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EMBRYOGENESIS/SOMATIC EMBRYOGENESIS

Field performance of *Theobroma cacao* L. plants propagated via somatic embryogenesis

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Abstract Somatic embryogenesis is an *in vitro* clonal propagation method with potential to contribute to the improvement of cacao varieties. Before using this technology for commercial production, it is essential that somatic embryogenesis-derived plants be tested in field conditions. Therefore, we established a field test at Union Vale Estate, Saint Lucia. Thirty- to 50-yr-old trees were selected for clonal propagation as potentially high yielding based on local farmers observations. Clonal plants were propagated *in vitro* from immature flowers by embryogenesis and micropropagation. Multiple plants from nine genotypes were acclimated to greenhouse conditions then returned to Saint Lucia and planted in a field. Orthotropic rooted cuttings and locally propagated open pollinated seedlings were also planted for a total of 214 trees. Growth data were collected every 4–6 mo. including: stem diameter, stem height, length of the longest jorquette branch, number of jorquette branches, and dates of first flowering and fruiting. At 4.5 yr after planting in the field there were no major differences in all growth parameters among the propagation methods evaluated with exception of the orthotropic rooted cuttings. Trees grown from seeds were slightly taller then trees propagated by the other methods. Trees propagated as orthotropic rooted cuttings exhibited smaller average stem diameters, shorter stem heights to the jorquette, and shorter jorquette branches. We concluded that somatic embryo-derived plants demonstrated normal phenotypes in field conditions and have growth parameters similar to plants propagated by traditional methods.

Keywords Cocoa · Somatic embryos · Micropropagation · Orthotropic rooted cuttings

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Introduction

Theobroma cacao L. trees are grown in the humid tropics to produce cocoa beans, which are a cash crop and an important raw material for the chocolate industry. Cacao trees demonstrate a high degree of segregation for many traits when propagated by seeds. For this reason, clonal propagation systems such as rooted cuttings and grafting have been applied for multiplication of elite varieties but a vast majority of cacao plants in production were derived from seeds (Eskes 2005). The use of *in vitro* propagation methods for cacao could potentially contribute to efforts at crop improvement, germplasm conservation, and rapid distribution of new improved varieties. Somatic embryogenesis using floral explants has been the only method successfully developed for *de novo* regeneration of cacao plants *in vitro* (Lopez-Baez et al. 1993; Alemanno et al. 1996a, b, 1997; Li et al. 1998). An integrated propagation system, which combines primary and secondary somatic embryogenesis with a secondary multiplication step of rooted cuttings, has been proposed as a potential method to increase the speed and minimize the cost of large-scale clonal propagation of cacao (Maximova et al. 2005). In addition, methods for cryopreservation of cacao somatic embryos have been developed and could be applied as a

solution for the long-term and secure conservation of cacao germplasm (Florin et al. 2000; Fang et al. 2004). Finally, the possibility of eliminating cacao diseases from infected plants (phytosanitation) has been explored and the application of tissue culture could help speed up the international transfer of important cacao genotypes.

In a few specific cases, it was demonstrated that plants of other species produced via tissue culture exhibit high levels of genomic instability and somaclonal variation (Rival et al. 1997; Tremblay et al. 1999). In severe cases, this has resulted in sterile or very poorly performing plants. Therefore, before *in vitro* propagated cacao are distributed on a wide scale, it is essential that field performance is carefully evaluated in multiple locations. Various research groups have established field tests of cacao somatic embryo-derived plants, including the one described in this manuscript, although to date, no publications exist which describe these tests. To establish the field test described in the present manuscript, flowers from selected trees in Saint Lucia were shipped to The Pennsylvania State University (Penn State) in the USA and used as a source of explants for somatic embryogenesis. After regeneration and acclimation to greenhouse conditions, bare-root plantlets were returned to Saint Lucia and planted in a randomized design along with seed grown controls. Other methods of propagation also evaluated included micropropagation and orthotropic rooted cuttings. In summary, the results demonstrated that during establishment and juvenile growth over a period of 4.5 yr, the seed-propagated plants grew slightly taller then the rest of the plants. No significant differences in the growth rates were observed among plants propagated by the different *in vitro* methods. In comparison, plants propagated by orthotropic rooted cuttings appeared to be somewhat more compact in growth habit.

