In Vitro Cell. Dev. Biol.—Plant 38:252–259, May–June 2002
DOI: 10.1079/IVP2001257

1054-5476/02 $10.00+0.00

EFFICIENCY, GENOTYPIC VARIABILITY, AND CELLULAR ORIGIN OF PRIMARY AND SECONDARY SOMATIC EMBRYOGENESIS OF *THEOBROMA CACAO* L.

SIELA N. MAXIMOVA[1], LAURENCE ALEMANNO[2], ANN YOUNG[1], NICOLE FERRIERE[2], ABDOULAYE TRAORE[1], AND MARK J. GUILTINAN[1]*

[1]*Department of Horticulture, College of Agricultural Sciences, The Pennsylvania State University, 103 Tyson Building, University Park, PA 16802-4200*
[2]*CIRAD, TA 80/03, Avenue Agropolis, 34398 Montpellier Cedex 5, France*

(Received 7 February 2001; accepted 8 October 2001; editor S. A. Merkle)

Summary

The development of efficient tissue culture systems for cacao holds the potential to contribute to the improvement of this tropical crop by providing a rapid and efficient vegetative propagation system for multiplication of elite genotypes. It may also find application in facilitation of germplasm movement across quarantine borders, enhancement of germplasm conservation via cryo-preservation, and development of genetic transformation systems. Somatic embryogenesis using floral tissue explants was previously the only tissue culture procedure for regeneration of cacao. We report the development of a secondary embryogenesis system utilizing primary somatic embryo cotyledon explants, which results in up to a 30-fold increase in somatic embryo production compared to primary somatic embryogenesis. The influence of genotype on the efficiency of the system was evaluated. To understand the cellular origins and developmental pathways operative in this system, we investigated the morphological changes occurring over time using light and scanning electron microscopy. While primary embryos arise from clusters of cells forming embryonic nodules, secondary embryos arise predominantly from the division of single cells, in a pathway reminiscent of zygotic embryogenesis. These results have important significance to the application of tissue culture to cacao improvement programs.

Key words: cocoa; somatic embryos; efficiency; morphology; regeneration.

Introduction

Theobroma cacao is a tropical tree, with origins in the Amazon basin, currently cultivated throughout the tropics to supply the global demand for cocoa. Although not well documented, a high degree of yield variation is a generally observed characteristic of cacao worldwide. This is in part due to the predominant use of seed propagation in this mostly self-incompatible and highly heterozygous tree crop (Figueira and Janick, 1995). A recent study of the early yield of five high-producing cacao families grown in full sun in Puerto Rico indicated that 2–3% of the trees in a population accounted for more than 60% of the yield (Irizarry and Rivera, 1998).

Through plant breeding, improvement via additive genetic gain is rapidly lost, as farmers propagate plants through seeds, and segregation results in a highly heterozygous population of plants. Vegetative propagation systems could provide a means to capture such additive genetic gain. Although vegetative propagation systems including grafting and rooted cuttings are used in some countries, they have not been widely adopted in developing countries. Additionally, the dimorphic architecture of the cacao tree and the predominance of plagiotropic material in the field had made these traditional propagation techniques inefficient and costly (Figueira and Janick, 1995). Furthermore, it is widely thought (although definitive data supporting this belief are not available) that plagiotropic rooted cuttings, which lack taproots and require extensive formative pruning, do not perform well in the field, especially during early establishment and during dry seasons or in high winds.

Modern biotechnology offers a suite of new approaches to speed up the development and deployment of genetically improved genotypes. These include enhancing breeding programs with quantitative trait mapping and marker-aided selection, the introduction of novel genes through genetic engineering and rapid propagation of clean stock through tissue culture (Timmis, 1998). The main advantages of tissue culture methods include the possibility of rapidly generating asexually propagated, uniform plants with highly valued genetic traits. Additionally, for cacao, somatic embryogenesis offers a system for the clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. The production and testing of disease-free materials and germplasm conservation via cryo-preservation are other important potential contributions of plant tissue culture to the improvement, preservation and distribution of cacao germplasm.

Cacao tissue culture research has focused on somatic embryogenesis (SE), which has been developed in several laboratories. The first report of cacao SE was by Esan (1977), who described a method using immature zygotic embryo tissue explants.

*Author to whom correspondence should be addressed: 306 Wartik Lab., Biotechnology Institute, The Pennsylvania State University, University Park, PA 16802. Email mjg9@psu.edu

Similar methods were later reported by others (Pence et al., 1979; Villalobos and Aguilar, 1990). However, while a step forward, these methods did not produce clonal material that replicates the genotype of the source tree, and thus, genetic gain is lost. Subsequently, increased efforts were directed towards the development of tissue culture systems from somatic tissues including leaves (Litz, 1986), nucellus (Chatelet et al., 1992; Figueira and Janick, 1993; Sondahl et al., 1993, 1994) and floral explants, including petals and staminodes (Lopez-Baez et al., 1993; Alemanno et al., 1996a, b, 1997). These systems, while partially successful in producing somatic embryos, were still of limited usefulness, being applicable to only a few genotypes and suffering from low conversion rates. More recently, an efficient SE method was reported which was capable of propagating a wide variety of cacao genotypes (Li et al., 1998). Primary somatic embryos were produced from floral explants at high frequencies using thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D). With optimization of conversion media, large numbers of plants were successfully acclimated and grown to sexual maturity (Traore, 2000). Field-testing of SE-derived plants produced using these methods has been initiated at field sites in St. Lucia, Brazil and in West Africa.

To increase the efficiency of the method reported by Li et al. (1998), we have developed a protocol for secondary embryogenesis using cotyledon explants from primary somatic embryos. This study was conducted to evaluate the potential of secondary embryogenesis to increase the multiplication rate of this propagation method and to compare embryo production of primary versus secondary embryogenesis. The results indicated that embryogenic capacity for both primary and secondary embryogenesis exhibits a high degree of genotypic variation. A study of the anatomy of embryo initiation and development was completed. The histological analysis of early stages of embryogenesis revealed a predominantly multicellular origin of primary SEs, which was in contrast to a predominantly unicellular origin for secondary SEs.

