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Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L.

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The first objective of this study was to assess and optimize somatic embryo production in a genetically diverse range of cacao genotypes. The primary and secondary somatic embryogenesis response of eight promising cacao clones and a positive control was evaluated using modified versions of standard protocols. The second objective was to optimize the efficiency of primary somatic embryogenesis for a commercially important cacao clone, CCN 51, which has proven to be quite recalcitrant to standard protocols, relative to CCN 10, a clone also included in our analysis. The efficiency of the overall process was assessed by determining the number of somatic embryos produced per starting somatic tissue explant, as well as the quality of embryos (normal vs. abnormal) produced. Donor floral explants were subjected to five tissue culture steps, each 15-25 days in duration. Although all studied genotypes produced primary somatic embryos, most of them originated only from brown or brown-white callus. Overall, flower petals performed better than staminodes, and our best performing genotype yielded an average of 7-10 embryos produced in brown callus explants with embryogenic response during primary somatic embryogenesis procedures. In conclusions our analysis from a pilot in a small-scale are: 1) it is possible to achieve a high production of plants by somatic embryogenesis, although the efficiency is highly genotype-dependent; it is therefore necessary to optimize hormone balance and hormone type, as well as the explant type for each genotype, 2) through the use of secondary somatic embryogenesis, it is possible to increase somatic embryogenesis production at least ten-fold, and 3) the observed response variation between genotypes may reflect differences in endogenous and exogenously-supplied hormones. The importance of adapting the tissue culture protocol to the genotype is discussed.

Key words: Somatic embryogenesis, 2,4-D, cellular competence, propagation, abnormalities, ethylene, cacao.

INTRODUCTION

Theobroma cacao L. is a tropical understory tree originating from the Amazon and Orinoco valleys (Wood and Lass, 2008; Motamayor et al., 2008). Since the Mayas domesticated the cocoa tree, cacao has become a major commodity crop cultivated in numerous tropical countries, where it represents a significant source of

income for small farmers (Franzen and Borgerhoff, 2007). Until the 1980s, Brazil had the highest cocoa bean production in South America and it was the second highest producer worldwide, with an average production of 400,000 tons per year (FAO, 2013). By the late 1980s, annual Brazilian production decreased to 291,868 tons (IBGE, 2015), primarily due to outbreaks of devastating cacao diseases, in particular witches' broom (*Moniliophthora perniciosa*), which caused massive economic loss and much social distress (Marelli, 2008). In response to this crisis, numerous government agencies and universities were enlisted to develop disease-resistant, high-yielding cacao clones (CEPLAC, 2009).

Once superior clones are developed, large-scale propagation will be required. Cacao is traditionally propagated using rooted cuttings or by grafting. The use of rooted cuttings is constrained by biology: a cacao tree has two types of branches: orthotropic (vertical growing) ones that generate trees of desirable architecture, but which are limited in number; the second type are plagiotropic (horizontal growing) ones. Although, more numerous, plagiotropic grafts produce trees of undesirable architecture that require long time and labor investments (Miller, 2009).

The solution for cacao, like other commercially important trees, is somatic embryogenesis (SE), which is a method for generating plant embryos asexually. Somatic embryogenesis relies on the ability of somatic plant cells to de-differentiate and be reprogrammed along an embryonic developmental pathway (reviewed by Fehér, 2015). Under specific tissue culture conditions, a single somatic cell (or groups of cells) can be converted into a single embryo that develops into a plant that is genetically identical to the donor plant from which the original cell was derived (Konieczny et al., 2012).

Importantly, SE affords opportunity for amplification, from a relatively small number of explants into hundreds or thousands of somatic embryos (SEs) per experiment. Although a half-century has passed since SE was first demonstrated in the model system *Daucus carota* (wild carrot) (Steward, 1958), the molecular mechanisms underlying the complex cellular reprogramming required to achieve totipotency are just beginning to be elucidated (Fehér, 2015; Mahdavi-Darvari et al., 2015). Luckily, early identification of critical parameters controlling SE induction, such as endogenous and/or exogenously supplied plant growth regulators (PGRs, for example, 2,4-dichlorophenoxyacetic acid (2,4-D)) have enabled this largely empirical science to evolve into the foundation of large-scale vegetative propagation for a large range of plant species.

Somatic embryogenesis induction and expression are only possible if totipotent somatic plant cells can acquire the competence necessary to respond to embryogenic signals and initiate the embryogenesis process (Fehér, 2015); despite the broad success of SE methodology across the plant kingdom, within a single species, a high degree genotype-to-genotype variation in response has been observed, thereby necessitating protocol customization. Therefore, to be commercially viable in cacao, or any other species, somatic embryogenesis requires: 1) donor material with a high rate of competent cells to express the embryogenic pattern, 2) donor material relatively unlimited in supply and physically accessible, 3) genetic and epigenetic uniformity in the SEs generated, and 4) efficient conversion of SEs into plantlets.

In early cacao studies (for example, Sondahl et al., 1989, 1993), SEs were generated from a variety of somatic cell type explants, although yields were low. The first moderately successful cacao SE protocol was developed by Li et al. (1998); their exhaustive study highlights the importance of genotypic variation in SE response. Staminodes from 19 cacao genotypes were evaluated for their ability to undergo somatic embryo-genesis; the diverse genotypes produced primary somatic embryos (PSEs) at very different rates. Explant response (callus growth) ranged from 1-100%, and the number of SEs per responsive explant ranged from 1-46. This basic cacao SE protocol was further optimized by Maximova et al. (2002), who developed a key method for secondary somatic embryogenesis (SSE) using cotyledon explants from PSEs.

In this study, eight diverse genotypes were tested, and from 4.8 to 24.7, secondary somatic embryos (SSEs) were generated per explant, within a 12-month SSE protocol. Yet another SE protocol (using flower petals) was developed by Lopez-Báez et al. (2001), using Murashige and Skoog 1962 (MS) salt-based induction medium that included testing of carbohydrate (sucrose, glucose and maltose), PGRs (2,4-D or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and kinetin), in 12 diverse genotypes. Again, high genotype response variance was noted. Although the collective impression from cacao SE research indicates that an ideal, genotype-independent protocol has not yet been developed, several key efficiency determinants have been identified, including the concentration and type of sugar used in culture medium (Solano-Sanchez, 2008; Chanatásing-Vaca, 2004; Tan and Furtek, 2003). To enhance the efficiency of primary somatic embryogenesis (PSE) production and plant regeneration, we attempted to further optimize cacao SE protocols.

The overall objectives of the present study were therefore: 1) to evaluate a pilot scale the production

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efficiencies (number of embryos/explant) in both PSE and SSE procedures for eight commercial cacao clones and SCA 6 as a positive control, using a standard protocol with some modifications, and 2) we also attempted to improve the yield and quality (Increase the number of normal embryos/explant) of PSE generated from two cacao clones, Coleccion Castro Naranjal-51 (CCN 51) and Coleccion Castro Naranjal-10 (CCN 10), which are of commercial interest to Brazil. In these clones, we will use two types of somatic donor explants (petals and staminodes), and variation in glucose concentration and hormone balance.

MATERIALS AND METHODS

This study is divided into two parts. First is an evaluation of pilot scale production using a single protocol with some modifications in eight genetically diverse *T. cacao* clones (MCCS 14-056, POUND 7, TSH 1188, TSH 565, VB 1151, CCN 51, UF 613 and PS 1319) as well as Scavina 6 (SCA 6) as a control. SCA 6 is a clone that has historically performed well in a variety of somatic embryogenesis procedures (Solano-Sanchez, 2008; Chanatásing-Vaca, 2004; Maximova et al., 2002; Li et al., 1998). The second is an experiment aimed at improving a specific protocol for PSE in CCN 51 and CCN 10 clones. All the clones used in this work are from the Mars Center for Cocoa Science (MCCS) germplasm collection.

Evaluation of a pilot scale production of plantlets from nine commercial cacao clones by somatic embryogenesis

Primary somatic embryogenesis procedures

Immature cacao flowers (approximately 900 in total), 6-8 mm in length, from MCCS 14-056, POUND 7, TSH 1188, TSH 565, VB 1151, SCA 6, CCN 51, UF 613 and PS 1319 were collected in the morning in the field and kept in cold sterile distilled water (4°C), and transported to the laboratory, where they were submerged in a fungicide solution (per liter: 9 g Ridomil Gold Mz (Syngenta), 10 mL of carbendazim solution (0.1% in MeOH)) for 20 min. After rinsing three times with sterile water, samples were then surface-sterilized with 1% calcium hypochlorite for 25 min in a laminar flow hood, and again rinsed three times with sterile water. After sterilization, petals and staminodes were dissected. A total of 50 explants per Petri dish were cultured using the protocol for PSE published by Li et al. in 1998 (standard), with some modifications.