Materials and Methods

Union Vale Estate is located on the Island of Saint Lucia, West Indies (Lat/Long: 13° 48′ 40.8″ N, 061° 03′ 22.2″ W), on the East side of the Gros Piton Mountain. With the assistance of the farm manager Eustus George, mature trees that were observed to consistently produce high yields were identified and tagged. Most of the selected trees were large, old trees (approx. 30–50 yr old) and appeared to be of Trinitario heritage based on pod shape. A number was assigned to each tree in the order it was identified. Each tree number, along with the abbreviation UV (Union Vale) was used as a unique identifier of the clone during propagation and field planting. Immature flowers were collected from the mother trees and transported on ice to Penn State as previously described (Maximova et al. 2005). The flowers were first sterilized then staminodes and petal bases were dissected and cultured on tissue culture media for induction of primary somatic embryos (PSE) as previously described (Li et al. 1998). Primary somatic embryogenesis was followed by secondary somatic embryogenesis (SSE), micropropagation (MP), and orthotropic rooted cutting (ORC) propagation also as previously described (Maximova et al. 2002; Traore et al. 2003; Maximova et al. 2005). Acclimated plants from nine individual genotypes were returned to Saint Lucia as bare-root plants, 5 to 12 in. in height. The plants were transplanted to a 1:1 mix of sand and soil in black plastic bags and placed in a greenhouse with 50% shade under misting (30 s every 8 min) for 3 to 4 d. Plants were then grown in the greenhouse with shade for 11 to 18 mo. prior to planting in the field. Open pollinated seedlings from randomly selected trees were also planted in bags and grown as described above. In January of 2001, a total of 214 trees were planted in the field in a randomized design with 3-m spacing under established banana and coconut palm as shade. The field was drip irrigated with river water for the first 2 yr as needed during dry periods. Growth data were collected every 4–6 mo. from 6/2001 to 11/2005. The growth parameters measured were stem diameter at 10 cm above the soil, height of the main stem from the soil to the first jorquette, and length of longest jorquette branch. Means for the individual propagation methods and data collection points were calculated and variation was established by Fisher Protected LSD test at $p<0.05$ level of significance. The time of jorquette development, number of jorquette branches, and flowering and fruiting were recorded for each individual tree. To test for significant differences for these parameters among the propagation methods, we applied Chi-square tests for homogeneity ($p<0.05$).

Results and Discussion

The main objective of this study was to evaluate SE-derived cacao plants grown in the field for any adverse effects of the somatic embryogenesis process. We successfully collected flowers in St. Lucia, transported them to Penn State to use as tissue culture explants and regenerated plantlets via somatic embryogenesis. A large genotype effect on the primary somatic embryo regeneration was observed that was consistent with previous reports (Li et al. 1998; Maximova et al. 2002; Traore et al. 2003; Maximova et al. 2005). However, all genotypes that produced one or more primary embryos were successfully multiplied by secondary embryogenesis. After regeneration and acclimation in a greenhouse at Penn State, bare-root plants were wrapped in wet paper towels, packed in suitcases, and transported to Saint Lucia as carry-on luggage. Plants were transplanted and acclimated in a greenhouse in black plastic

soil container bags with nearly 100% survival rate. Plants were then transplanted to the field under banana and coconut palm shade with drip irrigation as shown in Fig. 1*A*. The field was steeply sloped and south facing, consisting of rocky, weathered, volcanic soils. Field growth data were collected in intervals of 4 to 6 mo. for a total of 4.5 yr. Early survival rates were approximately 95%, but during the first 2 yr of establishment, 32 plants died mainly due to drought conditions and severe insect damage. One border row in particular was nearly completely destroyed by insects, apparently migrating from an adjacent row of infested Glory Cedar trees (*Gliricidia sepium*).

