Somatic Embryogenesis in Yam (*Dioscorea rotundata*)

Embriogenesis Somatica en Ñame (*Dioscorea rotundata*)

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Abstract. Embryogenic yam (***Dioscorea rotundata***) cultures *were induced from petioles of leaves of* ***in vitro*** *grown plants on medium supplemented with different 2.4-D concentrations. Cultures were maintained either on semisolid or in liquid MS medium supplemented with 4.52 µM 2.4-D. The effect of sucrose concentration on somatic embryo development was also evaluated and the effects of different BAP concentrations on somatic embryo conversion were determined. Treatments were distributed using a complete randomized design. The highest rate of induction occurred with 4.52 µM 2.4-D. Sucrose at 131.46 mM significantly enhanced somatic embryo development. The conversion rate was not affected by BAP.*

Key words: Auxin, conversion, tropical species, food security.

Resumen. *Cultivos embriogénicos de ñame (****Dioscorea rotundata****) fueron inducidos a partir de explantes consistentes de hojas con peciolos, aisladas de plantas establecidas en condiciones* ***in vitro****, en presencia de diferentes concentraciones de 2,4-D. Los cultivos inducidos fueron mantenidos en medio MS líquido o semisólido suplido con 4,52 µM 2,4-D. El efecto de las concentraciones de sacarosa sobre el desarrollo de embriones somáticos y el efecto de varias concentraciones de BAP sobre la tasa de conversión de embriones somáticos en plantas también fueron evaluados. Todos los tratamientos fueron distribuidos usando un diseño completamente al azar. El mayor porcentaje de inducción de tejidos embriogénicos ocurrió con 4,52 µM de 2,4-D. La adición de 131,46 mM de sacarosa incrementó significativamente el desarrollo de embriones somáticos. La tasa de conversión de embriones somáticos en plantas no fue afectada por las concentraciones de BAP.*

Palabras clave: Auxina, conversión, especies tropicales, seguridad alimentaria.

White yam (*Dioscorea rotundata*) is a staple food for rural inhabitants of the Caribbean Coast of South America. It has great cultural value for people in tropical regions (Sanchez and Hernández, 2000). Yams are propagated from seeds and from tuber cuttings; however, flowering and seed production occur rarely for most cultivars in the tropics. Commercial crops are planted using tuber pieces as propagules. Vegetative propagation, and the poor flowering biology of the species, has eroded the genetic base of the species and breeding to overcome anthracnose, which is caused by the fungus, *Colletotrichum gloeosporiodes*, and the main limiting factor of yam production, has been very difficult. The disease attacks the entire plant, prevents tuber formation and causes plant death (Mignouna *et al*., 2001; Pérez *et al*., 2003).

Biotechnology technics can be used when traditional breeding is ineffective for crop improvement. One approach involves the *in vitro* selection of mutated cell lines in the presence of a selective agent (Royero *et al*., 2007). Embryogenic cultures have been utilized to obtain disease-resistant off-types following their challenge by the culture filtrates produced by *Colletotrichum gloeosporioides* and *Elsinoe ampelina*, respectively (Jayasankar *et al*., 1998; 2000). Plant regeneration from cells is necessary to apply *in vitro* selection or genetic transformation.

The objective of the present study has been to develop a protocol for somatic embryogenesis of *Dioscorea rotundata* as a plant regeneration technique from cellular tissues.

MATERIALS AND METHODS

Embryogenic culture induction and maintenance. Explants, consisting of leaf petioles (Espitia and

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Quintero, 1999), were isolated from *in vitro*-established 9811-090 *Dioscorea rotundata* plants growing on semi solid MS (Murashige and Skoog, 1962) medium supplemented with (in mg L^{-1}) sucrose, (30000), myo-inositol (100), thiamine-HCl (0.1) and TC- Agar (6000) (Sigma®). Leaf petiole explants were inoculated into sterile 30 mL semi solid induction medium (IM) (De Wald *et al*., 1989; Witjaksono and Litz, 1999a) in (60 x 15 mm) Petri dishes. Induction medium consisted of B5 (Gamborg *et al*., 1968) major salts, MS minor salts and (in mg L^{-1}) sucrose, (30000), myo-inositol (100), thiamine-HCl (0.1) and TC- Agar (6000). To evaluate the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on embryogenic culture induction, IM was independently supplemented with four (0.0; 4.52; 9.05 and 18.1 µM) 2,4-D concentrations. Treatments were distributed using a complete randomized design with 20 replicates per treatment. Every experimental unit consisted of a single Petri dish that was inoculated with three petiole explants. The Petri dishes were sealed with Parafilm® and were stored in darkness at 25 °C for 4-8 weeks. The number of induced explants as well as the type of embryogenic culture induced was registered weekly.