Materials and Methods

Explant material. Tissue culture experiments were conducted with 14 different cacao genotypes at two different locations, The Pennsylvania State University (PSU), University Park, PA, USA and CIRAD BIOTROP Laboratory, Montpellier, France. At PSU, immature flowers from four genotypes (UVE 1, 5, 9, and 22) were collected at Union Vale Estate, St. Lucia and two genotypes were provided by Almirante Experimental Farm, M&M Mars, Brazil (EEG 29 and TSH 565). Immature flowers were stored in sterile water on ice during transportation. Cultures were also initiated from greenhouse-grown Scavina-6 plants. The eight genotypes that were evaluated at CIRAD were Lower Amazonian Forastero varieties (GU 143, GF 23, IFC 5, and KER 1) and Upper Amazonian Forastero varieties (IFC 705, NA 32, NA 79, and Scavina-6). Floral material was collected from field-grown plants at Paracou-Combi, French Guyana.

Tissue culture. Primary somatic embryos from cacao staminodes were produced from all genotypes as previously described (Li et al., 1998). Cotyledon explants for secondary embryogenesis were selected from primary embryos (from staminodes). Only well-developed cotyledons that were light yellow in color were used. The cotyledons were cut with a scalpel into approximately 4 mm^2 pieces. Between 50 and 200 explants were cultured for 14 d on modified secondary callus growth medium (SCG) containing 2.4 μM 2,4-D and 1.4 μM 6-benzylaminopurine (BA) (Li et al., 1998). The modifications of SCG medium included substitution of kinetin with BA and exclusion of the coconut water. After 14 d culture on modified SCG the explants were transferred and maintained on plant growth regulator- (PGR) free embryo development medium (ED) (Li et al., 1998). At PSU, secondary embryogenesis from Scavina-6 primary embryo cotyledons was initiated at 20 (W20), 22 (W22), 24 (W24), 26 (W26), and 28 (W28) wk after staminode culture initiation. Cotyledons of primary embryos from UVE genotypes and genotypes TSH 565 and EEG 29 were selected from 11-mo.-old staminode cultures. The total number of embryos produced was counted every 2 wk for up to 40 wk after culture initiation. Additionally, for genotype Scavina-6, the number of normal embryos produced was recorded.

The mean number of total embryos produced per explant was calculated for all genotypes. Data were analyzed using analysis of variance for a completely randomized design with 16–23 levels of treatment (weeks after culture initiation). We compared the means for the number of total versus normal embryos produced per explant, normal embryos produced from staminode versus cotyledon explants, and the number of normal embryos produced from cotyledons initiated at different times after primary culture initiation for Scavina-6. The differences among the means of the total number of embryos produced for the six genotypes were also analyzed. Fisher Protected LSD test at $P < 0.05$ level of significance was used for mean separation (StatView5, SAS Institute Inc., Cary, NC).

At CIRAD the total number of embryos produced from eight genotypes via primary and secondary embryogenesis was recorded every 2 wk. The percentage of embryo-producing explants and the mean number of somatic embryos per explant were calculated. The differences among the means of the genotypes for percentage of explants producing embryos and the mean number of embryos produced per explant from primary and secondary embryogenesis were analyzed by one-way ANOVA. Significance was determined at $P < 0.05$ using ANOVA (StatView5).

Light microscopy. Bright field images of embryos were taken with a Nikon SMZ-4 dissecting microscope equipped with 3 CCD video camera system (Optronics Engineering, Goleta, CA). The video camera was connected to a Macintosh computer with NIH Image 1.6 image processing and analysis software.

Scanning electron microscopy (SEM). Five to eight staminode and cotyledon explants were sampled at 0, 5, and 30 d after culture initiation. The explants were fixed in 2.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.2) (Electron Microscopy Science, Fort Washington, PA) for 2 h at room temperature under vacuum and stored at 4°C overnight. The specimens were washed three times in 0.1 *M* sodium cacodylate buffer, post-fixed in 1% osmium tetroxide (Electron Microscopy Science) in 0.1 *M* sodium cacodylate buffer and dehydrated through a graded alcohol series (25%, 50%, 70%, 85%, 95%, and 100% × 3) for 15 min each step. Samples were stored overnight at 4°C in 100% ethanol and critical-point dried using liquid carbon dioxide in a Polaron E3000 critical-point dryer (Energy Beam Sciences, Agawam, MA). Dried explants were mounted onto aluminum stubs (Electron Microscopy Science), coated with gold and palladium in a BAL-TEC SCD050 sputter-coater (Boston, MA) and examined with a JEOL (JSM 5400) scanning electron microscope at 5–10 kV accelerating voltage. Digital images were collected using PGT's IMIX-PC system (Princeton Gamma-Tech, Inc., Princeton, NJ). At 30 d after culture initiation different tissue samples were selected from staminodes and cotyledons (three samples each) and the diameter of 28 individual cells per treatment was measured using the same image-processing system. Additionally, at 30 d after culture initiation the sizes of 10 primary and 10 secondary globular embryos from different tissue samples ware measured. The data were analyzed using an unpaired *t*-test with an equal number of samples and unequal variances. For this analysis, significance was determined using half the usual degrees of freedom (Snedecor, 1948).

Histological analysis. Samples from primary (Scavina-6) and secondary explants (Scavina-6, KER 1, and GU 143) were collected every 4 d after culture initiation. They were fixed for 24 h in phosphate buffer (0.2 *M*, pH 7.2) containing 2% paraformaldehyde, 1% glutaraldehyde and 1% (w/v) caffeine at 4°C. After dehydration in an alcohol series: 70% for 60 min; 95% for 15 min; 95% for 30 min; and 100% for 15 min; samples were subsequently embedded in Technovit 7100 resin (Kulzer, Wehrheim, Germany). Three-μm thick sections were cut using an LKB Historange microtome (Leica, Heidelberg, Germany). A double stain PAS- (periodic acid–Schiff) naphthol blue-black was used. Proteins were stained with naphthol blue-black (Fisher, 1968) and polysaccharides stained red with PAS (Martoja and Martoja, 1967). Images were collected using Leica DMRXA microscope and camera.