Plants were measured and photographed at regular intervals (Fig. 1). The majority of the plants grew normally as compared to seed controls and after 4.5 yr most had flowered and were producing pods (Fig. 1*C*,*D*). Stem diameters gradually increased at a near linear rate from about 15 mm diameter 6 mo. after planting to an average size of about 60 mm at 57 mo. (Fig. 2*A*). Trees propagated by various methods showed no significant differences in stem diameter (p values>0.06) with the exception of the plants propagated by ORC. The ORC plants had significantly smaller stem diameters compared to the rest of the methods for the first 2 yr (p values<0.0001). After 28 mo.

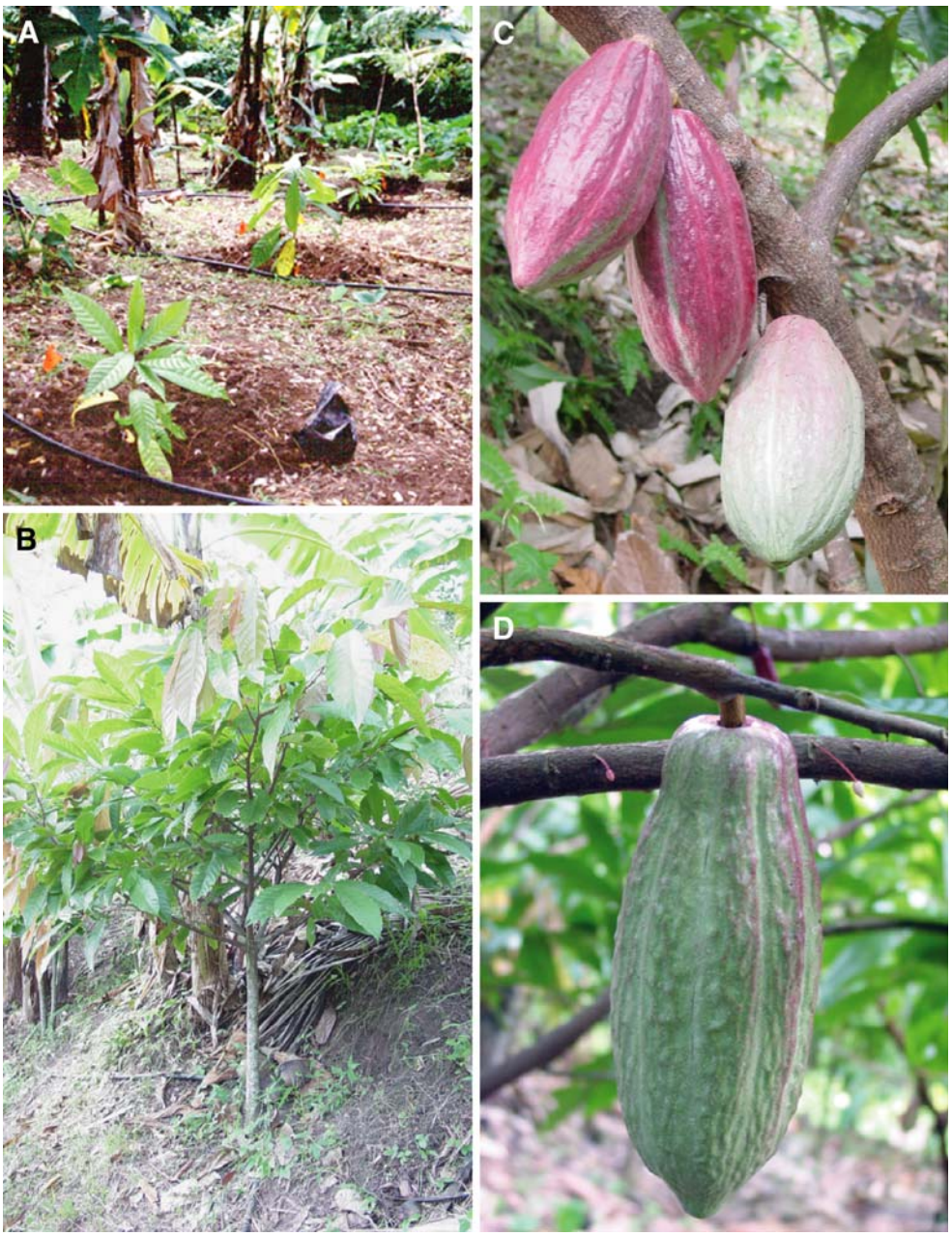

Figure 1. Somatic embryo-derived plants growing in the field at Union Vale Estate, St. Lucia. (*A*) Row of cacao plantlets shortly after planting with drip irrigation, inter-planted with banana and coconut shade, Jan. 2001; (*B*) plant #408, UV1 genotype, secondary somatic embryo plant, Dec. 2003; (*C*) plant # 354, UV1 genotype, secondary somatic embryo plant, Nov. 2005; (*D*) plant 412, UV56 genotype, primary somatic embryo plant, Nov. 2005.