The PSE modified protocol follows four steps, while the standard protocol follows three steps. In the modified protocol, primary and secondary callus growth steps were done in the same way as in the original protocol. Primary Callus Growth (PGC) medium, which is comprised of 1X Driver and Kuniyuki Walnut Basal Salt (DKW) plus vitamins (Driver and Kuniyuki, 1984) supplemented with 2 mg L^{-1} 2,4-D and 5 µg L^{-1} thidiazuron (TDZ), 250 mg L^{-1} L-glutamine, 200 mg L^{-1} myo-inositol, and 20 g L^{-1} glucose. The DKW vitamin stock solution yielded final concentrations of 1 mg L^{-1} nicotinic acid and 2 mg L^{-1} glycine, 0.1 g L^{-1} myo-inositol, 2 mg L^{-1} thiamine–HCl. The pH of the solution was adjusted to 5.8 and supplemented with 2 g L^{-1} phytagel prior to autoclaving. After two weeks of culture at 27±2 °C in the dark, explants were transferred to Petri dishes containing secondary callus growth medium (SCG), comprised of 1X woody plant medium (WPM) salts (Lloyd and McCown, 1980) and 1X Gamborg's vitamin solution (Gamborg, 1966), 2 mg L^{-1} 2,4-D and 50 µg L^{-1} 6-benzylaminopurine (BAP), 20 g L^{-1} glucose, plus 2 g L^{-1} phytagel, pH 5.8. After a further two weeks of culture, calli were transferred to embryos development-4 medium (ED4) for another two weeks and finally to embryos development-3 medium (ED3) for eight weeks. ED4 and ED3 media are comprised of 1X DKW salts and vitamins, supplemented with 40 g L^{-1} or 30 g L^{-1} sucrose, respectively, 2 g L^{-1} phytagel and pH 5.8. ED3 and ED4 cultures were 8-10 weeks maintained in darkness at 27±2°C until embryos were about 1 cm in in length but they are not reached their maturity. Embryo Development medium both ED4 and ED3 are a modification from ED medium used in Li et al. (1998) protocol. The modifications are in the carbohydrate source. In our protocol, we used 40 g L^{-1} and 30 g L^{-1} of sucrose in ED4 and ED3 medium, respectively, instead of 1 g L^{-1} of glucose and 30 g L^{-1} of sucrose as it is used in ED medium in the original one. The other components in the medium are the same that are used in the original protocol. Data were collected every 14 days between each transfer during the ED3 culture steps. At the end of the procedure (two to three months of ED3 culturing) the total and average numbers of embryos produced per explant were calculated for the statistical analysis. The PSEs that were obtained in this step we used as a source of explants for SSEs production in a pilot scale.

Secondary somatic embryogenesis procedures

Secondary somatic embryogenesis procedures were initiated using the original protocol stablish by Maximova et al. (2002) with some modifications. Basically, the Maximova´s protocol follows the same steps as Li et al. (1998) protocol with the difference that there is not primary callus growth step, and the initial explants are cotyledons from PSEs instead of flowers parts (petal or staminodes). The embryos development step is the same for both protocols mentioned before. In our protocol the modifications are only in embryos development step where we have used ED4 and ED3 instead of ED medium. Cotyledons from PSEs with ~1 cm in length and light yellow in color were used as donor explants.

Approximately 1,080 PSEs were source of explant to the aim to produce 10,000 SSEs in this pilot scale production (roughly 120 PSEs per genotype). The cultures for pilot scale production were done in three batches per year (40 PSEs per each genotype were used to start the each batch of production). They were cut with a scalpel into small pieces (~30 pieces/cotyledon; each square piece measured approximately 1 mm^2 on a side), and introduced into SCG for two weeks to induce callus formation (two cotyledons per Petri dish); during this step two different cytokinins were tested: kinetin (300 µg L^{-1}) or BAP (50 µg L^{-1}). Half of the initial explants were treated with kinetin and the other half with BAP. Callus formed in SCG were transferred to ED4 medium for two weeks and then to ED3 medium for more 12 weeks, for embryo development step until SSEs formed and matured (as the same way in the PSE procedure mentioned before). It typically required 12-14 weeks of dark incubation at 27 ± 2°C for SEs emerge and reach their maturation. SEs was deemed mature when their leaves developed pink and purple pigmentation, their stems were white and a radicle has started to show up. These features reflect the ability of the embryos to store substances required for subsequent adaptation and germination.

Efficiency of embryogenesis calculation

Our objective was to determine the efficiency of embryogenesis (*EE)* values for nine cacao genotypes in both PSE and SSE protocols in pilot scale. Genotypes were compared in their relative responsiveness to SE procedures by determining the ratio of the number of SEs produced at the end of an experiment to the number of initial explants in each experiment (Maximova et al., 2002). This ratio, termed the *EE* or efficiency index, is a measure of the net efficiency of a given clone to the numerous, sequential culture steps and conditions.

For PSE procedures, 50 explants per Petri dishes were used in *EE* value calculations from a total of 1000 explants (500 petals and 500 staminodes) per each genotype (20 Petri dishes as experimental units) and the *EE* is calculated from the ratio: number of PSEs produced/number of donor explants. Whereas for SSE procedures, the SSE *EE* is calculated from: the number of SSEs produced/number of PSEs used as source of donor explants. Approximately, 120 Petri dishes were used in SSE *EE* value calculation from a total 120 PSEs as explants (two cotyledons per Petri dish) per each genotype (1,080 Petri dishes as experimental units). Data from PSE and SSE for these experiments were collected every two weeks for up to 28 weeks after culture initiation from petals or staminodes. At the end of primary and secondary embryo production (seven to eight months of culture), the total and average numbers of embryos produced per explant in PSE and SSE was calculated for statistical analyses.

Conversion and regeneration of plants

After incubation for about 12-14 weeks in ED3 medium, SSEs showed morphological characteristics of mature embryos, and were transferred to embryo development in light (EDL) medium for 12 weeks at 27±2°C under a 16-h light/8-h darkness photoperiod regimen, in which the photosynthetically active radiation (PAR) used ranged from 50 – 190 umol m^{-2} s^{-1} during the light phase. EDL medium is the same Primary Embryo Conversion (PEC) medium from cacao tissue culture protocol book (Young et al., 2003) that we adapted to our protocol. EDL medium is comprised of 1 X DKW salts plus 1 X vitamins supplemented with 20 g L^{-1} glucose, 0.3 g L^{-1} KNO_3, 45.65 mg L^{-1} L-lysine, 32.80 mg L^{-1} L-leucine, 51.05 mg L^{-1} L-tryptophan, 43.55 mg L^{-1} L-arginine, 18.76 mg L^{-1} glycine, 2 g L^{-1}phytagel, at pH 5.8. The modification in this medium was only in the carbon source, where we have used 20 g L^{-1} glucose unlike the PEC that use 20 g L^{-1} glucose and 10 g L^{-1} sucrose. Embryos were maintained in EDL medium until they developed into plantlets with 2-3 leaves, after which they were transferred to a modified version of EDL medium: half-strength of DKW basal salts, 1 X DKW vitamins, 0.3 g L^{-1} KNO_3, 3.0 mg L^{-1} indole-3-butyric acid (IBA) and 1.8 g L^{-1} phytagel at pH 5.8 with the aim to develop roots. Data were collected every three to four months until the plantlets had 4-5 sets of permanent leaves and a well-developed root system. Plantlets from SSE take seven to eight months to get ready for acclimatization since cotyledons from PSSEs are introduced in SSE process.

Plant acclimatization

Plant acclimatization was achieved by incubating individual plantlets in plastic cone tubes (290 cm^3 in volume) covered with polyethylene bags in a greenhouse with environmental conditions of 70% of shading in the first two months, then 50% of shading for the remainder of the process, a temperature range of 29 ± 5°C in the day and 19 ± 5°C at night. Lighting was provided for 12 h per day. Data were collected every three months until the plantlets became acclimatized. Three-month-old plantlets with 4-6 leaves were washed with sterile distilled water to remove residual phytagel; their roots were then submerged in a 0.2 mg L^{-1} solution of Derosal fungicide followed by a two-minute immersion in IBA (6 g L^{-1}). Plants were then transferred to pots containing substrate comprised of: 1 part coconut fiber, 1 part Holambra (organic substrate) and ¼ part of perlite. During acclimatization, plants were watered every 30 days with 1X modified Hoagland solution (Hoagland and Arnon, 1950). The solution was modified in the micro-nutrients stock salt solution, where Manganese (II) Sulfate Monohydrate ($MnSO_4.H_2O$), zinc sulfate Heptahydrate ($ZnSO_4.7H_2O$) and sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) concentration were increased (from 1.690, 2.875 and 0.404 g L^{-1} to 6.760, 5.750 and 0.605 g L^{-1}, respectively). The plants were completely acclimatized with three months in the greenhouse.

Primary somatic embryogenesis protocol optimization in two commercial cacao genotypes

The PSE protocol developed by Li et al. (1998) used in the first section of this work was adjusted. The objective of this experiment was to develop an improved protocol for PSE in CCN 51 and CCN 10 clones by optimizing hormone balance and glucose concentrations in PCG medium. The experiments comprised a factorial design with five independent variables as follows: two explants (petals and staminodes) X two clones (CCN 51 and CCN 10) X five 2,4-D concentrations (0.25 - 2 mg L^{-1}) X five TDZ concentrations (0.003 - 0.009 mg L^{-1}) X five glucose concentrations (20 - 80 g L^{-1}). A total of 500 treatments with three replicates per treatment were done. Each replicate was comprised of 25 explants (25 petals or staminodes per Petri dish), and the experiment was repeated two times. Data were collected every 14 days (at the ED3 transfer steps). At the end of the embryos production (three to four months of culture in ED3), the total and average of embryos (normal and abnormal) produced per explant was calculated for the statistical analysis. The procedures and environmental conditions in this section are the same that were used in PSE procedures in the first section. The rest of PCG medium components were the same as the original protocol.