Results

Efficiency of primary and secondary embryogenesis. Embryonic

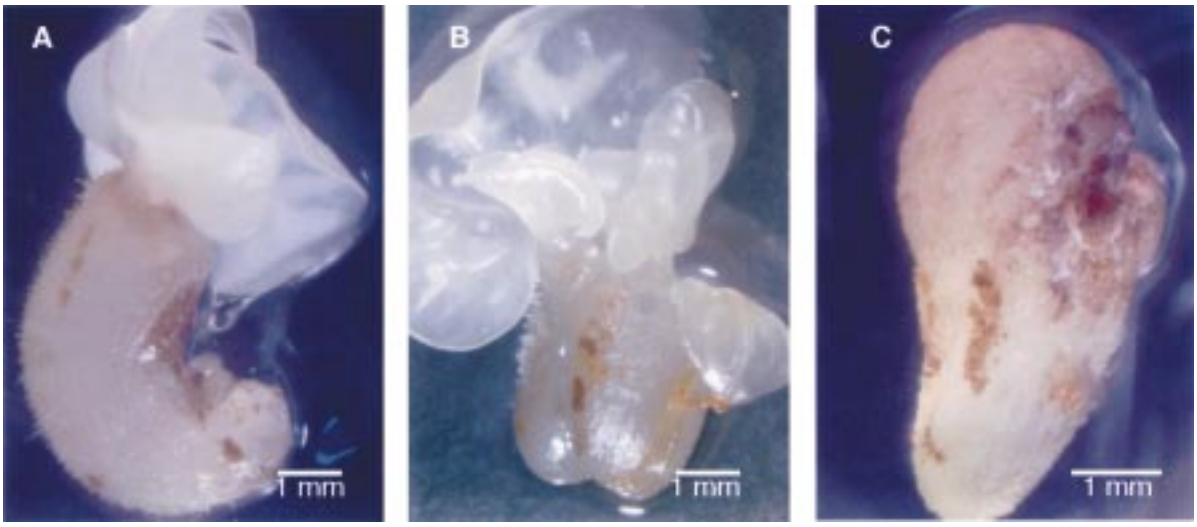

FIG. 1. Embryonic structures observed during primary and secondary embryogenesis of cacao. Representative embryos from genotype Scavina-6: *A*, normal embryo; *B*, fasciated embryo; *C*, abnormal embryo-like structure (*bar* = 1 mm).

structures produced from staminode and cotyledon explants were separated into three morphological categories—normal embryos (Fig. 1*A*), fasciated embryos (Fig. 1*B*) and abnormal embryo-like structures (Fig. 1*C*). Normal embryos later converted to plants with high success rates (data not shown). Fasciated embryos consisted of two or more fused embryos with multiple cotyledons (Fig. 1*B*), very few of which converted to plants. Abnormal embryo-like structures were formed in groups, missing apical meristem development and lateral symmetry, and never developed into plants (Fig. 1*C*).

The total number of embryonic structures produced reached a maximum between 28 and 32 wk after primary culture initiation (Fig. 2*A*). No significant differences in the total number of embryos per explant were observed from week 22 to week 38. At that time the maximum number of embryonic structures produced from a single explant ranged from 12.5 to 23. However, only a small portion of the total number of these were normal embryos (Fig. 2*A*). The mean number of total embryonic structures formed per staminode was 13.8, compared to 2.86 normal embryos; these means were significantly different ($P < 0.0001$). A minimal number of normal embryos were observed during the first 12 wk after culture initiation. The largest increase (8-fold) in the production of normal embryos was observed between weeks 12 and 14 after culture initiation. Statistical analysis indicated that there were no significant differences in the number of normal embryos from week 24 to week 38, suggesting that the system had reached maximum normal embryo production at 12 wk after culture initiation.

The number of normal embryos per explant was significantly higher ($P < 0.0001$) for cotyledons versus staminodes (Fig. 2*B*). The number of normal embryos produced from cotyledons increased very quickly and, at 10 wk after secondary culture initiation, the cotyledon explants had greatly exceeded the normal embryo production of the staminodes.

Our results demonstrated that the efficiency of embryogenesis from cotyledons was affected by the age of the staminode culture used for explant source. Despite the observed morphological and physiological similarities at the time of placement in culture, the cotyledon explants selected at different times after staminode culture initiation produced variable numbers of normal embryos (Fig. 2*C*). At 20 wk after cotyledon culture initiation there were no significant differences between cultures W20 and W22 ($P < 0.7586$), or W26 and W28 ($P < 0.5637$). The maximum number of embryos was produced from W24 culture and was significantly higher than the other four cultures ($P < 0.01$). At 40 wk the average number of embryos produced per cotyledon from the W24 culture was significantly different only from W20 and W22 ($P < 0.0220$, $P < 0.0140$) due to increased standard errors for W26 (SE = 38.16) and W28 (SE = 44.99).

Evaluation of embryogenic potential of staminode and cotyledon explants from different cacao genotypes. To evaluate the effect of genotype on embryogenesis frequency, cotyledon explants from six different genotypes were cultured for secondary embryo initiation and the number of embryos produced was counted every 2 wk (Fig. 2*D*). All genotypes produced embryos and the formation of embryos was first observed between 4 and 6 wk after culture initiation. The peak in embryo production for all genotypes was between weeks 8 and 18. After week 18 few new embryos were formed. Analysis of variance indicated that cotyledon embryogenesis varied among genotypes. Genotype UVE 1 produced significantly more embryos per explant (5.571) compared to the other five genotypes ($P < 0.01$) (Fig. 2*D*). UVE 5 and TSH 565 produced very low numbers of embryos (0.282 and 0.236). Overall, multiplication rates ranged from 1 to 11.2 embryos per explant.

Primary embryos were produced in cultures initiated from eight genotypes at CIRAD and a significant level of variation was also observed between different genotypes ($P < 0.01$) (Table 1). During staminode embryogenesis the genotype Scavina-6 produced both the highest percentage of embryogenic explants (75%) and total number of embryos per embryogenic explant (42) (Table 1). The genotype IFC 5 exhibited low embryogenic potential, with only 6% of explants producing embryogenic cultures with an average of only four embryos per explant. Overall, an approximately 10-fold difference in efficiencies was observed between Scavina-6 and IFC 5 (Table 1).

