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INTEGRATED SYSTEM FOR PROPAGATION OF *THEOBROMA CACAO L.*

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1. INTRODUCTION

Theobroma cacao L. is a tropical tree, with origins in the Amazon basin, currently cultivated throughout the tropics to supply the global demand for cocoa, the main ingredient for the manufacture of chocolate. A high degree of yield variation is a general characteristic of cacao plantations worldwide, due in part 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 to 3% of the trees in a population accounted for more than 60% of the yield (Irizarry and Rivera, 1999).

Improvements made by plant breeders are often rapidly lost, as farmers propagate plants through seeds, and segregation results in a highly heterozygous population of plants. Vegetative propagation systems provides a means to capture such additive genetic gain, and has been used to some degree with cocoa with the development of grafting, and rooted cutting techniques. Modern biotechnology offers a suite of new approaches to speed up the development and deployment of genetically improved genotypes. Recently, research conducted at Plant DNA Technology, (Sondahl et al., 1994) CIRAD, Montpellier, France (Alemanno et al., 1996a, b, c; Alemanno et al., 1997); Nestles Inc., Tours, France; Almirante Cacao Research Station, Bahia, Brazil (Lambert et al., 2000); and at The Pennsylvania State University (Antúnez de Mayolo et al., 2003; Li et al., 1998; Maximova et al., 2002; Traore et al., 2003), has led to the development of efficient methods for somatic embryogenesis of cacao. 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 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, which is currently preserved only in living collections in the tropics.

Building on work of others, our group has optimized a protocol for cacao somatic embryogenesis using floral staminode and petal base explants. This system is relatively expensive, and thus its utility limited primarily as a research tool. To address this limitation, we have incorporated three propagation steps that increase the multiplication rate and thus reduce the cost of production of orthotropic plants. These steps include secondary embryogenesis (Maximova et al., 2002), micropropagation (Traore et al., 2003) and production of orthotropic rooted cuttings in non-sterile greenhouse or field conditions (Guiltinan et al., 2000). The sequential application of these systems could provide a low cost, rapid clonal multiplication system. This chapter will give detailed protocols for each of these methods. Updated and further information including PDF versions of our publications containing detailed photographs of the protocols can be found at our website (guiltinanlab@cas.psu.edu). Instructions for joining an email discussion group on this topic are also available at the web site. While we have extensively tested and verified these protocols in the lab and greenhouse, they are a work in progress; new adaptations will be posted at the web site. Furthermore, field-testing of plants produced by this method is underway to assess the long-term sustainability of these plants in field production conditions.

2. PRIMARY SOMATIC EMBRYOGENESIS FROM CACAO FLOWER EXPLANTS

Culture Conditions

Growth chambers: 25º-30ºC, cool white fluorescent lights with an intensity of 50-100 $\mu mol*m^{-2}*s^{-1}$ measured at shelf height, 16/8 hour photoperiod (light/dark). Greenhouse conditions are maintained at: 60-65% humidity, 30ºC day, 24ºC night (+/- approx. 5ºC), 50% shade under full sun (250-500 $\mu mol*m^{-2}*s^{-1)}$, supplemental HID 400W sodium halide lights are utilized to supplement during low light periods and to extend photoperiod to 14 hours in short daylight periods of the year. Gentle horizontal airflow is applied continuously.

Simplified protocol for collection and surface-sterilization of flower buds from greenhouse or field near to the dissection site

Immature flower buds are collected early in the morning (between 8 and 9 a.m.) in clean containers containing cold water (Fig. 1). Prior to collection, observe and evaluate the growth and development of flower buds and select unopened flowers of medium-large size (relative to the size of the flowers on an individual tree). Flowers in advanced developmental stages or harvested in the afternoon often open during surface sterilization, contributing to contamination of the tissue culture explants. Surface-sterilize the flowers in a 1% (v/v) Bleach solution (Clorox—6% sodium hypochlorite) using sterile water in a sterile plastic or glass container (Fig. 1). Perform the following steps of this protocol inside sterile tissue culture transfer hood. Decant the cold water from the container and transfer all the flower buds into new container with Bleach solution. Immerse flowers in the Bleach solution and sterilize for 20 min. During the sterilization step assure contact of the solution with the flower buds. This can be achieved by gently inverting the tube a few times every 5 minutes. After 20 min, completely remove the Bleach solution and add 50 ml room temperature (RT) autoclaved water to rinse the flower buds. Invert tubes several times and decant water. Repeat the rinse with fresh

water 2 more times. Decant the last rinse and remove as much water as possible. With sterile forceps transfer flowers to a sterile Petri dish inside the transfer hood. Close the Petri dish to prevent desiccation of the flower buds and place in the hood until dissection as soon as possible (not longer than the end of the day).

