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OPTIMIZATION OF SOMATIC EMBRYOGENESIS PROCEDURE FOR COMMERCIAL CLONES OF *Theobroma cacao* L.

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ILHÉUS – BAHIA – BRASIL

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CLAUDIA YANET GARCIA ROJAS

OTIMIZAÇÃO DOS PROCEDIMENTOS DE EMBRIOGÊNESE SOMÁTICA PARA CLONES COMERCIAIS DE *Theobroma cacao* L.

Dissertação apresentada à Universidade Estadual de Santa Cruz, como parte dos requisitos para a obtenção do grau de Mestre em Genética e Biologia Molecular.

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DEDICATION

My dear Shirley Viviana, because you are the fire that warms my soul, I dedicate it.

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Claudia Garcia

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EXTRATO

GARCIA ROJAS, Claudia Yanet, C.Y., Universidade Estadual de Santa Cruz, Ilhéus, Setembro de 2016. O**timização dos procedimentos de embriogênese somática para clones comerciais de *Theobroma cacao* L.** Orientador: Prof. Dr. Alex-Alan Furtado de Almeida. Coorientador: Prof. Dr. Márcio Gilberto Cardoso costa.

Embriogênese somática é uma ferramenta com grande potencial em *Theobroma cacao* L. para a propagação em massa de plantas; no entanto, existem ainda muitos problemas a serem resolvidos. O principal deles é a produção de grandes quantidades de embriões com morfologias anormais que não conseguem desenvolver em plantas maduras. Há estudos moleculares em plantas modelo como Arabidopsis, onde foi observado o papel da metilação do DNA na regulação da expressão do gene e sua influência na produção de morfologias normais ou anormais em embriões somáticos. Além disso a metilação do DNA é um dos problemas epigenéticos mais encontrados na propagação da planta por cultura in vitro. A metilação de ADN desempenha um papel importante na capacidade da célula de planta para levar o processo de morfogénese, activando ou reprimindo os genes que a controlam. Variações somaclonais e padrões de metilação podem ser influenciadas principalmente pela acção da auxina 2,4-D. Este hormônio é conhecido por ter grande influência em alguns genes e fatores de transcrição que são altamente dependentes da atividade da auxina além de sua própria interação. E também estes genes têm funções importantes na expressão da embriogênese detectado em embriões zigóticos e somáticos nas suas fases de indução, desenvolvimento e maturação.

Portanto, o primeiro objetivo deste estudo foi avaliar e otimizar a produção de embriões somáticos em uma grupo geneticamente diversificado de genótipos de cacau. A resposta da embriogênese somática primária e secundária de oito clones de cacau promissores e um controle positivo foi avaliada utilizando versões modificadas de protocolos padrão. O segundo objetivo foi otimizar a eficiência da embriogênese somática primária para um clone de cacau com importância comercial o CCN 51, que tem provado ser muito recalcitrante com protocolos convencionais, em relação ao CCN 10 também incluído na nossa análise. A eficiência do processo foi avaliada pela quantificação do número de embriões somáticos produzidos a partir dos explantes de tecido somático usados, assim como a qualidade dos

embriões (normais vs. anormais) produzidos. Explantes florais foram submetidos a cinco passos de cultura de tecidos, cada um com 15-25 dias de duração. Apesar de todos os genótipos estudados produzirem embriões somáticos primários, a maioria deles foi originada somente a partir de calos marrom ou marrom-branco. No geral, as pétalas da flor tiveram melhor produção de embriões do que os estaminódios. O genótipo MCCS-14056 teve o melhor desempenho rendeu uma média de 7-10 embriões por explantes com calos marrons na embriogênese somática primária. Na embriogênese somática secundaria UF-613 foi o genotipo que apresento a maior producao de embrioides de 75-80 embriões usando kinetine como fonte de citocinina. As conclusões da nossa análise do piloto de produção a pequena escala são: 1) é possível alcançar uma elevada produção de plantas por embriogênese somática, embora a eficiência seja altamente dependente do genótipo; por conseguinte, é necessário otimizar o equilíbrio hormonal e fazer uma correta seleção do tipo de hormônio a ser usado, assim como o tipo de explante para cada genótipo; 2) através da utilização de embriogênese somática secundária, é possível aumentar a produção de embriões somáticos, pelo menos, dez vezes; 3) a variação da resposta observada entre genótipos podem refletir diferenças dos hormônios endógenos e os hormônios exogenamente fornecidos no meio de cultura, assim como o balanco hormonal entre a auxina e a citocinina e a concentracao de glicose no meio de inducao de embriogenese somatica. Discute-se a importância de adaptar o protocolo de cultura de tecidos para o genótipo.