Embryogenic cultures were transferred onto semi solid maintenance medium (MM) (De Wald *et al*., 1989; Witjaksono and Litz, 1999a) that was supplemented with 4.52 µM 2.4-D and stored as indicated for induction with subculture to fresh medium at four-week intervals. Maintenance medium consisted of MS and (in mg L^{-1}) sucrose (30000), myo-inositol (100), thiamine-HCl (0.1), TC- Agar (6000) and 4.52 µM 2.4-D. Approximately 200 mg of a two-week-old embryogenic culture that had been maintained on MM were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of liquid maintenance medium (MM without TC- Agar). Erlenmeyer flasks were covered with aluminum foil, sealed with Parafilm® and maintained on a rotary shaker at 120 rpm in darkness at 25 °C. Cultures were transferred to fresh medium biweekly.

Somatic embryo development. Embryogenic cultures consisting of proembryonic masses (PEMs) from liquid medium were sieved through sterile 1.8 mm mesh nylon filtrate fabric. The smallest fraction was air-dried on several layers of sterile Kimwipes® to remove excess liquid medium, and the embryogenic tissue was inoculated into 20 mL semi solid somatic embryo development medium (SED) in sterile (100 x 20 mm) Petri dishes. SED consisted of MS medium with (in mg L^{-1}) thiamine HCl, (0.4), myo-inositol (100) and Phytagel® (3000) (Sigma®). To evaluate the effect of sucrose concentration on somatic embryo development, SED also supplemented with three (87.64; 131.46 and 175.28 mM) sucrose levels. Approximately 100 mg of PEMs was evenly spread over the medium surface of each Petri dish and incubated for eight weeks in darkness at 25 °C. Treatments were distributed using a complete randomized design with 30 replicates per treatment. After eight weeks, the total number of opaque-white somatic embryos (>0.2 cm diam) that developed on each Petri dish was determined. Data were analyzed with ANOVA and means were separated by Duncan test.

Plant recovery. Opaque-white somatic embryos (>0.2 cm diam) were inoculated onto somatic embryo germination medium (SEG) supplemented with 2.89 µM GA_3. SEG consisted of MS with (in mg L^{-1}), thiamine HCl (4), myo-inositol (100), sucrose (30000) and Phytagel® (3000). In order to evaluate the effect of benzylamino purine (BAP) on somatic embryo conversion, four (0; 1.11; 2.22 and 4.44 µM) BAP levels were examined. The medium was autoclaved and dispensed in 25 mL aliquots into 125 mL sterile baby food jars. Seven white-opaque somatic embryos were inoculated into each jar (75 jars). Treatments were distributed using a complete randomized design with 20 replicates per treatment. The jars were covered with heavy duty aluminum foil, sealed with Parafilm® and were stored in translucent plastic boxes in a 16-h photoperiod provided by cool white fluorescent tubes (40–50 µmol s^{-1} m^{-2}) at 25 °C until roots and/or shoots were visible. Plants were recovered from opaque-white somatic embryos two-three months after inoculation onto SEG, and were maintained on MS basic medium. Data were analyzed with ANOVA and means were separated with Duncan test.

Medium sterilization. After all components were supplied, the pH of all media was adjusted to 5.7–5.8 prior to addition of gelling agent. Media were sterilized by autoclaving at 121 °C at 1.1 kg cm^{-2} for 15 min.

RESULTS AND DISCUSSION

Embryogenic culture induction and maintenance. Embryogenic cultures consisted of PEMs (Figure 1A). Auxin (2.4-D) was necessary for induction and PEMs were visible after two weeks (Figure 2). The collected data showed that the lowest 2,4-D

concentrations were sufficient for induction, while high concentrations of 2.4-D suppressed induction. Embryogenic cultures proliferated as PEMs on semisolid and in liquid medium (Figure 1B).

Somatic embryo development. Opaque-white somatic embryos developed from embryogenic cultures after transfer onto SED medium. The somatic embryos were opaque-white and round (Figure 1C).