Alternative protocol for collection, sterilization and transportation of material from field to the dissection site locations

If the flowers will not be dissected the same day, the flowers can be treated in the field with bleach then shipped in cold conditions. After arrival at a suitable laboratory within 3 days, the material is re-sterilized and dissected for culture induction.

Field Sterilization: Follow with the standard sterilization protocol but use water at 4ºC and maintain at 4ºC (keep on ice) throughout the procedure. For this pre-sterilization, filter sterilized tap water can be used without negative effect on the culture. After rinsing the flowers with 4ºC cold water three times decant the water and add 50 ml of 4ºC DKW basal, sugar and hormone media free medium (Table 1). Gently invert the tubes few times to wet the flowers. Pour out the liquid from the tube and drain for 5 seconds. Hold the tube with the sterile flowers horizontally and disperse the flowers across the tube so none of the flowers are bathed in any residual water. If possible performed the sterilization inside sterile tissue culture transfer hood. If the procedure is done on open air at the field site, repeating the procedure in sterile conditions prior to dissection may be necessary.

Shipping: Place the tubes with flowers in a horizontal position in a plastic bag. In the bottom of an insulated container place a bag of crushed ice and layer with few layers of paper towels or other absorbent material. Place the bag with the flower on the top of the paper towels and cover with more paper towels on the sides and the top. Place more bags with crushed ice on the sides and the top of the flowers keeping them separated from the ice with the paper towels. The temperature in the transportation container should be approximately 1ºC in the beginning and should not increase above 16-17ºC over the course of 3 days. After a maximum of 3 days the flowers are dissected following the standard protocol.

Dissection of flower buds and callus induction

Transfer 4-10 sterile flower buds to a sterile Petri dish. It is important not to transfer excess water from the tube when moving the flower buds to the new dish. The number of buds transferred depends on a personal preference and one's tissue culture skills. Prepare in batches that will take no longer than 5 min. to avoid desiccation of the cut surfaces. Slice the flower buds across at a position 1/3 of the flower length from the base using a sterile scalpel blade. A number 11 scalpel blade is recommended. Extract staminodes and petal tissues together through the opening at the cut end using sharp sterile forceps. Separate and discard the attached stamens and pistil. The two most commonly used explants for embryogenesis are staminodes and petal bases. Transfer staminodes and petal base explants into a Petri dish containing 25-30 ml of PCG medium (Li et al., 1998) (Table 1). Separate any fused staminodes and petal base explants and distribute explants evenly on the entire surface the medium (up to 50 explants per dish), assuring good contact of the explants with the medium without immersion. Separate the petal base explants and the staminodes into different Petri dishes to evaluate the

regeneration potential of the individual tissues. Seal the Petri dishes with parafilm and maintain cultures in the dark at 25-30ºC for 14 days (cardboard box in a growth chamber works well). Follow with transfer of the explants to a Petri dish containing 25 - 30 ml of SCG medium (Maximova et al., 2002) (Table 1). Seal the dishes and maintain cultures in the dark for another 14 days. Callus formation should be apparent by the end of this culture period. At this step, good contact of the explants with the medium without immersion is important.

