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Factibilidad técnica y económica para el escalado de la tecnología productiva de plántulas de cacao (*Theobroma cacao* L.) a nivel de Biofábrica.

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Sé firme en tus actitudes y perseverante en tu ideal. Pero sé paciente, no pretendiendo que todo te llegue de inmediato. Haz tiempo para todo, y todo lo que es tuyo, vendrá a tus manos en el momento oportuno.

M. Gandhi

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Una tesis es un camino que implica cambios en la persona que la realiza, y que requiere del apoyo de muchas personas para lograr el camino acertado. En mi caso, estos años han vislumbrado el evento más importante de mi vida que fue el nacimiento de mi hija, por lo que podría decir que soy una persona totalmente diferente al momento en el que empecé.

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Resumen

Factibilidad técnica y económica para el escalado de la tecnología productiva de plántulas de cacao (*Theobroma cacao* L.) a nivel de Biofábrica.

El cacao (*Theobroma cacao* L.) es un cultivo comercial en muchos países en desarrollo y provee la materia prima para la producción de chocolate. A medida que aumenta la demanda de alimentos, se requiere establecer cultivos óptimos y en este sentido, la producción de plantas a escala es necesaria para satisfacer la creciente demanda, convirtiéndose los sistemas de propagación de plántulas en un reto para el fortalecimiento agroindustrial. Para hacer frente a este obstáculo, se plantea el método de la embriogénesis somática (ES) y realizar la transición de una escala de investigación a una escala comercial. El tiempo estimado para la producción suele estar subestimado, lo que resulta que sea inviable para enfrentar los mercados. En el primer capítulo se presenta la producción del genotipo *CCN51* a escala piloto en laboratorio con miras a la reducción de tiempo en el proceso. Se lograron definir las diferentes etapas de producción de plántulas por SE: iniciación, multiplicación, maduración, germinación y aclimatación. El tiempo mínimo obtenido para la producción de *CCN51* fue de 30, 70, 50, 70 y 30 días respectivamente. Con el fin de continuar habilitando técnicamente el sistema de propagación vía ES fue necesario evaluar la fidelidad clonal de las plántulas regeneradas como un primer paso para responder a la calidad. En el capítulo 2 se estudió la estabilidad genética de plántulas de cacao propagadas por SE respecto al injerto convencional para los genotipos *CCN51* y *TSH565* usando 13 microsatélites (SSR). Los 13 loci analizados revelaron 25 alelos en el genotipo *CCN51* y 24 alelos en el genotipo *TSH565*. De acuerdo con los resultados, no se observaron diferencias en la composición alélica, esto indica que las plantas propagadas por SE no mostraron detrimento perceptible en su genoma con los SSR utilizados. Seguidamente, con miras a la producción a mayor escala como laboratorios comerciales o biofabricas, es necesario interconectar la propagación con una

gestión eficaz y eficiente del proceso productivo a escala industrial. En el capítulo 3 se presenta un sistema de control y planificación de la producción (PPC) para la multiplicación por SE a través de un piloto de producción de 100.000 plántulas para *CCN51*. Se detalla los principales indicadores del proceso en materiales, mano de obra, calidad y rendimiento. Finalmente, considerando la ES como una tecnología que suele ser costosa en comparación con otras técnicas de propagación, en el capítulo 4 se identificaron los factores claves en la viabilidad financiera para el proceso productivo. Se estimaron los costos del proceso, identificando los factores que influyen en el proceso con una Simulación de Monte Carlo (MCS). Los componentes del costo identificados fueron medio de cultivo (CM), costos indirectos de fabricación (IMC), mano de obra (directa e indirecta) y gastos operativos (GO). La mano de obra tuvo la mayor participación de los costos, con un 53%, seguida de los GO, con un 30%, CM, con un 12% e IMC, con un 5%. La MCS ayudó a definir que las variables con mayor impacto en el precio unitario fueron la respuesta de los embriones en la etapa de germinación y el coeficiente de proliferación. Esta proyección arrojó una cifra de US $ 0,73 por plántula.