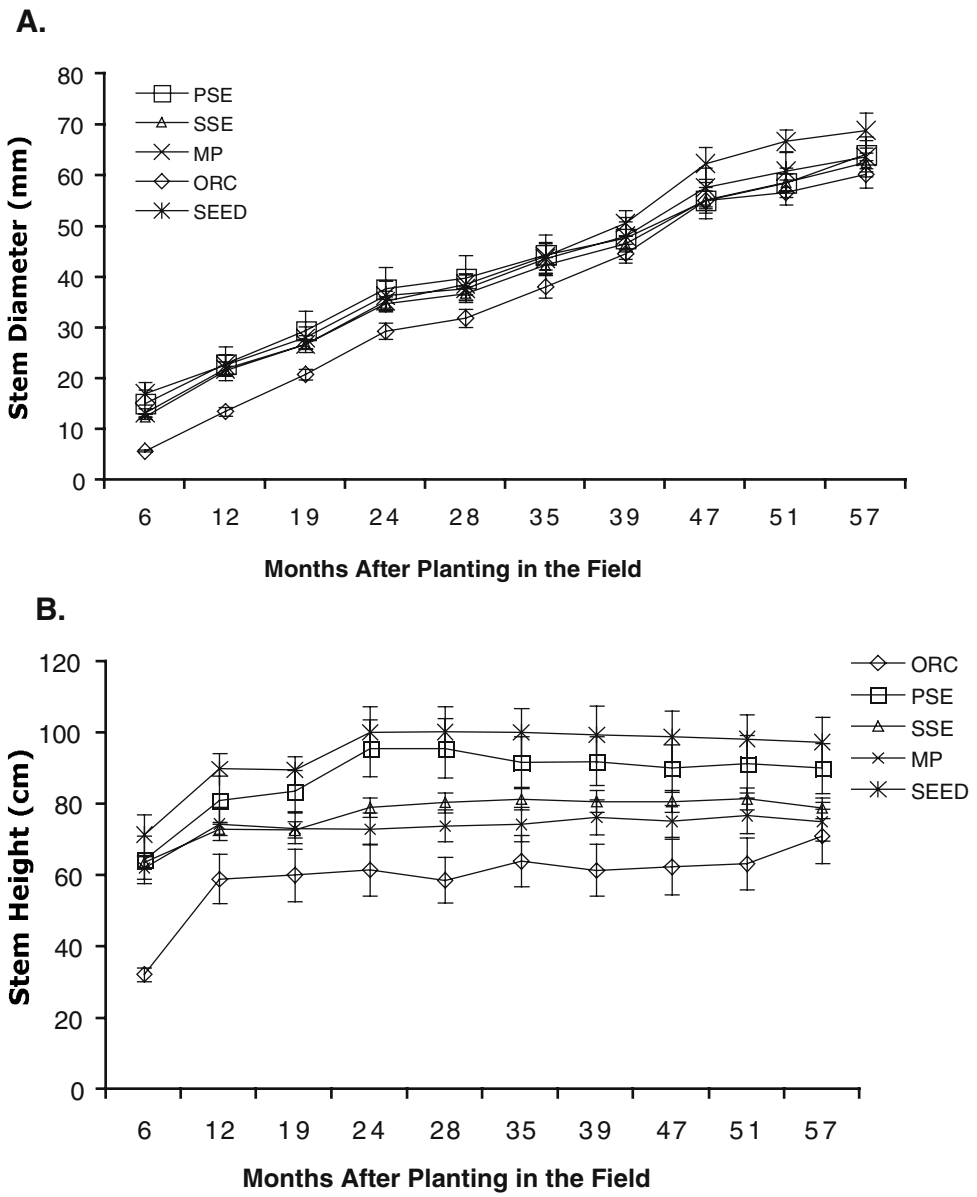

Figure 2. Stem diameter and height to the first jorquette of cacao trees propagated by different methods recorded over 4.5 yr. (*A*) Stem diameter: average stem diameter in mm±SE is presented for each propagation method. (*B*) Stem height to the first jorquette: average stem height in cm±SE is presented for each data point and each propagation method. *PSE* primary somatic embryogenesis, *SSE* secondary somatic embryogenesis, *MP* micropropagation, *ORC* orthotropic rooted cutting.

in the field, the mean stem diameters of ORC propagated plants were not significantly different from the rest of the trees (p values>0.06). After 39 mo. in the field, MP plants increasingly developed slightly larger mean stem diameters compared to the SSE and ORC plants ($p<0.04$). However, there were no significant differences recorded among all methods at the final data collection point of 57 mo. in the field (p values>0.1). Additionally, no significant variation in stem diameter correlated with different genotypes was observed (data not shown).

The height and timing of jorquette formation of young cacao trees, as well as the number and length of these branches, can be indicators of the normal development of a cacao tree and the transition from juvenile to adult phases of development. Thus, we measured the height of the main stem of each tree over time, from the soil surface to the shoot apex for young plants, and once a tree had formed a jorquette, from the soil to the jorquette. The results indicated that the majority of the trees had achieved their maximum stem height by the end of the first year after planting in the field (Fig. 2*B*), averaging between 59 and 90 cm. A minor increase of the average stem height was observed for the following 3.5 yr of growth. The comparison of the mean stem heights of the plants from the different propagation methods demonstrated that seed grown and PSE plants grew taller than the other plants during first 28 mo. ($p<0.02$). After 35 mo of growth, only the seed plants were slightly taller then the SSE, MP, and ORC plants ($p<0.02$) with an average difference of 19 to 26 cm depending on the propagation method. Seed plants were not significantly different from PSE plants for the rest of the measuring period ($p>0.2$), but remained taller than

the plants produced by other methods for the 57 mo. ($p<0.0079$). Plants propagated by SSE and MP had similar mean stem heights throughout the measuring period, which were slightly shorter than seedlings and the PSE propagated plants. The ORC propagated plants grew significantly shorter than the rest of the plants for the first 6 mo. ($p<0.0001$) but after 12 mo., the stem height of these plants differed only from the seed and PSE propagated plants ($p<0.02$). We have recorded this difference in greenhouse studies as well and noted that ORC plantlets often jorquette lower than normal (C. Miller and M. Guiltinan, unpublished results). Removal of a low jorquette immediately below the branching point results in subsequent activation of the immediate lower auxiliary meristems and reestablishment of the orthotropic growth, followed by new jorquette formation at a normal height. For this field test, the plants with lower jorquettes were not pruned, thus, the average jorquette heights reflect the difference.