Palavras-chave: Embriogênese somática, 2,4-D, competência celular, propagação, anormalidades, etileno, cacau.

ABSTRACT

GARCIA ROJAS, Claudia Yanet, C.Y., Universidade Estadual de Santa Cruz, Ilhéus, September, 2016. **Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L.** Advisor: Prof. Dr. Alex-Alan Furtado de Almeida. Co-advisor: Prof. Dr. Márcio Gilberto Cardoso costa.

Somatic embryogenesis is a tool with great potential in *Theobroma cacao* L. for mass propagation of plants; however, there are still many problems to be resolved. The principal one is a production of large quantities of embryos with abnormal morphologies which fail to develop into mature plants. There are molecular studies in model plants such as Arabidopsis where has been observed the role of DNA methylation in the regulation of gene expression and its influence in the production of normal or abnormal morphologies in somatic embryos. Also DNA methylation is one of the most epigenetic problems that has been found in plant propagation. DNA methylation plays an important role in the plant cell's ability to undergo morphogenesis by activating or repressing of genes that control it. Somaclonal variations and methylation patterns can be influenced mainly by the action of auxin 2,4-D. This hormone is known to have high influence in some genes and transcription factors that are highly dependent on auxin activity beyond their own interaction. And also these genes have important functions in the expression of embryogenesis detected in zygotic and somatic embryos in their induction, development and maturation stages.

Therefore, the first objective of this study was to assess and optimize somatic embryo production in a genetically diverse group 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 with 15-25 days in duration. Although all studied genotypes produced primary somatic embryos, most of them were

originated only from brown or brown-white callus. Overall, flower petals performed better than staminodes. The MCCS-14056 genotype had the best performance earned an average of 7-10 embryos per explant with brown calluses in primary somatic embryogenesis. In secondary somatic embryogenesis UF-613 genotype had the best result in embryos production with an average of 75-80 embryos per explant using kinetin as a source of cytokinin. 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 embryos production at least ten-fold; 3) the observed response variation between genotypes may reflect differences in endogenous and exogenously-supplied hormones, as well as hormone balance between auxin and cytokinin and glucose concentration in the medium to induce somatic embryogenesis. The importance of adapting the tissue culture protocol to the genotype is discussed.

Keywords: Somatic embryogenesis, 2,4-D, cellular competence, propagation, abnormalities, ethylene, cacao

1. INTRODUCTION

Theobroma cacao L. is a tropical understory tree originating from the Amazon and Orinoco valleys [1]. 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 [2]. 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 [3]. By the late 1980s, annual Brazilian production decreased to 291,868 tons [4], primarily due to outbreaks of devastating cacao diseases, in particular witches' broom (*Moniliophthora perniciosa*), which caused massive economic loss and much social distress [5]. In response to this crisis, numerous government agencies and universities were enlisted to develop disease-resistant, high-yielding cacao clones [6].

Once superior clones are developed, large-scale propagation is required. Cacao is traditionally propagated using rooted cuttings or grafting. The use of rooted cuttings is constrained by biology: a cacao tree has two types of branches: orthotropic (vertical growth ones that generate trees of desirable architecture, but which are limited in number; the second type are plagiotropic branches with characteristic horizontal growth. Although more numerous, plagiotropic grafts produce trees of undesirable architecture that require time and labor to obtain desirable trees [7].

One 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 [8]. 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 [9].

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) [10], the molecular mechanisms underlying the complex cellular reprogramming required to achieve totipotency are just beginning to be elucidated [8, 11]. Luckily, early identification of critical parameters controlling SE induction, such as endogenous and/or exogenously supplied plant growth regulators (PGRs, e.g., 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 [8]; 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 requiring 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 generated somatic embryos, and 4) efficient conversion of somatic embryos into plantlets.

In early cacao studies [12, 13], somatic embryos were generated from a variety of somatic cell type explants, although yields were low. The first moderately successful cacao SE protocol was developed by [14]; 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 embryogenesis; the diverse genotypes produced primary somatic embryos (PSEs) at very different rates. Explant response (callus growth) ranged from 1-100%, and the number of somatic embryos per responsive explant ranged from 1-46. This basic cacao SE protocol was further optimized by [15], 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 [16], using Murashige and Skoog

(MS) salt-based [17] in 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 [4]. 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 [18, 19, 20]. To enhance the production efficiency of primary somatic embryogenesis (PSE) and plant regeneration, we attempted to further optimize cacao SE protocols.

