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SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM FLORAL EXPLANTS OF CACAO (*THEOBROMA CACAO* L.) USING THIDIAZURON

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Summary

A procedure for the regeneration of cacao (*Theobroma cacao*) plants from staminode explants via somatic embryogenesis was developed. Rapidly growing calli were induced by culturing staminode explants on a DKW salts-based primary callus growth (PCG) medium supplemented with 20 g glucose per L, 9 μ*M* 2,4-D, and thidiazuron (TDZ) at various concentrations. Calli were subcultured onto a WPM salts-based secondary callus growth medium supplemented with 20 g glucose per L, 9 μ*M* 2,4-D, and 1.4 n*M* kinetin. Somatic embryos were formed from embryogenic calli following transfer to a hormone-free DKW salts-based embryo development medium containing sucrose. The concentration of TDZ used in PCG medium significantly affected the rate of callus growth, the frequency of embryogenesis, and the number of somatic embryos produced from each responsive explant. A TDZ concentration of 22.7 n*M* was found to be the optimal concentration for effective induction of somatic embryos from various cacao genotypes. Using this procedure, we recovered somatic embryos from all 19 tested cacao genotypes, representing three major genetic group types. However, among these genotypes, a wide range of variation was observed in both the frequency of embryogenesis, which ranged from 1 to 100%, and the average number of somatic embryos produced from each responsive explant, which ranged from 2 to 46. Two types of somatic embryos were identified on the basis of their visual appearance and growth behavior. A large number of cacao plants have been regenerated from somatic embryos and established in soil in a greenhouse. Plants showed morphological and growth characteristics similar to those of seed-derived plants. The described procedure may allow for the practical use of somatic embryogenesis for clonal propagation of elite cacao clones and other applications that require the production of a large number of plants from limited source materials.

Key words: somatic embryogenesis; cocoa; *Theobroma cacao*; plant regeneration; thidiazuron.

Introduction

Cacao trees (*Theobroma cacao* L.) are grown principally in rainforest areas in the tropical regions of the world. Cacao seeds are the source of cocoa powder and butter, which are important ingredients in chocolate and confectionery products. Cocoa butter is also used in a number of pharmaceutical and cosmetic products. According to recent statistics, approximately 2.76 million metric tons of cacao dried seeds were produced annually, with an export value of $3.86 billion (Wakeling, 1996).

In spite of its economic importance, genetic improvement of cacao has been hampered by the narrow genetic base and the long-term breeding cycle of the crop (Kennedy et al., 1987). The vegetative clonal propagation of superior cacao genotypes has long been recognized as a potential means to increase cacao production (Wood and Lass, 1987). However, progress in the development of improved methods for vegetative propagation of cacao has been slow. Currently, cacao trees are reproduced primarily by seeds and plagiotropic cuttings. Cacao seeds are usually produced through open pollination and thus have a highly heterozygous genetic background. As a result, cacao plants derived from seeds are highly variable in terms of their agronomic performance. In addition, there are a number of disadvantages associated with the propagation of cacao plants via the rooting or grafting of plagiotropic cuttings, including the intensive labor and associated costs, a generally low propagation rate, and an undesirable bush-like growth pattern which can occur (Figueira and Janick, 1995).

Although efforts have been made to develop organogenesis-based propagation methods, cacao has proven to be recalcitrant to *in vitro* shoot regeneration and plant micropropagation (Orchard et al., 1979; Passey and Jones, 1983; Flynn et al., 1990; Figueira and Janick, 1994). Plant regeneration via somatic embryogenesis provides an alternative approach for clonal propagation of cacao. Since somatic embryos are produced through bipolar development of somatic cells, plants derived from somatic embryos are genetically identical to their parental donor cells and have the growth characteristics of seed-derived plants.

Early attempts to develop a somatic embryogenesis-based system for cacao propagation focused on direct embryogenesis from immature zygotic embryos (Esan, 1977, 1992; Pence et al., 1979, 1980). Although somatic embryos were obtained from zygotic embryo-derived tissues, the conversion or germination of these somatic embryos into viable seedlings was problematical (Wang and Janick, 1984).