The development of jorquettes in all plants was further evaluated by recording the time of jorquette development for each tree (Table 1), the number of jorquette branches (Fig. 3*A*) and the length of the longest jorquette branch (Fig. 3*B*). After the first 6 mo. in the field, 50–54% of the SSE, MP, and seed plants had developed jorquettes compared to only 13% of the ORC and 43% of PSE (Table 1). By 19 mo. after planting, 100% of MP and seed plants had jorquettes and by the end of the second year 100% of PSE, SSE, and ORC plants had also developed jorquettes. Considering that 100% of the plants had jorquettes by 24 mo., we selected that date to report the percentage of trees developing different numbers of jorquette branches (Fig. 3*A*). The majority of the trees developed four or five jorquette branches regardless of propagation method, with a frequency of about 40% each, for a total of 80% (Fig. 3*A*). Chi-square analysis demonstrated that there were no significant differences among the propagation methods in the proportions of plants producing four- or five-branched jorquettes ($p=0.64$ for four-branched jorquettes and $p=0.42$ for five-branched jorquettes). With the exception of PSE plants, which did not produce any two-branched jorquettes, a small percentage of plants from all propagation methods produced two- or three-branched jorquettes at a frequency of about 5% and 15%, respectively. The length of the longest jorquette branch was recorded at 12 and 19 mo. after planting for all plants that had developed jorquettes at the time of the data collection (Fig. 3*B*). Significant increases in mean length of the longest jorquette branch (between 18 and 22 cm) were recorded from 12 to 19 mo. for all plants ($p<0.001$). When we compared the mean branch lengths among the propagation methods, we observed that the ORC plants had significantly shorter jorquette branches during both data collections ($p<0.01$ and $p<0.03$). No significant difference was observed among the rest of the propagation methods for both dates ($p>0.13$).

Another developmental process evaluated was the cyclical changes in flowering that occur in cacao as a result of seasonal changes in rainfall. Here, we present the percentage of flowering trees recorded from December 2003 (35 mo. in the field) to November 2005 (57 mo. in the field) (Fig. 4*A*). During that period, all trees evaluated had developed jorquettes. A significantly higher percentage ($p=8.47$ E-15) of plants, regardless of propagation method, flowered during the spring months (April and May) compared to the winter months (November, December, and January). Thus, the flowering occurred mainly at the end of the dry season (Jan to April) and the beginning of the rainy season (May to Nov) in Saint Lucia. No significant differences were observed in the percentage of flowering trees among the propagation methods (p values>0.5).

Another marker of cacao tree development measured was the age of first fruit formation. A cacao tree must reach a sufficient size and physiological vigor to support the energy demands of fruit and seed development. Younger trees, even when flowering, do not always immediately sustain fruit development, but instead fruit are often aborted at an early stage. As they mature, the trees can support fruit development, but the number of fruits is also regulated by physiological vigor. Thus, the date of first fruit production is indicative of the general physiological and developmental stage of a cacao tree. The percentage of trees producing mature fruit is presented in Fig. 4*B*. During this field test,

Table 1. Percentages of plants forming jorquettes recorded at different times after planting in the field

Propagation method	Total number of plants	6 mo. after planting (%)	12 mo. after planting (%)	19 mo. after planting (%)	24 mo. after planting (%)
PSE	15	43	79	79	100
SSE	91	51	88	94	100
MP	39	51	95	100	100
SEED	26	54	75	100	100
ORC	37	13	78	92	100

PSE primary somatic embryogenesis, *SSE* secondary somatic embryogenesis, *MP* micropropagation, *ORC* orthotropic rooted cutting.