Similarly, all genotypes tested produced secondary embryos, and significant genotype effects were also observed ($P < 0.01$) (Table 1). In some cases, the percentage of explants producing secondary embryos was higher than observed for primary embryogenesis (KER

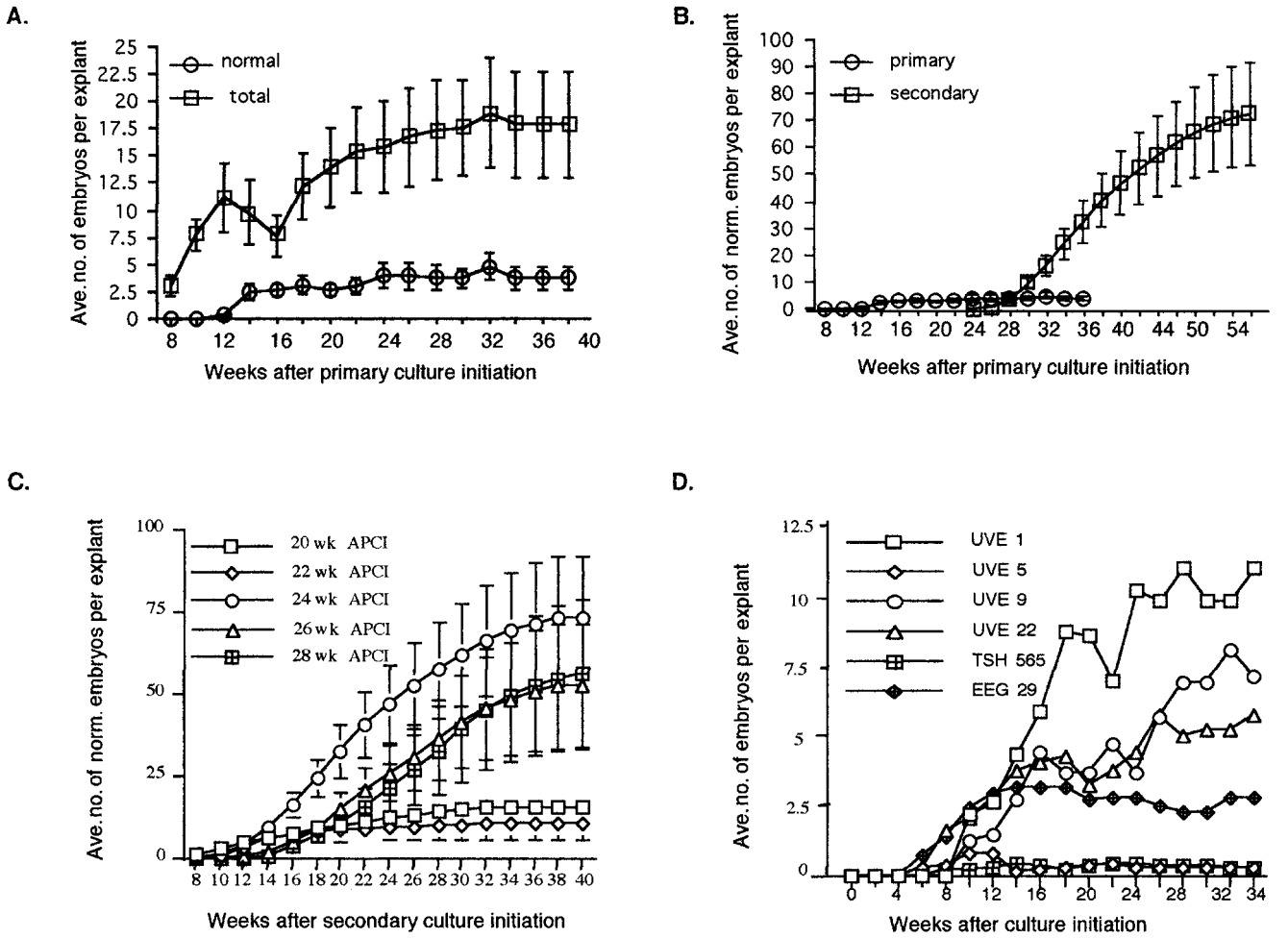

FIG. 2. Dynamics of primary and secondary embryogenesis. Primary and secondary embryos were produced from six genotypes at Penn State University. The total number of embryos produced was recorded every 2 wk after culture initiation. For Scavina-6, the number of normal embryos produced was also recorded. *A*, Average number of primary total and normal embryos produced per explant from Scavina-6. *B*, Average number of primary and secondary normal embryos produced per explant from Scavina-6. *C*, Average number of secondary normal embryos produced per explant from Scavina-6. Five different secondary cultures were initiated at 2-wk intervals using the same primary culture starting at 20 wk after primary culture initiation. *D*, Average number of secondary embryos produced from six cacao genotypes. Significance was determined at $P < 0.05$ using ANOVA. APCI, after primary culture initiation.

1 and GF 23) and in other genotypes the percentage was lower (Scavina-6). Overall, these results show that the methods we have developed support embryogenesis in a wide variety of genotypes, although efficiencies may vary with genotype.

Morphological comparison of developing somatic embryos. Prior to culture initiation, staminode surfaces appeared smooth with large numbers of long unicellular trichomes (Fig. 3*A*). After 5 d of culture multiple transverse parallel ruptures formed (Fig. 3*B*). Numerous individual, swollen cells were observed emerging from the ruptures (Fig. 3*B*, *C*). After 30 d of culture, the calluses proliferating from the staminode explants contained two morphologically distinct types of clustered cells. The first type consisted of elongated cells (Fig. 3*D*), which appeared white under a dissecting scope (not shown). Somatic embryos never developed from this type of cell cluster. The second type consisted of round cells approximately 40 μm in diameter (Table 2; Fig. 3*E*). These cell clusters were often found in association with somatic embryos (Fig. 3*F*). The sizes of primary embryos at the globular stage ranged from 608 to 2122 μm (Table 2). Heart-shaped embryos as well as poly-cotyledonous embryos were also observed (Fig. 3*F*). Cotyledons of mature embryos had stomata and multicellular trichomes (Fig. 3*G*, *H*). Primary embryo initiation and development were highly asynchronous.

