6-benzylaminopurine induces somatic embryogenesis in staminodia of new genotypes of Theobroma cacao L. from the Papaloapan Basin of Mexico and reveals differences with T. bicolor Bonpl.

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Research Article

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Abstract

*Theobroma cacao* and *T. bicolor* belongs to most important agricultural crops of the Mexican tropics. Currently, propagation of these crops is carried out by seed, which means that demand exceeds production. In this context, somatic embryogenesis is an alternative to these issues. Thus, we evaluated the presence of embryogenic genotypes of *T. cacao* and *T. bicolor* in the Papaloapan Basin of Mexico with the idea of implementing this technology in the region. The analysis of the phenotypic expression of the floral whorls revealed that 6-benzylaminopurine in combination with 2,4-dichlorophenoxyacetic acid, induces different morphogenetic responses of the genotypes evaluated in the primary callus phase of *T. cacao*, unlike *T. bicolor*. The staminodia presented the highest percentage of caulogenesis in *T. cacao*, while *T. bicolor* presented the highest frequency of caulogenesis in staminodia and carpels. Some calli differentiated in the root, being to a greater extent those derived from staminodes of *T. cacao*. The calli of *T. bicolor* did not differentiate. A parallel study using thidiazuron as an inducer revealed a similar behavior in obtaining callus in both species, however the rhizogenesis from staminodes was 50% lower in the evaluated genotypes of *T. cacao*. Staminodes were the only ones that presented primary somatic embryogenesis in 66% of the *T. cacao* genotypes evaluated using benzylaminopurine. Finally, the secondary somatic embryogenesis was evaluated from cotyledons, reaching a 60% success rate, of which 95.48% were normal somatic embryos. Both types of embryogenesis were characterized morphologically using optical and/or scanning electron microscopy.

Key message

The *in vitro* morphogenic capacity of wild genotypes of *Theobroma cacao* and *T. bicolor* was evaluated, identifying only embryogenic genotypes of *T. cacao* var. Forastero.

Introduction

The cacao *Theobroma cacao* L., (2n = 2x = 20) which is native to the tropical forests of South America, whose center of speciation and genetic diversification is the north of the Amazon (Coe SD and Coe MD, 1996, Motamayor JC et al., 2008) was domesticated in Mexico and Central America 3,000 years ago (Coe SD and Coe MD, 1996, Motamayor JC et al., 2002, Henderson JS et al. 2007) and was part of the political, economic, religious and social relations of the inhabitants of different regions of Mesoamerica (Motamayor JC et al., 2002, Henderson et al. 2007). Despite these historical antecedents, the drop in its production, as a consequence of the substitution of plantations for more profitable crops, has led Mexico to occupy the eleventh place in the world in the production of this grain. However, mexican cocoa producers have the potential to significantly increase their production, for which it is necessary to renew plantations and increase the arable area.

In addition, *Theobroma bicolor* Bonpl., another of the most important relatives of cacao, is the second culturally important species of the genus Theobroma in Mesoamerica. It has also been consumed and used for ritual purposes. It is distributed from Mexico to Brazil and Bolivia, sharing a range similar to that
of *T. cacao*. Partially domesticated, in Mesoamerica it is called *balam te*’ (the Jaguar Tree), *patakte* or *balamte*; the Spaniards found it in the markets of the Mexican lands, but it seemed to be less appreciated than *T. cacao*. The word "cacao" generally refers to the species *T. cacao*, although among the Mesoamerican Maya it is also sometimes applied to *T. bicolor*. It is morphologically similar to *T. cacao* and its fruits are very different from any other cultivated species of the genus Theobroma; as they mature their shell comes to resemble the pattern of the jaguar's skin. Its scant recorded ethnobotanical evidence indicates that it held a special niche in pre-Hispanic ritual life, particularly among the Maya, and continues to do so to this day (McNeil, 2006).

The state of Oaxaca, located in the southeast of Mexico, is one of those regions where cocoa continues to be part of the local culture since pre-Hispanic times. Therefore, and as part of an economic development strategy for the region, there is an interest to reactivate the economy of production of this grain, for which cultivation areas are being allocated in the north of this state, in a region called the *Cuenca del Papaloapan* or the Papaloapan Basin. In this tropical region, indigenous-speaking territories with Chinantec and Sotaventine roots where *T. cacao var. Forastero* and *T. bicolor* are cultivated, a problem faced is the lack of availability of elite clonal cocoa seedlings from which to increase productivity of the region. Currently, propagation is carried out by seed, which requires the establishment of clonal gardens with cocoa genotypes with elite characteristics and regenerative capacity via somatic embryogenesis for the massive *in vitro* production of clonal tree plants.

