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Biotechnology and Avocado Improvement

Introduction

Avocado breeding programs exist in all the major producing countries. With respect to rootstocks, selection of materials tolerant of *Phytophthora* root rot is the primary goal in California and South Africa, while tolerance of white root rot caused by *Rosellinia necatrix* is a major concern in Spain. The development of dwarfing rootstocks has been a major interest for the Fundación Sánchez Colín in Mexico, while extensive research was carried out in Israel to develop avocado selections that are salt tolerant (Litz *et al*., 2005). Scion breeding objectives include improved fruit quality, e.g., easy peeling, small seed size, medium fruit size (250-350 g), extended shelf life, and tolerance of postharvest fruit diseases, e.g., anthracnose (*Colletotrichum gloeosporioides*) and Cercospora black spot (*Pseudocercospora purpurea*) (Litz *et al*., 2005). Biotechnology could be used to accelerate breeding programs. In this paper, we review the state of the art in three biotechniques of great potential for the future in avocado plant improvement programs: zygotic embryo rescue, somatic embryogenesis and genetic transformation.

Avocado embryo rescue

The avocado fruit has a high abscission rate. As a result of controlled or hand-pollination, only a few fruits remain on the tree and few hybrid embryos develop to maturity. This is a serious obstacle for avocado breeding. Abscissed avocado embryos cannot germinate without intervention; however, the *in vitro* culture of immature embryos allows germination and plant conversion, i.e., embryo rescue, and enables the recovery of interesting crosses which would be lost otherwise (Sharma *et al*., 1996). *In vitro* embryo rescue of avocado was first reported by Skene and Barlass (1983). They observed that shoots could sprout after culturing immature embryos (40-140 days after pollination, DAP) in half strength MS liquid medium (Murashige and Skoog, 1962) supplemented with 2.22 μM benzyladenine (BA). They pointed out that embryos <6-weeks-old did not produce shoots *in vitro* with progressively better results being obtained with embryo age. However, the rooting capacity of the recovered shoots was low and it was necessary to graft them onto greenhouse grown seedlings.

Perán-Quesada *et al*. (2005) carried out histochemical studies with the avocado in order to define distinct phases during seed development; they were particularly interested in zygotic embryo development. They identified 1) the histodifferentiation phase, i.e., active cell division and differentiation which is completed approximately 100 DAP (16-18 mm long embryos) and 2) the maturation phase, i.e., starch accumulation and the appearance of protein bodies, which can be detected approximately 125 DAP (24-26 mm long embryos). Germination of very immature embryos (64-95 DAP) occurred at low frequency (< 20%), while for 125 DAP embryos it was 60% on semi solid M1 medium (Skene and Barlass, 1983). Based on these results, Márquez-Martín (2007) studied the effect of an *in vitro* maturation treatment on the subsequent germination capacity of embryos. She used embryos 65 DAP and cultured them on B5 medium (Gamborg *et al*., 1968) with MS micronutrients and organics and with two supplements: 30 g l^{-1} sucrose and Jensen's amino acids (Jensen, 1977) (Figure 1). As expected, this *in vitro* maturation step greatly enchanced the conversion rate allowing 65% plant recovery vs. 6% for the control, in which embryos were directly cultured on semi solid M1 medium (Figure 2). This technique can be used as a reliable method