Statistical analysis

In the first part of this work, the differences among the means of the total number of PSEs and SSEs produced per explant were analyzed for nine genotypes. We compared the means for the number of total PSEs produced per embryogenic explant (only calli with embryos formation) from staminodes versus petals, and total SSEs produced per PSEs as initial explant as a function of cytokinin type. The *EE* was calculated from the mean of the counting the number of PSEs produced per initial explant (per Petri dish as an experimental unit), and the mean of the total SSEs produced per PSEs as initial explant (using one PSE (cotyledons) per Petri dish as an experimental unit) from three batches of production. *EE* (as an efficiency index) is a decimal value where the value embryos number with relation of total explants used for all the experiment with either calli formation or not. Data were analyzed using descriptive statistics, calculating means and variances. The interval of confidence was calculated with respect to the mean with 5% of error.

In the second part, data were processed using deviance analysis for completely randomized design with five independent variables. The dependent variables observed were the numbers of normal and abnormal embryos counting in a fixed interval of time. A generalized Poisson model was adapted to link function to estimate the number of normal embryos produced in function at the best treatment in combinations of: hormone balances, carbohydrate concentration and the types of explants used for each genotype. Both, first and second section analyses were performed with the aid of R 3.2.5 software.

RESULTS

Evaluation of a pilot scale production of plantlets from nine commercial cacao clones by somatic embryogenesis

After eight weeks in culture, both petal and staminodes

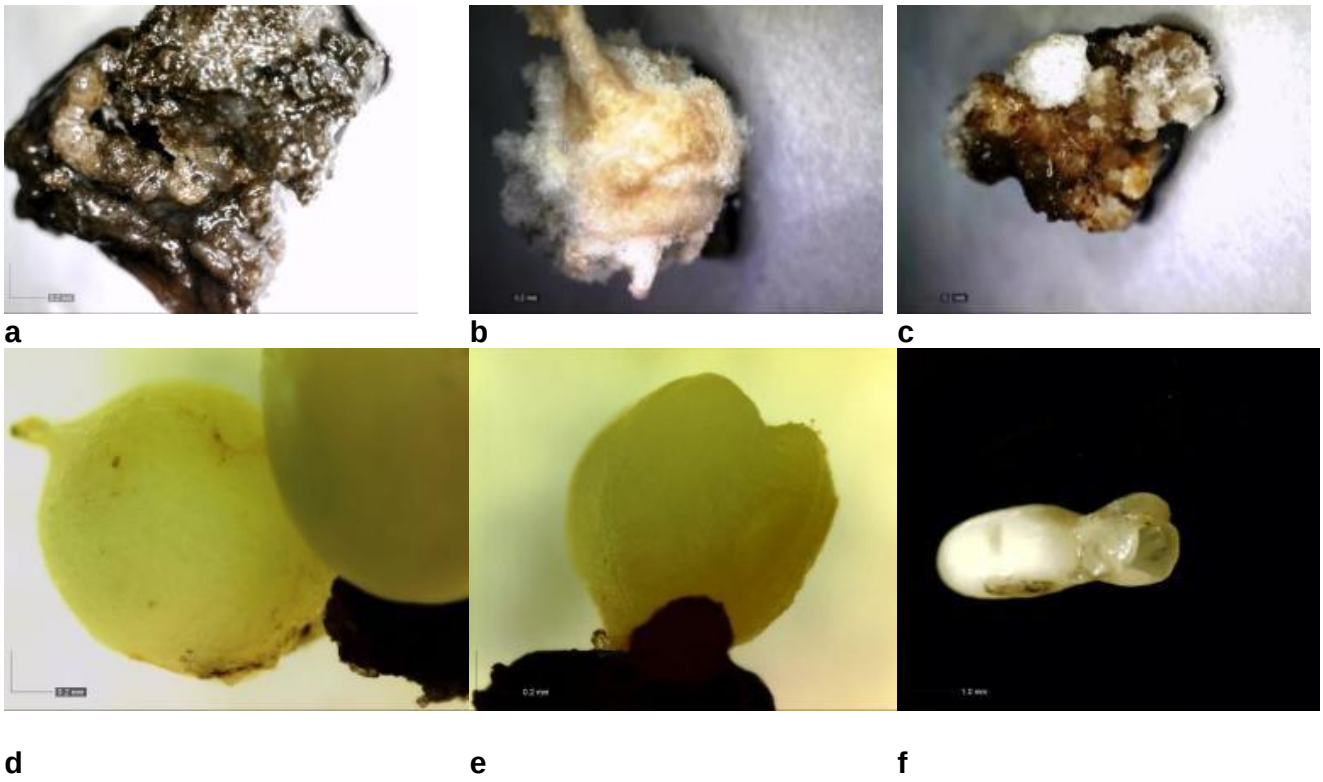


Figure 1. Three callus types in *T. cacao* somatic embryogenesis (a) Brown callus, (b) White callus, (c) Mixed Brown/White callus. SSE stage: globular (d) heart (e) and torpedo (f).

explants formed callus in most genotypes. During the ED3 culture steps, explants developed into three distinct types of callus: brown, white and mixed (brown and white). Over time, brown callus becomes coated with a dark brown material (oxidized phenolic compounds) that covers the entire explant surface (Figure 1a); whereas white callus has a compact, uniform dry appearance (Figure 1b); mixed callus has both brown and white areas (Figure 1c).

Each callus type (Figure 1a to c) was subsequently evaluated for embryogenic response. Once callus had formed, PSEs began to appear, and developed through three distinct developmental stages: globular, heart, and torpedo (Figure 1d-f). All genotypes produced PSEs, the highest number of which developed from brown or brown-white callus, for both petal bases and staminodes. Petals had the best overall performance compared with staminodes; the average of number of callus exhibiting and embryogenic response ranging from 20-70% (data not shown). MCCS-14-056 had a better response in petal bases, with an average of 7-10 embryos produced per brown callus with embryogenic response. For staminodes, Pound 7 was the most productive, with an average of six embryos produced per each brown-white callus with embryogenic response. As expected from published studies (Maximova et al., 2002), the positive control SCA 6 gave good results in petals, producing six embryos per each brown-white callus with embryogenic response. It should be noted that all embryos produced from brown-white callus originated from the brown portion of the mixed callus. The overall results are shown in Figure 2; values are given as mean ± interval of confidence with 5% of error.

Secondary somatic embryogenesis response

To evaluate the production of embryos in the SSE procedure, we assessed callus formation and SE production using PSE-derived explants. Specifically, we first counted the three different callus types in secondary embryogenesis under culture conditions that used two different cytokinins (BAP and kinetin). BAP induced a higher overall response in callus formation for most (77 %) clones (data not shown). Only TSH 1188 and UF 613 showed a (slightly) higher response with kinetin compared to BAP (data not shown). For SSE, we calculate production of embryos per responsible explant (brown or brown-white calli). All clones had a high proportion of brown calli, irrespective of the cytokinin used. For clones TSH 1188 and TSH 565, however, only small amounts of white and mixed callus formed. Analysis of SSE production as a function of callus type and cytokinin revealed that large numbers of embryos