Somatic embryo induction and maintenance

After the first 4 weeks, transfer all explants to Petri dishes containing 30 ml of ED medium (Li et al., 1998)(Table 1). Incubate in the dark for 14 days. At this step the size of the individual explants will increase and calli will develop (Fig. 1). Two morphologically distinct types of callus are produced as a result of the induction. The first type, consist of elongated cells and appears white under a dissecting microscope. Somatic embryos almost never develop from this type of cell clusters. The second type consists of round cells and appears light to dark brown and friable. These cell clusters were often found in association with somatic embryos (Maximova et al., 2002). Subculture explants onto fresh ED medium every 14 days, and maintain cultures in the dark. As the explants increase in size, reduce their number per plate to 20-25. During the ED culture period, large numbers of somatic embryos develop. The initiation of new embryos continues for up to 10 months and embryos at different developmental stages (globular, heart torpedo and mature) are present on individual explants at the same time. Cultures can be maintained on ED medium for up to one year. This is not a synchronous system; new embryos will continue to develop for about 8-10 months with maximum production at 24 weeks post culture initiation. At each subculture mature individual embryos could be selected and transferred to conversion medium (see section on conversion).

3. INDUCTION OF SECONDAY EMBRYOGENESIS

Secondary embryogenesis is used to increase greatly the number and quality of embryos produced (Maximova et al., 2002). For secondary embryogenesis, select recently matured, primary embryos with developed cotyledons (Fig. 1). Cotyledons with high embryogenic potential are light yellow or sometimes pink in color. Avoid using very young cotyledons, which are transparent/white or old, thickened hairy cotyledons with long, visible trichomes. The optimal time frame for secondary embryo initiation is 21 to 26 weeks after primary culture initiation (Maximova et al., 2002). Separate the cotyledons from the embryo hypocotyl and slice with a sharp scalpel blade (#10 or 11 is recommended) into 4 by 4 mm pieces. Culture the explants in a Petri dish containing 25 - 30 ml of SCG medium (Table 1). At this step good contact of the explants with the medium without immersion is very important. Seal the dishes and maintain cultures in the dark for 14 days. Callus formation should be apparent by the end of this culture period. Transfer cotyledon explants to Petri dishes containing 30 ml of ED medium and culture in the dark for 14 days. Subculture explants onto fresh ED medium every 14 days, and maintain cultures in the dark. Secondary embryos should form within 2 to 3 months after culture initiation with no callus or minimal callus development. The embryos produced by secondary embryogenesis are of better quality and have more synchronized development than the primary embryos (Maximova et al., 2002). Maintain the individual embryos in the dark with a subculture interval of 14 days on ED medium until they reach maturity and are ready for conversion.

4. SOMATIC EMBRYO CONVERSION AND PLANT ESTABLISHMENT

Select mature somatic embryos (up to 2 cm in length) with distinctive cotyledons and an extended axis. Place the embryos on PEC medium (Traore, 2000) (Table 1) at a 90º angle, root tip first, up to the base of the cotyledons at a density of 6 to 10 embryos per Petri dish. For this step use deep Petri dishes (100 x 20 mm) with 30 ml PEC medium. Seal the culture dishes with parafilm and maintain cultures under light (16/8h photoperiod) at 25-30ºC. Subculture embryos to fresh PEC medium every 30 days until shoots with one or two leaves develop. Transfer shoot-producing embryos with 2 leaves of at least 1 cm in length and developed primary root into Magenta GA3 vessels or glass jars containing 143 ml of RD medium (Traore, 2000)(Table 1). Place the embryos root tip first, at a density of 4 to 5 embryos per vessel. Maintain cultures under light with a 16/8h photoperiod for 30 to 90 days. At this stage young plants are sensitive to ethylene buildup, which could cause leaf drop. The recommendation is to use magenta GA3 vessels with vented lids (Sigma Chemical, St. Louis, MO Cat. #C3430) and to seal the container with Petri seal (Diversified Biotech, Boston, MA) to prevent contamination. Transfer embryos to fresh RD medium every 2 months. Before transferring gently break up the new media with sterile forceps then insert plants into medium. To preserve the fragile roots, hold the plant with one pair of forceps, press the plant into the fresh media gently inserting the roots into the media. Occasionally, plantlets produce abnormal shoots, which continue to produce cotyledon like leaves with long stems/internodes. To promote normal development of these plantlets, prune (decapitate) with a sharp scalpel by cutting the apical shoot approximately 0.5 cm above the first node. If the shoot is extraordinarily long, decapitate between any of the lower nodes, assuring the presence of at least one node for adventitious shoot proliferation (Traore et al., 2003).