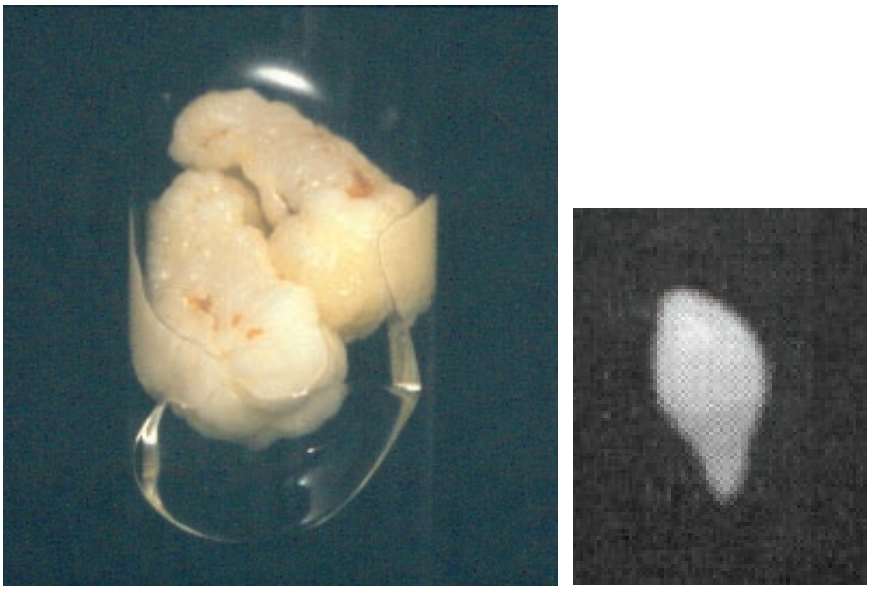

Figure 1. a. Immature zygotic embryo 65 DAP. (b) Immature zygotic embryo following culture in maturation medium.

Figure 2. Avocado seedling derived from a zygotic embryo that developed and germinated *in vitro*.

for recovery of plants from hand pollinations which otherwise would be lost due to the high rate of immature fruit abscission.

Somatic embryogenesis

The development of efficient transformation systems requires the establishment of reliable regeneration protocols from single cells. Plant regeneration can occur via adventive organogenesis, e.g., development of unipolar structures (shoot meristems), which after elongation can be rooted and a whole plant recovered, or somatic embryogenesis, the development of bipolar structures with shoot and root meristems (somatic embryos), from which plants can be obtained after proper maturation treatments. In the case of the avocado, the embryogenic regeneration pathway is used.

Induction

Induction of embryogenic cultures of avocado was first reported by Pliego-Alfaro (1981) and Pliego-Alfaro and Murashige (1988) using immature 'Hass' embryos. Since then, it has been observed in zygotic embryos $\leq$1 mm of several different cultivars (Witjaksono and Litz, 1999a), although Raviv *et al*. (1998) also observed the process using 7-10 mm long embryos. Witjaksono *et al*. (1999a) also induced embryogenic cultures using maternal tissues as explants, i.e., the nucellus, under the same conditions used for zygotic embryos. The nucellar embryogenic response is much less than the response of zygotic embryos and severe browning problems occur following removal of this tissue from the fruit and establishment of it on induction medium.

Induction of embryogenic cultures from both types of explants occurs on semi-solid MS medium supplemented with 0.41 μM picloram. Induction occurs 8-25 days after explanting, and generally starts at the embryo's hypocotyl region, although, later on, response can be observed from the entire embryo. Frequency of response falls within the range 0-15% and it is clearly genotype-dependent. Initial experiments for induction of embryogenic cultures were carried out either in darkness or under intermittent light/dark conditions. Currently, incubation under constant darkness is the general rule since better responses are generally obtained (Witjaksono and Litz, 1999a; Perán-Quesada *et al*., 2004).

Maintenance

Conditions for maintenance and proliferation of embryogenic cultures are those indicated for induction, although Witjaksono and Litz (1999a) observed that more somatic embryos develop on medium consisting of B5 major salts with MS micronutrients and organics. In the course of development, embryogenic and non-embryogenic cultures can be distinguished and separated. According to Witjaksono and Litz (1999a) two types of embryogenic cultures with very distinct morphologies can be distinguished:

- PEM type cultures (Figure 3), mainly consisting of proembryonic masses (PEMs) with very rare differentiation of embryos beyond heart stage, in the presence of auxin.
- SE type cultures (Figure 4), a mixture of somatic embryos (SEs) at different developmental stages with low frequency appearance of PEM-type structures in auxin-containing medium.