Cotyledon explants were excised from primary embryos and reintroduced in culture on a secondary callus growth medium with 2,4-D and BA for induction of secondary embryogenesis. Five days after culture initiation the surface of the cotyledons was ruptured and swollen cells were observed emerging from the openings (Fig. 3*I*, *J*). The ruptures on the cotyledon surface did not occur in a particular orientation (Fig. 3*I*). During secondary embryogenesis the majority of calluses formed were similar to the second category described above, with round, translucent-waxy-yellow cells, which were often found in close association with large numbers of somatic embryos (Fig. 3*K*, *L*).

Cell and embryo size were measured for staminode and cotyledon explants (Table 2). The mean size of embryogenic cells from cotyledons was 10 times larger ($t = 22.97$, $P < 0.001$) and showed

TABLE 1

GENOTYPE RESPONSE OF CACAO STAMINODE AND SOMATIC EMBRYO COTYLEDON EXPLANTS DURING PRIMARY AND SECONDARY EMBRYOGENESIS

	Primary embryogenesis		Secondary embryogenesis	
Cacao genotype	Explants producing embryos ± SE (%)	Average number of embryos per explant	Explants producing embryos ± SE (%)	Average number of embryos per explant
GF 23	10.0 ± 5.2 c	9.0 ± 2.5 b	61.8 ± 27.0 a	8.8 ± 1.7 ab
GU 143	27.1 ± 6.3 bc	18.9 ± 3.5 bc	26.2 ± 4.2 b	20.8 ± 4.7 ab
IFC 5	6.1 ± 2.5 c	4.0 ± 0.8 c	20.4 ± 1.3 b	4.8 ± 1.8 b
IFC 705	4.9 ± 1.5 c	8.7 ± 2.2 bc	17.6 ± 5.3 b	10.2 ± 3.8 ab
KER 1	15.1 ± 6.8 c	14.6 ± 3.1 a	71.8 ± 15.6 a	24.7 ± 2.1 a
NA 32	9.2 ± 5.3 c	35.2 ± 8.0 b	23.6 ± 9.0 b	12.9 ± 2.7 ab
NA 79	37.2 ± 3.2 b	15.9 ± 1.6 a	43.7 ± 6.2 ab	16.9 ± 4.6 ab
Scavina-6	71.6 ± 4.9 a	42.9 ± 3.9 a	20.4 ± 2.1 b	10.2 ± 1.9 ab

Number of explants producing embryos and total number of embryos were counted every 2 wk until embryo production ceased (approx. 12 mo. after culture initiation).

Data are presented as percentage of embryo-producing explants ± standard errror (SE) and as average number of embryos per producing explant ± SE. *Values* that are significantly different within *columns* at the 5% level of significance are indicated with different *letters*.

TABLE 2

CACAO EMBRYOGENIC CALLUS CELL AND PRIMARY/SECONDARY EMBRYO SIZE

Tissue culture explant type	Single callus cell diameter (μm) ± SE; variance	Somatic embryo size (μm) ± SE; variance
Staminodes	41 ± 2; 90.85	1193 ± 191; 364 113.69
Cotyledons	402 ± 16; 6839.55	907 ± 59; 31 619.31

At 30 d after culture initiation the diameter of callus cells ($n = 30$) and globular embryo ($n = 10$) size of primary and secondary tissue samples were measured. The cell and embryo size variation were analyzed using an unpaired t-test with equal number of samples, unequal variances, with 27 and 9 degrees of freedom, respectively.

approximately 70-fold more variation then embryogenic cells observed in staminodes. In contrast, the measured sizes of globular primary embryos produced by 30-d-old cultures show approximately 10-fold more variation than did secondary embryos, although the mean sizes were not significantly different ($t = 1.36$, $P < 0.1896$).

Origin of primary somatic embryos. Prior to induction of primary somatic embryogenesis, sectioning of staminode explants revealed distinct layers of epidermal cells, parenchyma cells, vascular bundles, and large vesicles full of mucilage (Fig. 4*A*). After 10 d of culture, callus derived from active division of parenchyma and epidermal cells was observed (Fig. 4*B*). After 2 wk growth on SCG media, callus containing embryonic cells was visible (Fig. 4*C*). At that time the calluses were composed of numerous meristematic zones with diffuse division activity similar to that found in primary meristems (Fig. 4*D*). After transfer to ED medium, the meristematic cells accumulated a large number of protein globules and acquired embryonic characteristics (Fig. 4*E*). The resulting callus was frequently observed to fragment into embryonic masses, spatially isolated from the surrounding cells (Fig. 4*F*). Subsequently, these cellular groups developed into immature somatic embryos (Fig. 4*G*). Thus, somatic embryos developing from staminode explants appeared to originate primarily from multiple cells.

Origin of secondary somatic embryos. Cross-sections of cotyledon explants excised from primary somatic embryos prior to culture revealed a single-layered epidermis with uniform cells. Well-defined vascular tissue was interspersed among the parenchyma cells (Fig. 4*H*). Some parenchyma and vascular bundle sheath cells contained polyphenols (Fig. 4*H*). After transfer of the explants to ED medium division of single epidermal cells was observed (Fig. 4*I*). The following continuous division led to formation of meristematic nodules and friable calluses consisting of detached, dedifferentiating cells (Fig. 4*J*). Further development gave rise to globular (Fig. 4*K*, *L*), heart (Fig. 4*M*), torpedo, and cotyledon stages similar to zygotic embryogenesis (Fig. 4*N*). Secondary embryos were frequently observed to be loosely attached to the parent tissue by a suspensor-like organ (Fig. 4*M*).