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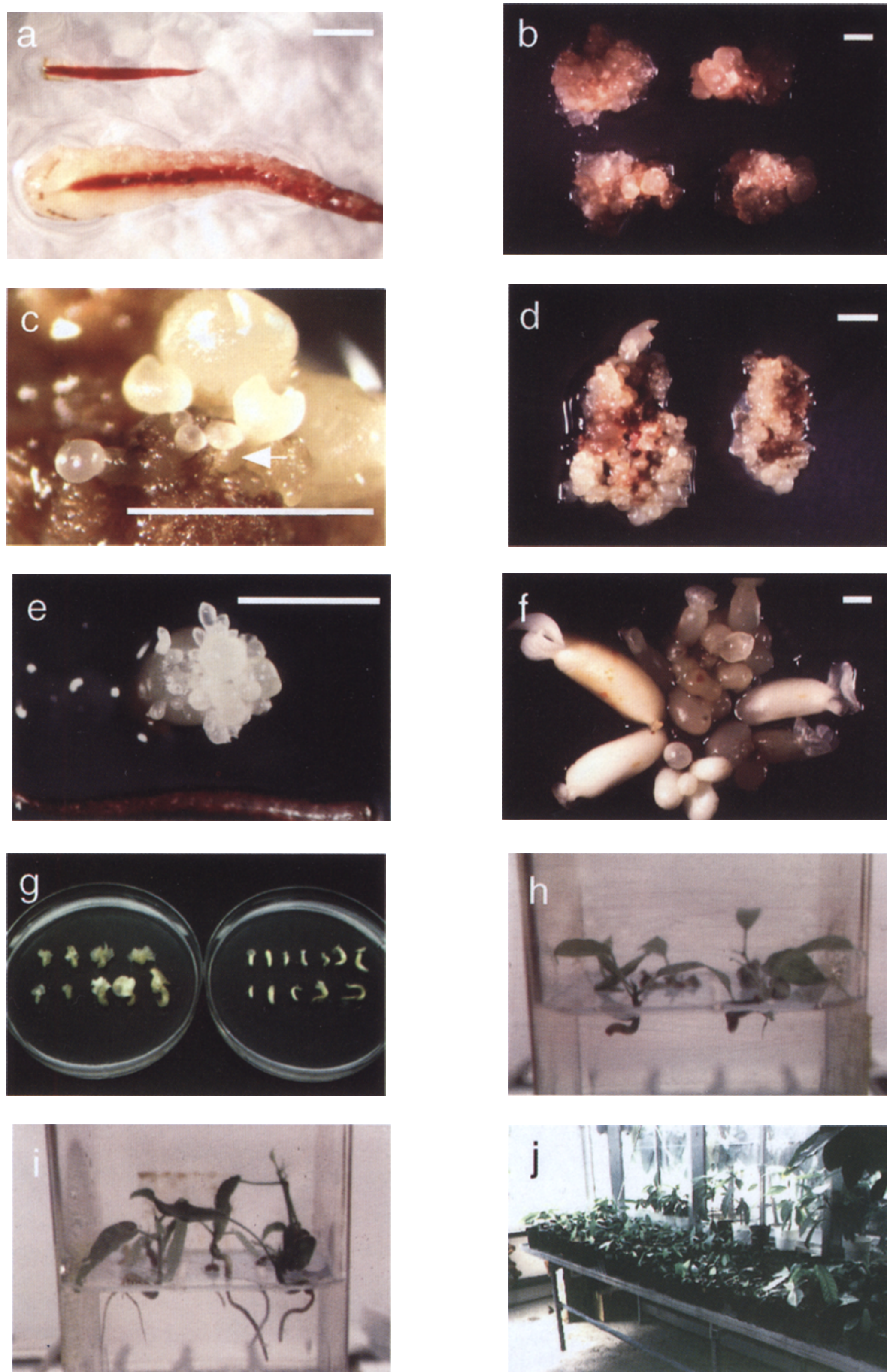

FIG. 1. Somatic embryogenesis and plant regeneration from cultured staminode explants of cacao. *a*, Staminode explants prior to (*top*) and 1 wk after culture on PCG medium containing 22.7 n*M* TDZ (*bottom*). *b*, Embryogenic callus induced from the entire staminode explant 14 d after culture on SCG medium. *c,d*, Somatic embryos at various stages of development (globular and heart-shaped embryos). *Arrow* indicates a suspensor structure of a developing embryo. *e*, Secondary somatic embryos being produced from a primary embryo. A staminode was included for size comparison. *f*, Torpedo-shaped somatic embryos. *g*, Type I (*left*) and II (*right*) somatic embryos at early to late cotyledonary stages on 100 × 15-mm petri dishes. *h,i*, Plantlets produced from Type I and II somatic embryos, respectively. *j*, Established somatic embryo-derived plantlets in the greenhouse. *Bar* = 2 mm.

More recently, efforts were made to induce somatic embryos from floral and nucellar somatic tissues to alleviate the genetic heterogeneity problem associated with the use of zygotic tissues (Sohndahl et al., 1989, 1993; Figueira and Janick, 1993; Lopez-Baez et al., 1993; Alemanno et al., 1996a, 1996b). In spite of the recent progress in this area, the reported efficiencies of somatic embryogenesis and plant regeneration obtained remained low. Furthermore, the practical utilization of this technology for clonal propagation remains hindered by an inability to induce somatic embryogenesis from a majority of elite cacao genotypes.

In this study, we report a procedure for somatic embryogenesis and plant regeneration from floral tissues of a large number of cacao genotypes. The effects of thidiazuron (TDZ), a phenylurea derivative with strong cytokinin-like activity, on somatic embryogenesis in cacao were also evaluated.

Materials and Methods

Plant materials and explant preparation. Staminodes, needle-like structures fused with a stamen at the base, were obtained from unopened immature flowers of greenhouse-grown cacao (*Theobroma cacao* L.) trees. Immature flowers about 4 to 5 mm long were collected in the morning, surface-sterilized by immersion in 1% (wt/vol) calcium hypochlorite for 20 min without agitation, and then rinsed three times with sterile water. Explants were prepared by briefly blotting the immature flowers with sterile paper towels and then slicing them perpendicular to their longitudinal axis about 1/3 of the flower length from its base. Staminodes were extracted from the upper part of the flower bud and placed on the culture medium.

Culture media and growth conditions. Chemicals for medium preparation were purchased from Sigma Chemical Co., St. Louis, MO, or Fisher Scientific, Pittsburgh, PA, unless noted otherwise. All culture media were adjusted to pH 5.8 with 1 *M* KOH and autoclaved at 121° C for 20 min.