Embryogenic avocado cultures can proliferate on semi solid medium for several years by subculturing the smallest PEMs; however, as culture age progresses, a tendency to form smaller and more disorganized structures is observed; e.g., SE-type cultures lose the ability to form somatic embryos beyond the heart stage, and acquire the PEM-type appearance. PEM-type cultures also show a progressive deterioration with time and completely lose the ability to regenerate embryos.

It is also possible to maintain embryogenic cultures in suspension, although under these conditions they can rapidly lose their embryogenic competence if not properly handled. Suspension cultures of SE-type must be sieved prior to subculture and only the fraction $\leq$0.8 mm is used for subculturing onto fresh medium at 2-week-intervals. For suspension cultures, MS medium is modified by lowering the contents of NO_4NO_3 and KNO_3 to 12 and 30.3 mg/l, respectively (Litz *et al*., 2005).

Embryo development and maturation

Cailloux *et al*. (1996) pointed out that during somatic embryo maturation, the accumulation of storage products is responsible for changes in embryo appearance from translucent to white opaque; hence this trait can be used as an indicator of the efficiency of treatments employed to mature avocado somatic embryos.

Figure 3. PEM-type embryogenic avocado culture on induction medium.

Figure 4. SE-type embryogenic avocado culture on induction medium.

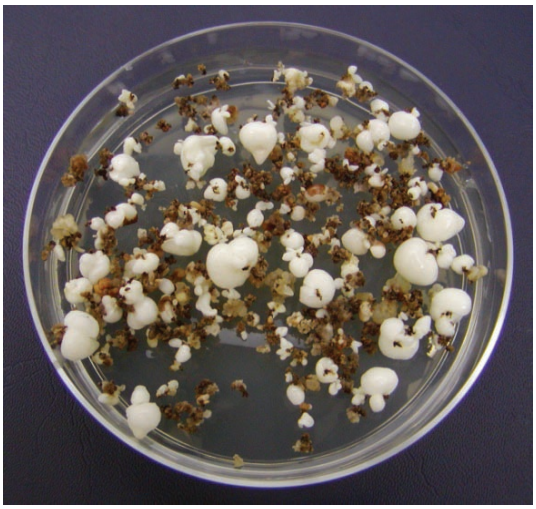

Figure 5. White opaque avocado somatic embryos on maturation medium.

The embryogenic suspension fraction that passes through a 1.8 mm screen mesh opening and retained on a 0.8 mm screen mesh opening, has been used by Witjaksono and Litz (1999b) as the inoculum for initiating somatic embryo development. Filtration is a very important step since it allows culture synchronization favoring development of uniform somatic embryos. Inoculum size and time in culture have been shown to be important for recovery of white opaque embryos, e.g., nine days in liquid medium appear to be optimum for SE-type cultures while up to 14 days is optimum for PEM-type suspensions. Optimum inoculum size also varies for both types of material ranging from 0.4 g per 40 ml in SE-type cultures to 4 g per 40 ml for PEM-type cultures (Sánchez-Romero *et al*., 2005). In any case, the capacity for recovery of white opaque somatic embryos is much greater with SE-type than PEM-type cultures.

In early reports, somatic embryo development was described on auxin-free medium; however, Raviv *et al*. (1998) and Witjaksono and Litz (1999b) observed that somatic embryo development from SE-type cultures occurs on auxin-containing medium. Other factors, e.g., gelling agent type and concentration, are of critical importance for proper development of avocado somatic embryos. Litz *et al*. (2005) recommends that high concentration of gelling agent, e.g., 6-7 g l^{-1} gellan gum, should be used to stimulate the development of high quality somatic embryos (Figure 5); similarly, Sánchez-Romero *et al*. (2005) used 10-12 g l^{-1} agar. Sugar concentration is also a key factor for somatic embryo maturation, Witjaksono and Litz (1999b) obtained more white opaque somatic embryos on medium with 90 g l^{-1} sucrose, although embryo-size was significantly reduced. According to Litz *et al*. (2005) optimum development of somatic embryos to full maturation occurs in darkness at 25°C, and the incorporation of coconut water (20% v/v) seems to improve the quality of the recovered somatic embryos.

