was tested on the basis of the reduction of TTC (2,3,5-triphenyltetrazolium chloride) to form formazan by cellular respiration (Jain et al., 1996). After thawing and discarding the cryoprotectant, 1.0 ml of TTC solution was added to the vials and maintained at room temperature (25^{o}C) for 24 h. The TTC solution consisted of 0.6 % TTC powder (w/v) in phosphate buffer consisting of 3.752 g l^{-1} Na_2HPO_4 and 4.896 g l^{-1} potassium KH_2PO_4 and a drop of Tween® 80.

Viability was recorded after 24h using a dissecting microscope (8X-10X). One sample from each vial was placed on a small Petri dish and divided into three groups. The number of viable (red) and non-viable (white) PEMs were counted, and viability was expressed as the percentage of total red PEMs to total white + red PEMs. The average of

three counts for each vial with 4 to 5 replications for each treatment were used for ANOVA (SAS Institute, 2000).

Regrowth

Cryopreserved embryogenic cultures were plated on MSP medium for recovery. The cultures from each vial were divided into 5 clump inocula and plated at five spots on 15 ml medium in a 60 x 15 mm Petri dishes, with one inoculum in the middle of the dish and four others around it. Petri dishes were sealed with Parafilm® and maintained in darkness at 25^{o}C.

Data were collected as the number of clumps that showed regrowth in each Petri dish. Percentage regrowth referred to the percent of proliferating embryogenic culture clumps out of 5 clumps. Data were analyzed with ANOVA to determine treatment effects (SAS Institute, 2000). Data were plotted using Sigma Plot™ (Jandel Scientific, San Raphael, CA).

Cultures that proliferated after removal from liquid nitrogen were subcultured either onto semi solid MSP or into liquid MS 3:1P medium, and were subcultured monthly on semisolid and biweekly in liquid media. Embryogenic cultures consisting of PEMs up to 0.9 mm in diameter were used as inocula to form 0.2-0.4 cm diameter colonies. Seven inocula were plated on each Petri dish, one in the middle and six around it.

'Fuerte', 'Suardia' and 'T362'

The experiment above was repeated with 'Fuerte' F3.2, 'Suardia' SA1.1 and 'T362' T1.8 embryogenic lines, with 5% DMSO and 5% glycerol in MS3:1 medium as the cryoprotectant. Experiments involving 'Fuerte' F3.2 and 'Suardia' SA1.1 were to compare the rate of proliferation, while the experiment with 'T362' T1.8 addressed type

of embryogenic culture that could grow on the recovery media. The protocols were similar to the previous experiment, except for the type of cryogenic vial and the liquid nitrogen container. In this experiment, 1.2 ml O-ring internal thread Corning® cryogenic vials were used instead of externally threaded vials. A 20 Liter 'Bio Cane™ 20 can and cane' system was used instead of the 'Taylor-Wharton 10LD' Dewar container.

Effect of 5% DMSO+5% glycerol+1.0 M sucrose

The combination of 5% DMSO and 5% glycerol was used for 'Suardia' SA1.1 and 'Fuerte' F3.2; however, MS3:1 media was supplemented with 1.0 M sucrose (Kobayashi et al., 1990; Engelmann and Etienne, 1995), instead of 0.13 M sucrose of regular MS3:1. Approximately 200 mg air-dried embryogenic culture was mixed with 1.0 ml cryoprotectant in 1.2 ml O-ring internal thread Corning® cryogenic vials and the vials were maintained on ice for 30 min. Vials were inserted into Nalgene® "Mr. Frosty" containers that contained 250 ml isopropanol and were placed in a –80^{o}C freezer for 2h. The vials were removed and directly immersed into liquid nitrogen (-196^{o}C) in a 'Bio Cane™ 20 can and cane system' for 24h. Except for the cryoprotectant combination, other protocols were similar to the previous experiment.

The recovery of cryopreserved 'Suardia' SA1.1 and 'Fuerte' F3.2 with respect to recovery media and washing treatments were observed. Two different media, i.e., semi solid MSP maintenance medium and B5^{+} induction medium, were tested. Two washing treatments also were tested; 1) washing the cultures with liquid MS3:1 medium directly after thawing and removal of cryoprotectant; and 2) directly plating without washing. There were 18 vials each for 'Fuerte' F3.2 and 'Suardia' SA1.1: nine vials for washing and nine for non-washing treatments. From these nine vials, the cultures of three vials

were used for TTC staining, and the content of three vials were plated on MSP medium and three vials on $B5^{+}$ medium. Recovery data, which was the number of PEM clumps that proliferated, were collected two weeks after plating.

Fast Cooling Cryopreservation (Vitrification)

Vitrification Solution

The loading solution consisted of 2.0M glycerol and 0.4M sucrose (Sakai et al., 1990). Modified Plant Vitrification Solution Number 2 (PVS2) (Sakai et al., 1990) consists of 15% (v/v) DMSO, 30% (v/v) glycerol and 15% (v/v) ethylene glycol in MS3:1 medium supplemented with 0.4M sucrose. Both loading solution and PVS2 were filter-sterilized.

Vitrification

Suspension cultures of 'Booth 7' line B7.1, 'Fuerte' line F3.2 and 'Suardia' line SA1.1 were sieved through sterile 1.8 mm nylon fabric. The small PEM fraction was collected, and air-dried on 16-20 layers of sterile Kimwipes® EX-L in Petri dishes under a laminar hood for 1h. Approximately 200 mg of PEMs of each line were transferred into 1.8 ml internal thread cryovials and 1.0 ml sterile loading solution was added. After 15 min, the loading solution was discarded and 1.0 ml of PVS2 vitrification solution was added. After the cryovials had been maintained on ice for 1h, the PVS2 solution was discarded using an Eppendorf pipette. Vials were inserted into cryosleeves, and then directly immersed into liquid nitrogen for 24h.

Thawing

Cryosleeves were thawed by slow warming at room temperature inside a laminar flow hood. For osmoconditioning of cryopreserved cultures, 1.0 ml liquid MS3:1

medium containing 1.0M sucrose was added to each cryovial and incubated for 1h after which the medium was discarded.

Regrowth

Cultures were plated after 1h on semisolid MSP. Cultures from each vial were divided into 5 inocula and plated onto 5 spots on 65 x 15 mm Petri dishes containing approximately 10–15 ml medium. Petri dishes were sealed with Parafilm® and maintained in darkness at room temperature (25^{o}C). The total number of growing inocula out of 5 inocula from each vial was observed at two-day intervals. Percentage of recovery, i.e., the ratio of proliferating inocula out of 5 inocula 2 weeks and one month after plating were analyzed using ANOVA (SAS Institute, 2000).

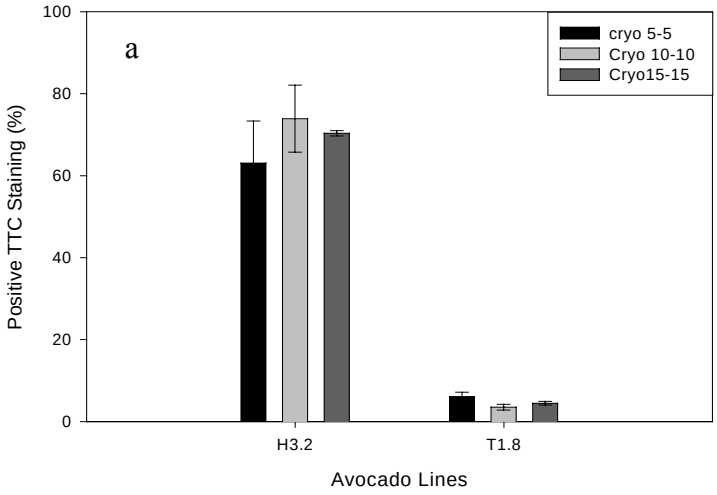
Results

Slow Cooling Cryopreservation

Cryopreservation with 5% DMSO+5 % glycerol

Comparison of the effect of three different cryoprotectant mixtures on viability of 'Hass' H3.2 and 'T362' T1.8 is shown in Figure 5-1a. Viability of 'Hass' H3.2 was 63.1±10.3%, 74.0±8.0% and 70.4±0.6% (viability±SE) which corresponds to cryoprotectant mixtures of 5% DMSO+5% glycerol, 10% DMSO+10% glycerol and 15% DMSO+15% glycerol, respectively. The corresponding values of cryopreserved embryogenic cultures of 'T362' T1.8 was 6.2±1.0%, 3.6±0.7% and 4.6±0.4% (viability±SE), which corresponds to cryoprotectant mixtures of 5% DMSO+5% glycerol, 10% DMSO+10% glycerol, 15% DMSO+15% glycerol, respectively. There were no significantly different responses for 'Hass' H3.2 and 'T362' T1.8 to different mixtures of cryoprotectant; however, there were significantly different responses ($p=0.01$) between 'Hass' H3.2 and 'T362' T1.8 with respect to cryoprotectant treatment.

Cryopreserved ‘Hass’ H3.2 showed 69.2% viability irrespective of cryoprotectant mixture, which was 14-fold higher than the viability of cryopreserved ‘T362’ T1.8 (4.8%).



b

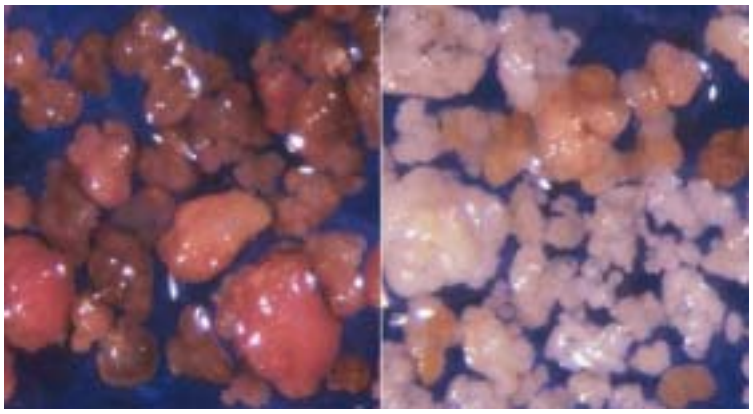


Figure 5-1. Effect of cryoprotectant mixtures on TTC staining. Cryo 5-5, Cryo 10-10 and Cryo 15-15 indicate 5% DMSO+5% glycerol, 10% DMSO+10% glycerol and 15% DMSO+15% glycerol, respectively on MSP semi solid medium (Figure a; Bar = ± SE). Figure 1b shows a positive staining (left) and negative staining reaction (right). Actual regrowth of ‘T362’ line T1.8 treated with 5% DMSO+5% glycerol (4% regrowth)

Cryopreserved cultures were maintained on semi solid recovery medium for > 4 months before they were discarded if they did not demonstrate proliferation. Only one

clump of ‘T362’ line T1.8 proliferated on recovery medium with cryoprotectant mixture 5% DMSO + 5% glycerol. There was no correlation between the TTC staining test result and the actual regrowth of cryopreserved cultures. Therefore, staining does not provide accurate data about survival.