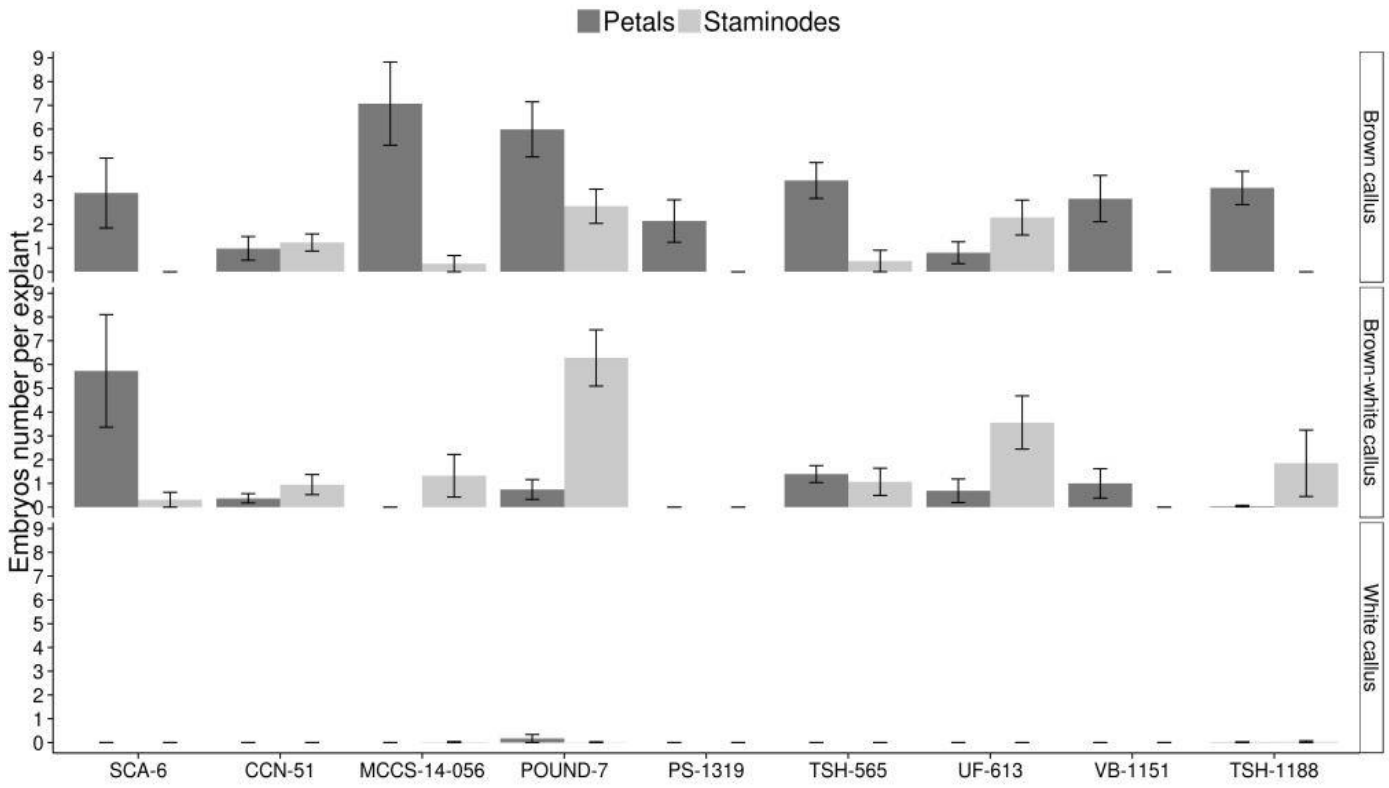


Figure 2. Average number of PSEs produced by each callus type and explant type that had response to the treatment (petals and staminodes) for the nine studied genotypes. Values are given as mean ± interval of confidence with 5% of error.

can be produced on brown callus, in all genotypes. TSH 1188 and UF 613 showed better response with kinetin; whereas SCA-6 had the highest embryo production in BAP-containing medium (Figure 3).

Embryo efficiency in primary and secondary somatic embryogenesis

As shown in Figures 4 and 5, SSE production was higher than PSE production for all clones studied. An average of almost 10-fold enhancement of SE production was achieved using SSE compared with PSE procedures (Figure 5a, b); thus, each PSE with two cotyledons cut into pieces has the capacity to produce between 5-58 SSEs (Figure 4b). In contrast, petals or staminodes can produce 1-5 embryos per embryogenic explant (Figure 4a). TSH 1188 and UF 613 generated the largest numbers of embryos, with *EE* values of 12 and 58, respectively in SCG medium supplemented with kinetin (Figure 4b). The remaining clones responded better to BAP, with an average *EE* of 19 embryos per PSEs (Figure 4b). Secondary embryos with good maturation/ morphology were able to convert efficiently into plantlets (Figure 5c, d) that developed 4-6 leaves prior to acclimatization.

Plant acclimatization

A total of 4,912 in vitro-generated plants were greenhouse-acclimatized using protocols developed at MCCS, from 9,000 SSE-derived plantlets. Clones MCCS 14-056 and Pound-7 had the highest acclimatization success rates (99.6 - 94.4% respectively); whereas other genotypes were lower: TSH 565, VB 1151, SCA 6 and PS 1319 had 35.8, 31.0, 11.2 and 8.3% acclimatization success rates, respectively. Table 1 shows the percentages of acclimatized plants with respect to total production per year from nine cacao genotypes. In total, 4,088 (45.43%) plants were lost, and in mean 54.57% plants were successfully acclimatized.

Primary somatic embryogenesis protocol optimization in two commercial cacao genotypes

Two commercial clones (CCN 10 and CCN 51) were used to study the effect of genotype, hormone balance and glucose concentration on PSE production. For both genotypes, PCG medium supplemented with glucose (80 g L^{-1}) had the best response in most treatments with various hormone combinations using petals as explant source. The other treatments using staminodes as

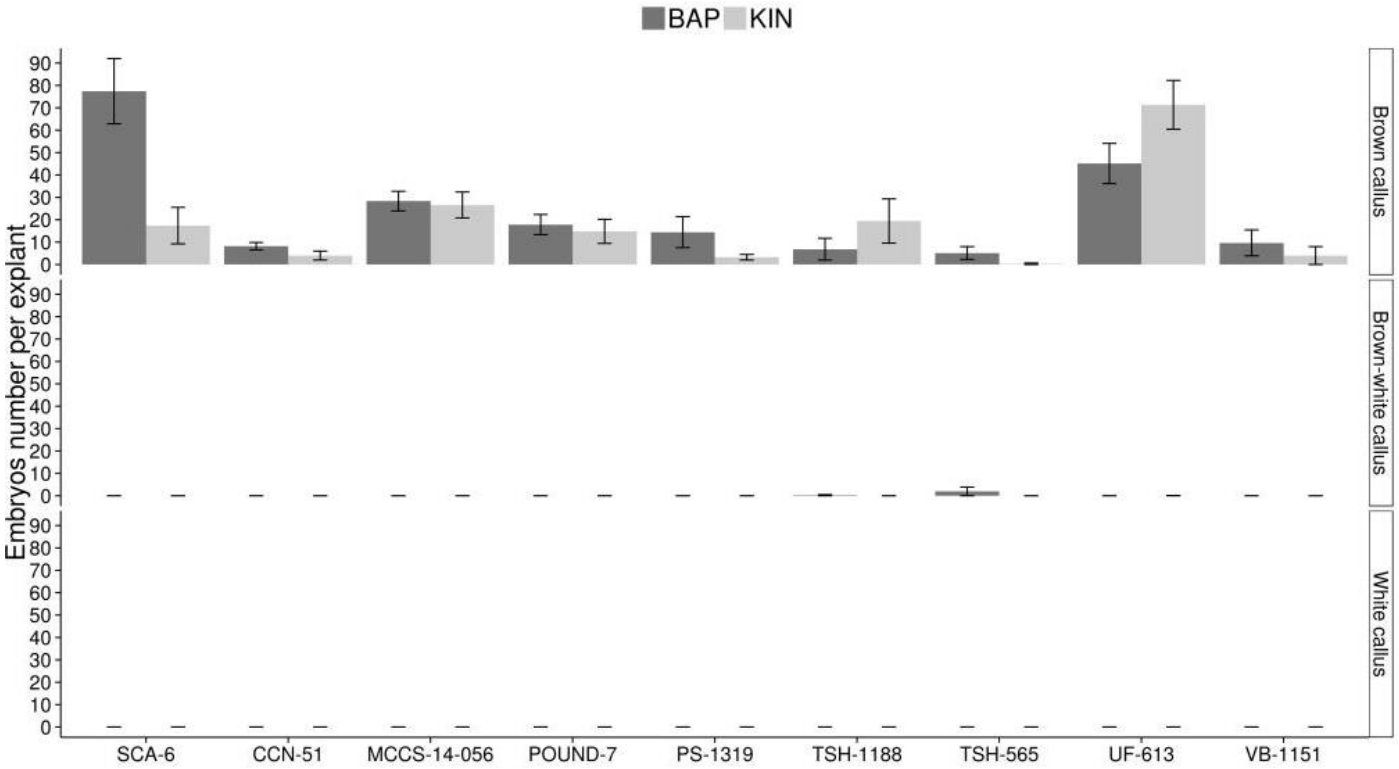


Figure 3. Average number SSEs formed from cotyledons as a function of cytokinin type. Values are given as mean ± interval of confidence with 5% of error.

explants and glucose concentration inferior to 80 g L^{-1} were discarded in the statistical analysis, because there was not embryogenic response. Figure 6 is a graphic representation of embryogenic efficiencies of these experiments. Values (SEs per explant) for CCN 10 ranged from ≥ 10 (dark red) to ≤ 3.0 (dark blue); whereas CCN 51 values ranged from ≥ 3.0 (dark red) to ≤ 1.0 (dark blue). Production of SEs for CCN 10 was optimal (10 SEs per explant) with 0.25 mg L^{-1} 2,4-D plus 3.0 µg L^{-1} TDZ; whereas CCN 51 was (3 SEs per explant) with 2.0 mg L^{-1} 2,4-D with 3 µg L^{-1} TDZ; only 12.5% of SEs were normal for CCN 10, and 60% for CCN 51 (Supplementary Tables 1 and 2). When 2.0 mg L^{-1} 2,4-D and 5.0 µg L^{-1} TDZ were used in PCG medium, using an original protocol by Li et al. (1998), in CCN, 51's staminodes had higher production of embryos than petals, although both explant types had low indices (10 PSE/100 callus) (Figure 2).