Staminodes were first cultured on primary callus growth (PCG) medium that contained DKW basal salts as described by Driver and Kuniyuki (1984) and Tulecke and McGranahan (1985), supplemented with 250.0 mg glutamine per L, 200.0 mg *myo*-inositol per L, 2.0 mg thiamin-HCl per L, 1.0 mg nicotinic acid per L, 2.0 mg glycine per L, 20.0 g glucose per L, 9 μ*M* 2,4-D, various concentrations of TDZ, and 2.0 g phytagel per L. Plastic petri dishes (100 × 15 mm), containing 30 mL of medium, were used as culture vessels. Cultures were maintained in the dark at 25° C for 14 d. Various concentrations (0, 22.7, 45.5, 113.6, 227.3, and 454.5 n*M*) of TDZ, in combination with 9 μ*M* 2,4-D, in PCG medium were evaluated for their ability to stimulate callus growth with three genotypes (Fig. 2) and to enhance somatic embryo production with five cacao genotypes (Figs. 3 and 4). The optimal TDZ concentration identified through these evaluations was subsequently utilized to test somatic embryogenesis response from other available cacao genotypes (Table 1), representing three major genetic groups of cacao (Toxopeus, 1985; Lerceteau et al., 1997). In these experiments, each treatment contained 20 staminodes per culture vessel, with three replicate vessels. Experiments were repeated two to three times depending on the availability of floral explants.

After 14 d on PCG medium, explants were transferred onto petri dishes containing 30 mL of secondary callus growth (SCG) medium and maintained for another 14 d under the culture conditions described above. SCG medium was composed of basal salts of the low salt McCown's woody plant medium (Lloyd and McCown, 1980, Sigma M-6774), Gamborg's vitamin solution (Gamborg, 1966, Sigma G-1019), 20.0 g glucose per L, 9 μ*M* 2,4-D, 1.4 μ*M* kinetin, 50.0 mL coconut water per L and 2.2 g phytagel per L. Coconut water was obtained from immature coconut fruits purchased locally and was filtered through activated charcoal sandwiched between layers of Whatman #4 filter paper, prior to use. Coconut water was added to medium prior to autoclaving.

Somatic embryos were induced by transfer of floral tissue-derived calli onto petri dishes containing 30 mL of embryo development (ED) medium. ED medium was composed of DKW basal salts, 100.0 mg *myo*-inositol per L, 2.0 mg thiamin-HCl per L, 1.0 mg nicotinic acid per L, 2.0 mg glycine per L, 30.0 g sucrose per L, 1.0 g glucose per L, and 2.0 g phytagel per L. Cultures were maintained in the dark at 25° C and subcultured at intervals of 14 d. Somatic embryos at the torpedo-shaped stage of development were

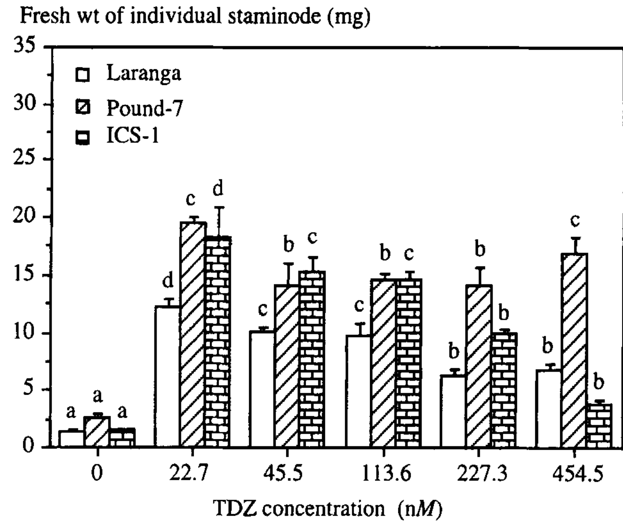

FIG. 2. Callus growth of staminodes in response to different concentrations of TDZ used in PCG medium. Fresh weight of each staminode was determined 14 d after culture initiation. Each treatment contained 20 staminodes per plate with three replicate plates. *Bar* values with standard errors (*vertical lines*) represent means collected from two experiments. Means with different letters (a,b) within a genotype are significantly different from each other at the 0.05 probability level.

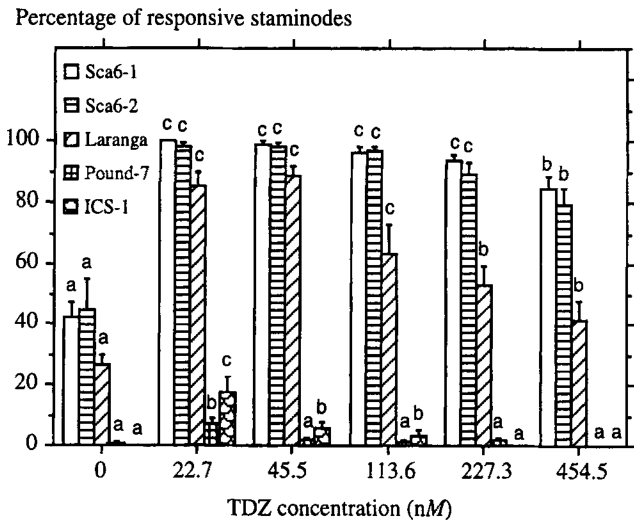

FIG. 3. Effects of TDZ concentration in PCG medium on the frequency of somatic embryogenesis with staminode explants of five cacao genotypes. Embryogenic calli were subcultured onto SCG medium and somatic embryos were subsequently induced by culturing calli on ED medium. Data were collected 2 mo. after culture initiation. Each treatment contained 20 staminodes per plate with three replicate plates. *Bar* values represent the average percentages of embryo-producing staminodes from three repeated experiments. *Vertical lines* indicate standard errors. Means with different letters (a,b) within a genotype are significantly different from each other at the 0.05 probability level.