Germination and plant recovery

For somatic embryo germination, fully enlarged, white opaque embryos 0.5-1 cm diameter are used. Witjaksono and Litz (1999b) recommended semi solid MS medium supplemented with 4.44 μM BA and 2.89 μM gibberellic acid (GA_3) for germination; Perán-Quesada *et al*. (2004) used semi solid half strength MS medium with 2.22 μM

BA, and also recommended the partial removal of the cotyledons. Low germination rates, usually much less than 10%, are typical of avocado somatic embryos, and is largely genotype-dependent. Unipolar somatic embryos that have either a shoot or a root are obtained (Figure 6); very rarely do both poles differentiate in the same somatic embryo. This could be due to suboptimal maturation conditions, particularly at the earliest stage of maturation. Shoots obtained from germinated somatic embryos are weak and grow slowly. Raharjo and Litz (2005) improved shoot recovery by micrografting somatic embryo shoots onto *in vitro* germinated seedlings (Figure 7). Sprouted shoots, 0.5-1 cm long, can be multiplied *in vitro* following either the procedure of Barceló-Muñoz *et al*. (1990) or that of Witjaksono *et al*. (1999b); shoot proliferation occurs on medium containing 4.44 μM BA. For rooting, shoots $\geq$1.5 cm long are dipped in 122.6 μM indole-butyric acid (IBA) for 3 days and then transferred to 33% MS basal medium.

Genetic transformation

Genetic transformation allows the incorporation of specific genes coding for important agronomical traits without modifying the rest of the plant genome. Genetic transformation is viewed as having great potential for protecting avocado from infection by avocado sunblotch viroid (ASBVd), *C. gloeosporioides* and *P. cinnamomi*, and for addressing certain horticultural problems, e.g., controlling fruit ripening, particularly in the Antillean (West Indian) cultivars (Litz et al., 2005).

Fruit of Guatemalan and Mexican cultivars and their hybrids mature but do not directly ripen, and can be stored on-the-tree for 2-4 months (Whiley, 1992); ripening occurs after the fruit have been harvested. This has enabled nearly year-round production of 'Hass' in many parts of the world. In contrast, West Indian and West Indian x Guatemalan cultivars mature and ripen on-the-tree, and therefore must be picked at maturity. Year-round production of West Indian and West Indian x Guatemalan fruit in the tropics requires several cultivars, each with a different maturity date. According to Crane *et al*. (1996), more than 20 cultivars, each with a specific ripening period, are grown in Florida to provide a constant supply of fruit. Consequently, there is no market standard for tropical avocados. Blocking fruit ripening of tropical avocados would enable on-the-tree storage.

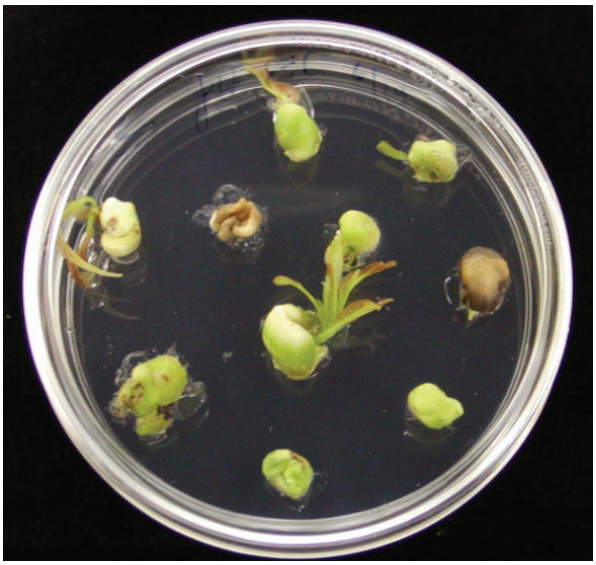

Figure 6. Shoot development from unipolar avocado somatic embryos.