Plants that develop at least 4-5 leaves (each greater than 2 cm long) and healthy primary and secondary roots (more than 2 cm long) are ready for acclimation in the greenhouse (Fig. 1). Transfer these plants into D40 Deepots (plastic, 62.5mm x 250mm; Hummert, Earth City, MO, USA) or bags containing autoclaved, pre-moistened soil with good drainage or pure sand. Saturate the soil mixture/sand with water prior to planting. Observe the plants every day during the first week and water carefully or as needed. If sand is used, it is best to set up a drip irrigation system programmed to water the plants 5 or more times a day. However, it is important not to water excessively, since at this stage the plants are very sensitive to over watering, which reduces oxygen access to the roots. Immediately after transplanting, it is necessary to start application of diluted fertilizer with each watering. Our fertilizer of choice is 1/10 Hoaglund's solution (Table 1), however, other fertilizers may be employed. The young plants are placed on a misting bench with 100% humidity and 70% shade (approximately 100 $\mu mol*m^{-2}*s^{-1}$), and are misted for 10 seconds every 15 minutes. After 4 weeks they are transferred and maintained to maturity at 60-65% humidity with 50% shade (250-500 $\mu mol*m^{-2}*s^{-1}$). By the end of the acclimatization period the plants should reach approximately 5-10 cm in height. At this point, the rate of growth should be similar to a seed grown plant. The acclimatization and/establishment periods vary for individual plants dependent on the quality of the embryos and the condition of the plantlets (number of roots, leaves and height) at the time of the transfer to the greenhouse. Plantlets produced by this method have been grown to maturity in greenhouse conditions (Fig. 1). Bare rooted plantlets up to 12 inches in length have been transported to field conditions and also grown to maturity (Fig. 1).

5. MICROPROPAGATION OF SOMATIC EMBRYO-DERIVED PLANTS

Somatic embryos can be micropropagated *in vitro* to increase the number of plants of an individual clone. This system was previously described in detail (Traore et al., 2003).

Harvest of micro cuttings and root induction

For apical and nodal stem micro-cuttings, select healthy somatic embryo-derived plantlets grown on RD medium with developed roots and leaves. Plantlets ready for micropropagation should have green healthy leaves at least 3 cm long. Young purple leaves often drop after the micropropagation, reducing the rooting ability of the shoots. Identify a cutting point at an internode approximately 1 to 2 cm below the apex and the first 2 or 3 leaves of the shoot. If the shoots have more than 5 or 6 nodes and leaves, nodal micro cuttings can also be harvested and rooted successfully. Using a sterile scalpel, trim only the green leaves above the cutting point to1/3 of their size. Holding the tip of the shoot with sterile forceps cut the stem at the selected point/s. (see section below for further instructions on culturing the remaining stock plantlet). Transfer micro-cuttings into a Magenta box or glass jar containing 50 ml or less of root induction (RI) medium (Table 1) (Traore et al., 2003). The depth of the medium in the container needs to be 1 cm or more. Place the micro cutting vertically with the basal cut end immersed 3 to 5 mm into the medium. Root induction period varies from 48h to 7 days depending on the genotype. Incubate cultures under light (16/8 h photoperiod). Longer exposure to IBA could cause formation of excessive callus and/or a large number of roots with stunted growth, although some genotypes may require an extended root induction period.

Root development, maintenance and plant acclimation

After the root induction period, transfer explants into a Magenta box or glass jar containing 140-150 ml of RD medium (Table 1). Assure that the basal ends of the micro-cuttings are always immersed in the medium. Incubate under light (16/8 h photoperiod) and transfer explants as necessary every 2 months into fresh RD medium. Roots emerge and grow 2-3 cm within 30 - 40 days after root initiation. Some plants fully develop within the two-month period and can be transferred to the greenhouse. For acclimation of these plants follow the protocol described above (Sec. 4). Plants produced via orthotropic micro cuttings have a tendency to jorquette prematurely when established in the greenhouse. If this is undesirable the jorquette height can be corrected by removing/decapitation of the apical, jorquette bud/s as soon as it appears. Following removal, an axillary orthotropic shoot from below the cut will be released and will grow to achieve a standard height (1 – 1.5 m) prior to the appearance of the next jorquette (Bertrand and Agbodjan, 1989).