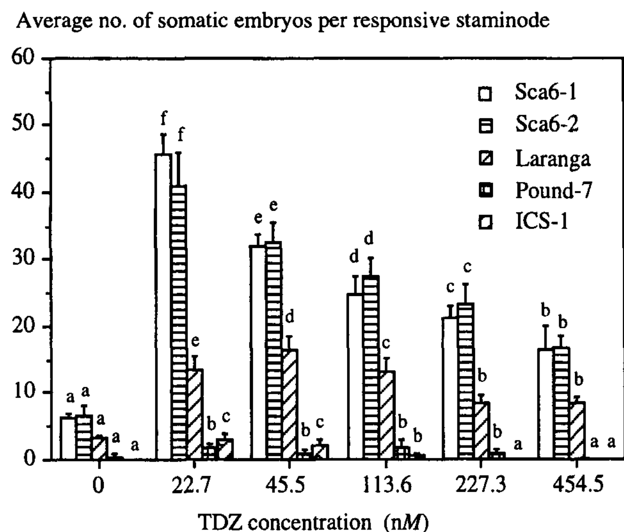

FIG. 4. Effects of TDZ concentration in PCG medium on the average number of primary somatic embryos produced from each responsive staminode explant of five cacao genotypes. Embryogenic calli were subcultured onto SCG medium and somatic embryos were subsequently induced by culturing calli on ED medium. Data were collected 2 mo. after culture initiation. Each treatment contained 20 staminodes per plate with three replicate plates. *Bar* values represent the average number of somatic embryos per responsive staminode from three repeated experiments. *Vertical lines* indicate standard errors. Means with different letters (a,b) within a genotype are significantly different from each other at the 0.05 probability level.

TABLE 1

RESPONSE OF SOMATIC EMBRYOGENESIS OF 19 TESTED CACAO GENOTYPES USING STAMINODE AS CULTURE EXPLANTS

Genotype	Genetic group	Percentage of RS[a,b]	Average no. of SE[a] per RS
Catongo	Forastero	5.8	4.8
Laranga	Forastero	85.6	13.5
Matina	Forastero	22.3	4.7
Sca 6-1	Forastero	100	45.7
Sca 6-2	Forastero	98.3	40.9
Sca 6 × ICS 1 #1	Forastero	23.2	8.1
Sca 6 × ICS 1 #3	Forastero	31.7	5.8
TSH 1112	Forastero	0.8	27.0
RB48	Forastero	5.0	6.4
EET 400	Forastero	0.8	1.0
Unknown 1	Criollo	6.7	4.3
Pentagonia F1	Criollo	20.6	2.0
ICS 1	Trinitario	14.2	3.9
ICS 16	Trinitario	45.8	6.4
ICS 39	Trinitario	10.0	2.0
ICS 67	Trinitario	0.8	42.0
Pound 7	Trinitario	7.2	2.8
Tomate Ceplac	Trinitario	1.7	2.0
UF 613	Trinitario	45.6	5.6

[a]RS, responsive staminode; SE, somatic embryo.
[b]Each treatment contains 20 staminodes per plate with three replicate plates. Values represent the average number from two to three repeated experiments, depending on the availability of explants.

separated from callus and cultured further on ED medium under similar conditions. The percentage of embryo-producing (responsive) staminodes over the total number of cultured explants, representing the frequency of embryogenesis, and the average number of primary embryos produced from each responsive staminode were determined 2 mo. after culture initiation. Data were analyzed with Duncan's new multiple range test (Duncan, 1955).

Mature somatic embryos with a fully developed embryonic axis or with an extended radical were selected for embryo conversion and plant regeneration. Five germinating embryos were inserted vertically into 80 mL of plant regeneration (PR) medium in a G7 Magenta vessel. Plant regeneration medium contained 1/5 × DKW basal salts, 100.0 mg *myo*-inositol per L, 2.0 mg thiamin-HCl per L, 1.0 mg nicotinic acid per L, 2.0 mg glycine per L, 10.0 g glucose per L, 5.0 g sucrose per L, 0.2 g KNO_3 per L, and 1.7 g phytagel per L. The cultures were maintained under a 16-h (light) photoperiod with a light intensity of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and were subcultured to identical medium every 14 d.

When plantlets had developed green leaves and healthy roots at least 3 cm long, they were transferred into 4-inch plastic pots containing sterile Metro-Mix 300 soil mixture and established in the greenhouse under 70% relative humidity at 27–30° C. After about 2 mo., plants were transferred to 15-inch pots containing Metro-Mix 300 soil mixture. Plants were watered with tapwater with pH value adjusted to 5.5 to 6.0 with diluted H_3PO_4 solution and fertilized once every 2 wk with Stern's liquid Miracle-Gro (Stern's Miracle-Gro Product, Inc., Port Washington, NY).