Regrowth occurred only on the treatment with 5% DMSO and 5% glycerol as the cryoprotectant. The TTC staining test also showed that there was no difference in the response between cryoprotectant mixtures, and therefore this cryoprotectant mixture was used for all subsequent experiments.

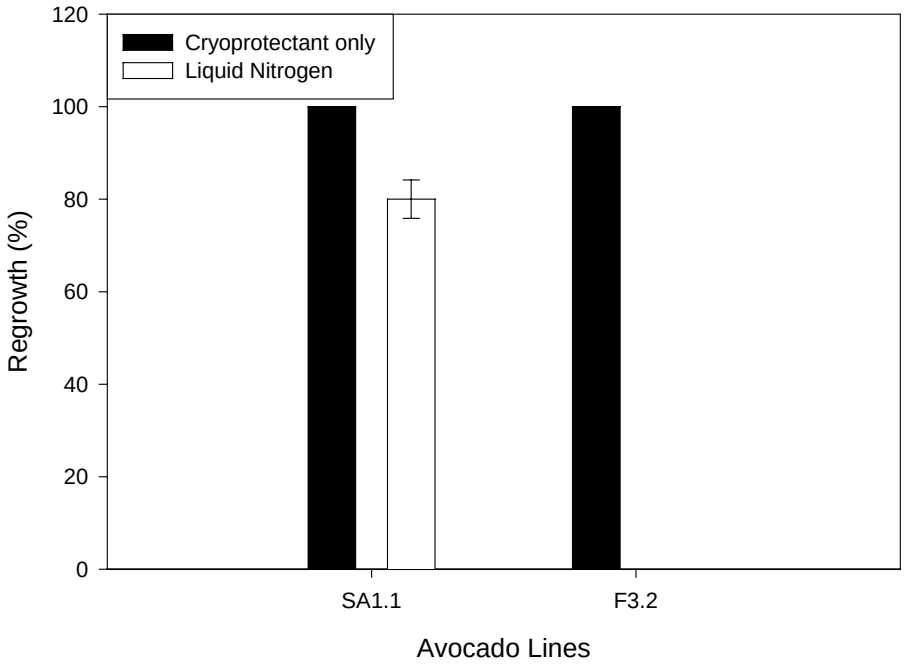


Figure 5-2. Effect of 5% DMSO and 5% glycerol cryoprotectant mixture on recovery and proliferation of ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2 embryogenic avocado cultures from liquid nitrogen. There was no recovery of ‘Fuerte’ F3.2. Cryoprotectant only indicates culture was treated 30 min with cryoprotectant but not frozen, and liquid nitrogen indicates treatment for 30 min with cryoprotectant and frozen at $1C^{o}$ min^{-1} for 2 h in –80 ^{o}C and then immersion for 24 h in liquid nitrogen.

Figure 5-2 shows that ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2 both can survive exposure to cryoprotectant with 100% recovery; however, after cryopreservation for 24 h in liquid nitrogen, only ‘Suardia’ SA1.1 survived and its recovery was approximately 80%, while cryopreserved ‘Fuerte’ F3.2 did not show any recovery. With avocado ‘T362’ line T2.11.1, regrowth was approximately 52% (Figure 5-3). The embryogenic response of cryopreserved embryogenic ‘T362’ T2.11.1 cultures was recorded by counting the number of clumps that produced proembryonic masses (PEMs), somatic embryos and disorganized cultures on semi solid recovery media. From the total regrowth of 52.3%, approximately 74.4% consisted of PEMs, 56.3% consisted of somatic embryos (SEs) and only 1.5% of the recovered growth was as disorganized cultures (Figure 5-3).

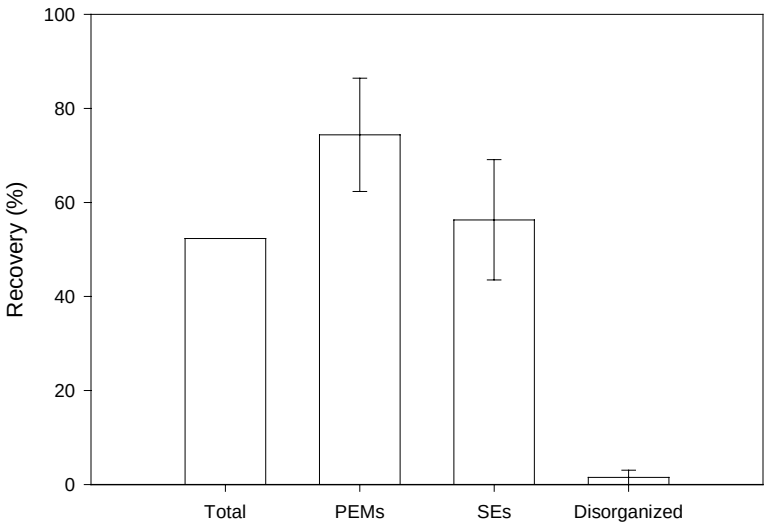


Figure 5-3. Recovery and proliferation of cryopreserved avocado embryogenic cultures of ‘T362’ line T2.11.1 with 5% DMSO+5% glycerol as cryoprotectant. Total recovery is 52%. ANOVA was done to the different categories of proliferation cultures in recovery media: PEMs, SEs and Disorganized.

Cryoprotection with 5% DMSO+5% glycerol + 1.0 M sucrose

Responses of Different Embryogenic Lines. Increasing the sucrose concentration in MS3:1 medium from 0.13 M to 1.0 M in the cryoprotectant mixture of 5% DMSO+5% glycerol caused a lower survival rate for cryopreserved 'Suardia' SA1.1, i.e., from 80% to 60% (Figure 5-2 and 6.4). On the other hand, "Fuerte' F3.2 embryogenic cultures benefited from the increased sucrose concentration, showing 0% recovery with 0.13 M sucrose and 75% recovery with 1.0 M sucrose (Figure 5-2 and 6-4). The differences in recovery of 'Suardia' SA1.1 and 'Fuerte' F3.2 with cryoprotectant supplemented with 1.0 M sucrose were not statistically significant.

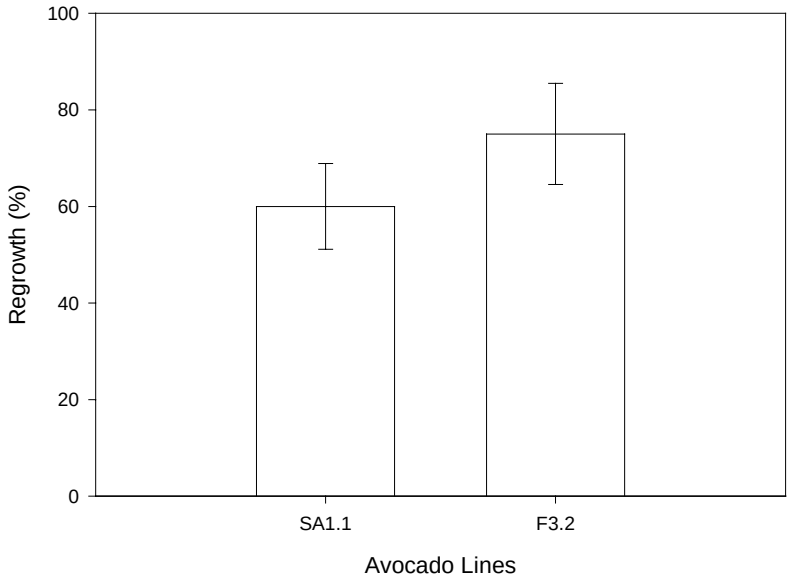


Figure 5-4. Regrowth of embryogenic avocado lines 'Suardia' SA1.1 and 'Fuerte' F3.2 after cryopreservation with 5% DMSO+5% glycerol on MS3:1 medium containing 1.0 M sucrose as cryoprotectant.

Effect of Recovery Media on Regrowth. There were no differences between the effects of MSP and $B5^{+}$ media on recovery of cryopreserved 'Suardia' SA1.1 and 'Fuerte' F3.2 cultures (Figure 5-5). Combined data for recovery of 'Suardia' SA1.1 and

'Fuerte' F3.2 indicated that MSP medium resulted in 61.8±10.9% recovery; whereas, $B5^{+}$ resulted 73.3±8.6% recovery. Since all avocado cultures are routinely maintained on semi solid MSP medium, this medium has been adopted as the recovery medium.

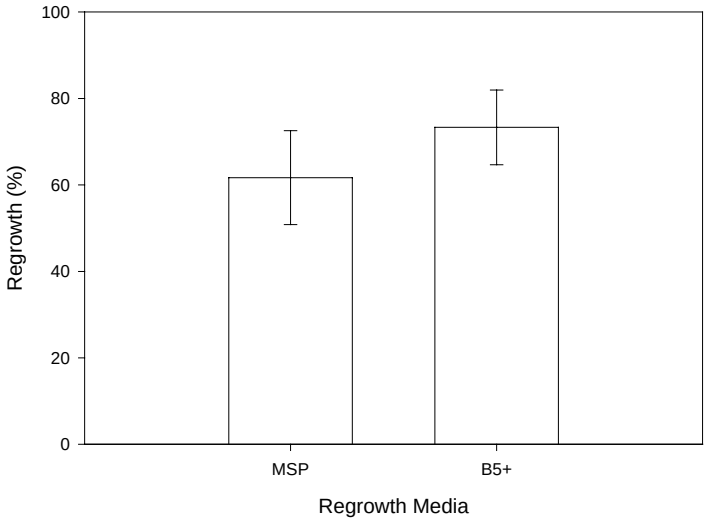


Figure 5-5. Effect of recovery media on proliferation of 'Suardia' SA1.1 and 'Fuerte' F3.2 embryogenic avocado cultures following cryopreservation with 5% DMSO+ 5% glycerol on MS3:1 medium containing 1.0 M sucrose. MSP = MS basal medium and B5 = Gamborg et. al. basal media, both of which are supplemented with 0.41 µM picloram. Data represent regrowth of 'Suardia' SA1.1 and 'Fuerte' F3.2.

Effect of washing embryogenic cultures following cryopreservation on recovery.

Washing of cryopreserved embryogenic cultures with MS3:1 liquid medium directly after thawing resulted in 51.7±9.4% recovery compared to 83.3±9.4% for the non-washed cultures (Figure 5-6). Washing cultures with MS3:1 medium significantly reduced the percentage of recovery of 'Suardia' SA1.1 and 'Fuerte' F3.2 by approximately 38%. Staining result of washing and without washing can be seen on Figure 5-7.