This study had the following biological questions: (i) Why do somatic embryos show abnormal morphologies? (ii) Is there influence of hormone balance (auxin-cytokinin) in the number of abnormal embryos produced? (iii) Is there influence of glucose concentration in the number of abnormal embryos produced? Furthermore, it presented the following hypotheses: Hypothesis H01: Glucose concentration does not significantly affect the production of somatic embryos of *Theobroma cacao* L.; Hypothesis H1: Glucose concentration significantly affect the production of somatic embryos of *Theobroma cacao* L.; Hypothesis H02: 2,4-Dichlorophenoxyacetic acid exogenous levels do not significantly affect normal and abnormal morphologies of somatic embryos of *Theobroma cacao* L.; Hypothesis H2: 2,4-Dichlorophenoxyacetic acid exogenous levels significantly affect normal and abnormal morphologies of somatic embryos of *Theobroma cacao* L.; Hypothesis H03: There is not a relation between hormone balance from 2,4-D and TDZ and abnormalities in *Theobroma cacao* L. somatic embryos; Hypothesis H3: There is a relation between hormone balance from 2,4-D and TDZ and abnormalities in *Theobroma cacao* L. somatic embryos.

1.1. Objectives

1.1.1. Overall

1) Evaluating a pilot scale the production 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;
2) Improving the yield (increase the number of normal embryos/explant) and quality (normal vs. abnormal) of PSE generated from two cacao clones, *Colección Castro Naranjal-51* (CCN 51) and *Colección 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.

1.1.2. Specific

1) Identify the best source of explants for primary somatic embryos production;
2) Evaluate the influence of hormonal balance and glucose concentration in the production of abnormal embryos;
3) Quantify and calculate the rate of production of primary normal and abnormal embryos;
4) Calculate the rate of plant conversion from normal secondary somatic embryos.

2. LITERATURE REVIEW

2.1. *Theobroma cacao* L.: Overview

2.1.1. Botanical and genetic aspects

Theobroma cacao L. belongs to *Theobroma* genus classified by Linnaeus in 1753 and it is also a Malvaceae family member [21, 22].Though this genus contain 22 species where *T. cacao*, *T. grandiflorum* and *T. bicolor* are the most important species known, *T. cacao* is the only one widely used in the chocolate industry as a raw material around the world [22]. *T. cacao* (2n = 20) is an allogamus and perennial plant that was morphogeographically classified by Cheeseman in 1994 in Criollo, Forastero and Trinitario (hybrid between Criollo and Forastero) [22]. However, in 2008 was published a new classification by Motamayor where were found 10 new groups: Marañon, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional and Guiana [1]. The first report about the center of origin of cacao was the Upper Amazon near the Colombia-Ecuador border and there is new evidence that support the hypothesis of Amazon species diversification. Thus, it is believe that the cacao center of origin is South America, specifically the Amazon basin in the border region between Brazil, Colombia, Ecuador and Peru where high cacao diversity is found. Criollos are the only genetic group found in Central America showing a significantly lower diversity [1].

Morphologically, *T. cacao* has a dimorphism in the vegetative organs. The main stem and the adventitious orthotropic shoots have a radial structure, and the plagiotropic branches are monopodial and dorsiventral. The trunk is sympodial. When the plant reach a height of 1-2 m, the vertical growth ceases and, on the terminal end of the stem, five buds with short internodes grow forming the jorquette [22, 23]. The leaves show dimorphic characters depending upon the different types of stem from which they arise. The first leave on the chupons have long petioles and are symmetrical. The leaves on the fan branches have shorter petioles and are slightly asymmetrical. Leaves color may change as they age. The young

leaves can be light green or red to brown, but all the types turn dark green when they reach their mature age [22, 23, 24].

Cocoa plants possess hermaphrodite's flowers, which means that the cocoa flower has male and female sexual organs. Cocoa flowers are on the trunk and branches, habit referred as cauliflorous plants. The flowers are borne on long pedicels on wood of two or three years old trees under good growing conditions. They are small with about 15 mm in diameter and have five free sepals, five free petals, 10 stamens (five fertile and five non-fertile) and ovary of five united carpels. The fertile stamens bear two anthers which lie in the pouch of the corresponding petal. The petals have a shape that is strait at the base but expand into a cup-shaped pouch and end in a broad ligule. Cocoa trees produce large number of flowers sometimes two times a years or sometimes all the year around. It would depend of the geographic condition and genotype. In geographic regions as West Africa and Brazil, flowering occurs mainly between January and June [22, 23, 24].