RESULTS

Callus initiation from floral explants. Staminode explants cultured on PCG medium enlarged to two to three times the size of the original explant within 1 wk (Fig. 1 *a*). Following this period of tissue expansion, compact callus developed over the entire staminode. Frequently, globular callus clusters appeared over the surface of the entire explant by the end of the culture period on the SCG medium (Fig. 1 *b*).

The effect of TDZ on cell growth stimulation and callus induction, as determined by fresh weight increase of cultured staminodes, from staminode explants of three cacao genotypes (Laranga, Pound-7, and ICS-1) was evident within 14 d after culture initiation (Fig. 2). Staminode explants cultured on PCG medium lacking TDZ expanded only slightly in size and generated limited callus around the cut site. In contrast, staminode explants of all three genotypes increased significantly in fresh weight and produced compact callus in most instances when cultured on PCG medium containing TDZ. A TDZ concentration of 22.7 n*M* promoted the highest rate of callus proliferation which was occasionally up to 10 times the fresh weight of staminode explants maintained on TDZ-free medium (Fig. 2). Fresh weight of Laranga and ICS-1 explants gradually declined with increasing TDZ concentrations (Fig. 2). When cultured on PCG medium containing 454.5 n*M* TDZ, staminode explants of genotypes Laranga and ICS-1 produced only limited callus at their base, while the remainder of the explant ceased to grow and turned black. Staminodes of Pound-7 produced friable callus when cultured under these conditions. Callus growth rates were reduced slightly after transfer of staminode explants to SCG medium.

Somatic embryo induction and development. The production of somatic embryos from embryogenic callus was evident about 2 wk after transfer to ED medium. Initially, pre-embryonic protuberances emerged from the explant surface with suspensors attached to callus and these often occurred in clusters. Subsequently, these structures developed into globular-shaped embryos (Fig. 1 *c*). Four wk after culture on ED medium, or approximately 2 mo. after culture initiation, the upper surfaces of staminode explants were covered with heart-shaped-stage somatic embryos (Fig. 1 *d*).

The TDZ concentration in PCG medium significantly affected the percentage of explants which ultimately developed somatic embryos (Fig. 3) as well as the number of embryos per embryo-producing explant (Fig. 4). Culture of staminodes on TDZ-free PCG medium resulted in the production of somatic embryos from four of five tested genotypes with frequencies ranging from 0.6 to 44% (Fig. 3), resulting in between three and six somatic embryos per responsive explant (Fig. 4). Among the six TDZ concentrations tested, TDZ at 22.7 n*M* resulted in the highest percentage of responsive staminodes (Fig. 3) and the greatest number of somatic embryos per responsive explant (Fig. 4) with all five genotypes. Up to 100% and 98% of the staminodes from two Sca-6-derived clones (Sca-6-1 and Sca-6-2, respectively) produced somatic embryos, with an average of over 40 embryos per staminode explant. However, both the percentage of responsive staminodes and the number of embryos per explant were significantly reduced when staminodes were obtained from Pound-7 and ICS-1 (Figs. 3 and 4). The frequencies of somatic embryogenesis for all five cacao genotypes were reduced when explants were cultured on PCG medium containing more than 22.7 n*M* of TDZ. No somatic embryos were produced from ICS-1 staminodes when cultured on medium containing more than 227.3 n*M* TDZ (Fig. 3). The rate of callus proliferation in the presence of TDZ was positively correlated with the frequency of somatic embryo production (Figs. 2,3, and 4).

The observation that the lowest tested TDZ concentration (22.7 n*M*) resulted in the highest frequency of somatic embryogenesis prompted us to investigate the efficacy of concentrations of TDZ lower than 22.7 n*M*. Due to the limited supply of staminode explants from other previously tested genotypes and also according to the similar trend in culture response observed from all tested genotypes, we chose to use staminodes of Sca-6-1 in the subsequent experiments. Results from two replicated experiments indicated that fresh weights of callus from explants of Sca-6-1 cultured on media containing TDZ at concentrations of 0, 0.45, 4.55, 11.36, or 22.7 n*M* were 7.0, 26.3, 54.5, 56.2, and 65.8 mg per five staminodes, respectively, 2 wk after culture initiation. The average percentages of responsive staminodes were 55.8, 68.3, 95.8, 98.3, and 100%, with average number of embryos per responsive explant ranging from 6.2, 7.2, 19.1, 19.8 and 23.9, respectively, under these culture conditions. Thus, the use of TDZ at concentrations lower than 22.7 n*M* did not positively influence either callus growth or somatic embryo production when compared to explants cultured on medium containing 22.7 n*M* TDZ. Accordingly, a TDZ concentration of 22.7 n*M* in PCG medium was determined to be optimal for stimulation of somatic embryogenesis in cacao, and this concentration was used in all subsequent experiments.

Staminodes from 14 additional cacao genotypes were evaluated for their ability to undergo somatic embryogenesis under the previously defined culture conditions (Table 1). For comparison, data obtained from the five previously tested genotypes were also included in the table. All 19 tested cacao genotypes were responsive to the culture conditions and produced somatic embryos at various rates ranging from about 1% to 100% of the cultured explants, and 1 to 46 embryos per responsive staminode.