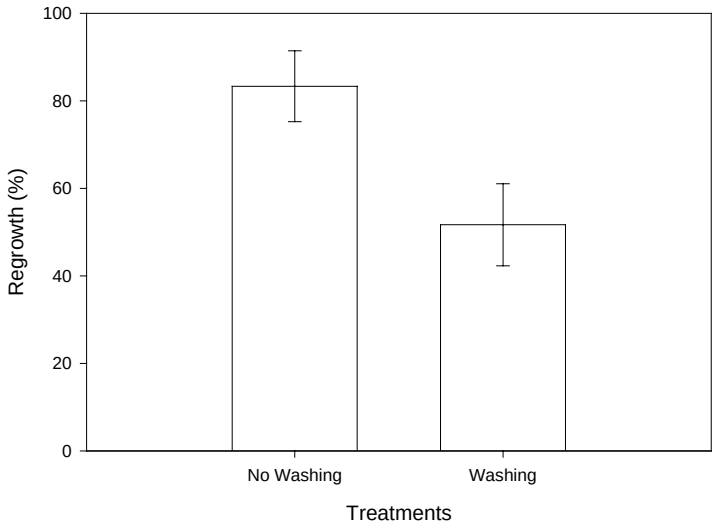


Figure 5-6. Effect of washing PEMs after thawing by MS3:1 media on recovery of cryopreserved 'Suardia' SA1.1 and 'Fuerte' F3.2 embryogenic avocado cultures. PEMs were washed with MS3:1 medium; while in the treatment without washing treatment, the PEMs were plated directly on semisolid MSP or B5 after thawing. Data represent regrowth of 'Suardia' SA1.1 and 'Fuerte' F3.2.

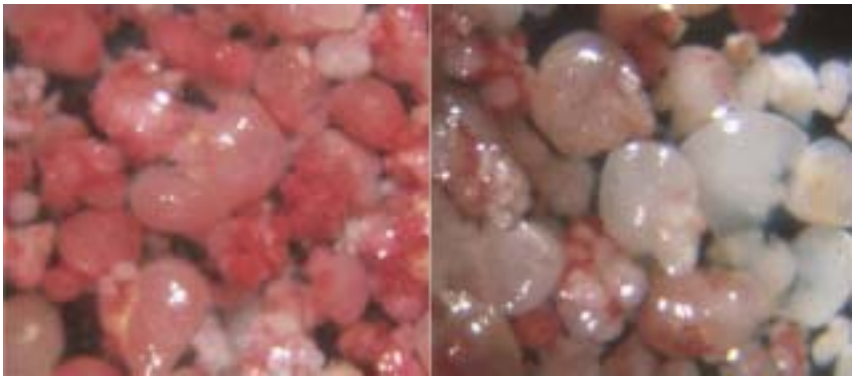


Figure 5-7. Staining after washing (right) and without washing (left) following thawing of cryopreserved 'Suardia' SA1.1 cultures.

Vitrification

Embryogenic cultures that had been exposed to PVS2 but which were not immersed in liquid nitrogen started to grow 12 days after plating on semi solid MSP medium. PVS2 solution alone caused a delay in recovery by >1 week. Cultures that

underwent complete vitrification, with immersion for three days in liquid nitrogen, followed by thawing and plating on recovery medium, began to proliferate after 17 days for 'Suardia' SA1.1 and one month for 'Booth 7' B7.1 and 'Fuerte' F3.2 (Table 5-2).

Table 5-2. Time required for recovery of embryogenic cultures after vitrification using PVS2 solution.

Avocado Lines	Regrowth on recovery medium (days after culturing)	
	PVS2, non frozen	PVS2 and frozen
'Booth 7' B7.1	12	30
'Suardia' SA1.1	12	17
'Fuerte' F3.2	12	30

Figure 5-8. Regrowth of embryogenic avocado cultures of 'Fuerte' line F3.2 (left) and 'Suardia' line SA1.1 (right) after vitrification with PVS2 solution.

Figure 5-8 shows the recovery of embryogenic culture of 'Suardia' SA1.1 (two weeks after plating) and 'Booth 7' B7.1 (four weeks after plating). Figure 5-9a demonstrates the effect of vitrification on TTC staining. Viable PEMs of 'Booth 7' B7.1, 'Suardia' SA1.1 and 'Fuerte' F3.2 were 87.1±0.6%, 94.1±1.6% and 81.8±4.9%, respectively. There were no significant differences with respect to TTC staining among the three genotypes (Pr>F = 0.24 and R^2=0.19). Figure 5-9b demonstrates the

percentage of actual recovery, i.e., 18.1±14.3%, 61.7±16.4% and 5.0±5.0, respectively for 'Booth 7' B7.1, 'Suardia' SA1.1 and 'Fuerte' F3.2. The recovery of 'Suardia' SA1.1 is statistically better than either 'Booth 7' B7.1 or 'Fuerte' F3.2 (Pr>F = 0.03 and R^2=0.54 for original data, and 0.02 and 0.56, respectively, for arcsin transformed data).

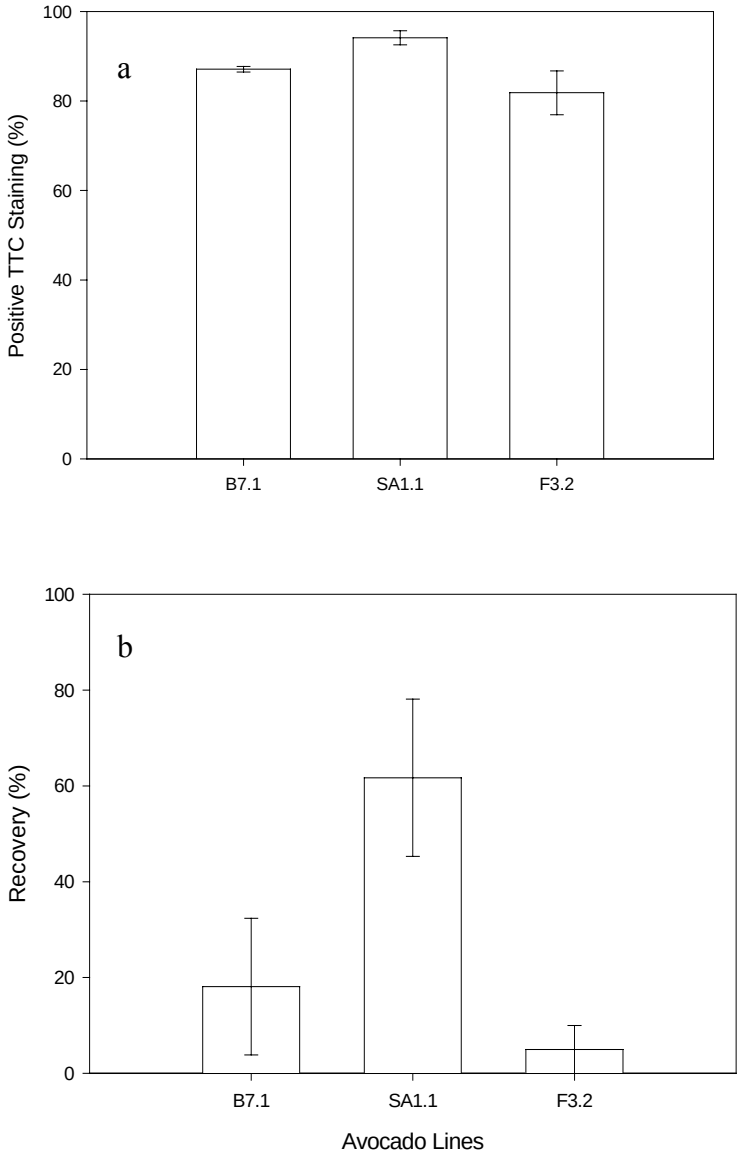


Figure 5-9. Effect of vitrification solution (PVS2) on TTC staining (a) and actual recovery (b) of 'Booth 7' line B7.1, 'Suardia' line SA1.1 and 'Fuerte' line F3.2 embryogenic avocado cultures. Bar = ± SE.

The correlation between viability based upon TTC staining and regrowth on recovery medium was low and was not statistically significant (The Pearson correlation coefficient = 0.54 and the probability = 0.13.)

Discussion

Dimethylsulfoxide (DMSO) and glycerol are most commonly used cryoprotectants for cryopreservation. A mixture of 5% DMSO and 5% glycerol was shown to be the best cryoprotectant for embryogenic cultures of cotton (Rajasekaran, 1996), while 10% (v/v) DMSO was used as the cryoprotectant for somatic embryos and embryogenic cultures of citrus (Marin *et al*., 1993; Duran-Vila, 1995; Pérez, *et al*., 1997; 1999). Preliminary studies indicated that a cryoprotectant consisting of either DMSO or glycerol in MS3:1 medium did not provide adequate cryoprotection, and the cultures failed to survive. Therefore a combination of DMSO and glycerol in MS3:1 (MS 3:1 P without picloram) supplemented with either 0.13M or 1.0M sucrose was used.

Viability of cryopreserved embryogenic avocado cultures based upon TTC staining ranges between 4.8 and 70% as a result of slow cooling (Figure 5-1) and 88% as a result of vitrification (Figure 5-9). These results are comparable to the viability based on FDA staining of cryopreserved embryogenic citrus cultures, which were 70-90% either by slow cooling or vitrification (Sakai *et al*., 1990, 1991; Kobayashi *et al*., 1991). In these reports, there was no attempt to establish a correlation between viability and recovery. Watanabe *et al*. (1999) reported that there is a good correlation between viability based on TTC staining and the recovery of cryopreserved embryogenic rice cultures. Ishikawa *et al*. (1997) reported similar results in which orchid zygotic embryos were cryopreserved following vitrification.

Pérez *et al*. (1997) noted that the staining test performed immediately after thawing overestimates viability, because some cells that are positively stained would later die on recovery medium (Bajai, 1995). Engelmann (1991) and Duran-Vila (1995) also concluded that TTC staining does not provide accurate data about survival, and therefore regrowth on recovery medium is the only reliable test for viability.

The lag period required for recovery of cryopreserved embryogenic avocado cultures in these experiments ranged from 12 days to about 6 weeks. These results lie within the range for cryopreserved citrus, which varies from 3 days (Kobayashi *et al*., 1990; Sakai *et al*., 1990, 1991) to 2-6 weeks (Marin and Duran-Vila, 1988; Marin *et al*., 1993; Duran-Vila, 1995; Pérez *et al*., 1997, 1999) and for Scots pine (*Pinus sylvestris* L.), which is 2-4 weeks (Häggman *et al*., 1998).

Recovery of cryopreserved embryogenic cultures was genotype-dependent and affected by the cryoprotectant or cryopreservation method. With slow cooling cryopreservation, recovery rate of embryogenic cultures of 'T362'varied from 4% with line T1.8 (Figure 5-1) to 53% for line T2.11.1 (Figure 5-3). These differences may be due to differences in genotype. The recovery rate of 'Suardia' SA1.1 was 60-80% using slow cooling (Figure 5-2 and 6.4) and 62% using vitrification (Figure 5-9). 'Fuerte' F3.2 recovery varied from 73 to 75% using slow cooling (Figure 5-4 and 5-5) to only about 5% with vitrification (Figure 5-9). Variation in recovery rate also was observed in citrus, in which the recovery rate varied from 3.7 to 5% for cryopreserved somatic embryos (Marin and Duran-Vila, 1988) to 30% (Marin *et al.*, 1993; Duran-Vila, 1995) and 100% for cryopreserved embryogenic cultures (Duran-Vila, 1995; Pérez *et al*., 1997, 1999). These differences in citrus recovery rate may be due to differences in protocol

and plant material since the response attributed to genotype was not very strong (Pérez *et al*., 1997, 1999, 2000). The recovery of cryopreserved embryogenic avocado cultures is similar to reports for other species. Recovery of cryopreserved embryogenic cultures of *Hevea brasiliensis* was 65% (Engelmann and Etienne, 1995), 70% for cotton (Rajasekaran, 1996) and 73-100% for interior spruce (*Picea glauca engelmanni* complex) (Cyr *et al*., 1994). Janeiro *et al*. (1996) reported that somatic embryos of *Camellia japonica* were unable to survive cryopreservation by several protective methods; whereas, embryonic axes were easily cryopreserved with simple desiccation and direct immersion into liquid nitrogen.