Discussion

We developed a protocol for secondary embryogenesis that utilizes primary embryos from somatic tissues. It is the first system of this kind reported for cacao. The new procedure has the potential to increase embryo production 30-fold and to help reduce its cost. The system used in this study differs from prior work in the use of TDZ to induce embryogenesis, and in its ability to regenerate various genotypes (Li et al., 1998). The secondary embryos produced from all genotypes were also more uniform and developed in shorter time periods compared to primary somatic embryos. One limitation of the protocol developed for cacao is that embryo production is confined to the first 8–9 mo. after culture initiation. Also, as in primary embryogenesis, secondary embryogenesis efficiency was genotype dependent. The results obtained for Scavina-6 indicated that further optimization of the age of the primary culture used as a cotyledon source for individual genotypes could improve efficiency.

Our results indicate that primary and secondary somatic embryogenesis of cacao result from two distinct ontogenetic pathways. Staminodes formed embryos of multicellular origin, while embryos from cotyledons developed primarily from unicellular origins. The formation of secondary embryos was greatly stimulated by transfer of primary embryos to PGR-containing SCG media,

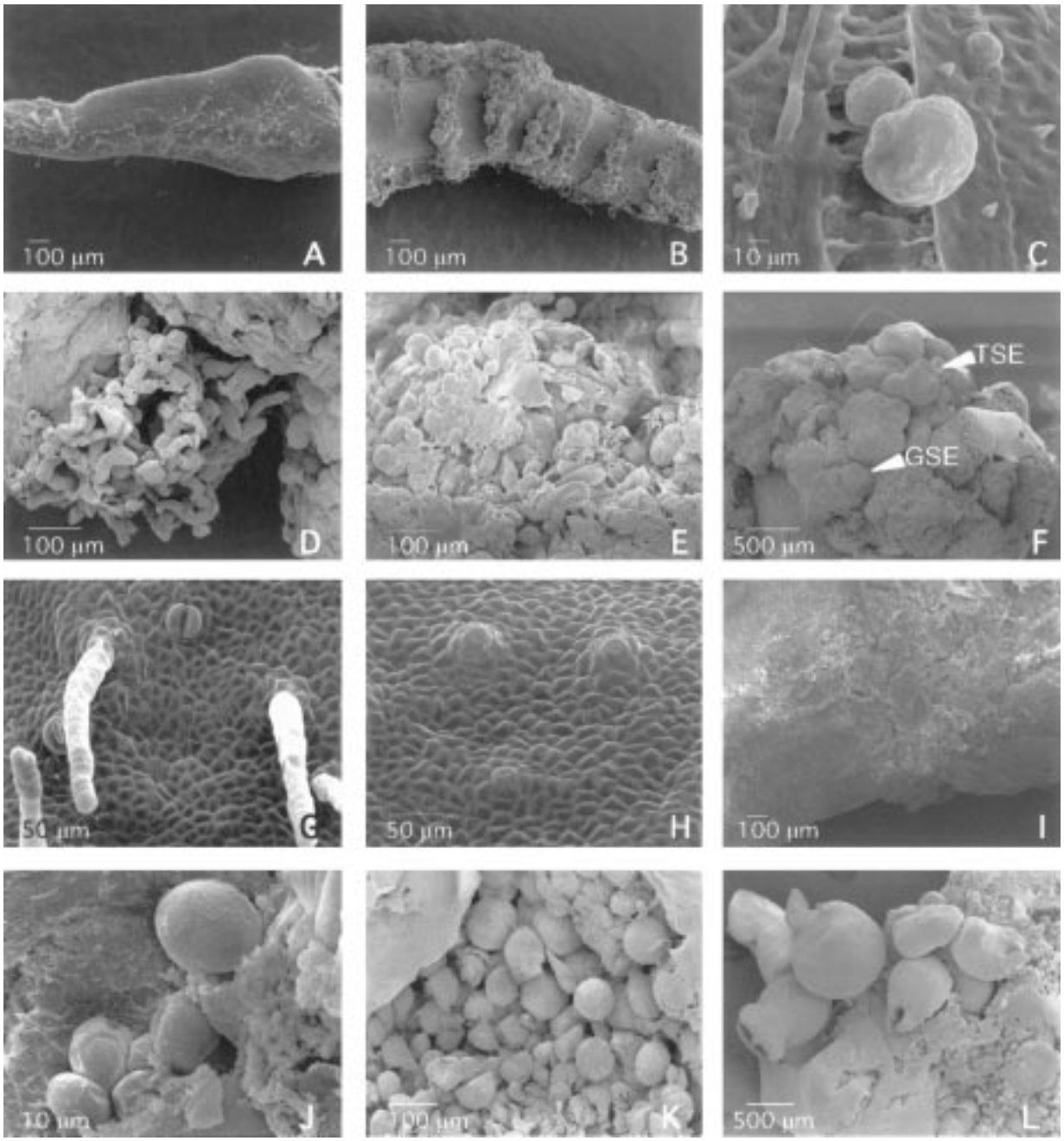

FIG. 3. Scanning electron micrographs of various stages of primary and secondary embryogenesis of cacao. *A–F*, Primary (staminode) embryogenesis; *G–L*, secondary (SE cotyledon) embryogenesis. *A*, Staminode at 0 d after primary culture initiation (APCI) (*bar* = 100 µm). *B*, Staminode at 5 d APCI (*bar* = 100 µm). *C*, Staminode at 5 d APCI (*bar* = 10 µm). *D*, Non-embryogenic callus from staminode at 30 d APCI (*bar* = 100 µm). *E*, Embryogenic callus from staminode at 30 d APCI (*bar* = 100 µm). *F*, Primary somatic embryos from staminode at 30 d APCI (*bar* = 500 µm); GSE, globular-stage somatic embryo; TSE, torpedo-stage somatic embryo. *G*, Cotyledon at 0 d after secondary culture initiation (ASCI) (*bar* = 50 µm). *H*, Cotyledon at 0 d ASCI (*bar* = 50 µm). *I*, Cotyledon at 5 d ASCI (*bar* = 100 µm). *J*, Cotyledon at 5 d ASCI (*bar* = 10 µm). *K*, Embryogenic callus from cotyledon at 30 d ASCI (*bar* = 100 µm). *L*, Secondary somatic embryos from cotyledons at 30 d ASCI (*bar* = 500 µm).