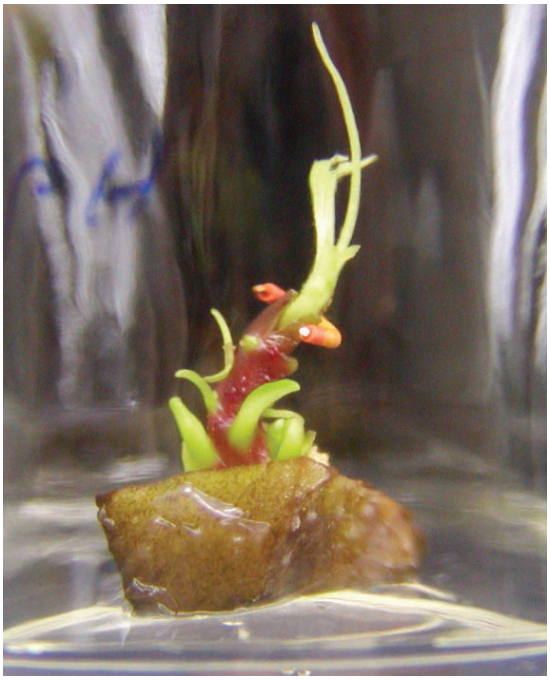

Figure 7. Micrografting of avocado somatic embryo shoot on decapitated *in vitro*-germinated seedling.

Efficient recovery of large numbers of avocado somatic embryos has been demonstrated; however, very few good quality, bipolar somatic embryos are produced (Pliego Alfaro and Murashige, 1988; Raharjo and Litz, 2005). This is a serious limitation for efficiently transforming this species. Nevertheless, Cruz Hernandez et al. (1998) successfully transformed embryogenic cultures and recovered somatic embryos; however, plant regeneration was not reported. Raharjo and Litz (2005) maximized the recovery of plants from genetically transformed cultures by micrografting transformed shoots on *in vitro*–grown seedling rootstocks. Approximately one year after micrografted plants had acclimatized in a greenhouse, transformed shoots could be air-layered in order to recover transformed roots (Raharjo *et al*., 2008) (Figures 8 and 9).

Extending shelf life of the avocado, a climacteric fruit

In order to address on-the-tree and postharvest storage of tropical avocado fruit by controlling ethylene biosynthesis, embryogenic cultures have been transformed with a modified bacteriophage gene S-adenosyl-L-methionine hydrolase (SAMase) under the control of a cellulase promoter isolated from avocado fruit (Hughes *et al*., 1987; Efendi, 2004). SAMase catalyzes the conversion of SAM to methylthioadenosine (Good *et al*., 1994). SAM is the metabolic precursor of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene; degrading SAM inhibits ethylene biosynthesis (Good *et al*., 1994). S-adenosyl-L-methionine hydrolase is in a binary vector, pAG4092; the pPZP200 binary vector was utilized as a backbone. The pAG4092 also includes the NPTII gene that encodes resistance to the antibiotic kanamycin under the AGT01 promoter.

Embryogenic suspension cultures in their logarithmic phase of growth were passed through sterile filtration fabric (1000 µmm pore size), and approximately 300 mg of the large fraction (>1000 µmm) was abraded with a sterile brush on sterile filter paper. Abraded embryogenic cultures were cocultured with log phase acetosyringone-activated *A. tumefaciens* containing pAG4092. An embryogenic suspension culture in its log phase of growth was sieved through sterile nylon filtration fabric (mesh size 1.8 mm), and the smaller fraction was subcultured into 80 ml fresh maintenance medium in 250 ml Erlenmeyer flasks supplemented with 50 mg litre^{-1} kanamycin sulfate

Figure 8. Adventitious roots from air layered avocado scion derived from a somatic embryo shoot.