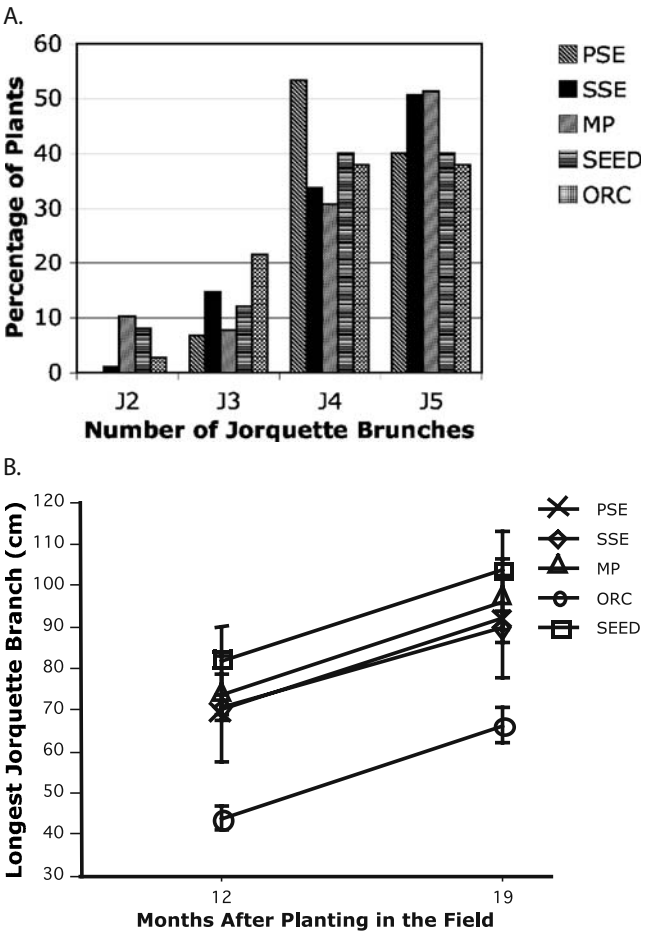

Figure 3. Evaluation of the numbers of jorquette branches and lengths of the longest jorquette branches of plants propagated by different methods. (*A*) The number of jorquette branches for each plant was recorded at 24 mo. after planting in the field and the percentages of plants with two-, three-, four-, or five-branched jorquettes were calculated for each propagation method. (*B*) The length of the longest jorquette branch was recorded for each tree at 12 and 19 mo. after planting in the field. Average length of the longest jorquette branch±SE is presented for each of the data points and each propagation method. *PSE* primary somatic embryogenesis, *SSE* secondary somatic embryogenesis, *MP* micropropagation, *ORC* orthotropic rooted cutting.

mature fruit development was observed as early as 19 mo. after planting on several of the PSE and seed-propagated plants. Overall, the percentage of trees producing fruit remained low initially then rose to about 40% of the trees (p=6.5 E-12) at 57 wk. This percentage will likely continue to increase as the trees mature. No significant differences were observed among the propagation methods at each time point throughout the entire period (p values>0.3). Thus, the onset of fruit development was approximately the same for each propagation method. In the future, we will collect individual plant yield data and compare the morphological characteristics of the flowers and the fruits to the parent trees.

In conclusion, the vegetative growth of plants propagated *in vitro* did not differ significantly from that of seed-derived plants. During the first 3.5 yr of growth, the ORC plants grew slower as indicated by the smaller mean values of stem diameters and heights. By year 4 these plants were still slightly smaller than PSE and seed plants but had achieved sizes similar to the other *in vitro* propagated plants. Observations of the other growth parameters also indicated no abnormalities or significantly different growth patterns among the different genotypes or propagation methods at the end of the measuring period with the exception of the ORC plants, which on average had shorter jorquette heights at the end of the third year.

Additional field tests of cacao somatic embryos by our group and in collaboration with the local research units and farmers have been established in Puerto Rico, Brazil, Ghana and in Ecuador. The multilocated trials will, in the near future, provide a comprehensive evaluation of the suitability of cacao somatic embryogenesis for commercial deployment.