To date, no embryogenic genotypes have been identified in Mexico of *T. cacao* and *T. bicolor* that allow for the design of strategies for the use of resources and the renewal of plantations. In this context, our efforts are aimed at locating and identifying them in the field, specifically in the Papaloapan Hydrological Basin of Mexico.

**Materials and methods**

**Geographical location**

The recognition and labeling was performed of six trees of *T. cacao var. Forastero* and six *T. bicolor* trees located in the municipalities of San Juan Bautista Tuxtepec, Santa María Jacatepec and San José Chiltepec in the region of the Papaloapan Basin in the state of Oaxaca, Mexico. Geopositioning was performed using a GARMIN E-Trex Vista DATUM ITRF92 device (Fig. S1).

**Plant material**

An average of 40 non-pollinated closed flower buds were collected from six adult *T. cacao* trees geopositioned as GPS1, GPS3, GPS4, GPS5, GPS6 and GPS8, following the protocol of Maximova et. al, 2002. The trees geopositioned as GPS7, GPS9, GPS10, GPS11, GPS15 and GPS16 corresponded to *T. bicolor*. From the GPS2, GPS12, GPS13 and GPS14 trees sufficient samples were not able to be taken for the replicas, for which they were discarded in this study.

**Establishment of the in vitro culture**
The flower buds were transported in 50 mL Falcon tubes containing 30 mL of cold sterile distilled H2O to the laboratory where they were disinfected using a commercial bleach solution at 50% (v/v) (2.5% hypochlorite sodium) for 10 minutes, followed by 70% ethanol (2 minutes). A second disinfection method using 1% calcium hypochlorite (20 minutes) was also used. After 10 days the results of contamination and necrosis were evaluated.

**Induction and expression of somatic embryogenesis**

Once the flower buds had been disinfected, the next step was the dissection of the flower whorls: staminodes, petals, stamens and carpels in order to evaluate their morphogenic potential. The induction and expression of primary somatic embryogenesis was carried out by a modification of the protocol of Guiltinan et. al 2003, substituting thidiazuron (TDZ) for 6-benzylaminopurine (BAP) at a concentration of 1.4 µM in both the primary callus growth medium (PCG) and secondary callus (SCG), in order to evaluate this regulator growth in induction of embryogenesis. Caulogenesis was evaluated four weeks after inoculum. Rhizogenesis and primary somatic embryogenesis were evaluated at 14 weeks in the expression medium (ED). Secondary embryogenesis was induced and expressed according to Guiltinan et. al 2003 without modifications from five-week-old mature cotyledons in expression medium. Primary embryogenesis was evaluated in parallel using TDZ at a concentration of 22.7 nM according to Guiltinan et. al 2003 in the genotypes GPS3, GPS5 and GPS8 corresponding to *T. cacao* and in the genotypes GPS7, GPS9, GPS10, GPS11, GPS13, GPS15 and GPS16 of *T. bicolor*.

**Characterization**

Primary somatic embryogenesis samples were directly observed by light microscopy with a HIHOTEK brand XTX-7C-W stereoscopic microscope. Secondary somatic embryogenesis samples were also prepared for analysis by scanning electron microscopy (SEM). Tissue fixation was carried out in 2% glutaraldehyde for 24 h; subsequently they were gradually dehydrated for 15 minutes with 60, 70, 80, 90, 96 and 100% ethanol. A critical point dryer was used to remove remaining moisture (Smadri®, Tousimis, USA). The dried samples were placed on graphite tapes and adhered to aluminum cylinders. Subsequently, the samples were covered with gold using the Vacuum Desk® II equipment (Denton, USA). Finally, the samples were observed and photographed with a JSM-59001v® SEM (Jeol, Japan).