Stock plant maintenance for continuous micro cutting production

Prepare fresh RD medium and pour 140-150 ml into Magenta GA3 vessels or glass jars. Before transferring decapitated stock plantlets, gently break the surface of the medium with sterile forceps. Pick up the decapitated plantlets by the stems with sterile forceps and slowly pull out of the old medium. Place on the surface of the fresh RD medium. Hold the roots close to the tip with second pair of sterile forceps and insert into the fresh medium until the roots are entirely immersed into the medium. Maintain cultures under light (16/8h photoperiod) at 25 -30°C for 14 days. New axillary shoots develop and are ready for harvest in 6-8 weeks after previous harvest. When the shoots grow to approximately 3 cm in height, harvest the cuttings and proceed using the above-described protocol for rooting. It is important to harvest the new shoots regularly to prevent the re-establishment of apical dominance and to promote axillary bud proliferation. It is not unusual that after decapitation one or more newly developed shoots grow faster and re-establish themselves as dominant shoots.

6. PRODUCTION AND ROOTING OF ORTHOTROPIC CUTTINGS UNDER GREENHOUSE CONDITIONS

Once orthotrpic plants have been acclimated and are growing well in the greenhouse,

a further amplification step can be applied using single node stem segments to produce rooted cuttings. Plantlets produced with this method grow similar to seedlings, with a orthotropic growth phase followed by jorquetting and transition to plagiotropic adult growth phase. This step can be performed at low cost, greatly reducing the overall expense of an integrated orthotropic propagation system starting from somatic embryos. These plants can be used as self-rooted stock, for budwood or as root stock as required.

Stock plant establishment

Juvenile somatic embryo-derived plants at sapling stage (at or near jorquette height) should be used as "bentwood" stock plants for generation of orthotropic cuttings for rooting. Prior to bending the stock trees, prune the jorquette branches down to only 1-2 cm of their length. This promotes further the release of dormant axillary orthotropic meristems leading to greater orthotropic shoot production. With the pots remaining upright, arch the plants over so that the main trunk is at a horizontal position and secure the shoots with a metal hook or rope. The stock plants can be planted and established directly in the ground with the same success. Many orthotropic shoots should begin to proliferate in 1 – 2 weeks along the topside of the trunk length near the basal, curving end. Two to three months after bending, these orthotropic shoots should achieve 0.7 – 1.0 m in height and be ready for harvest.

Alternatively stock plants for orthotropic shoots can be established without bending the stems. For this method newly acclimatized SE-derived plants are grown in the greenhouse in D40 Deepots (plastic, 62.5mm x 250mm; Hummert, Earth City, MO, USA) for approximately 6 months until they are 3-4 feet tall, but without jorquette. At that time excise the apical shoot leaving short stump with only 4-6 leaves. The excised shoot can be segmented into 4 cm single leaf cuttings for rooting as described above. New axillary shoots are released from the remaining stock plant and cuttings are produced and rooted following the above-described protocol. The advantage of this method compared to the "bentwood" garden is that the area for stock plant maintenance is reduced. However, since the stock plants initially have fewer axillary buds, our observations are that they produce fewer shoots (data not published).

Rooted cutting production

Excise the shoots at the base of the stem, close to the trunk of the stock plant. After the first and the consequent harvests new shoots are generated at or near the cut. Hence the stock plants can be harvested repeatedly. Excise hardened, semi-hardened and dark green orthotropic shoots early in the morning while leaf water potential is still high (Fig. 2). Cuttings from shoots, at all developmental stages root well, but the semi-hardwood cuttings showing beginnings of characteristic browning of the stem have demonstrated the highest rooting potential (Guiltinan et al., 2000). Prune all leaves to 1/3 of the length. Make single leaf cuttings with stems approximately 4 cm long. Stems should be cut such that 0.5 – 1 cm remains above the node/leaf axils and 2 – 3 cm of the stem remains below the node. If the internodes are shorter than 2-3 cm, still make the cuttings 4 cm long, but remove the lower leaves completely. Dip the base of the stems in rooting solution for 5 seconds (Fig. 2). The quick dip method as described by Evans has been quite successful for the propagation of single-leaf cuttings under our conditions (Evans, 1951). Rooting solution consists of a 1:1 mixture of α-Naphthalene acetic acid (NAA) and α - Indole-3-butyric acid (IBA) at a total combined concentration of 4 g/L. The hormones are dissolved in 50% ethanol and the solution should be prepared fresh prior to treatment. After hormone treatment, insert the cutting in to a 0.5 cm wide and 3 cm deep opening in wet sand or rooting media of choice (Fig. 2). Gently press the sand around the stem of the cutting. Any well draining growth media could be used for this rooting method, however fine play sand or a 1:1 mix of sand and soil has demonstrated excellent results in greenhouse conditions. Place the new cuttings under intermittent mist (10 sec. every 10 or 15 min.) or a fog for 4-6 weeks. Relative