The cocoa fruit is a pod with the inside seeds embedded in mucilaginous pulp. The seeds number and colors depend of the genotype. Pod production starts when trees are 3 years old, reaching maximum productivity when the trees are between 20-40 years old [24]. The seed is formed after flower fecundation, however, self-incompatibility is observed in some cacao genotypes. Even though the pollen tube grows normally, the mechanism of abortion occurs during the fecundation of the ovule and it seems to be regulated by auxin pathways [23,24,25]. The cacao zygotic embryos are formed between 9 – 21 weeks after pollination. Ten weeks after pollination, the embryos start developing and, they pass throughout different stages: globular, heart, torpedo and cotyledonary [26,27]. The mature seed is extracted from the pod, fermented and dried to produce the chocolate and his derivatives.

2.1.2. Cultivation, propagation and economic importance

Mexico city the capital of the Aztecs was discovered by Hernán Cortes in the sixteenth century where, for the first time cocoa beans were used to make a special drink: the "chocolatl" prepared by roasting the beans, then crushing them and mixing with vanilla, chili and maize meal. Chocolatl was consumed by Montezuma and his court in largest quantities,

but the cacao was not cultivated in this unsuitable region for the cocoa tree, they always received the beans from Mayas who send the beans as a tribute to the capital [24].

Historically, the origin of cultivation of "wild" cacao was in the north of Yucatan peninsula, specifically in the Yucatec Maya zone (in the sacred groves**)** where cacao was grown by the ancient Mayas [28]. Probably the "wild" cacao may be an ancient Criollo that his individuals represent the original Criollo group. Molecular studies using microsatellites suggest that probably originated from a few individuals in South America that may have been spread by humans within Central America [29]. Although cacao Criollo was the first genetic group domesticated by humans, nowadays, it is not the most widely cultivated in the world. Amelonado and mixed hybrid types are commonly known in the old classification as Forasteros. Forastero is the principal genetic group that ~~it~~has been cultured in West Africa, the largest producer of cacao beans with 73% of the world production [30, 31].

The cacao tree is grown in the tropics, specifically in the zone called "Cocoa Belt", with most of the production in a band within 20° south or north of the equator with altitudes between 300 – 1,200 m. Cacao requires hot, moist conditions to grow. Farmers need to protect the culture from wind**,** direct sun, pests, and diseases. Prolonged drought conditions seriously damages the vegetative parts of the trees and their reproductive functions [32]. The environment of cacao farms has two important components such as climate and soil. The temperature range in cocoa-growing areas lies on average between a maximum of 30 – 32°C and a minimum of 18 – 21°C, annual rainfall is between 1,200 – 3,000 mm depending of the country. Relative humidity in coca-growing areas is high (100%) at night and (70 – 80%) by day. In dry days, the relative humidity falls to 50-60%. The amount of radiation on the cocoa tree will affect its growth and yield and that depend of the cocoa-growing area too. For example, radiations between 0,86 – 1,38 W m^{-2} are found in cocoa-growing counties such as Brazil, Ecuador, Ghana and Trinidad [24].

Cocoa has been known as a crop grown largely by smallholder farmers in the lowland tropics, including Latin America (Belize, Mexico, Ecuador, Peru, Costa Rica, Colombia, Venezuela and Brazil), West Africa (Cote d'Ivoire, Cameroon, Ghana, Nigeria, and São Tome), and Indonesia (Sulawesi, Central Sumatra) with 70% of total global cocoa production. Between 2013 and 2014 Cote d'Ivoire was reported as the first worldwide cocoa producer

with 1,449 thousand tons per year and Ecuador the first one with 192 thousand tons per year in the American continent leaving Brazil in second place with 185 thousand tons per year [30]. Until the 1980s Brazil was known as the highest cacao bean producer in South America and the second highest producer worldwide, with an average production of 400,000 tons per year [3]. By the late 1980s, annual Brazilian production decreased to 291,868 tons [4], primarily due to outbreaks of cacao diseases, particularly witches' broom which caused severe economic, ecological and social losses [5].

Cacao farmers use variable systems of production. Some authors described three principal cacao production systems such as *rustic shade* (in Latin America, West Africa and Indonesia), *cabruca* (in Brazil) and *full-sun* (in Brazil and Ecuador). *Rustic shade* and *cabruca* are similar traditional systems of cacao production, where primary or secondary forests are thinned and cocoa is planted beneath the remaining canopy of native tree species or under planted shade or "canopy" which is often composed of planted fruit or woody trees rather than remnants of the native forest [2]. Although *rustic shade* and *cabruca* are both agroforestry systems that maintain production and were evaluated as eco-friendly cacao production possibilities. However, farmers have contributed to the destruction of the tropical rainforest in production countries by practicing deforestation, which results in additional greenhouse gas emissions and the current climate change and global warming [33].