**Growth and development of secondary somatic embryos**

Once secondary embryogenesis has been induced from the cotyledons obtained from the primary somatic embryos in SCG medium following the protocol of Guiltinan et. al 2003, the expression of the secondary embryos was carried out in liquid ED medium coupling the RITA® temporary immersion system; 5 cycles of 5 min every 24 hours under dark conditions until they reached maturity. The conversion of secondary somatic embryos to seedlings was carried out under *in vitro* conditions in liquid PEC medium according to Guiltinan et. al 2003, using the RITA® temporary immersion system; 5 cycles of 5 min every 24 hours. The incubation conditions of the explants were 12/12 hours photoperiod of MAGG white fluorescent light of 40 watts (54 µmol.m-2s) and at 26 ± 2º C. The stage prior to transplantation to a warehouse with organic substrate under shade house was carried out in liquid RD
medium using agrolite as a solid support in Pyrex® 25x100 mm, 39.0 mL culture tubes, under a photoperiod of 16/8 hours of light at 26 ± 2º C. The shade house has 40x25 hpp black antiaphid mesh on the roofs and sides, with an inverted micro-sprinkler irrigation system. The weather conditions correspond to the location of the T. cacao trees geopositioned as GPS-1, GPS-2 and GPS-3.

Statistical analysis

The experimental design used was completely random, the experimental unit was the floral whorl and the number of independent experiments was three. A one-way ANOVA analysis was performed to determine the statistical difference between the different types of response of each genotype and floral whorl evaluated, with 95% reliability without data discrimination. In cases where there was a significant difference, the Tukey-Kramer multiple comparison of means test was performed.

Results

The collection of non-pollinated flower buds (Fig. 1) was made from trees that are grown in the field and backyard (Fig. S2), of which the efficiency for the establishment of the in vitro culture was evaluated through the quantification of contamination in the tissue. When using sodium hypochlorite, an average of 99.9% asepsis is achieved and maintained, while when using calcium hypochlorite it varies depending on the collection obtaining an average of 88.5%. In both cases the establishment of the culture was carried out in triplicate. The results with T. cacao are shown in Fig. 2.

At four weeks of in vitro culture, the percentage of caulogenesis was evaluated in the different types of explants: staminodia, petals, stamens and carpels, which allowed us to establish the response capacity of these organs to callus induction. From the results shown in Fig. 3, it can be stated that there is a significant difference in the caulogenic capacity, with staminodes presenting the highest probability of scrutiny for the evaluation of the morphogenic response in T. cacao and the carpels presenting the lowest. In T. bicolor, in addition to the staminodia, the petals and carpels were those that presented high values of caulogenesis, and the stamens those that presented significantly the lowest morphogenic potential (Fig. S3 and S4). In parallel, TDZ was evaluated, obtaining similar results as with BAP in T. cacao; (Fig. S5) shows what was obtained with the GPS-3 genotype. The same behavior was observed in T. bicolor (Fig. S6).

After the evaluation of caulogenesis, the phenomenon of necrosis was observed in the calli obtained. Necrosis followed an inverse trend to caulogenesis, with carpels showing the highest percentage of this phenomenon in T. cacao and stamens in T. bicolor; Fig. 4 shows the results in T. cacao.

Figure 5 shows the first morphogenic result from the calli obtained. The calli whose origin were staminodes presented a higher percentage of rhizogenesis with respect to the rest of the cell lines in T. cacao (Fig. 6a and 6b). The effect of TDZ with respect to this morphogenic capacity decreased in staminodia of T. cacao (Fig. S7). Morphogenesis did not occur in the calli of T. bicolor.
The behavior of the in vitro culture of each floral whorl is highly conserved; Fig. S8 to S11 show how reproducible the cultures of *T. cacao* induced with BAP are, and in Fig. S12 using TDZ. The reproducibility of *T. bicolor* can be seen in Fig. S13 to S17 using BAP, and in Fig. S18 and S19 the effect of the use of TDZ is seen, observing noticeable differences in the appearance of the callus compared to *T. cocoa*.

Finally, the embryogenic response was revealed only in *T. cacao* (Fig. 6c and 6d), specifically in cell lines from staminodia of the GPS-1, GPS-3, GPS-5 and GPS-8 genotypes, observing up to 3% somatic embryogenesis in the GPS-3 genotype (Fig. 7). These results allowed us to identify individuals with embryogenic capacity, observing that 66% of the trees sampled presented the embryogenic phenotype. In addition, it was observed that the greater response capacity to hormonal induction was presented by staminodia in *T. cacao* (Fig. 8).

From the light microscopy images it was possible to differentiate two types of primary somatic embryos of *T. cacao var. Forastero*, which were called normal (71.3%, Fig. 9) and abnormal (28.7%. Figure 9). The first comprises model embryos with two cotyledons (Fig. 6e) and the abnormal ones those that do not present or have more than two cotyledons (Fig. 6f).