Influence of hormone ratios in abnormal and normal morphologies

The final range of morphology (proportions of normal and abnormal) of the SEs produced (for both genotypes) was influenced by individual hormone treatments (Supplementary Tables 1 and 2). Figure 7 shows the proportions of normal SEs, ranging from high (with values 1.0) to low (value of 0). For CCN 10, the highest proportion of normal SEs (five to seven normal SE/callus, respectively) was achieved with 2.0 mg L^{-1} 2,4-D in combination with 7.0 or 9.0 µg L^{-1} TDZ and 0.5 mg L^{-1} 2,4-D with 9.0 µg L^{-1} TDZ (Figure 7a). CCN51 had the best proportion (value 1.0) of normal SE using 0.25 mg L^{-1} 2,4-D with 4.0 or 9.0 µg L^{-1} TDZ, (1-3 SE per callus) where one petal had the capacity to form callus (Figure 7b). We had two objectives for the experiments summarized in Figure 6. The first was to identify specific, optimal PGR levels/balance necessary to achieve efficient SE production in the genotypes under study. The second was to determine if we could discern specific effective PGR ranges from the comprehensive analysis of the matrix of 25 conditions tested for each genotype. Although the first aim was achieved for both genotypes, the patterns generated in Figure 7 were unexpectedly complex; with numerous discontinuities of values (more continuous gradients were expected). Possible explanations for this include a sampling size that is not large enough to overcome experimental and/or biological variation. Such variation could also reflect variation of endogenous auxin levels in the explants, perhaps due to flower position within a tree (Tan and Furtek, 2003), or to environmental/seasonal fluctuation over the course of the extended flower collection times required for this large-

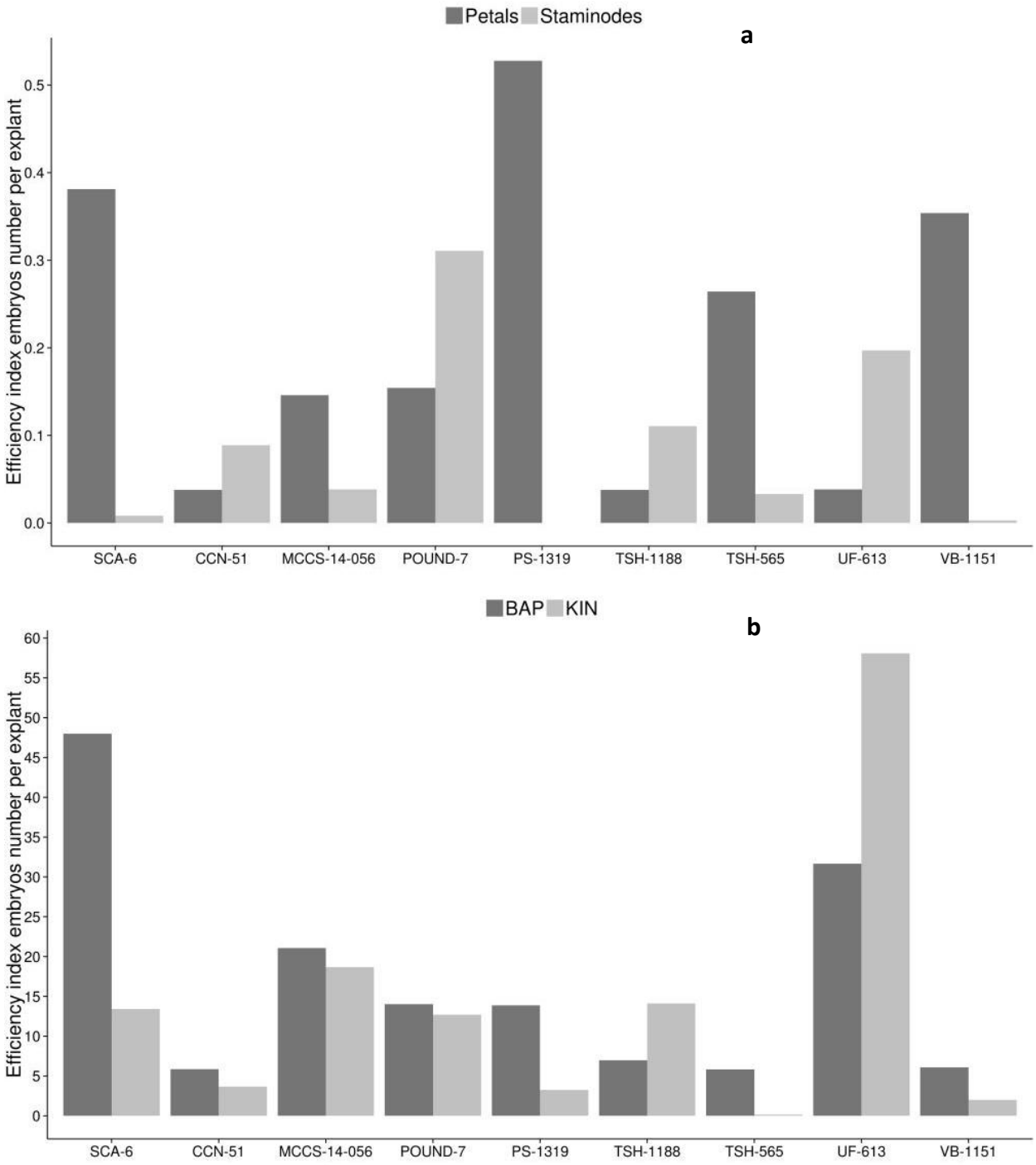


Figure 4. (a) Embryogenic efficiency index of primary somatic embryogenesis as a function of explant type (b) Embryogenic Efficiency Index of secondary somatic embryogenesis as a function of cytokinin type.

scale experiment.

DISCUSSION

Development of an efficient tissue culture-based cocoa propagation system is crucial for the much-needed large-scale dissemination of elite *T. cacao* genotypes; however, existing protocols show high genotype-to-genotype variation in SE production rates (Maximova et al., 2002; Li et al., 1998; Lopez-Báez et al., 1993). This technical hurdle must be overcome through development

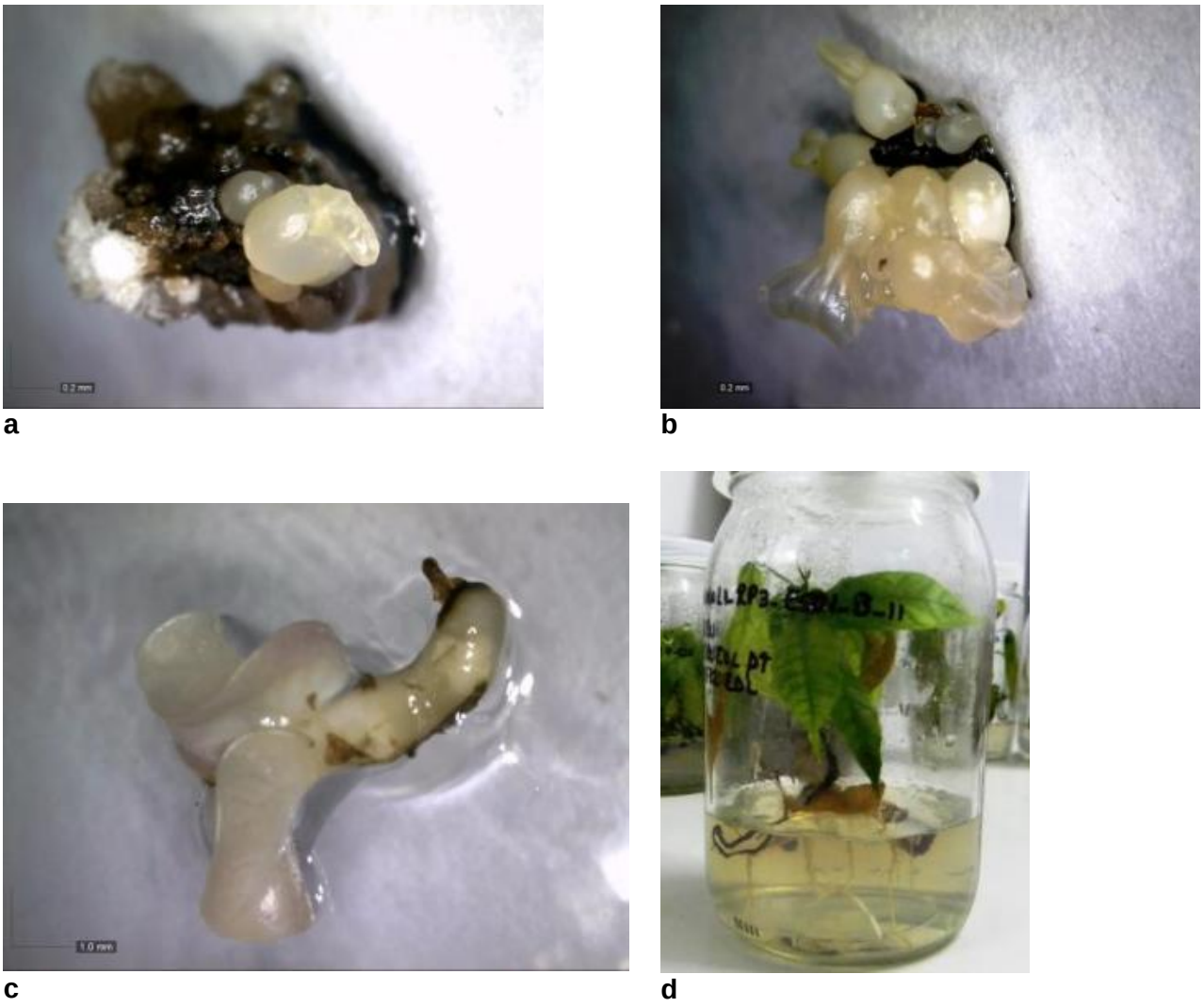


Figure 5. Somatic embryogenesis production response of brown callus in PSE and SSE: **(a)** Primary brown callus with PSEs and **(b)** secondary brown callus with SSEs. Germination and plant conversion of the SSEs. **(c)** Mature embryo and **(d)** young plantlets in EDL medium.