Differences in recovery rate may also reflect different morphologies of embryogenic cultures. Witjaksono and Litz (1999a) reported that there are two types of embryogenic avocado culture: proembryogenic masses type (PEM-type) and somatic embryo type (SE-type). PEM-type cultures proliferate as PEMs; whereas heart and later stages of somatic embryos develop in the presence of picloram with SE types. 'Booth 7' line B7.1 is an SE-type and 'Fuerte' line F3.2 is a PEM-type. 'Suardia' line SA1.1 is an intermediate form between both 'Booth 7' B7.1 and 'Fuerte' F3.2, and behaves as an SE-type for a few months after induction and then changes to a PEM-type over time. PEM-type embryogenic cultures respond better than SE-type to cryopreservation. Sieving the embryogenic cultures with sterile nylon fabric (mesh 1.8 mm) will increase uniformity of SE type and increase its recovery after cryopreservation.

Washing embryogenic cultures with MS3:1 liquid medium directly after thawing has a negative effect on recovery. Watanabe *et al*. (1999) reported that washing cryopreserved embryogenic rice cultures can alter the osmotic pressure of the cultures

and can be harmful. In other reports, washing 3x with culture medium can increase regrowth rate to 100% for cryopreserved embryogenic citrus cultures (Duran-Vila, 1995 and Pérez *et al*., 1997, 1999) compared to lower recovery without washing (Marin and Duran-Vila, 1988; Sakai *et al*., 1990). Dussert *et al*. (2000) also reported that post-thawing osmoconditioning increases the percentage of seedling recovery from cryopreserved coffee seeds.

$B5^{+}$ and MSP semi solid media resulted in similar recovery and MSP, which is a standard maintenance medium for embryogenic avocado cultures, has therefore been used for recovery of cryopreserved cultures. Other researchers also use semi solid maintenance medium for recovery of cryopreserved cultures. Cryopreserved citrus cultures were either plated directly on semi solid medium (Marin and Duran-Vila, 1988; Marin et al., 1993; Duran-Vila, 1995; Perez *et al*., 1997, 1999; Gonzales-Arnao, 1998) or were plated on top of filter paper on the medium (Sakai *et al*., 1990, 1991; Kobayashi *et al*., 1990). In conifer, cryopreserved embryogenic cultures also have been recovered on semi solid maintenance medium on filter paper on the medium, [*Pinus caribaea* (Lainé *et al*., 1992), *Picea mariana* (Klimaszewska *et al*., 1992), *P. glauca* (Cyr *et al*., 1994), *P. abies* and *P. sitchensis* (Find *et al*., 1998), and *Pinus sylvestris* (Häggman *et al*., 1998)]. Ford *et al*. (2000), on the other hand, plated cryopreserved *Pinus patula* cultures directly on top of maintenance medium. Lainé *et al*. (1992) suggested that using Millipore filters can reduce the lag phase of recovery because it helps to drain off excess cryoprotectant from the cultures.

Conclusion

Avocado embryogenic cultures can be cryopreserved by slow and rapid cooling techniques. The slow cooling protocol involves the use of a cryoprotectant consisting of

5% DMSO+5% glycerol in MS3:1 medium supplemented with either 0.13 M or 1.0 M sucrose. Fast cooling or vitrification involves the use of modified of Plant Vitrification Solution (PVS2). The ability to withstand cryopreservation appears to be genotype-dependent.

CHAPTER 6
SOMATIC EMBRYO DEVELOPMENT AND GERMINATION FROM CRYOPRESERVED EMBRYOGENIC AVOCADO (*Persea americana* MILL.) CULTURES

Introduction

The diversity of avocado germplasm collections provides a prerequisite for plant breeding programs. Traditionally, avocado genetic resources are preserved as whole plants in *ex situ* field gene banks. The plants in field collection are subject to losses caused by biological and climactic hazards. Germplasm collections are also expensive to maintain. Avocado genetic resources also can be preserved for short to medium term as embryogenic cultures (Witjaksono, 1997; Witjaksono and Litz, 1999a, 1999b); however, embryogenic competence is lost as early as four months after induction for some cultivars (Witjaksono 1997; Witjaksono and Litz, 1999a).

Cryopreservation is considered to be an ideal method for storage of germplasm since it offers long-term storage capability with stability of genotypic and phenotypic characteristics of the stored germplasm, and only minimal space and maintenance are required (Engelmann, 1997). Cryopreservation can be used as a back-up to support field plant gene banks as well as to continuously supply embryogenic cultures for research requiring *in vitro* cultures, i. e., and genetic transformation.

Embryogenic avocado cultures have been successfully cryopreserved by slow cooling and vitrification protocols (Efendi *et al*., 2001; Efendi, Chapter 5). However, the effect of cryopreservation of embryogenic avocado cultures on proliferation,

embryogenic competence, somatic embryo germination and plantlet conversion has not been addressed.

The reports on growth and embryogenic capacity of cryopreserved cultures of several plant species are somewhat contradictory. With citrus, Sakai *et al*. (1991) reported that growth of cryopreserved embryogenic cultures of naval orange is lower than nonfrozen control until 12 days after culturing after thawing; while Pérez *et al*. (1997) reported that appearance and growth rate of cryopreserved cultures of several genotypes of citrus were the same as the nonfrozen controls. With rice, cryopreservation did not affect the competence for plant regeneration (Carnejo *et al*., 1995; Moukadiri, 1999). With conifers, the effect of cryopreservation has varied. Cryopreserved somatic embryos grew better than nonfrozen controls for *Pinus patula* (Häggman *et al*., 1998; Ford *et al*., 2000) and for *Picea sitchensis* (Häggman *et al*., 1998); less than the nonfrozen control for *Abies normanniana* (Nørgaard *et al*., 1993); or comparable to the nonfrozen control for *Picea mariana* (Klimazzewska, 1995) and *Picea sitchensis* (Find *et al*., 1998).

This study was undertaken to measure growth of cryopreserved embryogenic cultures in suspension, and to determine the parameters for somatic embryo development and germination.

Materials and Methods

Embryogenic Culture Materials

Embryogenic 'Suardia' SA1.1 and 'Fuerte' F3.2 cultures were used in these experiments. Fruit origin, culture induction and maintenance have been described in Chapter 5.

Cryopreservation

Embryogenic avocado cultures that were successfully cryopreserved as described in Chapter 5 were used. 'Suardia' SA1.1 was treated with a cryoprotectant mixture consisting of a combination of 5%, DMSO (dimethylsulfoxide) and 5% glycerol in MS3:1 (MS3:1P without picloram) supplemented with 0.13M sucrose. 'Fuerte' F3.2 did not survive cryopreservation with this combination of cryoprotectant, and was treated with a cryoprotectant mixture consisting of a combination of 5% DMSO and 5% glycerol in MS3:1 supplemented with 1.0M sucrose. The cryopreservation procedures, i.e., cryoprotection, slow cooling, immersion in liquid nitrogen and thawing, have been described in Chapter 5.

Figure 6-1 shows the methodology. Samples were removed at every stage in order to determine viability. These cultures were transferred onto semi solid and into liquid medium in order to compare their proliferation, somatic embryo development and germination and to test for the residual effect of cryopreservation. There were two cryopreservation treatments and two controls: 1) Control 1 (noncryoprotected and nonfrozen); 2) Control 2 (cryoprotected and nonfrozen); 3) –80^{o}C (slow cooling to -80^{o}C with cryoprotectant); and 4) Cryopreserved at –196^{o}C (cryoprotectant, slow cooling to –80^{o}C and in liquid nitrogen for 24h). Cultures that showed growth were subcultured onto semisolid MSP medium as well as in liquid as described in Chapter 5. Cultures were subcultured monthly on semi solid medium and biweekly in liquid medium.

Growth of Cryopreserved Cultures in Suspension

After three subcultures in liquid medium, growth of cryopreserved embryogenic cultures was determined by inoculating 1.0 ml of suspension culture into 40 ml MS3:1P medium in 125 ml Erlenmeyer flasks. Flasks were capped with aluminum foil and sealed

with Parafilm® and maintained on a rotary shaker (125 rpm) under diffuse light at room temperature (25°C).

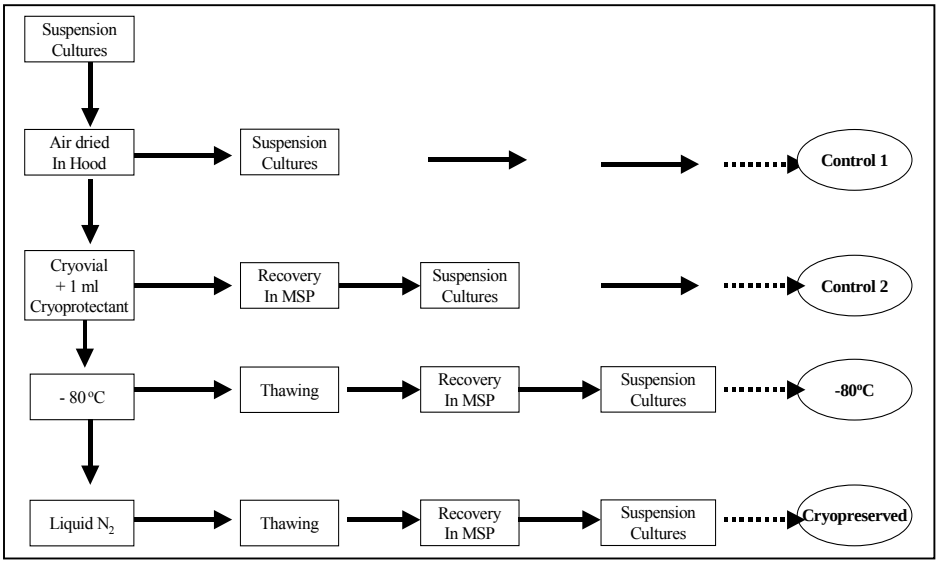


Figure 6-1. Methodology for cryopreservation of embryogenic avocado cultures. Embryogenic cultures were collected at every step and maintained in suspension for later experiments of residual effect, i. e., (1) control 1 (noncryoprotected, nonfrozen); (2) control 2 (cryoprotected but nonfrozen); (3) –80°C (cryoprotected and 2h exposure to –80°C); and (4) Cryopreserved (cryoprotected, 2h exposure to–80°C and 24h at -196°C).