The cotyledons of the primary somatic embryos obtained from staminodia of the GPS3 genotype were exposed to the process of secondary embryogenesis, which showed 60% embryogenic capacity, which contrasts with the results obtained for primary embryogenesis (Fig. 10).

Secondary somatic embryogenesis was induced from mature cotyledons of normal and abnormal primary somatic embryos with more than two cotyledons, reaching 91.61% of normal embryos in contrast to 8.39% of abnormal (Fig. 9), with an average production rate of 183 and 38 secondary embryos, respectively.

The secondary embryos obtained matured successfully and their conversion to seedlings was carried out under in vitro conditions following the protocol of Guiltinan et al. 2003, implementing the RITA® temporary immersion system. The stage prior to transplantation was carried out in liquid RD medium using agrolite as a solid support in Pyrex® culture tubes. The conditions of the shade house under the local climatological conditions (warm wet with abundant rains in summer (Fig. S1) allowed to complete the protocol until obtaining seedlings. Figure 11a and 11b shows the appearance of the secondary somatic embryos where their unicellular origin is confirmed by the presence of the suspensor. The subsequent maturation process in the RITA bioreactor is shows in Fig. 11c and 11d. The cotyledons of a zygotic embryo (Fig. 11e) and the cotyledons of a somatic embryo (Fig. 11f and 11g) showed no differences. Figure 11h-11j show the last stage of the process to finally obtain a seedling. From secondary somatic embryos it was possible to obtain the morphological characterization using SEM (Fig. 12a-12f and Fig. S20 to S22).

**Discussion**

**Geographic location, plant material and establishment of in vitro culture**
The geographical area and the season of the year are decisive for the efficiency in the establishment of the in vitro culture when it comes to field crops or plantations. Due to the above, the results reported in this research correspond to the cultivation of cocoa floral pieces collected in the months of June, July and August of the year 2014, which corresponds to the summer rainy season in the municipalities of Santa María Jacatepec, San José Chiltepec and San Juan Bautista Tuxtepec belonging to the Papaloapan Region, Oaxaca, Mexico, and may vary with the dry and cold seasons that coincide with flowering. In addition, the efficiency can vary depending on the year due to climatic variations that can influence the growth of microbial populations (Vargas-Gastélum et al., 2015). The results obtained with sodium hypochlorite fluctuated from 99.7–100% asepsis, while using calcium hypochlorite the efficiency ranged from 80 to 100%. These results could not be compared with previous works due to the lack of information in this regard. Other similar studies carried out using explants from the field are those by Tan and Furtek 2003, which indicate that the season of the year, the rainy season, and the physiological age of the flower affect the frequency of somatic embryogenesis. Our study, therefore, could also be influenced by the climatic conditions and the physiological age of the flowers, which should be taken into account in the future for both species of Theobroma.

Induction of primary somatic embryogenesis

In the evaluated genotypes of T. cacao var. Forastero, the use of BAP in combination with auxin 2,4-D induced caulogenesis in a high percentage as expected (Alemanno et al., 1996, Tan and Furtek 2003, Minyaka et al., 2008b); however, there was a significant difference between the floral whorls evaluated. The caulogenesis evaluated four weeks after the inoculum showed a higher average of callus in staminodes of 98.4%, followed by the petal 82.2%, stamen 68.6% and finally carpel 54.6%. The macroscopic appearance of the callus in our study was classified as having a compact consistency, a white-crystalline color, a friable consistency and a yellowish-brown color. The qualitative observation of caulogenesis, friable or compact callus, abundant or scarce, coloration, etc., allows us to deduce in many cases the probability of the appearance of embryogenesis pathways (Minyaka et al., 2008b). Tan and Furtek 2003 classified the callus according to its consistency and color as white compact and yellow friable, without determining which is embryogenic; only one case was presented in which the appearance of compact callus coincides with embryogenesis; the rest of the embryogenesis events coincide with both types of callus. T. bicolor showed a specific quantitative response according to the genotype despite no statistically significant difference between each of them, even when compared to the T. cacao genotypes. Induction with TDZ presented similar results to BAP in both species of Theobroma. Unlike T. cacao, the friable callus of T. bicolor did not differentiate in rhizogenesis or embryogenesis. The greatest advances have been achieved with T. cacao, for which it is necessary to continue working with the rest of the species of the Theobroma genus, especially with the Jaguar Tree.