Figure 9. Self-rooted avocado plants rescued by micrografting followed by air layering.

and 100 mg litre^{-1} spectinomycin to which acetosyringone-activated *A. tumefaciens* was added. After three days, the cultures were transferred into fresh maintenance medium supplemented with 200 mg litre^{-1} cefotaxime and 500 mg litre^{-1} carbenicillin for eight days with a change of medium after two days. Embryogenic cultures were then transferred into fresh maintenance medium supplemented with 50 mg litre^{-1} kanamycin sulfate and then four days later to fresh medium with 100 mg litre^{-1} kanamycin sulfate. Cultures were maintained in maintenance medium supplemented with 100 mg litre^{-1} kanamycin sulfate. Somatic embryo development occurred on semi solid MS medium supplemented with 30 g litre^{-1} sucrose, 20% (v/v) filter-sterile coconut water and 100-300 mg litre^{-1} kanamycin sulfate (Witjaksono and Litz, 1999b; 2002). Shoots from transformed cultures were micrografted in vitro and transferred to the nursery; these plants have been under assessment in a screenhouse since 2005.

Disease resistance

In the absence of candidate genes that might confer resistance to PRR and other diseases, e.g., avocado sunblotch, avocado has been transformed with an antifungal defensin gene pdf1.2 (Raharjo *et al*., 2008) and the pac1 ribonuclease gene (Perea and Litz, unpublished data), respectively. The pdf1.2 gene was originally isolated from *Arabidopsis thaliana*, and is induced by different phytopathogenic fungi: *Alternaria raphans*, *A. brassicola*, *Fusarium oxysporum f. sp. matthiolae, F. oxysporum f. sp. raphans*, etc. (Epple *et al*., 1997). The defensin gene was driven by the CaMV 35S promoter in binary vector pGPTV; the construct also contained the *uidA* reporter gene (GUS) and the *bar* gene for resistance to phosphinothricin, the active ingredient of the herbicide Finale®.

Embryogenic cultures were transformed with log phase acetosyringone-activated EHA105/pGPTV-BPDF1.2 (see above). Suspension cultures were sieved through sterile nylon filtration fabric (mesh size 1.8 mm), and approx. 3.0 g of the small fraction was plated on semi-solid maintenance medium and gently abraded with a sterile camel hair brush. Embryogenic cultures and EHA105/pGPTV-BPDF1.2 were co-cultivated for 3 days, and then transferred to liquid maintenance medium supplemented with filter-sterilized 200 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin. After 2 wk, the

proembryonic masses were transferred into fresh liquid maintenance medium supplemented with filter-sterilized 200 mg l^{-1} cefotaxime, 500 mg l^{-1}carbenicillin and 3.0 g l^{-1} phosphinothricin, and subcultured at 2-wk intervals for 3 months (Figure 10). Somatic embryo development occurred on semi solid MS medium supplemented with 30 g $litre^{-1}$ sucrose, 20% (v/v) filter-sterile coconut water and filter-sterilized 3.0 mg l^{-1} phosphinothricin. Somatic embryo shoots derived were excised and micrografted in vitro (Raharjo and Litz, 2005). After the micrograft unions had formed, the transgenic shoots were removed and side-grafted onto seedling rootstocks in the greenhouse (Raharjo and Litz, 2005). The transgenic scions were successfully air-layered after approximately 1 year in order to recover plants on transgenic roots. These plants have been under assessment in a greenhouse for more than 5 years (Figure 11).