There were two treatments and two controls with four replications, i.e., 16 Erlenmeyer flasks. Volume of embryogenic cultures was measured at three-day-intervals by decanting the contents of flasks into sterile Nalgene® graduated plastic centrifuge tubes. The proembryonic masses (PEMs) and media were decanted into a 50 ml sterile graduated cylinder and were left in a laminar flow hood for approximately 1 min until the PEMs precipitated. After measuring the PEM volume, they were returned into the original flasks, which were resealed. Cultures were maintained on a rotary shaker (125 rpm), with diffuse light at room temperature (25°C).

Somatic Embryo Development

Embryogenic cultures that were grown in liquid maintenance medium for 7-14 days were used as an inoculum. 'Suardia' SA1.1 cultures were sieved and the smaller fraction (<1.8 mm) was used. Embryogenic cultures were dried on 16-20 layers of sterile Kimwipes® in open plastic Petri dishes and air-dried in a laminar flow hood for 1h. Air-dried embryogenic cultures were plated on somatic embryo development (SED) medium.

SED medium is MS3:1 medium supplemented with 20% (v/v) filter-sterilized coconut water (CW) and solidified with 6 g l^{-1} Gel-Gro gellan gum (Witjaksono 1997; Witjaksono and Litz, 1999b). Approximately 40-50 mg of air-dried embryogenic cultures was plated on 50 ml SED semisolid medium in 100 x 20 mm Petri dishes. The cultures were evenly spread on the surface of the medium by tapping the forceps on the ridge of the dishes. The plates were closed but not sealed and were maintained in total darkness at room temperature (25^{o}C) for two months.

The number of opaque somatic embryos in three categories (<0.5, 0.5-1.0, and >1.0 cm in length), and hyperhydric and disorganized SEs were counted after one and two months. Data were analyzed using analysis of variance (SAS Institute, Cary, N.C.).

Somatic Embryo Germination

Two months after plating onto SED medium the somatic embryos were harvested. Seven good quality opaque somatic embryos from composite harvesting were plated in Petri dishes (100 mm x 25 mm) containing 50 ml germination medium. Each dish was sealed with Parafilm®. Germination medium consisted of MS 3:1 medium supplemented with 1.0 µM benzyladenine (BA) and 10 µM gibberellic acid (GA_3) and solidified with 3 g l^{-1} Gel-Gro gellan gum (Witjaksono and Litz, 1999b).

The number of somatic embryos (SEs) forming shoots and/or roots, and the number of somatic embryos that were dead were determined three months after plating. Data were analyzed using analysis of variance (SAS Institute, Cary, N.C.).

Results

Growth of Cryopreserved Cultures In Suspension

Embryogenic 'Suardia' SA1.1 cultures that had been frozen at –80^{o}C only or subsequently cryopreserved in liquid nitrogen (-196^{o}C) grew better than the controls. 'Suardia' SA1.1 cultures that received the complete cryopreservation procedure, i.e., cryoprotectant, slow cooling to –80^{o}C and immersion in liquid nitrogen grew significantly faster than nonfrozen controls. These cultures reached the maximum volume of 11.5 ml per flask on day 18, while the nonfrozen control cultures had 7.4 ml PEMs per flask at this time (Figure 6-2 above and Table 6-1).

At the end of the 21-day culture period, the volumes of the cultures from the two controls and two treatments were significantly different. The lowest volume of 2.0 ml occurred with control 2 (cryoprotected but nonfrozen). The culture volume of control 1 (noncryoprotected and nonfrozen) reached 8.4 ml. The highest volume, consisting of 12.0 ml/flask, was reached with cultures that originated from the –80^{o}C but noncryopreserved treatment. The cultures that originated from cryopreserved cultures reached a volume of 10.8 ml, a 0.7 ml drop compared to their maximum volume of 11.5 ml at day 18.

The average daily growth of 'Suardia' SA1.1 cultures ranged from 0.05 ml day^{-1} to 0.52 ml day^{-1}. Cultures that originated from cultures frozen at –80^{o}C but not immersed in liquid nitrogen showed the highest daily growth of 0.52 ml, while cultures from

cryopreserved ‘Suardia’ SA1.1 had a daily growth of 0.46 ml day^{-1}. Average daily growth was higher than the controls.

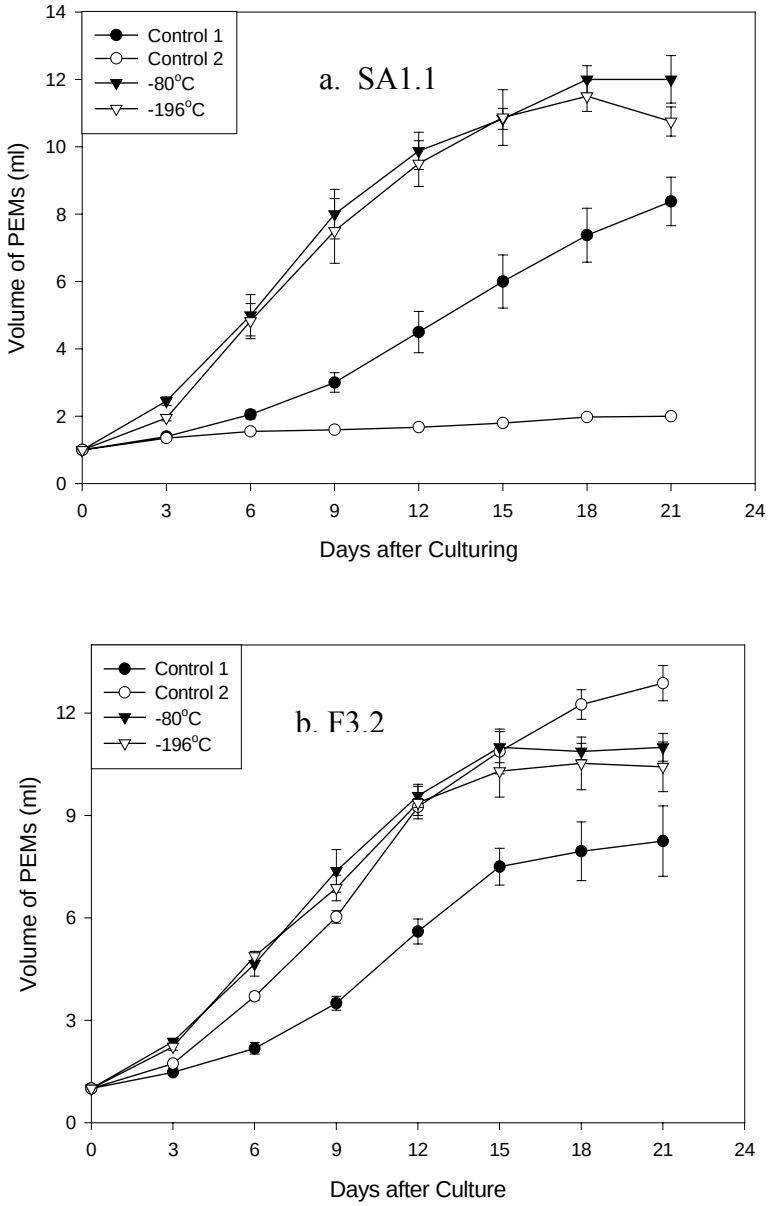


Figure 6-2. Growth of cryopreserved ‘Suardia’ SA1.1 (a) and ‘Fuerte’ F3.2 (b) in suspension cultures. Cryoprotectant is a mixture of 5% DMSO and 5% glycerol on MS3:1 medium supplemented with 0.13 M sucrose for SA1.1 and 1.0 M sucrose for F3.2.

Embryogenic ‘Fuerte’ F3.2 culture growth patterns were similar to those of ‘Suardia’ SA1.1. Cultures that received the cryoprotectant treatment and slow cooling to –80^{o}C with and without cryopreservation in liquid nitrogen grew better than the noncryoprotected-nonfrozen control 1 beginning 3-days after culturing. The PEM volume of these two treatments was higher than the PEM volume of the cryoprotected but nonfrozen (control 2) at 3, 6 and 9 days of culturing, but at days 18 and 21 the PEM volume of control 2 was higher (Figure 6-2 and Table 6-1).

The highest PEM volume of ‘Fuerte’ F3.2 was 12.9 ml after 21-days of culture with control 2 (cryoprotected but nonfrozen), followed by 11.0 ml in the –80^{o}C treatment (cryoprotected and slow cooled to –80^{o}C but not cryopreserved in liquid nitrogen) and 10.4 ml with cultures that received the cryopreservation treatment. The lowest volume, 8.2 ml, was recorded for the control 1 culture (noncryoprotected-nonfrozen).

The average daily growth was 0.6, 0.5, 0.4 and 0.3 ml flask^{-1} day^{-1} for control 2 (cryoprotected but nonfrozen), frozen at –80^{o}C, cryopreserved in liquid nitrogen and control 1 (noncryoprotected nonfrozen), respectively (Table 6-1).

Maximum volume and daily growth of ‘Suardia’ SA1.1 was similar to that recorded for ‘Fuerte’ F3.2 (Table 6-1). Volume after 21 days of culture was 8.4 and 8.2 ml for control 1 of SA1.1 and F3.2, respectively. Volume at day 21 of cultures that originated from cryopreserved PEMs was similar for SA1.1 and F3.2, i.e., 10.8 and 10.4 ml, respectively. Average daily growth of control 1 was 0.4 and 0.3, and for cryopreserved cultures was 0.5 and 0.4 for ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2, respectively (Table 6-1).

Table 6-1. Volume at day 21 and average daily growth of cryopreserved 'Suardia' SA1.1 and 'Fuerte' F3.2

Controls/Treatments	Volume at day 21 (ml)		Daily growth (ml)	
	SA1.1	F3.2	SA1.1	F3.2
Control 1 (noncryoprotected-nonfrozen)	8.4	8.2	0.35	0.34
Control 2 (cryoprotected-nonfrozen)	2.0	12.9	0.05	0.57
Frozen (–80°C)	12.0	11.0	0.52	0.48
Cryopreserved (–196°C)	10.8	10.4	0.46	0.45

Figure 6-3. Somatic embryos produced from cryopreserved cultures two months after plating on SED medium. Control 1 = noncryoprotected-nonfrozen ; Control 2 = cryoprotected-nonfrozen ; -80°C = cryoprotected and frozen at –80°C freezer; Cryopreserved = cryoprotected, frozen at –80°C and immersed in liquid nitrogen (–196°C)

Somatic Embryo Production from Cryopreserved Culture

The total number opaque somatic embryos produced in each Petri dish for 'Suardia' SA1.1 and 'Fuerte' F3.2 is demonstrated in Figure 6-4. There were more

‘Suardia’ SA1.1 somatic embryos than ‘Fuerte’ F3.2. Cryopreserved ‘Suardia’ SA1.1 produced significantly more somatic embryos compared to the nonfrozen control with or without cryoprotectant treatments, but the number of somatic embryos was similar to the cultures frozen at –80^{o}C (Pr>F, <0.0001). The average number of opaque somatic embryos produced on each plate after two months on SED medium was 17.4±1.1, 39.3±3.1, 46.9±2.7, and 52.3±3.0 for control 1 (noncryoprotected-nonfrozen), control 2 (cryoprotected-nonfrozen), frozen at -80^{o}C (after cryoprotection) and cryopreserved at –196^{o}C (after cryoprotection and frozen at -80^{o}C) treatments, respectively. The origin of embryogenic cultures of control 2 for SE development experiment (Figure 6-4) was not the same with the origin of control 2 for experiment of growth in suspension (Figure 6-2a). Opaque somatic embryos of ‘Suardia’ SA1.1 are shown in Figure 6-3.