Rhizogenesis was evaluated at 14 weeks in the embryo development medium (ED), presenting significant differences between the whorls and genotypes evaluated. The average rhizogenesis was the highest in staminodes at 49.6%, followed by stamens at 23.3%, carpels at 14.6% and finally petals at 7.5%. Callus
rhizogenesis is common as a regeneration pathway (Minyaka et al., 2008b); however it has been little evaluated.

Primary somatic embryogenesis was assessed at 14 weeks in embryo development medium (ED). The total number of somatic embryos obtained in this study is not comparable to culture week 38 in the study by Maximova et al., 2002. These authors emphasize that the highest production of embryos occurs between weeks 12 and 14 after the start of culture, while the peak of production occurs in the range of 8 and 18 weeks for most of the genotypes they evaluated. Other authors report embryogenesis at 6 weeks from the start of culture (Traore and Guiltinan 2006). However, in our study the result of the induction of primary somatic embryogenesis revealed the embryogenic phenotypes of Forastero cacao using BAP from the first stage of induction, primary callus, unlike previous works that propose the use of TDZ at this stage (Li et al., 1998, Maximova et al., 2002, Guiltinan and Maximova 2010). The average primary embryogenesis was 1.19%, and this only occurred in staminodes in 66.6% of the genotypes evaluated. Our results contrast with higher averages, 8.21% and 10.19%, in highly embryogenic genotypes cataloged IMC67 and Sca6, reported by Minyaka et al., 2008a, 2008b, among others (Li et al., 1998, Alemanno et al., 1996, Maximova et al., 2002, Traore and Guiltinan 2006). The frequencies of somatic embryogenesis obtained in this work are comparable with other works carried out under similar field conditions, such as Tan and Furtek 2003, which evaluated new, uncatalogued genotypes. In our study, somatic embryogenesis was not manifested with TDZ during this time period. It has been reported that in the secondary callus stage the time of appearance of embryogenesis can vary depending on the sulfur content, which at optimal concentrations inhibits caulogenesis and promotes direct differentiation into staminodes and petals (Minyaka et al., 2008a, b).

The quantification of necrosis allows evaluation of yields in regeneration pathways (Minyaka et al., 2008b). However, as is the case with rhizogenesis, tissue necrosis during in vitro cocoa culture has been rarely documented. We report a higher frequency in carpels from 11.7–60%, followed by stamens from 2–31.9%, petals from 0.9–23.1% and staminodes from 0–3.3%, this being inversely proportional to caulogenesis and somatic embryogenesis.

**Induction of secondary somatic embryogenesis**

Secondary embryogenesis induced from mature cotyledons of primary somatic embryos of the most prolific GPS3 genotype reduced the time to 5 weeks from induction and increased its frequency nearly twenty-fold, that is, from 3.1–60%. The increased rate of embryogenesis was consistent with most of the previous work (Maximova et al., 2002, Niemenak et al., 2008).

**Characterization of primary and secondary somatic embryogenesis**

Primary somatic embryogenesis was characterized under a common stereoscopic microscope, generally used in these millimeter-scale processes, being able to group embryos as normal, off-type or abnormal. The characterization of secondary embryogenesis was carried out under a common stereoscopic
microscope and under the scanning electron microscope (SEM), which allowed identifying the tissue that originated it, the stages of embryonic development, the frequency of off-type embryos (three and four cotyledons), the observation of the characteristic stomata, unicellular and multicellular trichomes of the epidermis as indicative of the quality of morphogenesis, as well as the presence of the suspensor, an embryonic structure that allows defining its unicellular origin (Maximova et al., 2002), which coincided with the first report of this type in cocoa primary somatic embryogenesis from zygotic embryos (Santos and Machado 1989), which suggests that both morphogenic processes are similar. However, the frequency of normal embryos from vegetative tissue in this study was the majority, contrasting with zygotic tissue. No other type of anomaly was determined such as the fusion of hypocotyls reported by Tan and Furtek 2003. Li et al. 1998, differentiates somatic embryos with respect to their pink or white coloration, marking them as a determining phenotypic characteristic, and also reported the appearance of off-type somatic embryos with fused hypocotyls, without cotyledons or with additional cotyledons, which are dismissed as determining factors for the following phases of growth and development.