humidity should be maintained at or near 100%, keeping the leaves wet without inundating the cuttings. Light intensity should remain approximately 100 μmol * m^{-2} * sec. $^{-1}$ PAR, or 85% shade. During the misting period water the cuttings with fertilizer every 3-4 days. A Hoaglund's fertilizer at 150ppm nitrogen works well. The first roots will appear at approximately 3 weeks after hormone treatment. By the end of the 6-week misting period all cuttings with rooting potential should form roots. When using play sand as a rooting media and applying the described conditions we routinely produce rooted cuttings with 90-95% success (Carter Miller, data not published).

Acclimation, establishment, and general cultural management for plants produced by rooted cuttings methods

Any rooted cutting with 2 or more roots and a growing axillary bud may be transplanted to plastic bags or pots with soil (Fig. 2). After transplanting, the plants are removed from the mist and should remain under 50% shade, and RH of 60-65%. Water and fertilizer are applied as needed. Best results are observed when drip irrigation system is used and the plants are fertilized 5 or more times daily. It is important when watering not to damage the new tender shoots. After the first flush has hardened the plants can be transferred to the greenhouse, shade house or field with increased light and lower RH conditions. If cuttings jorquette prematurely, the jorquette bud may be removed as soon as it appears similar to method applied for plants produced via micropropagation.

7. CONCLUDING REMARKS

Since the early work of cacao researchers in the late 1800s, propagation systems have been an important tool, enabling the multiplication of wild or breeding genotypes for distribution, germplasm collection and for replicate performance trials. Rooted cutting and grafting have been used throughout the world for propagation of cacao, however to date, a large percentage of production stocks are still grown from seed. In the 50s and 60s plant tissue culture methods were developed for the propagation of a wide variety of species, but were not applied to cacao until the late 70s, and then, with very limited success.

The protocol presented here is the current optimization of the various experiments we and others have performed on cacao somatic embryogenesis (see introduction). In addition, we also present a number of related propagation systems (Guiltinan et al., 2000). Although the somatic embryogenesis technology has been successfully utilized in multiple research laboratories around the world these methods should be regarded as a work in progress. To date, embryos have been produced from more than 100 different *Theobroma cacao* genotypes at multiple locations including laboratories in Ghana, Ivory Coast, Brazil, Malaysia, Puerto Rico and others.

However, the initial protocol was developed and optimized for Scavina-6 genotype only (Li et al, 1998, Maximova et al., 2002). We have observed that there are genotypic variations observed in the response to the protocol. In most cases genotypes with Scavina-6 parentage respond very well and produce a large number of embryos. Other genotypes produce very few embryos during primary embryogenesis, but the number dramatically increases during secondary embryogenesis. Finally, there are genotypes that need further optimization of the conditions to produce embryos. While these methods are potentially very powerful, we have estimated that by using them it is

possible to produce 800,000 plants from a single flower in two years, it should be noted that this is new technology that has yet to be fully tested in the field. Although our early field tests in Saint Lucia, Puerto Rico and Brazil are promising, the use of plants for large scale propagation and production must clearly await full validation of the methods through large scale field testing over multiple harvests and in several environments and with multiple genotypes. Our group and others are beginning to establish such tests. We invite our partners, the readers of this manuscript, to work together to set up additional field tests to help validate this system.