There were no significant differences in somatic embryo production between the two controls and two treatments with ‘Fuerte’ F3.2 embryogenic cultures (Pr>F = 0.6686). The numbers of opaque ‘Fuerte’ F3.2 somatic embryos produced two months after culturing on SED medium were 0.1±0.1, 0.3±0.1, 0.7±0.5, and 0.4±0.3 for control 1 (noncryoprotected-nonfrozen), control 2 (cryoprotected-nonfrozen), frozen to -80^{o}C (after cryoprotectant treatment) and cryopreserved at –196^{o}C (after cryoprotectant and frozen to -80^{o}C), respectively. There was no significant effect of cryopreservation treatment on production of opaque ‘Fuerte’ F3.2 somatic embryos.

‘Fuerte’ F.2 embryogenic cultures produced fewer somatic embryos than ‘Suardia’ SA1.1. The noncryoprotected-nonfrozen control of ‘Suardia’ SA1.1 produced 17 somatic embryos, while the control 1 of ‘Fuerte’ F3.2 produced only 0.1 somatic embryos per Petri dish. The highest opaque ‘Suardia’ SA1.1 somatic embryo production

occurred from cryopreserved PEMs, with 52 somatic embryos on each Petri dish, whereas in 'Fuerte' F3.2 there were only 0.7 somatic embryos on each Petri dish from PEMs that were frozen at -80°C.

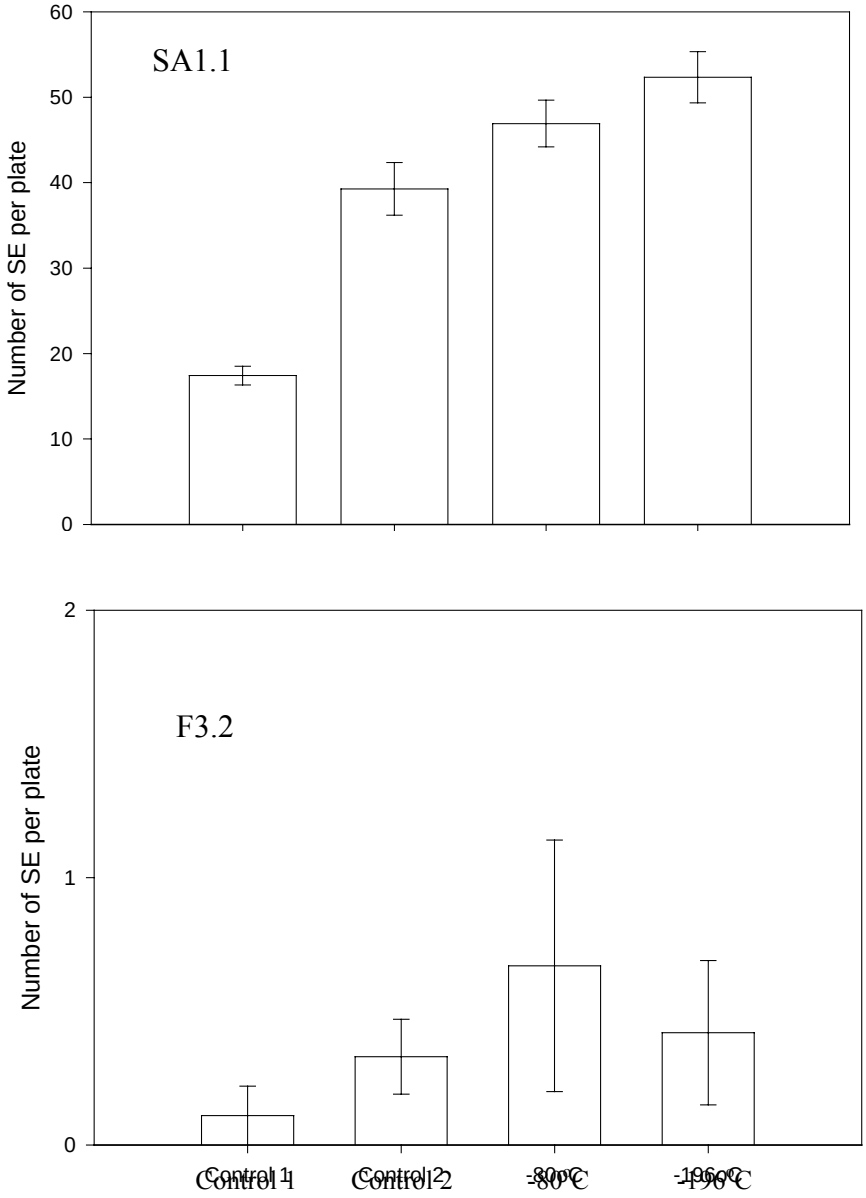


Figure 6-4. Somatic embryo production from cryopreserved SA1.1 (above) and F3.2 (below) two months after plating on SED media. Cryoprotectant is a mixture of 5% DMSO and 5% glycerol on SED media with 0.13 M sucrose for 'Suardia' SA1.1 and 1.0 M Sucrose for 'Fuerte' F3.2.

Production of different sizes of opaque somatic embryos is indicated in Figure 6-5 (above). Cryopreservation significantly affected opaque somatic embryo size. Approximately 50% of the total number of opaque somatic embryos was in the small category (SE < 0.5 cm in length). The frequency of small SEs was 42.7±3.7, 56.5±3.3, 51.4±1.8 and 54.0±3.4% for control 1 (noncryoprotected-nonfrozen), control 2 (cryoprotected-nonfrozen), frozen at -80^{o}C, and cryopreserved in liquid nitrogen (-196^{o}C), respectively. Frequency of production of medium opaque somatic embryos (SE 0.5-1.0 cm in length) was 13.8±1.7, 23.0±2.0, 18.7±0.7 and 16.9±0.9% for control 1, control 2, –80^{o}C treatment and cryopreserved (-196^{o}C) treatment, respectively. Large opaque somatic embryos (SE>1.0 cm in length) ranged in size from 4.2±1.1, 5.2±0.7, 8.9±1.0 and 11.1±1.5% for controls and treatments as above, respectively. Cryopreserved 'Suardia' SA1.1 cultures produced significantly more large opaque somatic embryos than the nonfrozen control with or without cryoprotection, but the number were comparable to the –80^{o}C treatment.

Hyperhydric somatic embryos developed as a result of all treatments and controls. The frequency of hyperhydric somatic embryo was 20.8±2.7, 15.2±2.2, 20.9±1.7 and 18.0±2.3% for control 1, control 2, –80^{o}C, and cryopreservation (-196^{o}C) treatment, respectively (Figure 6-5a). Only noncryoprotected-nonfrozen control 1 resulted in the appearance of disorganized somatic embryos at a frequency of 18.6±3.7% two months after plating on SED medium (Figure 6-5b).

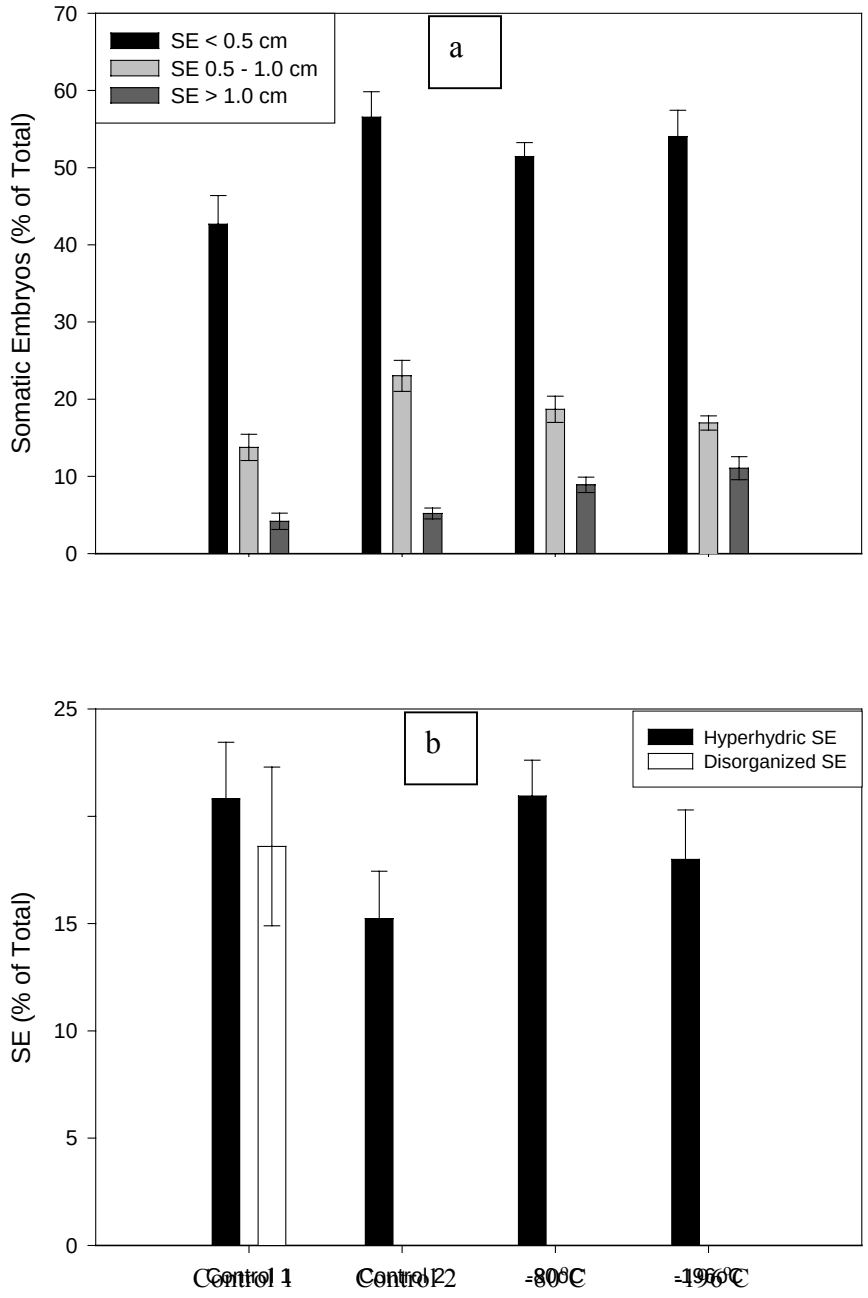


Figure 6-5. Effect of cryopreservation on percentage of different sizes of opaque somatic embryos (a) and hyperhydric and disorganized somatic embryos (b) of 'Suardia' SA1.1 two months after plating on SED medium.