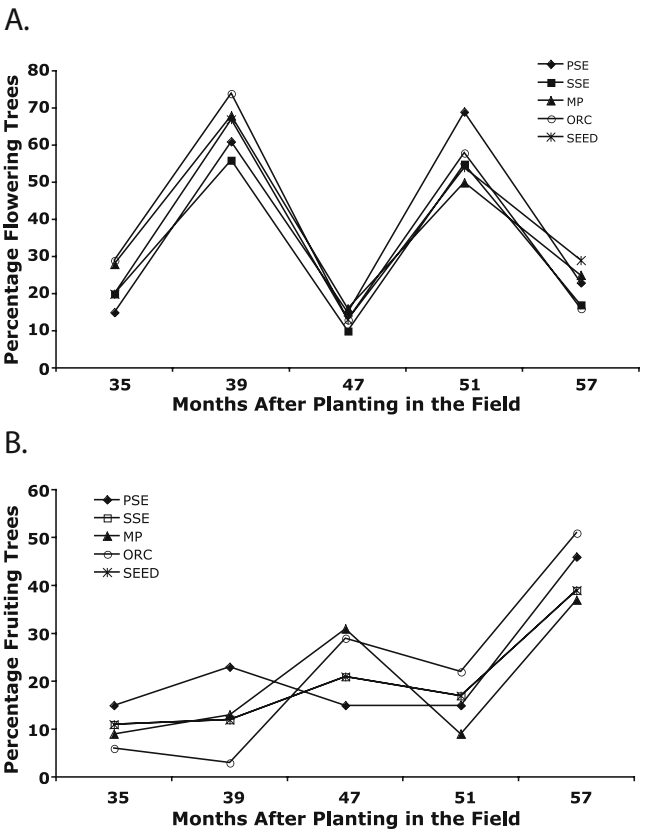

Figure 4. Evaluation of the flowering and fruiting of cacao trees. The flowering and fruit production of each tree was recorded every fall and spring from 35 to 57 mo. after planting (2–03 to 11–05) and the percentages of flowering and fruiting trees were calculated for each propagation method. (*A*) Percentage flowering trees. (*B*) Percentage fruiting trees. *PSE* primary somatic embryogenesis, *SSE* secondary somatic embryogenesis, *MP* micropropagation, *ORC* orthotropic rooted cutting.

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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 Tissue Organ Cult* 46: 187–194; 1996b.

Eskes, A. Proceedings of the International Workshop on Cocoa Breeding for Improved Production Systems: INGENIC International Workshop on Cocoa Breeding, F. Bekele, M. End, & A. Eskes, eds (INGENIC and Ghana Cocoa Board, Accra, Ghana), pp. 1–10, http://ingenic.cas.psu.edu/proceedings.htm; 2005.

Florin B.; Brulard E.; Pétiard V. *In vitro* cryopreservation of cacao genetic resources. In: Engelman F; Tagaki H (eds) Cryopreservation of Tropical Plant Germplasm. Japanese International Research Centre for Agricultural Sciences and IPGRI, Rome, pp 344–347; 2000.

Fang J. Y.; Wetten A.; Hadley P. Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. *Plant Sci* 1663: 669–675; 2004.

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.

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.

Maximova S. N.; Alemanno L.; Young A.; Ferriere N.; Traore A.; Guiltinan M. J. Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In vitro Cell. Dev. Biol. Plant* 38: 252–259; 2002.

Maximova S. N.; Young A.; Pishak S.; Miller C.; Traore A.; Guiltinan M. J. Integrated system for propagation of Theobroma cacao L. In: Jain SM; Gupta PK (eds) Protocols for somatic embryogenesis in woody plants. Springer, Dordrecht, The Netherlands, pp 209–229; 2005.

Rival A.; Berlenc F. A.; Morcillo F.; Tregear J.; Verdeil J. L.; Duval Y. Scaling-up *in vitro* clonal propagation through somatic embryogenesis: the case of oil palms: the example of oil palm (*Elaeis guineensis* Jacq.). *Plant Tissue Cult. Biotech* 32: 74–83; 1997.

Traore A.; Maximova S. N.; Guiltinan M. J. Micropropagation of *Theobroma cacao* using somatic embryo-derived plants. *In vitro Cell. Dev. Biol. Plant* 39: 332–337; 2003.

Tremblay L.; Levasseur C.; Tremblay F. M. Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, pinaceae) and white spruce (*P. glauca*, pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *Am. J. Bot* 86: 1373–1381; 1999.