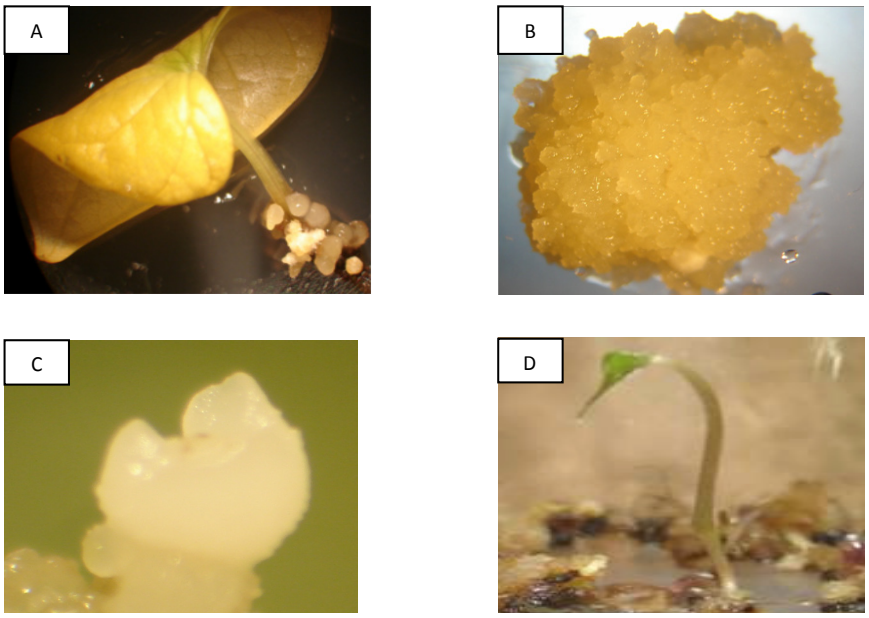

Figure 1. *Dioscorea rotundata* somatic embryogenesis. A: embryogenic culture induction, B: embryogenic culture maintenance, C: somatic embryo development and D: embryo conversion.

Results of ANOVA demonstrated that the number of developed somatic embryos was significantly affected ($P< 0.0001$) by sucrose concentration in the medium. Sucrose concentration at 131.46 mM resulted in more opaque-white somatic embryos per Petri dish (60) than sucrose at 87.64 mM (43). The lowest number of opaque-white somatic embryos (13) was observed with 175.28 mM sucrose.

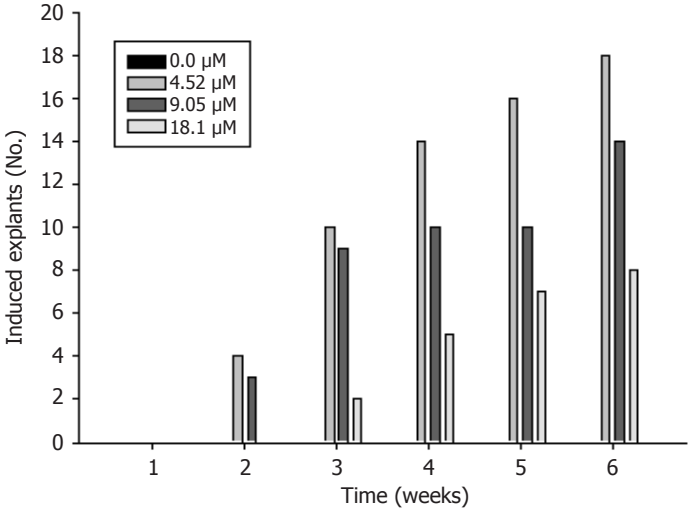

Figure 2. Effect of different auxin (2,4-D) µM on *Dioscorea rotundata* embryogenic tissue induction.

Plant recovery. Opaque-white somatic embryos (≥0.3 mm diameter) germinated (i.e., root and shoot emergence) after four weeks on SEG medium. Recovered plants appeared to be normal with no evidence of morphological variation (Figure 1D). The analyzed data showed that BAP levels had no effect (P= 0.9399) on root and shoot development from somatic embryos (Figure 3).

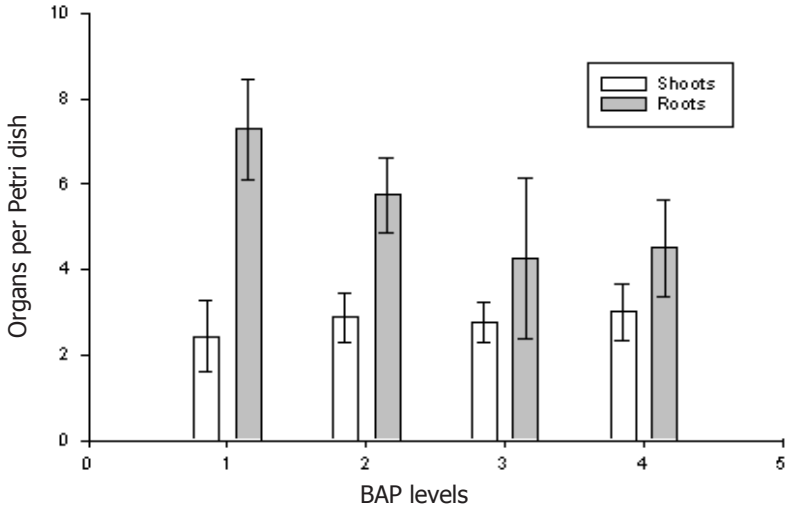

Figure 3. Effect of different BAP levels (1 = 0; 2 = 1.11; 3 = 2.22 and 4 = 4.44 µM) on shoots and root development means (bars=standard errors) from *Dioscorea rotundata* somatic embryos.