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Figure Legends

Figure 1. Production of cacao somatic embryos. A, Immature flowers prior to collection. B, Immature flowers in sterilization and shipping containers. C, Stages of somatic embryogenesis, top left, unproductive white callus, top right, dark callus with developing somatic embryo, middle left, globular somatic embryo, middle right, torpedo somatic embryo, bottom, mature cacao somatic embryo. D, Somatic embryo-derived plant in the field. E, Somatic embryo plantlet after conversion ready for acclimation in greenhouse. F-H, Fruit and seeds from somatic embryo derived plants grown in field conditions.

Figure 2. Production of single node rooted cutting from somatic embryo derived cacao plantlets. A, Selection of semi-hardwood stem sections (left) discard still fully green segments (right). B, Single node cuttings from stem sections 6-10 mm diameter. C, Cutting of leaves to 1/3 of original size. D, Single node segments with trimmed leaves. E, Dipping of stem segment in liquid root induction hormone solution. F, Placing a cutting in sand rooting media. G, Misting of cuttings in greenhouse. H, Finished rooted cutting ready for planting. I, Bed of rooted cuttings in containers in shade house prior to field planting.

Table 1. Composition of media used for cacao somatic embryogenesis.

Medium components	PCG mg/L	SCG mg/L	ED mg/L	PEC mg/L	RI mg/L	RD mg/L	Hoagland's Fertilizer mg/L
NH_4NO_3	1416	400	1416	1416	708	708	-
$Ca(NO_3)_2.4H_2O$	1969	-	1969	1969	984.5	984.5	-
$CaCl_2.2H_2O$	149	-	149	149	74.5	74.5	-
$CaCl_2$	-	72.5	-	-	-	-	-
$Ca(NO_3)_2$	-	386	-	-	-	-	47
K_2SO_4	1559	990	1559	1559	779.5	779.5	-
$MgSO_4.7H_2O$	740	-	740	740	370	370	12
$MgSO_4$	-	180.7	-	-	-	-	-
KH_2PO_4	265	170	265	265	132.5	132.5	-
$(NH_4)_2SO_4$	-	-	-	-	-	-	1.65
$NH_4N_2PO_4$	-	-	-	-	-	-	8.6
$Zn(NO_3)_2.6H_2O$	17	-	17	17	8.5	8.5	-
$MnSO_4.H_2O$	33.4	22.3	33.4	33.4	16.7	16.7	0.03
$CuSO_4.5H_2O$	0.25	0.25	0.25	0.25	0.125	0.125	$1.25X10^{-2}$
H_3BO_3	4.8	6.2	4.8	4.8	2.4	2.4	0.15
$Na_2MoO_4.2H_2O$	0.39	0.25	0.39	0.39	0.195	0.195	-
$FeSO_4.7H_2O$	33.8	27.8	33.8	33.8	16.9	16.9	-
Na_2-EDTA	45.4	37.3	45.4	45.4	22.7	22.7	-
KCL	-	-	-	-	-	-	0.37
$ZnSO_4.7H_2O$	-	8.6	-	-	-	-	0.06
$(NH_4)_6MO_7O_{24}$	-	-	-	-	-	-	0.06
Fe-EDTA	-	-	-	-	-	-	1.84
myo-Inositol	200	100	100	100	-	-	-
Glutamine	250	-	-	-	-	-	-
Thiamine-HCL	2	10	2	2	-	-	-
Nicotinic acid	1	1	1	1	-	-	-
Glycine	2	-	2	2.187	-	-	-
Pyridoxine	-	1	-	-	-	-	-
Arginine	-	-	-	0.435	-	-	-
Leucine	-	-	-	0.328	-	-	-
Lysine	-	-	-	0.456	-	-	-
Tryptophane	-	-	-	0.511	-	-	-
KNO_3	-	-	-	300	300	300	30
Glucose	20,000	20,000	1000	20,000	10,000	10,000	-
Sucrose	-	-	30,000	10,000	5000	5000	-
2,4-Dichloro-phenoxyacetic Acid	9.05uM	9.05uM	-	-	-	-	-
6-Benzylamino-purine	-	.22uM	-	-	-	-	-
Indole-3-Butyric Acid	-	-	-	-	12.4uM	-	-
Thidiazuron	.023uM	-	-	-	-	-	-
Phytagel	2000	2200	2000	1750	1750	1750	-

All chemicals are purchased from Sigma Chemical Co., St. Louis, Mo. Abbreviations: PCG – Primary Callus Growth; SCG – Secondary Callus Growth; ED – Embryo Development; PEC – Primary Embryo Conversion; SEC – Secondary Embryo Conversion; RI – Root Induction; RD – Root Development.