As a joke says; *"A new British survey has revealed that 9 out of 10 people like chocolate. The tenth lies. – Robert Paul –"* [34] cacao is the unique source of cocoa butter and powder for the 200 billion USD global chocolate business and it is cultivated around the world and everyone loves it [31]. About 5–6 million smallholder farmers gain most or all of their income from cocoa production. This aspect makes cacao a crop with a huge social and economic importance. But it is in danger of disappearing due to fields that abandoned be may converted into pasture or full-sun coffee when the beans' price is low or fluctuating. Also, due to lack of farmer organization and market power, small size of farms, uncertainty of land tenure, sharecropping, low productivity, lack of infrastructure and access to market and market information. Additionally, when the prices improve cocoa farmers do not have enough planting materials with good genetic quality to plant or they plant new trees often by clearing new forest rather than replanting existing cleared areas [35].

Cocoa beans have a worldwide demand. If a retrospective is performed, from 2000 until 2014 the consumption and production of the product have increased more than 33% in equal proportions year after year [36]. However, the demand keeps growing and production decays because of old plantations, problems of diseases and insects, high degree of genetic heterozygosity, problems of abiotic stress, low prices and decreased land with capacity to establish cultures. As a result, market experts and industry as a whole expect a substantial deficit between supply and demand in 2020 onwards, unless measures are taken to solve the main problems [37]. As a consequence, it is necessary to start implementing strategies, for example, establishing new plantations and restoring existing plantations, which need a lot of young plants with improved homogeneous genetic characteristics and organoleptic quality productions that meets market needs.

Currently there are traditional methods to propagate cocoa plants, that is, by seeds, rooted cuttings, budding, grafting and the latest hypocotyledonary grafting. These methods require access to large amounts of plant material to supply seeds, branches and rootstocks, and in vegetative methods the percentages are low (in average 35-85% depending of the methodology). Also, it is necessary large facilities such as nurseries for the establishment and adaptation of the new plants which make these methods costly. Furthermore, the capacity to produce seedlings in large scale is still limited in several important cocoa-producing counties [7,35,37].

In the search for solutions to increase cocoa seedlings supply, organizations such as Penn State University, USDA, Nestle and Mars Inc., among others, have developed protocols for mass propagation of cocoa plants by somatic embryogenesis (SE) with good results, [37]. Research on somatic embryogenesis of cacao, made for over 30 years; still presents many problems to solve. One of the most important is the low conversion of embryos into plantlets that is a result of high production of abnormal embryos with morphological characteristics that fail to develop into mature plants. To solve this problem it is necessary to know the mechanisms that allow proper morphogenic development of the embryos [37].

2.2. Somatic Embryogenesis

2.2.1. Background information

Somatic embryogenesis is an important developmental process in which somatic embryos originate from differentiated somatic cells that restructure themselves to obtain embryogenic competent cells under suitable *in vitro* conditions. This process does not involve meiosis or fertilizations and it is naturally known as apomixis. The genus most known with apomixis reproduction is *Bryophyllum*. Species of this genus reproduces asexually via new shoots from leaves. Plants derived from SE are genetically identical to the parent plants and are called clones. Somatic embryos may be derived from a single competent cell or a group of them, and these cells may be genetically pre-determinate or not [15].

Somatic embryogenesis as a morphogenetic event is the proof that plant cells are totipotent. Induction and SE expression is only possible if the somatic totipotent plant cells have acquired the necessary skills to meet the embryonic signs and start the process of embryogenesis [38]. For expression of SE several sequences of events are necessary as a morphogenetic phenomenon that occurred after different phases or stages, which can be characterized by distinct biochemical and molecular events [39]. The first and one the most important event in SE is the induction phase where cells acquire differentiated somatic embryogenic capacity by direct or indirect ways [40]. Direct SE is known as a developmental embryo process without callus formation as a previous step. Indirect SE needs a callus formation step before embryos production. Two types of inductive conditions are recognized that permit differentiated cells become undifferentiated competent cells, these are: 1) internal and/or external plant growth regulators levels and 2) stress factors as osmotic shock using substances having different concentration changes (sucrose, polyethylene glycol, abscisic acid etc), dehydration culture medium, water stress, ions of heavy metals, changes in pH of the culture medium, cold treatments and thermal shock, hypoxia, antibiotics, ultraviolet radiation and chemical or mechanical treatments [41, 42].