In vitro studies with *Dioscorea* species have included micropropagation from pre-existing meristems and organogenenesis of *D. zingiberensis* (Chen *et al*., 2003), shoot organogenesis form immature leaves of *D. opposita* (Kohmura *et al*., 1995), shoot culture and microtuber formation in *D. composita* (Alizadeh *et al*., 1998) and shoot multiplication and *in vitro* tuber formation from *D. alata* stem segments (John *et al*., 1993; Salazar and Hoyos, 2007). Somatic embryogenesis in *D. floribunda, D. composita, D. alata* and *D. bulbifera* has been previously reported (Ammirato, 1978; 1982). In the present study, embryogenic culture induction from *D. rotundata* leaf tissue was possible after two weeks culture on 2.4-D supplied medium in darkness, although low induction frequencies (<30%), indicates a strong recalcitrance of the species to induce embryogenic tissues. Differential effects of 2.4-D concentrations on embryogenic cell induction have been observed. Embryogenic cell masses from *D. alata* root explants were induced in liquid MS supplemented with 4.52 µM 2.4-D in the light (Twyford and Mantell, 1996), while the highest induction rate of induction of *D. opposita* embryogenic cultures was observed when stem segments were cultured in liquid medium supplied with 13.6 µM 2.4-D (Nagasawa and Finer, 1989). Strong auxins, such as 2.4-D and picloram, are normally used for embryogenic culture induction. They stimulate rapid cell division and induce a redetermination of cell type and function associated with differential changes in gene expression that is probably linked to demethylation of DNA (Kohlembach, 1978; LoSchiavo *et al*., 1989). In the current study, somatic embryo development from PEMs occurred on auxin-free medium; however, Ammirato (1982) reported somatic embryo development from *D. bulbifera* and *D. floribunda* embryogenic cultures on media supplied with either 0.1 µM zeatin or 0.1 µM ABA in the absence of 2.4-D, which indicates a possible genotype dependent response. Development of *D. opposita* and *D. alata* somatic embryos from PEMs was also observed following their transfer to auxin-free liquid media (Nagasawa and Finer, 1989; Twyford and Mantell, 1996). Somatic embryo formation can occur when low levels of weaker auxins are present in the medium; Shu *et al*. (2005) reported low frequency of *D. zingiberensis* somatic embryo development when PEMs were transferred onto semi solid medium supplied with 17.74 µM BA and 1.07 µM NAA.

A significantly larger number of somatic embryos occurred when development medium contained 4.5% sucrose while at 6.0% the value decreased indicating a possible overdose. Sucrose is the most suitable

carbohydrate source for *D. rotundata* plant recovery from somatic embryos (Okezie *et al*., 1994). Higher sucrose levels increase medium osmolarity in order to stimulate somatic embryo maturation by lowering water content in the developing embryo, similar to the maturation stage of zygotic embryogenesis, allowing a more normal development process and protein accumulation (Compton and Gray, 1996; Gray, 1995). Additionally, higher sucrose level means more carbon nutrition for a better embryo development (Lee and Thomas, 1985; Carman, 1989). GA_3 has been used to overcome somatic embryo dormancy and increase germination levels (Deng and Cornu, 1992). Twyford and Mantell (1996) observed enhanced *D. alata* plantlet recovery when somatic embryos were cultured on basal semisolid MS medium supplemented with 4.55 µM GA_3. Likewise, cytokinine addition to the germination medium improved organization of the apical meristem of carrot somatic embryos (Fujimura and Komamine, 1975). In the present study, BAP had no effect on the recovered of plants. Similar results were reported for avocado somatic embryos (Witjaksono and Litz, 1999b).

CONCLUSIONS

Dioscorea rotundata can be regenerated via somatic embryogenesis. The highest frequency of embryogenic culture induction was observed on medium supplemented with 4.52 µM 2.4–D. Sucrose at 131.46 mM significantly increased development of opaque-white somatic embryos, while BAP had no effect on somatic embryo conversion. This regeneration protocol can be utilized for non-traditional *in vitro* breeding techniques in yam.

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