The production of secondary somatic embryos from primary embryos frequently occurred when somatic embryos were maintained on ED medium for an extended period of time. These secondary embryos could have been derived from the epidermal or subepidermal cells of the primary embryos through a budding-like process as

TABLE 2

PLANT REGENERATION RESPONSE OF TWO TYPES OF SOMATIC EMBRYOS (SE) OF CACAO (GENOTYPE SCA-6-A1)

SE type	Total no. of SE	SE with root (%)	SE with shoot (%)
I	96	31[a] (32.3)	26 (27.1)
II	191	183 (95.8)	140 (73.3)

[a]All data were collected 2 months after transfer of somatic embryos onto PR medium.

demonstrated previously (Pence, 1989). Small primary embryos about 2 mm long were capable of producing up to 50 secondary embryos within 1 mo. of culture on ED medium (Fig. 1 *e*).

Morphological characteristics of somatic embryos. Somatic embryos cultured on ED medium developed rapidly. After two to three rounds of subculture, the majority of embryos had reached the torpedo stage of development. Some embryos developed well-defined hypocotyl and cotyledons, while others showed fused hypocotyl structures (Fig. 1 *f*). Two distinctive types of somatic embryos were identified among the normal embryos (Fig. 1 *g*). The first type (Type I) of somatic embryo had a yellowish and translucent appearance, and an expanded embryo axis with large yellow to pink cotyledons. During extended periods of culture on ED medium, mature Type I embryos did not produce roots and remained dormant with cotyledon expansion (Fig. 1 *g*). The second type (Type II) of somatic embryo was whitish and had an opaque but defined embryonic axis with small white cotyledons. These embryos were capable of precocious germination and produced roots with elongated hypocotyls on ED medium at maturity (Fig. 1 *g*).

Somatic embryos with fused hypocotyls and with underdeveloped or extra cotyledons were observed. However, these morphological abnormalities were not necessarily indicative of the subsequent development of an abnormal plant. Normal cacao plants were recovered from these somatic embryos, but they developed at a slower rate *in vitro* than somatic embryos with normal morphological characteristics.

Embryo conversion and plant regeneration. Mature somatic embryos expanded in size after transfer to PR medium. Typically, predominant cotyledonary expansion occurred in Type I embryos (Fig. 1 *h*), while rapid taproot elongation (up to 2 cm in 1 wk) occurred in Type II embryos (Fig. 1 *i*). With both embryo types, shoots with small green leaves were subsequently produced within 1 mo. on PR medium. The two types of embryos differed in their ability to produce shoots. Type I embryos tended to produce shoots at a lower frequency than Type II embryos (Table 2).

Well-developed cacao plantlets with more than three leaves and with healthy root systems measuring more than 4 cm long readily survived the transfer to potting soil in a greenhouse equipped with a misting system. To date, more than 270 somatic embryo-derived plants from six genotypes have been successfully established in a greenhouse (Fig. 1 *j*).

Three somatic embryo-derived plants have been maintained in the greenhouse for a period of more than 10 mo. These plants grew vertically to about 1.5 m in height with 50 to 65 leaves and then produced five lateral buds with shorter internodes from the apical end of the stem. Such dimorphic growth, or so-called "jorquetting," with the vertical stem being orthotropic and the branches being plagio-

tropic, is similar to the course of development typical of seed-derived cacao plants. These three, and other plants produced normal-sized leaves and internodes and brownish bark tissues around the lower portion of the stem, as observed in seed-derived cacao plants. Thus, the somatic embryo-derived plants appear morphologically indistinguishable from seed-derived plants of similar age.

Discussion

A prerequisite for the successful clonal propagation of plants via somatic embryogenesis is the availability of a culture procedure that results in both somatic embryo production and plant regeneration. Although several previous reports demonstrated the production of somatic embryos from various somatic tissues of cacao and the recovery of plants from these somatic embryos, the efficiency of these procedures was low (Sondahl et al., 1989, 1993; Lopez-Baez et al., 1993). The reported rates of primary somatic embryo production from the culture of a large number of petal and nucellar explants were 4.3 and 2.0%, respectively (Sondahl et al., 1993). In addition, a recent study indicated that when a modified procedure was used, only 5 among 25 tested cacao genotypes were capable of producing somatic embryos, while the rest remained nonresponsive and failed to produce any embryos (Alemanno et al., 1996a). The culture procedures described here stimulated the initiation of embryogenic callus from staminode explants. This procedure is also effective in stimulating somatic embryogenesis in cacao from flower petal base tissue explants (data not shown). To date, somatic embryogenesis has been induced with explants from 19 genotypes representing three major genetic groups (Forastero, Criollo and Trinitario) in cacao. Although the conversion rate varied between the two different types of somatic embryos observed, the routine recovery of a large number of viable plants from somatic embryos has been achieved. Somatic embryo-derived plants have been grown to an advanced stage in the greenhouse, and these showed agronomic traits comparable to seed-derived plants.

In previous studies of somatic embryogenesis in cacao, MS medium (Murashige and Skoog, 1962) was routinely used as the main source of inorganic nutrients (Pence, 1989; Figueira and Janick, 1993; Lopez-Baez et al., 1993). However, our findings indicated that the culture of various cacao tissue explants, including immature leaves, shoot apices, zygotic embryonic axes, and cotyledons derived from both zygotic and somatic embryos, on MS-containing media often resulted in reduced growth, rapid senescence, and eventually tissue necrosis. DKW medium, which was developed for *in vitro* propagation of woody perennial species, provided a significantly higher concentration of calcium, sulfur, and magnesium than MS medium. These elements are essential for cell differentiation and somatic embryogenesis (Pedroso et al., 1996). The use of DKW medium stimulated the rapid growth of embryogenic callus, the efficient induction and development of somatic embryos, and the enhancement of embryo-derived seedling growth in cacao.