Figure 1

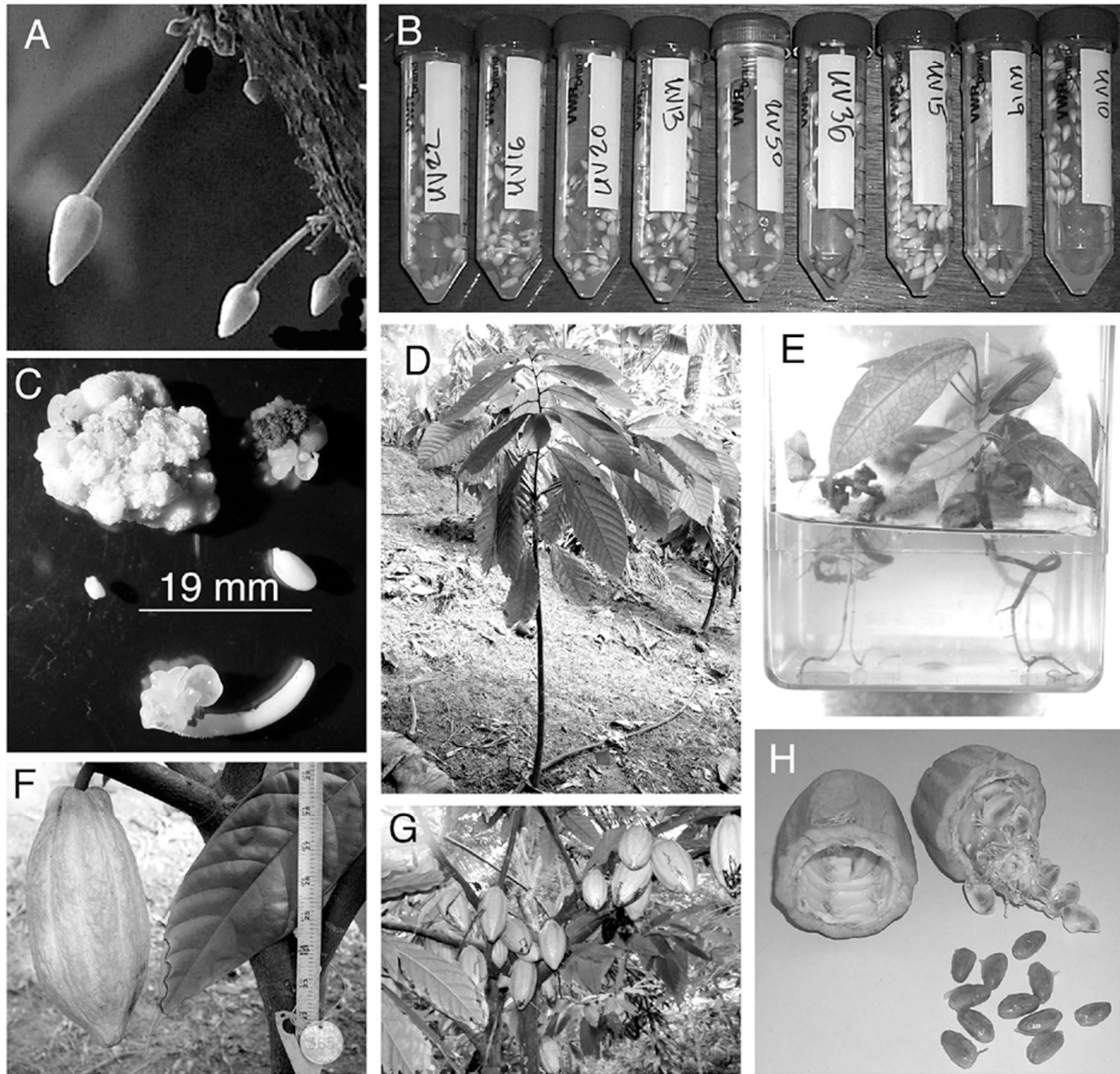

Figure 2