Auxins alone or in combination with cytokinins are considered to be the most important PGRs in cell division and differentiation, as well as induction of SE. In the indirect SE induction, 2,4-D plays an important role as a cell stressor and possibly in reprogramming cells by DNA methylation. There is evidence that SE follow highly organized patterns of methylation/demethylation of DNA, and acetylation/ deacetylation of histones were observed during stages of cell dedifferentiation/differentiation [41, 43]. In carrots, citrus fruits, coffee and olive, most systems studied, it was confirmed that 2,4-D is required to start the development of somatic embryo[44, 45]. It is possible that 2,4-D plays a role in cell polarity and asymmetric cell division, which is important because causes different changes in morphology, physiology, metabolism and gene expression in cells triggering epigenetic changes while somatic embryogenesis is starting or developing [8,46].When we talk about epigenetic changes, we are talking about those events that switch genes on and off (gene regulation) and affect how cells read genes instead of being caused by changes in the DNA sequence. The most important epigenetic events in SE are DNA methylation, histone modification or chromatin remodeling. [8]. In addition to the importance in the treatment of tissues with auxins to induce SE, it is important to make mention that the frequency of somatic embryogenesis induction also depends on the species, genotype, type of tissue, stage of development and physiology of the donor plant and endogenous hormone levels as well as the components of the medium (inorganic and organic compounds) that are used at this stage [8].

Somatic embryos' formation, as explained before, starts with an appropriate stimulus in cells to induce the SE. This event is followed by the initiation stage of SE in which pro-embryos start developing. The last event is maturation, where somatic embryos anticipate germination by desiccation, and accumulation of important substances required for normal plant regeneration [47].

2.2.2. Zygotic and somatic embryogenesis comparison

In Angiosperm sexual reproduction by zygotic embryogenesis (ZE) occurs following double fertilization in the female gametophyte found in the ovule of the flower. Fertilization gives rise to the embryo and endosperm compartments of the seed. Haploid cells from anthers

and ovules have been fused by double fertilization when the male pollen tube containing two sperm cells enters the ovule. Haploid sex cells that are product of meiosis and mitosis form the single-celled diploid zygote, which then undergoes cell division and pattern forming events to give rise to the diploid embryo by mitosis. The other haploid sperm cell fuses with the diploid central cell nucleus of the embryo sac initiates divisions to form triploid endosperm that provides resources to the developing embryo. Ovule tissues that surround the embryo and endosperm contribute to the seed coat [48]. ZE could be divided into three events: (1) embryogenesis *sensu strict* (after fertilization the morphogenesis of embryo and endosperm), (2) a growth period in which cotyledonary embryos reach their final form and size, and (3) a maturation period in which embryos accumulate protein and starch reserves, then they lose water until they get 70% of dehydration, and underwent a modification in soluble sugar composition to prepare the embryo for germination and conversion in plantlet [8].

Asexual embryogenesis can occur without fertilization, both *in vitro* and *in vivo*. Either somatic or zygotic cells are able to differentiate into embryos *in vitro* under the effect of the application of plant growth regulators or stress treatments [49]. SE *in vitro* is possible to induce in anther, isolated microspores, cultured ovules or ovaries applying various stress treatments such as cold/heat shock and starvation. The resulting embryos are haploid, possessing either maternal or paternal chromosomes depending on the gametophytic precursor cell. Haploid plants product from SE *in vitro* is a powerful mechanism to generate homozygous lines much faster that using conventional breeding. Treatments with colchicine can induce chromosome doubling of haploid embryos resulting in homozygous double-haploid plants which are useful tools in trait discovery and plant breeding applications [48]. As well, as it was explained before, SE also can be induced in vegetative explants or cells following treatment with PGRs or stress such as osmotic shock, dehydration and alteration of pH among others. Plantlets from SE *in vitro* are genetically identical to precursor material and this process is known as *in vitro* cloning [48]. SE is a process that starts with an induction stage in which the cells are reprogrammed to produce embryos controlled by exogenous signals. After the induction stage the pro-embryogenic cells go into an expression stage where the newly differentiated embryonic cells develop into embryo without any further exogenous signals [8, 48].

Asexual embryogenesis also occurs naturally in some plant species *in vivo*, from either ovule cells or somatic leaf tissues as part of a process defined as apomixis [48]. There are two mechanisms in apomixis known as gametophytic and sporophytic based upon the location of the precursor cell which develops into the embryo. In gametophytic apomixis or parthenogenesis an egg cell found inside an embryo sac that has formed mitotically without prior meiosis and is also chromosomally unreduced. Then, this mature egg cell directly develops into embryo. Sporophytic apomixis is also called as adventitious or nucellar embryony, embryos develop without fertilization directly from diploid ovule cells surrounding an embryos sac. Apomixis can happened in surface of plant tissue in nature. Somatic embryos can arise in leaves surface as happened in plants of the genus *Kalanchoë.* The *Kalanchoë* plantlets arise following proliferation of cells described as dormant meristem that are found in notches along their leaf margin. The embryos resulting from SE are diploid and genetically identical to the somatic cells from which they were formed [48, 49].