Growth and development of somatic embryos

The growth and development stage until conversion to seedlings in secondary embryogenesis was carried out according to the Guiltinan and Maximova 2010 protocol, adapted to RITA® temporary immersion systems, four cycles of 5 min every 24 hours. The optimization was reported by Niemenak et al., 2008, using TIS, four cycles of 1 min, where they observed that embryonic development improved in the jump to the torpedo stage, obtaining the conversion to seedlings.

Somatic embryogenesis in cocoa

Since the first reports of somatic embryogenesis in cocoa from immature zygotic embryonic tissue (Esan 1975; Pence et al. 1979; Santos and Machado 1989), the regenerative pathway that is deduced as being of a non-clonal nature and that which is very frequently used both in angiosperms (Peña-Ramírez et al., 2011) and in gymnosperms (Salaj et al., 2019), or from nucellar tissue, the clonal pathway (Söndahl et al., 1993, Chatelet et al., 1992, Figueira and Janick 1993), efforts in cocoa have focused on floral pieces, which represent a clonal regeneration pathway in cocoa (Söndahl et al. 1993; Lopez-Baez et al. 1993; Alemanno et al. 1996; Li et al. 1998; Maximova et al. 2002; Tan and Furtek 2003, Minyaka et al. 2008a, b) due to the high degree of heterozygosity and variation in cocoa yield (Figueira and Janick, 1995, Irizarry and Rivera, 1998, Maximova, et al., 2002). Despite these efforts, the main problem continues to be the great genetic variability of this crop, which has repercussions on its ability to respond to in vitro culture and is recalcitrant to the development of a universal protocol as occurs in other botanical species such as the case of tobacco (Häkkinen, et al., 2018) and the model plant Arabidopsis thaliana (Che, et al., 2006). Our research followed the protocol of Guiltinan and Maximova, 2010, for which the frequencies of somatic embryogenesis reported may vary using other referenced proposals. Previous work in cocoa shows that the basal culture medium, the MgSO4 concentration, and the K2SO4/MgSO4 ratio can by themselves promote differentiation to embryogenesis in cocoa staminodes and petals (Minyaka et al., 2008a, 2008b, Tan and Furtek 2003). In addition, the carbon source seems to be decisive, which is influenced by the type of explant (Traore and Guiltinan 2006). Embryogenesis in cocoa has been
presented in medium free of growth regulators (Li et al., 1998). However, the use of auxin 2,4-D as an inducer of caulogenesis in cocoa is widespread in combination with a wide range of cytokinins reported for the first phase, BAP (Chatlet et al. 1992), KIN (Lopez-Baez et al. 1993; Alemanno et al. 1996), TDZ (Li et al. 1998; Maximova et al. 2002) and 2-iP (Lopez-Baez et al. 1993; Tan and Furtek 2003). For the second phase, differentiation, it commonly occurs in medium free of regulators (Lopez-Baez et al. 1993; Alemanno et al. 1996, 1997; Tan and Furtek 2003). An intermediate stage has been proposed in which a hormonal substitution is included, called secondary callus. Hormonal replacement consists of changing the type of cytokinin TDZ/KIN (Li et al., 1998), TDZ/BAP (Maximova et al., 2002, Niemenak et al., 2008).