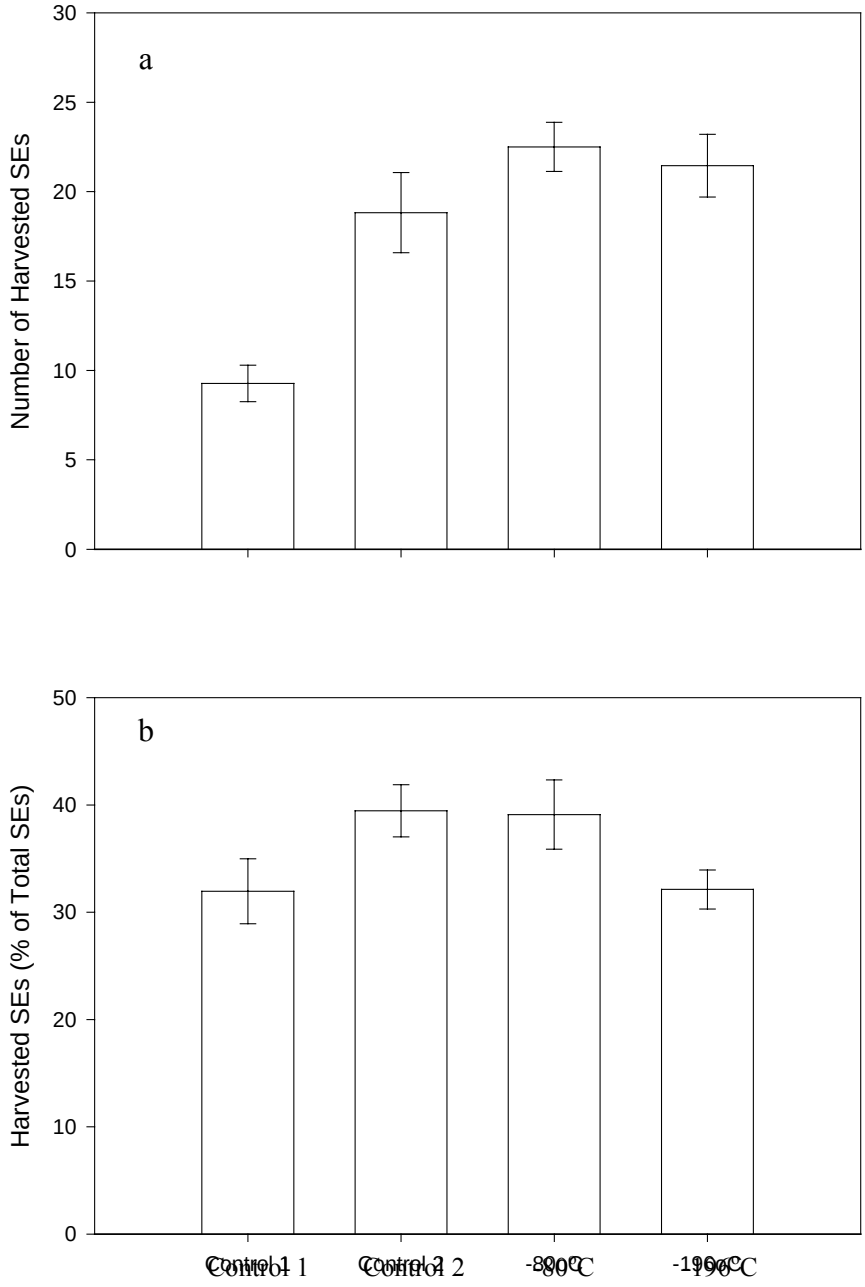


Figure 6-6. Effect of cryopreservation treatments on total number (a) and percentage (b) of somatic embryos harvested two months after plating on SED medium. Control 1 = noncryoprotected-nonfrozen ; Control 2 = cryoprotected-nonfrozen ; -80°C=cryprotected-frozen at –80°C; Cryopreserved= cryoprotected, frozen at –80°C and immersed in liquid nitrogen (-196°C).

Opaque and ⩾0.8 cm in length somatic embryos were harvested one week after somatic embryos were counted s (Figure 6-4 and 6-6). The total number of harvested opaque SEs was 9.3±1 SEs/plate for noncryoprotected-nonfrozen control 1, 18.8±2.2 SEs per plate for the cyoprotected-nonfrozen control 2, 22.5±1.4 for the –80°C treatment, and 21.4±1.8 per plate for the –196°C treatment. There was no significant difference between control 2, –80°C and –196°C treatments with respect to good quality somatic embryos. The number of harvested opaque somatic embryos from control 2 and the two treatments were significantly higher than from the noncryoprotected-nonfrozen control 1.

Data for percentage of good quality opaque SEs (Figure 6-6b) shows that there were no significant differences between the two controls and the two treatments (significant only at 10%). The percentages were 32.0±3.0, 39.4±2.4, 39.1±3.2, and 32.1±1.8% for control 1, control 2, –80°C and –196°C treatments, respectively.

Somatic Embryo Germination from Cryopreserved Cultures

Figure 6-7 shows the percentage of somatic embryos that formed a shoot only (a), a root only (b) and that were dead (c) three months after culturing on germination medium. There was a significant effect of cryopreservation on the ability of somatic embryos to form shoots. Only somatic embryos that originated from cultures that were exposed to –80°C with or without immersed in liquid nitrogen (-196°C) formed shoots. Somatic embryos from cultures derived from the nonfrozen control with without cryoprotectant did not form shoots. Shoot formation was 6.7±2.7 and 6.1±2.5% from somatic embryos that originated from cultures from –80°C and -196°C treatments, respectively.

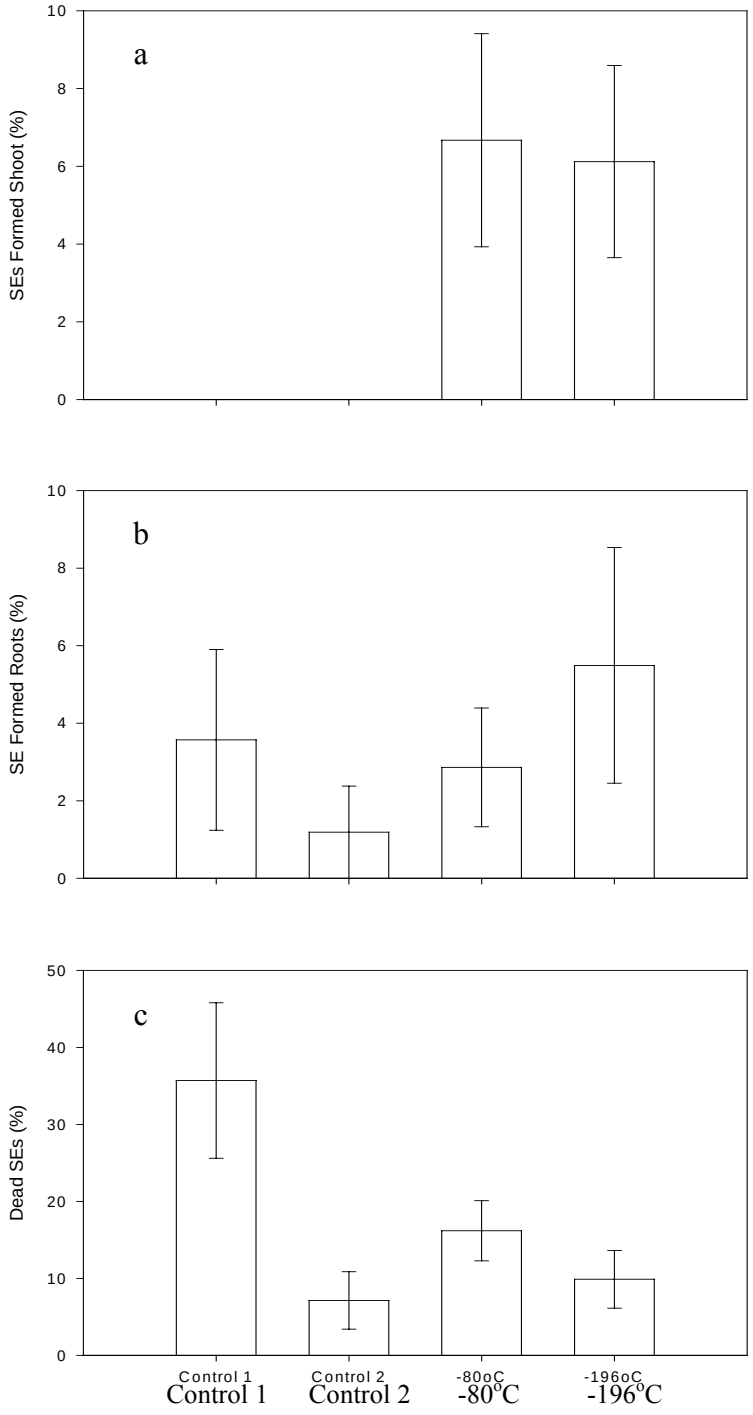


Figure 6-7. Percentage of somatic embryos that originated from cryopreserved cultures that formed shoots (a), roots (b) and that were dead (c) after three months on germination medium. Control 1 = noncryoprotected-nonfrozen; Control 2 = cryoprotected-nonfrozen; -80°C = cryprotected-frozen at –80°C; -196°C= cryoprotected, frozen at –80°C and immersed in liquid nitrogen (-196°C).

Root formation occurred in the two controls and the two treatments at a lower frequency than shoot formation. Percentage of somatic embryos that formed shoots three months after culturing on germination medium was 3.6±2.3,1.2±1.2, 2.9±1.5 and 5.5±3.0% for control 1, control 2, –80^{o}C and –196^{o}C treatments, respectively. There was no significant difference between these responses (Figure 6-7).

Three months after plating on germination medium, 35.7±10.1% of somatic embryos that originated from the noncryoprotected-nonfrozen control 1 were dead. This value is significantly higher than that of cryoprotected-nonfrozen control 2, -80^{o}C and –196^{o}C treatments that ranged from 7.1±3.7, 16.2±3.9 and 9.9±3.8%, respectively.

Discussion

Maximum SCVs of embryogenic avocado suspension cultures of the non-cryoprotected–nonfrozen control were 8.4 and 8.2 ml and were reached 21 days after recovery for ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2, respectively. The average daily growth was 0.4 and 0.3 ml per flask for ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2, respectively. The time to reach the maximum volume is comparable to that of ‘Esther’ embryogenic avocado suspension cultures (Witjaksono, 1997; Witjaksono and Litz, 1999a). The maximum volume and the daily growth rate of ‘Suardia’ SA1.1 and ‘Fuerte’ F3.2 are higher than those of ‘Esther’ whose maximum volume was 6.5 ml and whose daily growth rate was approximately 0.29 ml day^{-1} (Witjaksono, 1997; Witjaksono and Litz, 1999a). The differences may be genotype-dependent or due to the amount of explanted embryogenic tissue. In these experiments 1.0 ml of PEMs were explanted, whereas in ‘Esther’ experiments only 0.5 ml of PEMs were explanted (Witjaksono, 1997; Witjaksono and Litz, 1999a).

The growth rate of embryogenic cultures that originated from cryopreserved embryogenic cultures exceeded those of the noncryoprotected-nonfrozen control for both 'Suardia' SA1.1 and 'Fuerte' F3.2. The beneficial effect of cryopreservation may be related to selection, i.e., less vigorous embryogenic cultures may have been killed during cryoprotection, freezing or thawing.