Embryo morphologies in SE or ZE in dicot plants follow the same development with well-described stages. The first stage is the globular stage; the embryo is spherical and attached via the suspensor to the maternal tissue. In the second stage takes place the formation of cotyledonary primordia in the spherical-shape embryos and is developed the heart-shape embryos. Following the longitudinal expansion of the cotyledons, hypocotyl and root tip gives the embryo its torpedo shape allowing, at the final stage, development into the cotyledonary embryo [50]. Both SE and ZE start embryo development with asymmetrical cell division (but sometimes in SE it is not always the case) resulting in a more elongated basal cell that gives rise to the suspensor and the hypocotyl and a small apical cell that generates the embryo. The suspensor has a role in cell polarity and conducts nutrients to the developing embryo, then it is eliminated by programmed cell death between globular and torpedo stage in angiosperms and in late embryogenesis in gymnosperms. Also the suspensor could be the source of plant hormones as auxins, predominant PGR that has been reported to be involved in polarity and pattern formation especially in the PIN formed proteins regulation [51]. These proteins are directly involved in the regulation of cell polarity process in early embryogenesis [51]. Cytokinins and brassinosteroids, another plant hormones, were also reported to be important in polarity cell process as auxins, but the mechanism are not clearly resolved yet [52].

Biochemical comparison between somatic embryos and zygotic embryos, mainly analyses of major storage compounds (e.g. proteins, carbohydrates, and lipids) are found. Somatic embryos are able to accumulate storage proteins as zygotic embryos but in much lower concentrations and the protein composition can be different between them depending of the stage of embryo development (e.g. globulins, cytosolic glutamine synthase). Another types of proteins like arginases are expressed in somatic embryos indicating that storage proteins breakdown obviously started before germination. It is possible that storage protein accumulation is improving by abscisic acid (ABA) treatments for example in *T. cacao* SE [26] or by increasing sucrose concentrations in maturation media for *Pinus strobus* [48].

Analyses on carbohydrates and lipids concentrations among somatic embryos and zygotic embryos point to the fact that somatic embryos often contained lower total amounts of soluble sugars as well as lipids, in later stages and its derivatives that are considered to be important for desiccation tolerance in maturation stage. In consequence, maturation in SE is the major bottleneck for somatic embryogenesis in several species [48].

Embryo-specific genes expression and signaling regulation are another significant similarity between SE and ZE. Many publications in these aspects reported that transcription factors, PGR-related proteins, cell cycle-related proteins, cell wall-related proteins and proteins involved in signaling are similarly expressed in SE and ZE in different species as cotton, cacao and *Cyclamen persicum* for example [27,53,54]. In *Arabidopsis* and others species the well-characterized transcription factors affecting embryogenesis comprise the *LEAF COTYLEDON* genes (*LEC1* and *LEC2*), *BABY BOOM* (*BBM*), *WUSCHEL* (*WUS*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*), *FUSCA3* (*FUS3*), *YUCCA*, *LEA*, *AINTEGUMENTA-like* (*AIL*) and *ENHANCER OF SHOOT REGENERATION* (*ESR1*) between others [50, 55, 56, 57]. In *Theobroma cacao* L., transcriptome analyses comparing somatic and zygotic embryos identified numerous differentially regulated genes (e.g., *TcSERK, TcBBM, TcABI5, TcABI3, TcLec1, TcLec2, TcWRI1, TcAGL15, and TcFUS3*) whose *Arabidopsis thaliana* orthologues play important roles in embryo development [27]. In recent publications, it is reported that the most abundant transcription factors involved in SE are those involved in stress responses, synthesis of auxin, polyunsaturated fatty acids, and secondary metabolites that were more strongly expressed in somatic embryos relative to ZEs [27, 58].

2.2.3. Somatic embryogenesis in *Theobroma cacao* L.

Cacao is either traditionally propagated sexually from freshly harvested seed or asexually using rooted cuttings or grafting. The use of rooted cuttings is constrained by biology: a cacao tree has two types of branches: orthotropic shoots with vertical growth that generate trees of desirable architecture, but which are limited in number; the second type are plagiotropic shoots with horizontal growth. Although more numerous, plagiotropic grafts require time and labor investments to produce trees of desirable architecture [7].