The use of TDZ and glucose as sources of cytokinin and carbon, respectively, was essential for the initiation of embryogenic callus and the subsequent production of somatic embryos of cacao. TDZ was developed in 1976 as a cotton defoliant (Arndt et al., 1976), and its effect in the induction of leaf abscission was believed to be mediated by an increase in endogenous ethylene production (Suttle, 1985). Subsequent studies demonstrated that TDZ, a phenylurea derivative, possesses a strong cytokinin-like activity exceeding that of most other commonly used adenine-type cytokinins including zeatin, benzylaminopurine, and kinetin, possibly due to its capacity to stimulate endogenous cytokinin biosynthesis or alter endogenous cytokinin metabolism (Mok et al., 1982). TDZ is highly resistant to degradation by cytokinin oxidase (Mok et al., 1987). Recently, Murch et al. (1997) revealed that TDZ treatment could result in significant changes in tissue accumulation of minerals, including manganese, iron, copper, calcium, magnesium, and potassium, and in the increased levels of a large number of biological compounds involved in plant stress response. TDZ has been used to induce somatic embryogenesis, adventitious shoot formation, and axillary shoot proliferation in numerous crop genera, including woody plant species (Huetteman and Preece, 1993; Lu, 1993). However, TDZ had not been previously tested for its ability to stimulate somatic embryogenesis in cacao. Results of this study established that a TDZ concentration at 22.7 n*M* or 5 μg/l was sufficient to induce an optimal production of somatic embryos from staminode explants of various genotypes of cacao. This TDZ concentration is 15 to more than 400-fold less than that commonly used in other studies (Gill and Saxena, 1992; Gray et al., 1993; Lu, 1993). Our results also indicated that TDZ concentrations higher than 45.5 n*M* reduced callus growth and embryo production, and appeared to be toxic to some genotypes of cacao. The relatively narrow range of TDZ concentrations effective for somatic embryogenesis in cacao may be indicative of the high sensitivity of cacao tissues to the elevated levels of ethylene that can be induced by TDZ (Suttle, 1985; Wang et al., 1986; Lu, 1993).

Generally, sucrose and maltose have been used as carbon sources in studies attempting to induce embryogenic callus in cacao (Pence, 1989). Our selection of glucose as a carbon source resulted from previous observations that cacao tissues cultured on glucose-containing medium grew normally and did not show a hypersensitive reaction that resulted in tissue senescence or necrosis, as was frequently observed when these same tissues were cultured on medium containing other sugars (data not shown). In addition, in preliminary experiments we observed that continuous exposure of callus to PCG medium tended to induce excessive callus growth and reduced embryo production, while the use of low-salt WPM medium, in combination with kinetin, was important for enhancing the embryogenic response of callus cultures (data not shown).

The efficiency of somatic embryo production and plant regeneration achieved with the established procedure offers an avenue for the practical use of somatic embryogenesis for the clonal propagation of cacao. Plantlets produced from somatic embryos retained the orthotropic growth characteristics similar to seed-derived plants. Such a growth pattern facilitates efficient plantation management and harvest operation, and may reduce the cost associated with the extensive pruning required by plants derived from rooted cuttings. Although somatic embryo-derived plants have been used for large-scale crop production in some other plant species, the usefulness of somatic embryo-derived cacao plants in cocoa production awaits evaluation through comparative field trials. This improved somatic embryogenesis procedure may also provide an experimental tool for the genetic manipulation of cacao, through which foreign genes coding for useful traits such as resistance to pests, tolerance to abiotic stresses, and high product quality characteristics can be incorporated into cacao for crop improvement.

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References

Alemanno, L.; Berthouly, M.; Michaux-Ferriere, N. Embryogenése somatique du cacaoyer á partir de pièces florales. Plant. Rech. Dev. 3:225–233; 1996a.

Alemanno, L.; Berthouly, M.; Michaux-Ferriere, N. Histology of somatic embryogenesis from floral tissues cocoa. Plant Cell Tissue Organ Cult. 46:187–194; 1996b.

Arndt, F.; Rusch, R.; Stilfried, H. V. SN49537, a new cotton defoliant. Plant Physiol. 57:99; 1976.

Duncan, D. B. Multiple range and multiple F-test. Biometrics 11:1–42; 1955.

Driver, J. A.; Kuniyuki, A. H. In vitro propagation of Paradox walnut rootstock. HortScience 19:507–509; 1984.

Esan, E. B. Tissue culture studies on cacao (*Theobroma cacao* L.). A supplementation of current research. In: Proc. 5th Int. Cacao Res. Conf. 1975. Ibadan: Cacao Res. Inst. Nigeria; 1977:116–125.

Esan, E. B. Micropropagation of cacao (*Theobroma cacao* L.). In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry. Vol. 18. High-tech and micropropagation II. Berlin Heidelberg, New York: Springer-Verlag; 1992:96–122.

Figueira, A.; Janick, J. Development of nucellar somatic embryos of *Theobroma cacao*. Acta Hortic. 336:231–236; 1993.

Figueira, A., Janick, J. Optimizing carbon dioxide and light levels during in vitro culture of *Theobroma cacao*. J. Am. Soc. Hortic. Sci. 119:865–871; 1994.