Table 1. Percentage of plants for each genotype produced in the propagation process in 14 months, the reference total (4,912) is the total number of plants that survived the production process. All the genotypes were cultured in parallel over a 36-week culture period. The production of SSE started with an average of 40 PSEs per clone as the source of explants in three batches.

Genotype	Total plants acclimated	Total plants produced	Percentage of survival (%)
CCN 51	398	500	79.6
MCCS 14056	1494	1500	99.6
POUND 7	755	800	94.4
PS 1319	58	700	8.3
SCA 6	223	2000	11.2
TSH 1188	282	700	40.3
TSH 565	143	400	35.8
UF 613	1435	2000	71.8
VB 1151	124	400	31.0
Total	4,912	9,000	$\bar{X}$ 54,57

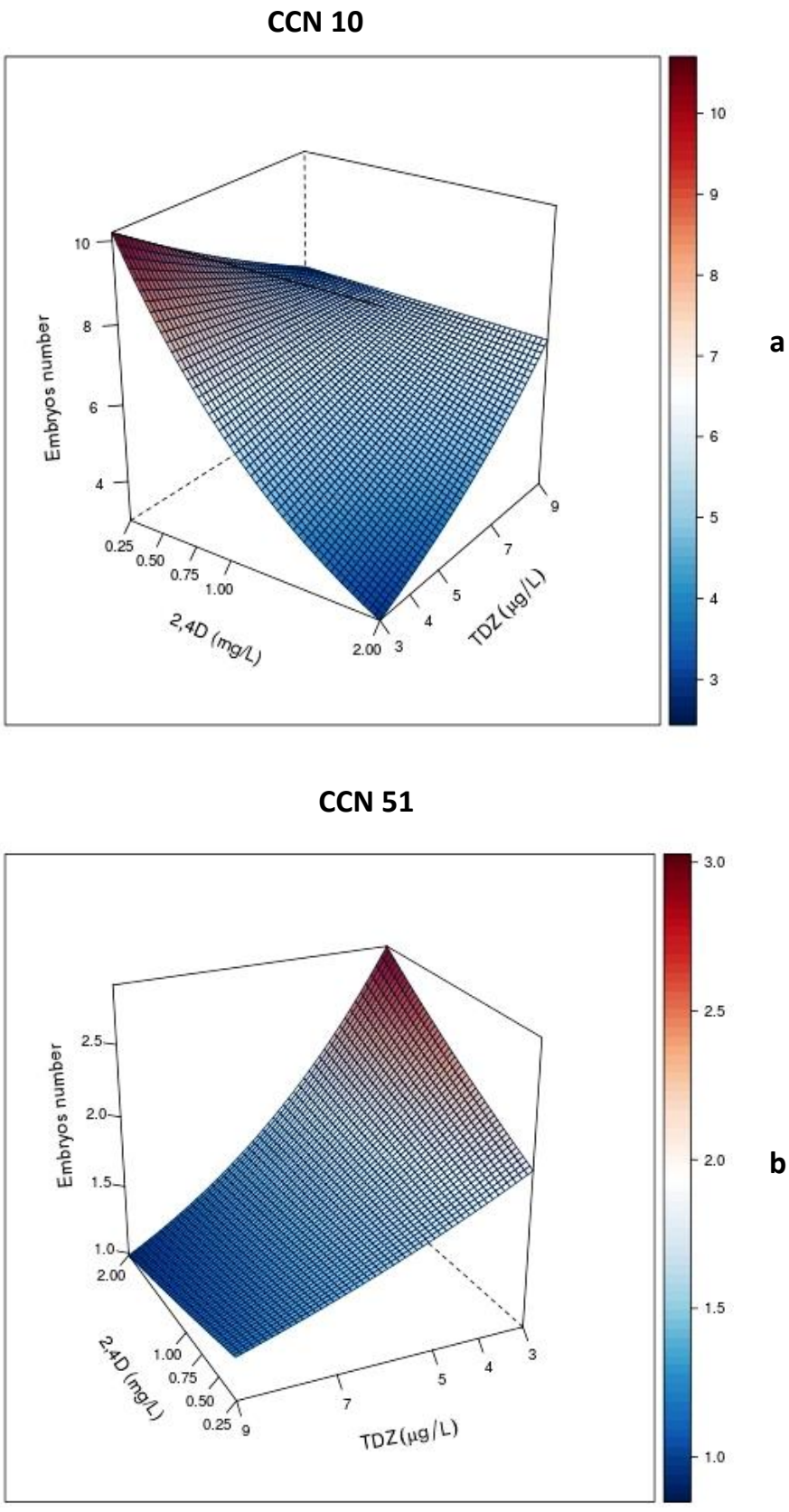
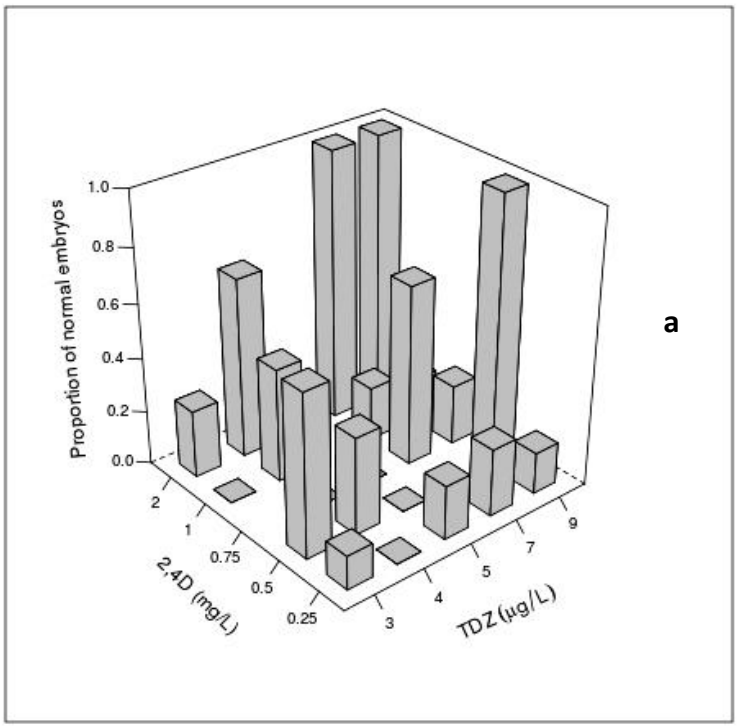


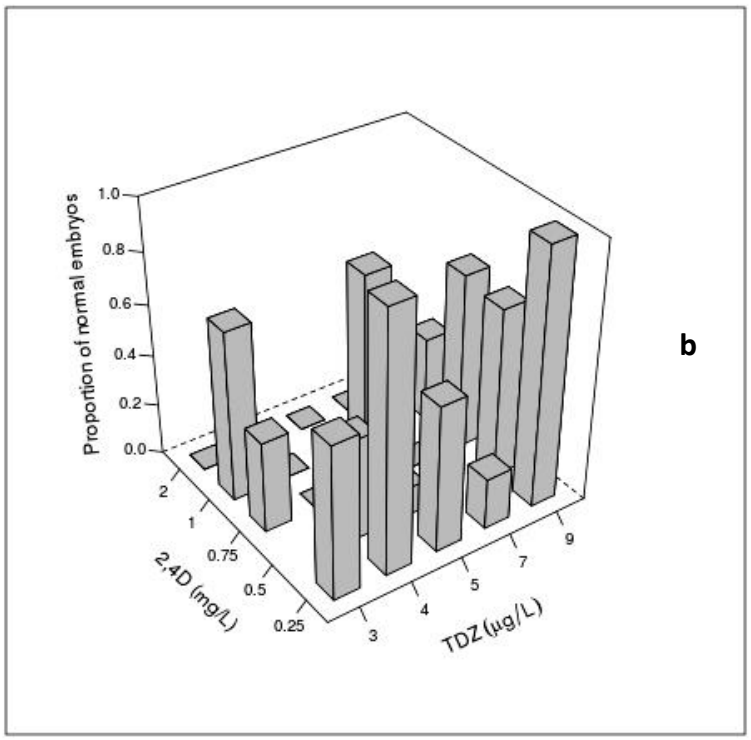
Figure 6. Total number of embryos produced as a function of hormone balance in petal explants using PCG medium with glucose 80 g L^{-1}. (a) PSE production in CCN 10 (b) PSE production in CCN 51.

CCN 10



a

CCN 51



b

Figure 7. Proportion of normal SE embryos obtained as a function of hormone balance in petal explants using PCG medium with glucose 80 g L^{-1}. (a) PSE proportion of normal embryos in CCN 10. (b) PSE proportion of normal embryos in CCN 51.

of optimized general procedures, which was the goal of our study. For all genotypes examined in our experiments, SSE protocols (using PSE explants) proved to be more productive than PSE protocols (using floral explants). This difference could be attributed to the fact that, within a given explant, cotyledonary cells have more competition than the presumably smaller populations of competent cells in petals or staminodes.