In parallel, new methodologies for asexual cacao propagation have been developed since 1954 by tissue culture. Propagation through organogenesis in *T. cacao* was reported for the first time by several authors where they used stalks, fragments of young orthotropic stems, apices, nodes and roots as a source of explants but all the attempts of regeneration failed [59]. Furthermore, propagation through somatic embryogenesis also has been strongly studied since 1975 until these days using several sources of explants as immature zygotic embryos, young leaves, nucellar tissue and finally floral explants [45].

In the late 1980s and 1990s considerable efforts have been made in tissue culture for production of cocoa plants by ES. These new procedures are complicated in its execution because they use different hormones in each different step (e.g. embryos germination and their conversion to plants) having many problems. Besides, the source of explant used (immature zygote embryos) was not suitable for initiation of cultures *in vitro* because it could lead to genetic variation in results [60, 61]. In early cacao studies (e.g., Sondahl et al., 1989, 1993), somatic embryos were generated from a variety of somatic cell type explants, although yields were low [12, 13]. The first moderately successful cacao SE protocol was developed by Li et al. in 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 embryogenesis; the diverse genotypes produced primary somatic embryos (PSEs) at very different rates. Explant response (callus growth) ranged from 1-100%, and the number of somatic embryos per responsive explant ranged from 1-46 [14].

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 primary somatic embryos (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 [15]. Yet another SE protocol (using flower petals) was developed by Lopez-Báez (2001) [16], using Murashige and Skoog (MS) [17] salt-based induction medium that included testing of carbohydrate (sucrose, glucose and maltose), PGRs (2,4-D or 2,4,5-T and kinetin), in 12 diverse genotypes [16]. 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 [18, 19, 20]. Besides the problem with genotype-independence, there are also morphology problems. Studies by CATIE using different protocols resulted in high variations in embryo expression and the propagation rates continued to be low due the high production of abnormal primary and secondary embryos. The principal abnormalities presented in cacao SE are predominant four types (fusion of two or more hypocotyl, lack of apical and root meristems, excess cotyledons growth and malformed hypocotyl. Those problems explain the low conversion rates of embryos in plants [18, 19].

The most recent work in propagating cocoa seedlings was the patent published by Nestec SA [62] using temporary immersion system and liquid culture. However, all these protocols use 2,4-D or 2,4,5-T as a source of auxin in the culture media to stimulate the embryogenic capacity in tissues via indirect embryogenesis. The use of these PGRs may have a negative effect on low conversion rates of somatic embryos into plants and higher amounts in the production of abnormal embryos. These PGRs are highly mutagenic. I have been reports in several SE procedures for different crops that it may generate somaclonal variation and morphological abnormalities [63, 64, 65].

3. CONCLUSIONS

In conclusion, our first set of experiments demonstrate that SE production is influenced by genotype and source of donor explant, PGR type and balance in 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.

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 the type and concentration of PGR is varied. 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 sources 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 on this theme using bio-reactors with no more than 1 L capacity with good results, but it is necessary to improve this process for large-scale production.

For the experiments that focused on CCN 51 and CCN 10, SE production was highest using a high concentration of glucose (80 g L^{-1}) in the induction medium. 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 low concentration of 2,4-D with combination of high concentrations of TDZ; whereas

CCN 10 showed more normal SEs productivity with high concentrations of 2,4-D in combination with high concentration of TDZ.

Embryogenic induction and the production of normal embryos appear to be influenced by the balance of applied and endogenously-produced PGRs. 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. Any variation in endogenous auxin levels would likely impact embryogenic capacity, and further compound underlying genotype-to-genotype variation in response.

4. NEXT STEPS

The present work is a part of macro-project from MARS Company in optimization of somatic embryo procedures in a genetically diverse range of cacao genotypes. The project **"Somatic embryogenesis in *Theobroma cacao* L.: Normal and abnormal morphotypes, DNA methylation profiles and somaclonal variation"** that was proposed in this master degree program is divided in three parts. The first part concerns the analysis of the influence of hormonal balance in the interaction with glucose concentration in the production of abnormal primary somatic embryos, this is the information that I present here in this dissertation with the title of **"Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L."** The second part of the project is a characterization of normal and abnormal secondary somatic embryos by microscopy analysis and DNA methylation profiles using the technique of bisulfite-sequencing of genomic DNA. And finally, the third part is SNP analysis for detection of mutations between normal and abnormal somatic embryos**.**

The next steps in this project is keep doing the experiment and write two manuscripts about the results from: "characterization of normal and abnormal secondary somatic embryos by microscopy analysis and DNA methylation profiles using the technique of bisulfite-sequencing of genomic DNA", and "SNP analysis for mutant detections between normal and abnormal somatic embryos".

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