followed by transfer to PGR-free ED media. However, at a much lower frequency, primary embryos also produced new embryos by repetitive embryogenesis without change in medium composition (data not shown). The multicellular origin of primary somatic embryos is similar to that previously reported by Alemanno et al. (1996a, b). Unicellular origin was also previously demonstrated for the development of embryos from cacao immature zygotic embryos (Pence, 1989). Other investigators have concluded that when meristematic cells from borage or ginseng are involved in somatic embryogenesis, embryos often originate from several cells (Quinn

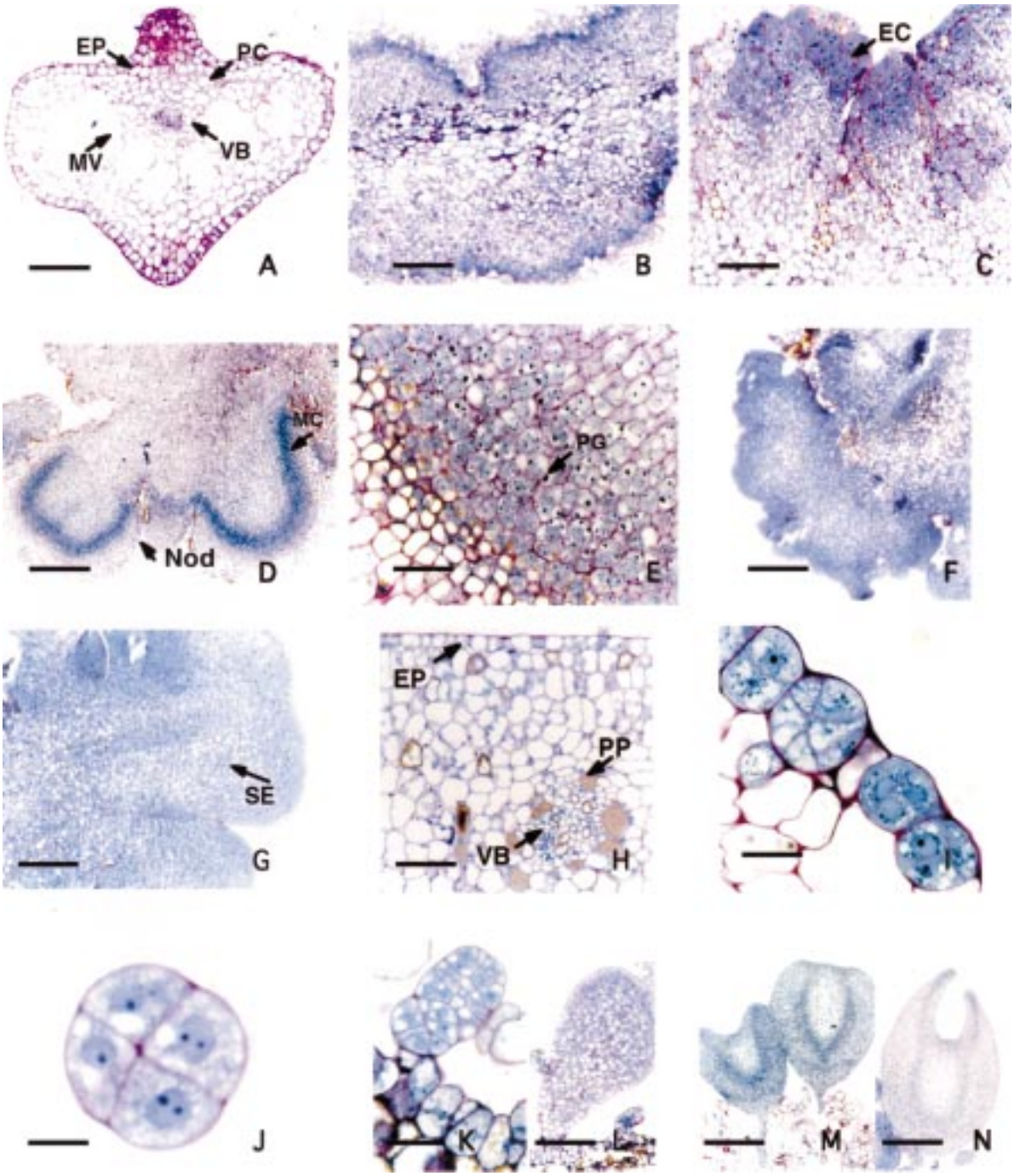

FIG. 4. Histological analysis of primary and secondary embryogenesis. *A–G*, Primary embryogenesis; *H–N*, secondary embryogenesis. *A*, NA 79; transverse section of a staminode, 0 d after primary culture initiation (APCI) (*bar* = 140 μm). *B*, Scavina-6, longitudinal section of staminode 10 d APCI (*bar* = 200 μm). *C*, Scavina-6, longitudinal section of an embryonic callus from staminode at 14 d APCI (*bar* = 90 μm). *D*, Scavina-6, longitudinal section of a callus containing nodules with meristematic cells, 24 d APCI (*bar* = 270 μm). *E*, Scavina-6, meristematic cells with increased protein content 32 d APCI (*bar* = 43 μm). *F*, Scavina-6, cellular masses formed as a result of active cell division of meristematic cells, 35 d APCI (*bar* = 280 μm). *G*, Scavina-6, primary somatic embryo, 35 d APCI (*bar* = 90 μm). *H*, KER 1, transverse section of somatic embryo cotyledon, 0 d after secondary culture initiation (ASCI) (*bar* = 140 μm). *I*, GU 143, first somatic embryo divisions of epidermal cells, 21 d ASCI (*bar* = 26 μm). *J*, Scavina-6, second somatic embryo cell division cells, 21 d ASCI (*bar* = 13 μm). *K*, Scavina-6, globular-stage somatic embryo, 21 d ASCI (*bar* = 39 μm). *L*, Scavina-6, late globular-stage somatic embryo, 35 d ASCI (*bar* = 130 μm). *M*, Scavina-6, heart- and torpedo-stage somatic embryos, 35 d ASCI (*bar* = 570 μm). *N*, KER 1, somatic embryo with developing cotyledons, 48 d ASCI (*bar* = 310 μm). Abbreviations: EC, embryonic cells; EP, epidermis; MC, meristematic cells; MV, mucilaginous vesicles; Nod, nodule; PC, parenchymatous cells; PG, protein globules; PP, polyphenolics; SE, somatic embryo; VB, vascular bundle.

et al., 1980; Choi et al., 1998). Conversely, cotyledons from *Trifolium repens* could also form meristematic zones followed by development of embryos with multicellular origin (Maheswaran and Williams, 1985).