Alternatively, a higher proportions of brown callus in the PSE-derived cultures might explain higher SE generation in SSE protocols, possibly due to larger populations of competent cells. The observed genotypic differences in SE productivity response likely reflect genetic variation in concentration/type of endogenously produced compounds (for example, polyamines, ethylene, phenolic compounds, auxin etc.), or to synergy of endogenously-expressed PGRs with medium-supplied PGRs (Niemenak et al., 2012; Lu et al., 2011; Li et al., 1998; Hatanaka et al., 1995). Additional variation in donor flower bud size, and/or the physiological stage might also underlie the observed performance variation (Batista et al., 2002).

Two general classes of SE procedures have been developed: direct and indirect. SE production via callus intermediate is a type of indirect somatic embryogenesis that has been achieved in numerous species (Ikeuchi et al., 2013). Embryogenic callus is typically produced from embryos or other immature tissue, such as flowers, leaves or microspores (Ahmed et al., 2013; Maximova et al., 2002). Three distinct callus types have been reported (Hoffmann et al., 1990). The first is light yellow in color, with a compact globular or nodular structure comprised of highly compacted small isodiametric cells. The second is a straw-colored, irregular callus consisting of loosely arranged cells with a semi-translucent appearance. The third type is also irregular in structure, and consists of extremely loose callus tissue containing giant translucent cells that are long, tubular and non-morphogenic. The second type, which is made up of aggregates of cytoplasmic-rich cells has been proposed to contain the so-called embryogenic units, the numbers of which reflect the embryogenic potential of this callus (Bajaj, 1995; Fransz and Schel, 1991).

The callus types described above are similar to those that we observed in *T. cacao* in our study: the first type is white callus, which is compact with a light yellow-white color; white callus never produces embryos. The second type is brown callus, an embryogenic callus that is similar to that described by Bajaj (1995); globular-stage embryos begin to arise at eight weeks of culture from brown callus, which contains small nodular clusters with lower proliferation rates. The third callus type that we encountered is brown-white callus, characterized by high cell proliferation, but which also contains sections of white callus; SEs only develops from the brown portions of mixed callus.

To optimize SE procedures for cacao, it is necessary to elucidate the biological processes underlying SE production. Numerous abiotic stressors have been demonstrated to impact somatic embryogenesis induction and subsequent development (Fehér, 2015; Jin et al., 2014; Karami and Saidi, 2010); such stressors include high sucrose concentrations, polyethylene glycol, abscisic acid, dehydration, water stress, heavy metal ions, pH, heat or cold shock treatments, hypoxia, antibiotics, ultraviolet radiation and mechanical treatment. In our experiments, SE response in *T. cacao* also appears to be influenced by the concentration of glucose in the induction medium (PCG); one explanation for this is that high sugar concentrations might induce osmotic stress, which in turn could enhance reprogramming cells to an embryogenic pathway (Fehér, 2015).

To achieve proper embryo development during SE procedures, auxin-related compounds must be omitted from tissue culture medium at some point to permit establishment of cell/tissue polarity; this is because, once dedifferentiation and totipotency have been achieved, polar auxin transport is a key step in meristem formation during subsequent embryo development (Nawy et al., 2008; Jiménez, 2005). In zygotic embryogenesis, polarity of cell division in the body axis is already apparent from the asymmetric division of the zygote (into a small apical and a large basal cell). This pivotal event is also presumed to occur in the somatic pathway of embryogenesis (Jiménez, 2005).

When 2,4-D is added to the SE induction medium, it increases indole acetic acid (IAA) concentration in the donor tissue and initiates reprogramming of genes that is mediated by DNA methylation and/or chromatin remodeling (Pasternak, 2002). In our study, depending on the concentration of 2,4-D added to the PCG medium, the percentage of abnormal SEs varied; this phenomenon might be related to endogenous IAA levels, which would be expected to impact SE morphogenesis (Leljak-Levanic et al., 2015; Abrahamsson et al., 2012).

In conclusion, our first set of experiments demonstrate that SE production is influenced by genotype and source of donor explant, PGR type and balance and sugar concentration; the nine genetically diverse genotypes tested showed different rates of PSE and SSE production. Flower petals, not staminodes, had the best response in PSE production for most of the tested clones; overall though, production of SSEs proved to be the more productive approach. Propagation of cacao by somatic embryogenesis in large-scale is possible, but first is necessary to know the embryogenic response in genotypes with commercial interest applying an efficient protocol. In consequence, it will be important to do the adjustments of the protocol for new clones without preliminary information.

Primary somatic embryogenesis is one of the principal problems to produce cacao plants in large-scale. Different studies in production of PSEs using petal and staminodes show low percentage of success including our study, even if it is varied the type and concentration of PGR.

Either the low or lack of production of PSEs per initial explant can increase the costs in large-scale production requiring high number of initial explant to start the cultures. It is important to look for new source of explants to make the PSE process more efficient. Secondary somatic embryogenesis in our work shows better results than PSE using solid medium. Next experiments might focus in production in large-scale using liquid medium and bioreactors bigger than 5L for SSE mass production because it might increase the efficiency. In the literature, there are few publications in this theme using bio-reactors with not more than 1 L of capacity with good results but it is necessary to improve this process for large-scale (Niemenak et al., 2008).

For the experiments that focused on CCN 51 and CCN 10, SE production was highest using a high concentration of glucose in the induction medium (80 g L^{-1}). As discussed above, high glucose in the medium might function as a biological stressor that synergizes with 2,4-D and/or TDZ, to enhance the embryogenic response in SE procedures. In addition to the quantity of SEs produced, SE quality (normality vs. abnormality) also appears to be impacted by the balance of 2,4-D and TDZ in the PCG medium. CCN 51 produced more normal SEs with 0.25 mg L^{-1} 2,4-D with 4.0 or 9.0 µg L^{-1} TDZ; whereas CCN 10 was more normal SEs productive with 2.0 mg L^{-1} 2,4-D in combination with 7.0 or 9.0 µg L^{-1} TDZ.

Importantly, SE morphogenesis, like embryogenic induction, appears to be influenced by the balance of applied and endogenously-produced PGRs (Jiménez, 2005). Thus, treatments that produce high numbers of SEs per explant have a low proportion of normal SEs; whereas treatments that produce low numbers of SEs per explant have high proportion of normal embryos. Although we identified optimized culture conditions for two genotypes (CCN 10 and CCN 51), and starting conditions for many other clones, our analysis suggests that somatic embryogenesis is a complex, multifactorial process that may be compounded by the fact that the floral explants used were produced from outdoor, greenhouse-grown trees. Thus, environmental as well as phenological variation may contribute to the response complexity uncovered in our analyses (for example, Figure 7).

Any variation in endogenous auxin levels would likely impact embryogenic capacity, and further compound underlying genotype-to-genotype variation in response. The relationship between phenology and somatic embryogenesis response has been studied, and suggests that both flowering and fruiting levels can seasonally impact somatic embryogenesis (Issali et al., 2009).

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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Abbreviations

2,4-D, 2,4-Dichlorophenoxyacetic acid; **2,4,5-T,** 2,4,5-trichlorophenoxyacetic acid; **BAP,** 6-benzylaminopurine; **TDZ,** thidiazuron; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **SE,** somatic embryogenesis; **SEs,** somatic embryos; **PGRs,** plant growth regulators; **PSE,** primary somatic embryogenesis; **PSEs,** primary somatic embryos; **SSE,** secondary somatic embryogenesis; **SSEs,** secondary somatic embryos; **PGC,** primary callus growth; **SCG,** secondary callus growth; **ED4,** embryos development-4; **ED3,** embryos development-3; **ED,** embryo development; **EE,** efficiency of embryogenesis; **EDL,** embryo development in light; **PEC,** primary embryo conversion.

REFERENCES

Abrahamsson M, Valladares S, Larsson E, Clapham D, von-Arnold S (2012). Patterning during somatic embryogenesis in Scots pine in relation to polar auxin transport and programmed cell death. Plant Cell Tissue Organ Cult. 109:391-400.

Ahmed W, Feyissa T, Disasa T (2013). Somatic embryogenesis of a coffee (*Coffea arabica* L.) hybrid using leaf explants. J. Hortic. Sci. Biotechnol. 88:469-475.

Bajaj YPS (1995). Biotechnology in Agiculture and forestry, somatic embryogenesis and synthetic seed I. Springer-Verlag. Berlin Heidelberg, New York. 30:78-81.

Batista J, Santos PA, De Oliveira M (2002). Otimização da metodologia de embriogênese somática visando a propagação clonal de genótipos elite de cacau (*Theobroma cacao* L.). Embrapa Brasilia. 79:1-34.

CEPLAC-Comissão executiva do plano da lavoura cacaueira (2009). Nota Tecnica: Programa de recuperação da lavoura cacaueira – 3ª e 4ª etapas. A crise da lavoura cacaueira, condicionantes, ação Governamental, Análise e Recomendações. Ministério Da Agricultura, Pecuária e Abastecimento Comissão Executiva do Plano da Lavoura Cacaueira. pp. 1-31.

Chanatásing-Vaca CI (2004). Inducción de la embriogénesis somática en clones superiores de cacao (*Theobroma cacao* L.), con resistencia a enfermedades fungosas. Escuela de Posgrado, Programa de educación para el desarrollo y la conservación del Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, ON Costa Rica. pp. 1-86.

Driver JA, Kuniyuki AH (1984). In vitro propagation of Paradox walnut rootstock. HortScience 19:507-509.