Our research demonstrated that hormonal substitution is not determinant for the appearance of BAP/BAP somatic embryogenesis. Lopez-Baez et al., 1993 evaluated staminodes, petals, stamens and the base of the floral pieces as explants from which somatic embryogenesis was obtained; based on this, our research evaluated staminodes, petals, stamens and carpels, reporting embryogenesis from staminodes. Several studies favor the induction of embryogenesis from staminodes (Li et al. 1998; Maximova et al. 2002; Tan and Furtek 2003), while others use stamens fused with the staminodes as an explant (Alemanno et al., 1996, 1997). Traore and Guiltinan 2006 report that staminodes produce 3 to 10 times more somatic embryos than petals. Minyaka et al., (2008a, b), also report embryogenesis from petals. The variety of cocoa seems to be decisive, with Forastero being the most documented (Alemanno et al. 1996, 1997; Li et al. 1998; Maximova et al. 2002; Traore and Guiltinan 2006). The influence of the genotype is documented as a strong limitation in the appearance of this morphogenic phenomenon in cocoa (Alemanno et al. 1996, 1997; Li et al. 1998; Maximova et al. 2002; Tan and Furtek 2003; Traore and Guiltinan 2006; Minyaka et al. 2008a, b). Significant advances have recently been made with the use of genetic engineering to introduce key genes involved in embryogenesis, which can counteract the aforementioned limitations, low efficiency and genotype dependence, increasing the frequency of somatic embryogenesis and being useful molecular markers during genotype screening. Key transcription factors in cell totipotentiality, involved in signaling pathways and regulation of plant growth and development, including embryogenesis, are some of them, which have been used to improve somatic embryogenesis. The cocoa LEAFY COTYLEDON factor 2, TcLEC2, was proposed as a molecular marker to increase the frequency of somatic embryogenesis and for the tedious and necessary screening of embryogenic varieties (Zhang et al., 2014). Another transcription factor identified in Brassica napus BABY BOOM (BBM), involved in the proliferation, growth and development of plant cells in conditions free of plant growth regulators (Jha et al., 2018), whose orthologs have been identified in Arabidopsis thaliana AtBBM and cocoa TcBBM, the latter showed a significant increase in somatic embryogenesis in cocoa, however it limited its development to seedlings, when its stable overexpression was of a constitutive nature. Its transient expression resolved the limitation of seedling development, a strategy that prevents obtaining transgenic cocoa trees. This strategy can be coupled to the conventional tissue culture procedure (Florez et al., 2015). Recently, the strategy of using transcription factors was improved, regulating their gene expression with the use of specific inducers, the glucocorticoid receptor TcBBM-GR/TcLEC2-GR, inducing somatic embryogenesis in transgenic cocoa flowers and leaves (Shires et al., 2017, Fister et al., 2018). Due to the above, even when protocols that induce somatic embryogenesis have

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been proposed for a wide range of genotypes, many of them previously cataloged (Pokou et al., 2019), it is crucial to identify new ones with elite characteristics that show advantages in the field, in order to optimize the particular conditions of *in vitro* culture for each one of them, taking into account the season of the year, the physiological age of the floral pieces, the components of the basal culture medium, and the cytokinin used for each of the stages of somatic embryogenesis and for each of the embryogenesis pathways (Minyaka et al., 2008a, b).

**Conclusions**

Currently, in Mexico there is no catalog of elite genotypes that are highly embryogenic and that present natural resistance to diseases, characterized, geopositioned and reported in the literature, in order to implement large scale clonal propagation. Therefore, this research is strategic to implement clonal propagation and meet the demand for this valuable resource for this region of Mexico, being of great relevance to increase the frequency and rate of somatic embryogenesis, the cloning route par excellence in forest species due to the advantages it represents as being a method of clonal propagation of plants with normal dimorphic orthotropic architecture. The main contribution of this work is the creation of the first catalog of cocoa embryogenic genotypes located in the geographic region of the Papaloapan Basin, Mexico. In terms of experimental observations, we demonstrated that BAP specifically induces the regeneration of staminodia until the obtainment and development of embryos, which allowed the localization and identification of new embryogenic genotypes of *T. cacao* L. *var. Forastero*. In this case, secondary somatic embryogenesis produces more and better quality embryos than primary somatic embryogenesis. Under the same experimental conditions (BAP as cytokinin), *T. bicolor* does not have morphogenic capacity. Finally, we observed that TDZ does not induce somatic embryogenesis in both species.

**Abbreviations**

2,4-D 2,4-dichlorophenoxyacetic acid  
BAP 6-benzylaminopurine  
ED Expression medium  
GPS Global Positioning System  
PCG Primary callus growth medium  
SCG Secondary callus  
SEM Scanning electron microscopy  
TIS Temporal Immersion System
TDZ Thidiazuron

Declarations

Supplementary Information The online version contains supplementary material available at Supporting Information.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nancy Carmín Martínez-Hernández, Carlos Antonio Dávila-Figueroa, José Antonio Morales-Serna, Nelda Xanath Martínez-Galero and Enrique Villalobos-Amador. The first draft of the manuscript was written by José Antonio Morales-Serna, Karla Viridiana Castro-Cerritos and Enrique Villalobos-Amador. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animal performed by any of the authors.

References


**Figures**

*Figure 1*
Scale and general appearance of the process of establishing the *in vitro* culture of flower whorls of *Theobroma* spp. Flower bud of *T. cacao* Forastero variety (1 cm) collected one hour before opening, a process which begins in the late afternoon and ends before sunrise (collection time 07:00 am). Flower bud before and after disinfection (top and bottom respectively) a); aspect of flower bud dissection revealing flower whorls; petals, gynoecium (carpel, stigma, style and ovary) and androecium (stamens; anther and filament, and staminodes; filament) b); stamens (2 mm) (top) and gynoecium (4 mm) (bottom) c); staminodes (5 mm) (top) and petal (5 mm) (bottom) d); and initial *in vitro* culture in a 90 mm Petri dish of all floral whorls e). Scale, 0.5 cm to 1 cm.