Transgenesis versus cisgenesis

Genetic transformation enables breeding programs to be accelerated, and there are several examples of successful products, e.g., ringspot virus resistant papaya, sharka virus resistant plums, etc. The testing and release of transgenic organisms must follow strict regulatory rules to prevent any negative effects on the environment or human health. Schouten *et al*. (2006) proposed that cisgenic plants could avoid the severe regulations and overcome public opinion resistance to genetically modified organisms (GMOs) in the EU. In cisgenesis, the same techniques described above are used; however, genes with promoters derived from the plant itself or from a sexually compatible relative are used. Genes used in cisgenesis could also be transferred through traditional breeding techniques. Compared to traditional breeding methods, cisgenesis is a simple step gene transfer without the drawbacks occurring in traditional breeding due to linkage drag (Jacobsen and Schouten, 2007). This supposes an advantage for cisgenesis and it could really speed up breeding programs of vegetatively propagated cross-fertilizing heterozygous plants. Genes could be introduced without changing the specific alleles combination of the recipient genotype. Schouten *et al*. (2006) refer to the the program for introducing the apple scab resistance gene from wild into cultivated apple, which began 50 years ago. Due to linkage drag effects, the obtained varieties still require further cross-

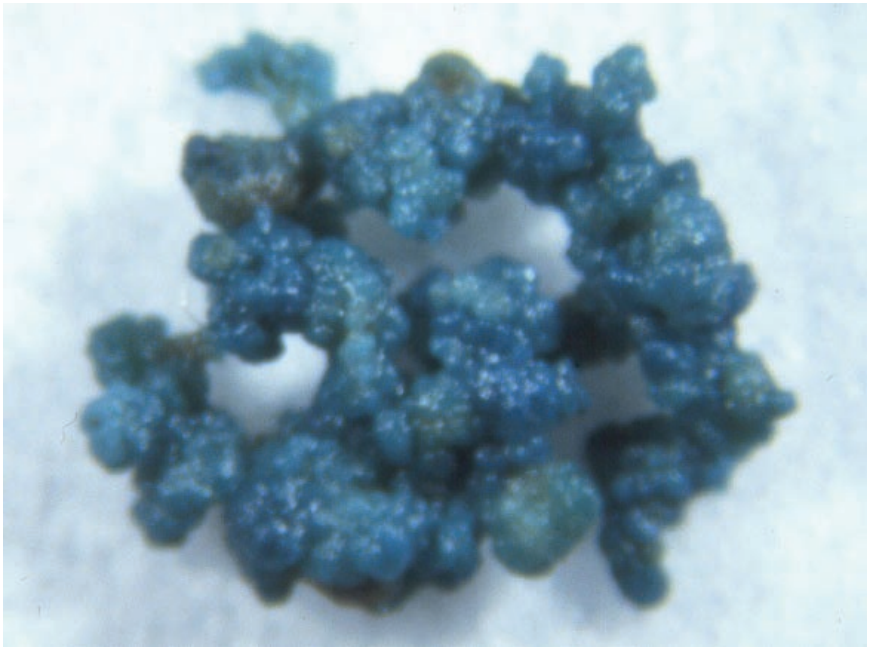

Figure 10. Genetically transformed embryogenic avocado cultures: GUS reaction.

Figure 11. Genetically transformed avocado plant (center) containing the following genes: bar for resistance to Finale®; pdf1.2 defensin; gus. The defoliated plants (left and right) are non transformed controls.

ing to acquire the same fruit quality traits as the sensitive parental cultivars. Schouten *et al*. (2006) propose that the regulations of the European Community that apply to genetically modified organisms should be changed and cisgenesis to be under the same regulations as mutagenesis or cell fusion, currently considered non-GMO breeding techniques. For this approach to be utilized for the avocado, we assume that understanding the genomics of avocado will have greater importance in avocado improvement.

In conclusion, important progress has been made in recent years towards the recovery of avocado material from tissue culture. The use of biotechnology approaches will assist in furthering the goals of all avocado plant improvement programs.

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