FAO (2013). Food and agriculture Organization of the United Nations. Accessed: October, 30, 2013. Available at: http://www.fao.org/corp/statistics/en/.

Fehér A (2015). Somatic embryogenesis-Stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta 1849:385-402.

Fransz PF, Schel JHN (1991). An ultrastructural study on the early development of *Zea mays* somatic embyos. Can. J. Bot. 69(4):858-865.

Franzen M, Borgerhoff MM (2007). Ecological, economic and social perspectives on cocoa production worldwide. Biodivers. Conserv. 16:3835-3849.

Gamborg OL (1996). Aromatic metabolism in plants. II. Enzymes of the shikimate pathway in suspension cultures of plant cells. Can. J. Biochem. 44:791-799.
Hatanaka T, Sawabeb E, Azuma T, Uchidab N, Yasudab Y (1995). The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora*. Plant Sci. 107:199-204.
Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. California Agric. Experiment Station Circular 347:1-32.
Hoffmann B, Schumann G, Kruger H-U (1990). Histological observation of morphogenesis from androgenetic tissues of *Triticum aestivum* L. I. Callus tissue. Arch Züchtungsforsch 20(3):179-186.
IBGE (2015). Instituto Brasileiro de Geografía e Estatistica. Accessed: October, 30, 2015. Available at: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/lspa_201504_5.shtm
Ikeuchi M, Sugimoto K, Iwase A (2013). Plant callus: mechanisms of induction and repression. Plant Cell 25:3159-3173.
Issali AE, Traoré A, Ngoran JAK, Koffi KE, Sangaré A (2009). Relationship between some phenological parameters and somatic embryogenesis in *Theobroma cacao* L. J. Crop Sci. Biotechnol. 11:23-30.
Jiménez VM (2005). Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul. 47:91-110.
Jin F, Hu L, Yuan D, Xu J, Gao W, He L, Yang X, Zhang X (2014). Comparative transcriptome analysis between somatic embryos (SEs) and zygotic embryos in cotton: evidence for stress response functions in SE development. Plant Biotechnol. J. 12:161-173.
Karami O, Saidi A (2010). The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol. Biol. Rep. 37:2493-2507.
Konieczny R, Sliwinska E, Pilarska M, Tuleja M (2012). Morphohistological and flow cytometric analyses of somatic embryogenesis in *Trifolium nigrescens* Viv. Plant Cell Tissue Organ Cult. 109:131-141.
Leljak-Levanic D, Mihaljeviç S, Bauer N (2015). Somatic and zygotic embryos share common developmental features at the onset of plant embryogenesis. Acta Physiol. Plant. 37(7):1-4.
Li Z, Traore A, Maximova S, Guiltinan MJ (1998). Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In vitro Cell. Dev. Biol. Plant 34:293-299.
Lloyd G, McCown B (1980). Commercially feasible micropropagation of Mountain Laurel, Kalmia latifolia, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30:421-427.
Lopez-Báez O, Bollon H, Eskes A (1993). Embryogènese somatique de cacaoyer *Theobroma cacao* L. á partir de pièces florales. C. R. Acad. Sci. 316:579-584.
Lopez-Báez O, Moreno J, Pacheco S (2001). Avanzos en Propagación de Cacao-*Theobroma cacao*- por Embriogénesis Somática en México. In. Ingenic. Proceedings of the International Workshop on new Technologies and Cocoa Breeding. Malaysia. Ingenic. pp. 163-176.
Lu J, Vahala J, Pappinen A (2011). Involvement of ethylene in somatic embryogenesis in Scots pine (*Pinus sylvestris* L.). Plant Cell Tissue Organ Cult. 107:25-33.
Mahdavi-Darvari F, Noor N, Ismanizan I (2015). Epigenetic regulation and gene markers as signals of early somatic embryogenesis. Plant Cell Tissue Organ Cult. 120:407-422.
Marelli JP (2008). *Solanum lycopersicum* as a model system to study pathogenicity mechanisms of *Moniliophthora perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. The Pennsylvania State University, Department of Plant Pathology. Pennsylvania, USA. pp. 1-143.
Maximova S, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan MJ (2002). 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.
Miller CR (2009). An integrated *in vitro* and greenhouse orthotropic clonal propagation system for *Theobroma cacao* L. The Pennsylvania State University. The Graduate School College of Agricultural Sciences. pp. 1-137.
Motamayor JC, Lachenaud P, da Silva e Mota JW, Loor R, Kuhn DN, Brown JS, Schnell RJ (2008). Geographic and Genetic Population Differentiation of the Amazonian Chocolate Tree (*Theobroma cacao* L). PLoS One 3(10):e3311.
Murashige T, Skoog FA (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479.
Nawy T, Lukowitz W, Bayer M (2008). Talk global, act local-patterning the *Arabidopsis* embryo. Plant Biol. 11:28-33.
Niemenak N, Awah TM, Lieberei R (2012). Establishment of suspension culture in *Theobroma cacao* and polyamines associated with cacao embryogenesis. Plant Growth Regul. 67:1-8.
Niemenak N, Saare-Surminski K, Rohsius C, Ndoumou DO, Lieberei R (2008). Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acids in different tissues. Plant Cell Rep. 27(4):667-676.
Pasternak TP (2002). The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol. 129:1807-1819.
Solano-Sanchez W (2008). Embriogenesis somatica en clones superiors de cacao (*Theobroma cacao* L.) obtenidos en el programa de mejoramientogénetico del CATIE," M.S. thesis, Escuela de Posgrado, Programa de educación para el desarrollo y la conservación del Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, ON Costa Rica. pp. 1-92.
Sondahl M R, Liu S, Bellato C (1993). Cacao somatic embryogenesis. Acta Hortic. 336:245-248.
Sondahl M R, Sereduk TB, Chen Z (1989). Somatic embryogenesis and plant regeneration of cacao, patent no. 88/'3078, Jen 25, Republic of South Africa.
Steward FC, Mapes MO, Mears K (1958). Growth and organized development of cultured cells. II. Organization in culture grown freely suspended cells. Am. J. Bot. 45:705-708.
Tan C, Furtek D (2003). Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues. Plant Sci. 164:407-412.
Wood GAR, Lass RA (2008). Cocoa. Fourth Edition. New York: Longman. pp. 1-620.
Young A, Miller C, Antunez de Mayolo G, Swanson JD, Pishak S, Maximova S, Guiltinan G (2003). Cacao Tissue Culture Protocol Book. Copyright The Pennsylvania State University. 1.4: 1-32.

Supplementary Table 1. CCN 10. Total number embryos produced as a function of hormone balance (relation 2,4D/TDZ) in petals explant using PCG medium with glucose 80 g L.

Concentration TDZ (µg L-1)	Concentration 2,4-D (µg L-1)	Rate 2,4D/TDZ	Average of embryos per explant	Average of normal embryos per explant	Normal embryos (%)
3	250	83	10.0	1.2	13
3	500	167	8.5	5.1	60
3	750	250	7.0	0.0	0
3	1200	833	5.0	0.0	0
3	2000	667	3.0	1.0	25
4	250	63	9.5	0.0	0
4	500	125	8.0	3.0	36
4	750	188	7.0	0.0	0
4	1200	300	5.5	2.3	42
4	2000	500	3.5	2.3	67
5	250	50	9.0	1.8	20
5	500	100	8.0	0.0	0
5	750	150	7.0	0.0	0
5	1200	240	6.0	0.0	0
5	2000	400	4.0	0.0	0
7	250	36	8.0	2.0	25
7	500	71	7.5	0.0	0
7	750	107	7.0	4.5	67
7	1200	171	6.5	1.3	20
7	2000	286	5.0	5.0	100
9	250	28	7.0	1.0	16
9	500	56	7.0	7.0	100
9	750	83	7.0	1.5	22
9	1200	133	7.0	1.1	16
9	2000	222	7.0	7.0	100

Supplementary Table 2. CCN 51. Total number embryos produced as a function of hormone balance (relation 2,4D/TDZ) in petals explant using PCG medium with glucose 80 g L.

Concentration TDZ (µg L-1)	Concentration 2,4-D (µg L-1)	Rate 2,4D/TDZ	Average of embryos per explant	Average of normal embryos per explant	Normal embryos (%)
3	250	83	2.0	1.2	60
3	500	167	2.0	0.0	0
3	750	250	2.0	0.7	36
3	1200	400	2.5	1.6	67
3	2000	667	3.0	0.0	0
4	250	63	2.0	2.0	100
4	500	125	2.0	0.8	40
4	750	188	2.0	0.0	0
4	1200	300	2.0	0.0	0
4	2000	500	2.5	0.0	0
5	250	50	2.0	1.0	57
5	500	100	2.0	0.2	11
5	750	150	2.0	1.6	83
5	1200	240	2.0	0.0	0
5	2000	400	2.0	0.0	0
7	250	36	1.5	0.0	0
7	500	71	1.5	0.3	20

Supplementary Table 2. Contd.

7	750	107	1.5	0.0	0
7	1200	171	1.5	0.0	0
7	2000	286	1.5	0.0	0
9	250	28	1.0	1.0	100
9	500	56	1.0	0.6	67
9	750	83	1.0	0.7	69
9	1200	133	1.0	0.3	33
9	2000	222	1.0	0.0	0

Total explants per Petri dish 25.