Reports of the growth rate of cryopreserved cultures of other species have shown various results. Sakai *et al.* (1991) reported that growth of cryopreserved navel orange cultures on recovery medium was lower than the non frozen control until 12 days after plating, after which rapid growth occurred which was comparable to the nonfrozen control. Cryopreserved embryogenic cultures of some citrus types, i.e., sweet oranges, lemon, Cleopatra Mandarin, sour orange and Mexican lime, presented the same growth rate as the nonfrozen control (Pérez *et al*., 1997; Pérez, 2000). The growth of cryopreserved cultures of rubber after the third subculture on proliferation medium is comparable to the nonfrozen control (Engelmann and Etienne, 2000).

The number of opaque somatic embryos produced by the noncryoprotected-nonfrozen control of 'Suardia' SA1.1 was 17.4 somatic embryos per Petri dish, which is comparable to 'Isham' that produces 15 opaque somatic embryos per plate and greater than that of 'T362' which only produces 3 opaque somatic embryos per plate (Witjaksono, 1997; Witjaksono et al., 1999b). 'Fuerte' F3.2 controls and treatments, however, produced very few somatic embryos, i.e., only 0.1 embryo per plate.

Embryogenic cultures of 'Suardia' SA1.1 that originated from cryopreserved cultures produced significantly more somatic embryos than noncryoprotected-nonfrozen and cryoprotected-nonfrozen controls. 'Fuerte' F3.2 cultures from the controls and

cryopreserved treatments produced statistically insignificant low numbers of somatic embryos. Cryopreservation appears to have a beneficial effect on somatic embryo production from genotypes that are morphologically competent but does not affect genotypes that have low morphogenic competence. Similar results have been reported by Pérez *et al.* (1997) for cryopreserved embryogenic citrus cultures. In this earlier study with sweet oranges, lemon, Cleopatra Mandarin and sour orange, cryopreserved cultures produced numbers of somatic embryos comparable to noncryoprotected-nonfrozen control. On the other hand, cryopreserved cultures of Succary sweet orange, Red Marsh grapefruit and Mexican lime did not produce somatic embryo, nor did their nonfrozen control. Marin and Duran-Vila (1988) and Pérez *et al*. (2000) reported that cryopreserved embryogenic citrus cultures produced somatic embryos with similar pattern as the control. Rajasekaran (1996) reported that somatic embryo production efficiency of cryopreserved cotton embryogenic cultures was similar to nonfrozen control. Helliot and Etienne (2000) also reported that cryopreserved rubber (*Hevea brasiliensis*) embryogenic cultures produce more somatic embryos than nonfrozen control.

The beneficial effect of cryopreservation on embryogenic capacity of some genotypes may be related to a selection process, as a result of which less-embryogenic and non-embryogenic cells are eliminated from the cultures, thereby increasing the synchrony of growth of the more embryogenic cultures. Helliot and Etienne (2000) and Gupta et al. (1995) speculated that non-embryogenic cells are preferentially destroyed during freeze-thaw cycle, leading to a selection of embryogenic material.

Recovery of bipolar somatic embryos was low, although similar to other reports of avocado (Pliego-Alfaro and Murashige, 1987; Mooney and Van Staden, 1988; Witjaksono, 1997). Witjaksono and Litz (1999b) reported that after culturing 9-10 months, only 2.5% of 'T362' and 5% of 'Booth 7' somatic embryos are able to produce shoots, while somatic embryos of 'Isham' did not develop any shoots. Somatic embryos that originated from noncryoprotected-nonfrozen and cryoprotected-nonfrozen controls of embryogenic avocado cultures did not produce any shoots, while 6.1-6.7% of somatic embryos that originated from embryogenic cultures frozen at –80°C with and without exposure to –196°C produced shoots. These results are at least comparable to report of shoot development from avocado somatic embryo in earlier studies (Pliego-Alfaro and Murashige, 1987; Money and Van Staden, 1988; Witjaksono, 1997; Witjaksono and Litz, 1999).

Conclusion

Embryogenic culture proliferation in suspension and somatic embryo development and germination were not adversely affected by cryopreservation treatments. Therefore, embryogenic avocado cultures can be cryopreserved without negatively affecting their proliferation rate, their capacity to form somatic embryos and the ability of somatic embryos to develop shoots and roots. Material for cryopreservation must be morphogenically competent since cryopreservation does not enhance the ability of cultures to form somatic embryos. Cryopreservation of embryogenic avocado cultures can be used 1) to back up field planting conservation, and 2) support long term availability of embryogenic cultures for *in vitro* research.

CHAPTER 7
SUMMARY AND CONCLUSIONS

Summary

In this study, two aspects of the embryogenic avocado culture system were investigated: genetic transformation and cryopreservation. Embryogenic avocado cultures were successfully transformed based upon an *Agrobacterium tumefaciens* protocol. The construct used in this experiment was a binary vector pAG4092 in *A. tumefaciens* strain EHA 101. The pAG4092 harbored the nptII gene for resistance to the antibiotic kanamycin under the control of the AGT01 promoter located near the left border and the *samK* gene driven by a fruit-specific avocado cellulase promoter located near the right border. The *samK* gene is a modified SAM-ase gene encoding for SAM hydrolase that catalyzes the conversion of SAM (S-adenosyl-methionine) to methylthioadenosin. Since SAM is the metabolic precursor of ACC (1-aminocyclo-propane-1-carboxylic acid), the proximal precursor of ethylene, a depleted SAM pool would inhibit ethylene biosynthesis.

Transformed embryogenic cultures were maintained and continuously selected in liquid medium supplemented with 100 mg l^{-1} kanamycin sulfate. Kanamycin sulfate at 200mgl^{-1} totally suppressed somatic embryo development and maturation of nontransformed cultures. However, growth and development of transformed somatic embryos carrying the *samK* and *nptII* genes were not affected by up to 400 mg l^{-1} kanamycin sulfate. Integration of the transgene into the avocado genome was

demonstrated by amplification of *nptII* and *samK* genes fragment from transformed embryonic cultures and somatic embryos.

Cryopreservation of avocado embryogenic cultures was achieved by slow (two-step) cooling and rapid cooling (vitrification) protocols. The slow cooling protocol involves the use of a cryoprotectant consisting of 5% DMSO (dimethyl sulphoxide) + 5% glycerol in MS3:1 medium supplemented with either 0.13 M or 1.0 M sucrose. Approximately 200 mg of the air-dried cultures was mixed with 1.0 ml cryoprotectant in 1.2 ml Corning® cryogenic vials and the vials were maintained on ice for 30 min. Vials were inserted into Nalgene® "Mr. Frosty" containers that contained 250 ml isopropanol and were placed in a –80^{o}C freezer for 2 h for slow cooling, where the temperature decreased at approx. 1^{o}C min^{-1}. The vials were removed and immersed directly into liquid nitrogen (-196^{o}C).

Fast cooling or vitrification involves the use of loading solutions and modified Plant Vitrification Solution (PVS2) and direct immersion into liquid nitrogen after embryogenic cultures are desiccated. Approximately 200 mg of air-dried cultures were transferred into 1.8 ml internal thread cryovials and 1.0 ml sterile loading solution was added. After 15 min, the loading solution was discarded and 1.0 ml of PVS2 vitrification solution was added. After the cryovials had been maintained on ice for 1 h, the PVS2 solution was discarded, and the vials were immersed into liquid nitrogen.

In slow cooling cryopreservation, the vials were removed from liquid nitrogen and immersed for 5 min in a 40^{o}C water bath for rapid thawing. For vitrification, the cryovials were slowly thawed at room temperature, and the cultures were incubated for 1 h with 1.0 ml liquid MS3:1 medium containing 1.0 M for osmoconditioning, after which

the medium was discarded and the cultures were plated on recovery medium. The cryopreserved cultures were also tested for viability by the tetrazolium chloride (TTC) test. Growth of cryopreserved embryogenic cultures in suspension cultures was determined, and somatic embryo development on semi solid medium was observed. The number of opaque, hyperhydric and disorganized somatic embryos were counted after one and two months, and the number of somatic embryos that formed shoots and/or roots was determined after three months.

Conclusion

Agrobacterium tumefaciens was used to deliver transgenes into the genome of embryogenic avocado. Integration of the transgene was evident by amplifying the *nptII* and *samK* genes fragment using PCR. Liquid MS3:1P medium supplemented with 100 to 300 mg l^{-1} kanamycin sulfate was used to select and maintain transformed embryogenic avocado cultures carrying the *nptII* kanamycin resistant gene. Somatic embryo development and maturation of nontransformed 'Suardia' SA1.1 was totally inhibited by 200 mg l^{-1} kanamycin sulfate. Development of transformed 'Suardia' SA1.1 somatic embryos harboring *nptII* gene was unaffected by up to 400 mg l^{-1} kanamycin sulfate.

Cryopreservation of avocado was genotype-dependent. Embryogenic culture proliferation was the only reliable criterion for determining recovery since vital staining with tetrazolium cloride immediately after thawing was not correlated with survival. Proliferation of embryogenic cultures in liquid medium and somatic embryo development and germination were not adversely affected by cryopreservation. Material for cryopreservation must be morphogenically competent since cryopreservation does not enhance the ability of cultures to form somatic embryos.

Cryopreservation of embryogenic avocado cultures can be used 1) to back up *ex situ* gene banks, and 2) support long-term availability of embryogenic cultures for avocado improvement programs based upon somatic cell genetics. Future studies with the avocado embryogenic system must account for the low recovery of bipolar somatic embryos, which affects the efficiency of plant recovery.

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BIOGRAPHICAL SKETCH

Darda Efendi was born on June, 16, 1963, in Silungkang, West Sumatra Province, Indonesia. He is son of the late Dahlan Rasyad (father) and the late Nuraya Rasyad (mother). After graduating from high school in 1982, he pursued his studies at Bogor Agricultural University ("Institut Pertanian Bogor" IPB), in Bogor, Indonesia, and earned a bachelor's degree, "Sarjana Pertanian," in agronomy in 1986. After graduating, he worked on vegetable research and production. Since 1988 he has been a faculty member in the Agronomy Department of the College of Agriculture at IPB. In 1992 he attended graduate school at IPB and in 1994 received the master's degree in agronomy.

In 1998 he received an overseas fellowship from the Center for Crop Improvement of IPB, and he began his Ph.D. degree in tropical fruit biotechnology in the Horticultural Science Department at the University of Florida (UF) under the supervision of Dr. Richard E. Litz. He spent five semesters in Gainesville taking classes before he began his research at the Tropical Research and Education Center, Homestead. He worked both as a graduate assistant and as a teaching assistant. Darda received support from the Harold E. Kendall, Sr. Endowed Scholarship and Miami-Dade County AGRI-Council Inc. His research on avocado transformation and cryopreservation has also been supported by the California Avocado Commission and by the College of Agricultural and Life Sciences (CALS).

Darda is married to Neneng Nurhasanah, who accompanied him during this Ph.D. program.