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CRYOPRESERVATION OF AVOCADO

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ABSTRACT

Avocado genetic resources are maintained ex situ in field repositories at great cost and always under threat of inclement weather, pests and disease. Cryopreservation is an important alternative method for long-term conservation of plant genetic resources. Moreover, it is an important storage method for biotechnology research, in which experimental materials, i.e., embryogenic cultures, lose morphogenic competence relatively quickly and cannot be stored reliably in vitro. Two cryopreservation procedures have been developed for avocado embryogenic cultures: 1) slow cooling at $-1^{o}C\ min^{-1}$ from 25°C to –80°C followed by rapid cooling to –196°C; and 2) vitrification or rapid cooling from 25°C to –196°C. Embryogenic cultures recovered from cryogenic storage demonstrate normal growth, and somatic embryos can be recovered. All of the avocado genotypes currently being genetically manipulated in our research program have been successfully introduced into liquid nitrogen. Cryogenic storage of avocado has important significance for management of avocado genetic resources.

Key Words: avocado, somatic embryo, cryopreservation,

INTRODUCTION

Somatic cell genetic studies of avocado are based upon the somatic embryogenic pathway. Witjaksono and Litz (1999a) demonstrated that embryogenic avocado cultures can lose their morphogenic competence as early as 3-4 months after induction. This loss of competence is genotype-dependent. As a result, it is impossible to carry out medium and long term research involving genetic transformation and protoplast culture with many avocado cultivars. Storage of embryogenic cultures in liquid nitrogen has been demonstrated with citrus (Sakai et al., 1990; 1991; Kobayashi et al., 1990; Duran Vila, 1995; Perez et al., 1997; 1999), and a few other tropical/subtropical fruit trees, e.g., rubber (Engelmann and Etienne, 1995) and oil palm (Dumet et al., 1993). This strategy ensures a continuous supply of embryogenic cultures to support somatic cell gene-

tic studies. Moreover, cryopreservation has become integral for the management of plant genetic resources. Germplasm collections are expensive to maintain and can be threatened by pests, diseases and weather. Cryopreservation is a relatively inexpensive method for backing up germplasm collections. The development of two protocols for cryogenic storage of embryogenic avocado cultures and recovery of somatic embryos and plants following cryopreservation have been developed: 1) slow or step-wise cooling (-1°C min^{-1}) to –80°C followed by rapid cooling to –196°C; and 2) rapid cooling to –196°C.

MATERIALS AND METHODS

embryogenic cultures

Embryogenic cultures were induced in petri dishes from zygotic embryo explants of several avocado cultivars on $B5^-$ medium, i.e., B5 major salts (without NH_4NO_3) (Gamborg et al., 1968), MS (Murashige and Skoog, 1962) minor salts, 0.41 mM picloram and (in mg $liter^{-1}$) thiamine HCl (4), myo-inositol (100), sucrose (30,000) and agar (8,000) (Witjaksono and Litz, 1999a). Cultures were maintained on MSP, i.e., MS basal medium, with 0.41 mM picloram, the organic components of $B5^-$ and agar (8,000) (Witjaksono and Litz, 1999a). After 3 to 6 subcultures on MSP, embryogenic cultures were transferred into liquid MS3:1P medium, i.e., MS basal medium containing 60 mM inorganic nitrogen (75% of nitrogen is NO_3^- and 25% is NH_4^+) supplemented with organic addenda (Witjaksono and Litz, 1999b) and the other components of MSP.

Petri dishes were sealed with Parafilm® and cultures were maintained in darkness at room temperature (25°C). Suspension cultures in Erlenmeyer flasks were sealed with aluminum foil and Parafilm, and incubated under ambient laboratory conditions at 125 rpm. The pH was adjusted to 5.7 with either KOH or HCl prior to autoclaving at 1.1 kg cm^{-2} and 120°C for 20 min.

cryopreservation

slow or step-wise cooling cryopreservation. Embryogenic cultures of avocado were successfully cryopreserved using a cryoprotectant mixture of 5% DMSO and 5% glycerol with either 0.13M or 1.0M sucrose (Efendi, 2003). Approximately 200 mg of air-dried embryogenic cultures were mixed with 1.0 ml of sterile cryoprotectant solution in 1.2 ml Corning®, cryogenic vials, and maintained on ice for 30 min. The vials were inserted into Nalgene®, "Mr. Frosty" containers containing 250 ml isopropanol. The containers were placed in a low temperature (–80°C) freezer for 2h for controlled cooling at the rate of -1°C min^{-1}. After 2 h, the vials were removed from "Mr. Frosty" and inserted into aluminum cryo-cane holders. They were then immersed directly into liquid nitrogen (-196°C) in a Bio-Cane 20 Dewar container.

rapid cooling or vitrification. Embryogenic cultures (approximately 200 mg) were suspended in 1.0 ml sterile loading solution (2.0M glycerol and 0.4M sucrose) in 1.2 ml cryovials for 15 min (Efendi, 2003). The loading solution was then replaced with 1.0 ml plant vitrification solution number 2 (PVS2) (Sakai et al., 1990), and maintained on ice. PVS2 consisted of 15% DMSO, 30% glycerol and 15% ethylene glycol. The vitrification solution was decanted after 60 min, and the cryovials were inserted into cryosleeves, and plunged into liquid nitrogen in a Bio-Cane 20 Dewar container.

recovery from cryopreservation. Following storage in liquid nitrogen, the cryovials were removed from liquid nitrogen and immersed for 5 min in a 40°C water bath (Efendi, 2003). The embryogenic cultures were decanted onto sterile semi solid MSP medium in Petri dishes. Vital staining with sodium tetrazolium chloride (TTC) immediately after thawing could not be correlated with survival. There was a strong cultivar-related difference in survival of cryopreserved cultures. Cryopreserved

embryogenic cultures proliferated on MSP medium and as suspension cultures in MS3:1P liquid medium. The growth rate of cryopreserved cultures exceeded that of the non cryopreserved controls.

Embryogenic cultures were plated on somatic embryo development (SED) medium, i.e., MS3:1 medium supplemented with 20% (v/v) filter-sterilized coconut water and solidified with 6 g liter^{-1} gellan gum (Witjaksono and Litz, 1999b). The plates were closed but not sealed and were maintained in total darkness at 25^{o}C for two months. The recovery of white-opaque mature somatic embryos was significantly greater from cryopreserved cultures than from the non frozen controls**.** Plants have been recovered from avocado somatic embryos derived from cryopreserved embryogenic cultures at the same frequency that has been reported for other studies involving avocado somatic embryogenesis.

DISCUSSION AND CONCLUSIONS

Avocado has been regenerated from non elite materials (zygotic embryos) (Mooney and Van Staden, 1987; Pliego Alfaro and Murashige, 1988; Raviv et al., 1998; Witjaksono and Litz, 1999a, b) and from elite or clonal materials (Witjaksono et al., 1999). The long term maintenance of clonal avocado genetic resources currently is based upon ex situ germplasm collections. Cryopreservation of elite avocado genetic resources is feasible, based upon the results of the current study. The establishment of long term cryogenic storage banks for avocado germplasm would be important back-ups for existing ex situ collections. Moreover, cryogenic storage of elite avocado germplasm will stimulate the exploitation of this material using somatic cell genetic approaches.

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