Our observations of the unicellular origin of cacao secondary embryos are the first report of such a developmental pathway in cacao. The pathway was very similar to those described for walnut (Polito et al., 1989) and Korean ginseng (Choi et al., 1997). This study also demonstrated a positive relationship between unicellular origin and higher embryo conformity. The larger number of abnormal embryos produced during primary somatic embryogenesis could be the result of variation in the number of cells that participate in the formation of each individual, and the possible lack of effective spatial coordination during development.

We have described a procedure for producing cacao secondary embryos that can be used to increase the efficiency and quality of embryos produced *in vitro*. This is of potential utility in the clonal propagation of elite genotypes of cacao for clonal breeding trials, and for scale-up of new varieties for large-scale propagation programs. Furthermore, the single cell origin of cacao secondary embryos offers a system useful in the development of a genetic transformation system. Long-term field conformity trials which monitor genetic stability, morphology and yield from plants produced by these methods are necessary to ensure the suitability of such plants in production systems.

Acknowledgments

We thank Dr. Zhijian Li for developing the modified SCG medium, Sharon Pishak for her technical assistance, Dr. Richard Craig for his assistance with the statistical analysis and Rosemary Walsh and Michelle Peiffer of the Electron Microscope facility at PSU for their technical assistance with the SEM analysis. We would also like to thank Dr. Smilja Lambert and Dr. Martin Aitken, Almirante Center for Cocoa Studies, Bahia, Brazil, and Pat and Edmond Opler Jr., Worlds Finest Chocolate, Chicago for providing cacao floral material for the experiments at PSU. This work was supported in part by the American Cocoa Research Institute Endowed Program in the Molecular Biology of Cocoa, by grants to M.J.G. from the American Cocoa Research Institute and the United States Department of Agriculture Office of International Research Programs, support from the Agricultural Experiment Station of The Pennsylvania State University (CRIS 3550) and by support from CIRAD, France to L.A.

References

Alemanno, L.; Berthouly, M.; Michaux-Ferrière, N. Embryogenese somatique du cacaoyer a partir de pieces florales. Plant. Rech. Dev. 3:225–233; 1996a.

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

Alemanno, L.; Berthouly, M.; Michaux-Ferrière, N. A comparison between *Theobroma cacao* L. zygotic embryogenesis and somatic embryogenesis from floral explants. In Vitro Cell Dev. Biol. Plant 33:163–172; 1997.

Chatelet, P.; Michaux-Ferrière, N.; Dublin, P. Potentialites embryogenes du nucelle et du tegument interne de graines immatures de cacaoyer (*Theobroma cacao* L.). C.R. Acad. Sci. Paris 315:55–62; 1992.

Choi, Y. E.; Yang, D. C.; Kim, H. S.; Choi, K. T. Distribution and changes of reserve materials in cotyledon cells of *Panax ginseng* related to direct somatic embryogenesis and germination. Plant Cell Rep. 16:841–846; 1997.

Choi, Y. E.; Yang, D. C.; Park, J. C.; Soh, W. Y.; Choi, K. T. Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormone-free medium. Plant Cell Rep. 17:544–551; 1998.

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

Figueira, A.; Janick, J. Development of nucellar somatic embryos of *Theobroma cacao*. Acta Hort. 336:231–238; 1993.

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

Fisher, D. B. Protein staining of ribboned epon sections for light. Histochemie 16:92–96; 1968.

Irizarry, H.; Rivera, E. Early yield of five cacao families at three locations in Puerto Rico. J. Agric. Univ. P.R. 82(3/4):163–171; 1998.

Li, Z.; Traore, A.; Maximova, S.; Guiltinan, M. J. Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell. Dev. Biol. Plant 34:293–299; 1998.

Litz, R. E. Tissue culture studies with *Theobroma cacao*. In: Dimick, P. S., ed. Cacao biotechnology symposium. Department of Food Science, College of Agriculture, The Pennsylvania State University, University Park, PA; 1986:111–120.

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

Maheswaran, G.; Williams, E. G. Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann. Bot. 56:619–630; 1985.

Martoja, R.; Martoja, M. Initiation aux techniques de l'histologie animale. Paris: Masson et Cie; 1967:345.

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. Hort. Sci. 104:145–148; 1979.

Polito, V. S.; McGranahan, G.; Pinney, C.; Leslie, C. Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans regia* L.): implications for *Agrobacterium*-mediated transformation. Plant Cell Rep. 8:219–221; 1989.

Quinn, J.; Simon, J. E.; Janick, J. Histology of zygotic and somatic embryogenesis in borage. J. Am. Soc. Hort. Sci. 4:516–520; 1980.

Snedecor, G. W. Statistical methods. Ames, IA: The Iowa State College Press; 1948:83.

Sondahl, M. R.; Liu, S.; Bellato, C.; Bragin, A. Cacao somatic embryogenesis. Acta Hort. 336:245–248; 1993.

Sondahl, M. R.; Sereduk, T. B.; Chen, Z.; Bellato, C. M.; Liu, S. J.; Bragin, C. H. Somatic embryogenesis and plant regeneration of cacao. US Patent #5.312.801; 1994.

Timmis, R. Bioprocessing for tree production in the forest industry: conifer somatic embryogenesis. Biotechnol. Progr. 14:156–166; 1998.

Traore, A. Somatic embryogenesis, embryo conversion, micropropagation and factors affecting genetic transformation of *Theobroma cacao* L. PhD thesis, The Pennsylvania State University, University Park; 2000.

Villalobos, V.; Aguilar, M. E. Plant regeneration of cacao (*Theobroma cacao* L.) by micrografting somatic embryos. Abstr. VII Int. Congr. Plant Tissue and Cell Cult., Amsterdam, Int. Assoc. for Plant Tissue Culture; 1990:140.