Figueira, A.; Janick, J. Somatic embryogenesis in cacao (*Theobroma cacao* L.). In: Jain, S.; Gupta, P.; Newton, R., ed. Somatic embryogenesis in woody plants. Vol. 2. The Netherlands: Kluwer Academic Publisher; 1995:291–310.

Flynn, W. P.; Glicenstein, L. J.; Fritz, P. J. *Theobroma cacao* L.: an axillary bud in vitro propagation procedure. Plant Tissue Organ Cult. 20:111–1E117; 1990.

Gamborg, O. L. Aromatic metabolism in plants. II. Enzymes of the shikimate pathway in suspension cultures of plant cells. Can. J. Biochem. 44:791–799; 1966.

Gill, R.; Saxena, P. K. Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea*): promotive role of thidiazuron. Can. J. Bot. 70:1186–1192; 1992.

Gray, D. J.; McColley, D. W.; Compton, E. High-frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. J. Am. Soc. Hortic. Sci. 118:425–432; 1993.

Huetteman, C. A.; Preece, J. E. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult. 33:105–119; 1993.

Kennedy, A. J.; Lockwood, G.; Mossu, G., et al. Cacao breeding: past, present, and future. Cocoa Grower's Bull. 38:5–22; 1987. Cadbury Schweppes, Birmingham, UK.

Lerceteau, E.; Robert, T.; Pétiard, V., et al. Evaluation of the extent of genetic variability among *Theobroma cacao* accessions using RAPD and RFLP markers. Theor. Appl. Genet. 95:10–19; 1997.

Lloyd, G.; McCown, B. Commercially feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30:421–427; 1980.

Lopez-Baez, O.; Bollon, H.; Eskes, A., et al. Embryogenèse somatique de cacaoyer *Theobroma cacao* L. á partir de pièces florales. C. R. Acad. Sci. Paris 316:579–584; 1993.

Lu, C. Y. The use of thidiazuron in tissue culture. In Vitro Cell. Dev. Biol. 29:92–96; 1993.

Mok, M. C.; Mok, D. W. S.; Armstrong, D. J., et al. Cytokinin activity of N-phenyl-N'-1,2,3-thiadiazol-5-yl urea (thidiazuron). Phytochemistry 21:1509–1511; 1982.

Mok, M. C.; Mok, D. W. S.; Turner, J. E., et al. Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. HortScience 22:1194–1197; 1987.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Murch, S. J.; KrishnaRaj, S.; Saxena, P. K. Thidiazuron-induced morphogenesis of Regal geranium (*Pelargonium domesticum*): a potential stress response. Physiol. Plant. 101:183–191; 1997.

Orchard, J. E.; Collin, H. A.; Hardwick, K. Culture of shoot apices of *Theobroma cacao*. Physiol. Plant. 47:207–210; 1979.

Passey, A. J.; Jones, O. P. Shoot proliferation and rooting in vitro of *Theobroma cacao* L. type Amelonado. J. Hortic. Sci. 58:589–592; 1983.

Pedroso, M. C.; Tavres, R.; Lino-neto, T., et al. Early events in somatic embryogenesis induction. In: Ahuja, M. R., et al., ed. Somatic cell genetics and molecular genetics of trees. The Netherlands: Kluwer Acad. Publishers; 1996:17–22.

Pence, V. C. Cacao (*Theobroma cacao* L.). In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry. Vol. 5. Tree II. Berlin Heidelberg New York: Springer-Verlag; 1989:203–221.

Pence, V. C.; Hasegawa, P. M.; Janick, J. Asexual embryogenesis in *Theobroma cacao* L. J. Am. Soc. Hortic. Sci. 104:145–148; 1979.

Pence, V. C.; Hasegawa, P. M.; Janick, J. Initiation and development of asexual embryos of *Theobroma cacao* L. in vitro. Z. Pflanzenphysiol. 98:1–14; 1980.

Sondahl, M. R.; Sereduk, T. B.; Chen, Z., et al. Somatic embryogenesis and plant regeneration of cacao, patent no. 88/3078, Jen 25, 1989. Republic of South Africa.

Sondahl, M. R.; Liu, S.; Bellato, C., et al. Cacao somatic embryogenesis. Acta Hortic. 336:245–248; 1993.

Suttle, J. C. Involvement of ethylene in the action of the cotton defoliant thidiazuron. Plant Physiol. 78:272–276; 1985.

Toxopeus, H. Botany, types and populations. In Wood, G. A.; Lass, R. A., ed. Cocoa. 4th edition. New York: Longman Scientific & Technical, and John Wiley & Sons, Inc.; 1985:11–37.

Tuleckle, W.; McGranahan, G. Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci. 40:57–63; 1985.

Wakeling, S. A. Review of production, consumption, stocks and prices. Cocoa Growers' Bulletin 50:3; 1996, Cadbury Schweppes, Birmingham, UK.

Wang, S. Y.; Steffens, G. L.; Faust, M. Breaking bud dormancy in apple with a plant bioregulator, thidiazuron. Phytochemistry 25:311–317; 1986.

Wang, Y. C.; Janick, J. Inducing precocious germination in asexual embryos of cacao. HortScience 19:839–841; 1984.

Wood, G. A. R.; Lass, R. A. Cocoa. 4th edition. New York: Longman Scientific & Technical and John Wiley & Sons, Inc.; 1987.