![Diagram](image)

**Figure 2**

Efficiency of aseptic culture establishment under *in vitro* conditions of *T. cacao* flower whorls collected in the field using sodium hypochlorite T1 and calcium hypochlorite T2. Values represent the mean ± standard deviation. Means with the same letters per column are not statistically different (Tukey, 0.05).
Figure 3

Evaluation of the caulogenesis induced with BAP (1.4 µM) in the different flower pieces; staminodia, petals, stamens and carpels of *T. cacao*. Values represent the mean ± standard deviation. Means with the same letters per column are not statistically different (Tukey, 0.05).
Figure 4

Evaluation of necrosis in the different floral pieces; staminodia, petals, stamens and carpels of *T. cacao*. Values represent the mean ± standard deviation. Means with the same letters per column are not statistically different (Tukey, 0.05).
Figure 5

Evaluation of the rhizogenesis induced with BAP (1.4 µM) in the different flower pieces; staminodia, petals, stamens and carpels of *T. cacao*. Values represent the mean ± standard deviation. Means with the same letters per column are not statistically different (Tukey, 0.05).
Figure 6

Appearance of *in vitro* morphogenesis from *T. cacao* flower whorls. Calli induced in ED medium according to Guiltinan et al. 2003 a) and induced with TDZ (22.7 nM) b); expressing rhizogenic phenotypes (bar = 1 cm); BAP-induced embryogenic calli (1.4 µM) (bar = 1 cm), c and d); friable embryogenic calli in proliferation originated from staminodes where a zone of differentiation can be seen e); primary somatic embryogenesis obtained from the culture of staminodes, cotyledonary stage f); and abnormal somatic embryos, without cotyledons (left) and with more than two cotyledons (right). The white bar is the scale, 0.2 cm.
Figure 7

Evaluation of the embryogenic capacity induced with BAP (1.4 µM) in the different flower pieces; staminodia, petals, stamens and carpels of *T. cacao*. 
Figure 8

Summary of the response capacity to induction of somatic embryogenesis with BAP (1.4 µM) in the different floral pieces; staminodia, petals, stamens and carpels of *T. cacao*. Values represent the mean ± standard deviation.
Figure 9

Comparison between the primary and secondary embryogenesis of *T. cacao* Forastero var. Values represent the mean ± standard deviation. Means with the same letters per column are not statistically different (Tukey, 0.05).
Figure 10

Comparison of the embryogenic capacity in staminodia of the GPS3 genotype and cotyledons of the somatic embryos obtained from it.
Figure 11

General appearance of the conversion of secondary somatic embryos to seedlings of *T. cacao*. Images showing abundant asynchronous secondary somatic embryogenesis from cotyledons of primary somatic embryos of *T. cacao*, noting the characteristic oxidation of the original explant (arrow) a) and b); expression of secondary embryos in liquid ED medium under the RITA® temporary immersion system, 5 cycles of 5 min every 24 hours under dark conditions, noting the characteristic pink to reddish coloration of mature embryos c); conversion of secondary somatic embryos to seedlings in liquid PEC medium under the RITA® system, 5 cycles of 5 min, 12/12 hours photoperiod of 40-watt MAGG fluorescent white light (54 µmol.m-2s) and at 26 ± 2º C (note the change to green color) d); detail of the maturation state of the somatic embryo showing cotyledon with characteristic venation pattern e); somatic embryo f); compared with a typical immature zygotic embryo (22.5 mm) (g); somatic embryo exposed to light h) and i); detail of developing seedling in RD medium presenting the characteristic trichomes j); and seedling growing in tray (4.5x4.5x9cm) under shady house conditions. The white bar is the scale, 0.2 cm.
Figure 12

Characterization of the secondary somatic embryogenesis of *T. cacao* Forastero var. through SEM. General appearance of the asynchrony of differentiated secondary somatic embryogenesis from cotyledons a); globular stage b); elongated globular stage c); heart stage d); cotyledonary stage (normal somatic embryo) e); and cotyledon stage (abnormal somatic embryo with four cotyledons) f).

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