Palabras clave: Análisis de costos, Cultivo de Tejidos Vegetales, Embriogénesis somática, Fidelidad genética, Propagación comercial, Simulación Monte Carlo, Tiempo de producción.

Abstract

Technical and economic feasibility for the scaling of the productive technology of cocoa plantlets (*Theobroma cacao* L.) at the Biofactory – commercial laboratory.

Cocoa (*Theobroma cacao* L.) is a cash crop in many developing countries and provides the main ingredients for chocolate production. As the demand for food increases, it is necessary to establish optimal crops and, in this sense, the production of plants on a scale is necessary to meet the growing demand, making propagation systems a challenge for agro-industrial strengthening. To overcome this obstacle, somatic embryogenesis (SE) is proposed and the transition from a research scale to a commercial scale is proposed. The estimated time to produce is usually underestimated, which results in it being unfeasible to face the markets. The first chapter presents the production of the *CCN51* genotype on a pilot scale in the laboratory with a view to reducing time in the process. It was possible to define the different stages of seedling production by SE: initiation, multiplication, maturation, germination, and acclimatization. The minimum time obtained to produce *CCN51* was 30, 70, 50, 70 and 30 days, respectively. To continue technically enabling the propagation system via ES, it was necessary to evaluate the clonal fidelity of the regenerated seedlings as a first step to respond to quality. In chapter 2, the genetic stability of cocoa seedlings propagated by SE with respect to conventional grafting was studied for the *CCN51* and TSH565 genotypes using 13 microsatellites (SSR). The 13 loci analyzed revealed 25 alleles in the *CCN51* genotype and 24 alleles in the TSH565 genotype. According to the results, no differences were observed in the allelic composition, this indicates that the plants propagated by SE did not show perceptible detriment in their genome with the SSR used. Next, with a view to larger-scale production such as commercial laboratories or biofactories, it is necessary to interconnect propagation with effective and efficient management of the production process on an industrial scale. Chapter 3 presents a production planning and control system (PPC) for SE multiplication through a pilot production of 100,000 for *CCN51*. The main indicators of the process in materials, labour, quality, and performance are detailed. Finally, considering ES as a technology that is usually expensive compared to other propagation techniques, in chapter 4 the key factors in the financial viability of the production process were identified. The costs of the process were estimated, identifying the factors that influence the process with

a Monte Carlo Simulation (MCS). The cost components identified were culture medium (CM), indirect manufacturing costs (IMC), labour (direct and indirect), and operating expenses (GO). Labour had the highest share of costs, with 53%, followed by GO, with 30%, CM, with 12% and IMC, with 5%. The MCS helped define that the variables with the greatest impact on the unit price were the response of the embryos in the germination stage and the proliferation coefficient. This projection gave a figure of US$0.73 per seedling.

Keywords: Commercial-scale propagation, Clonal Fidelity, Cost analysis, Monte Carlo Simulation (MCS), Plant Tissue culture, Somatic embryogenesis, Time production.

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1. Introducción

El cacao (*Theobroma cacao* L.) es un cultivo comercial de importancia a nivel mundial y constituye, para algunos países de América Latina, uno de los principales productos de exportación. En Colombia, este cultivo presenta condiciones prometedoras para expandir y fortalecer el sector agrícola, sin embargo, bajo las prácticas agrícolas actuales, su productividad es baja, principalmente debido a la baja tecnificación en el cultivo. En este contexto, es esencial el desarrollo científico innovador que involucre la modernización tecnológica de los procesos de producción de material vegetal, con el fin de contribuir a la competitividad e incidir efectivamente en la prosperidad de los productores. El presente trabajo de tesis interviene los sistemas de obtención de semilla donde se resalta el desarrollo de métodos eficientes de producción que permitan asegurar la disponibilidad de material vegetativo sano, al alcance de los cacaocultores. En este sentido, entendiendo la importancia de las nuevas biotecnologías como una alternativa de la bioeconomía mundial y el papel de Colombia como un país megadiverso y al mismo tiempo como una potencia de exportación de productos agroalimentarios, tales como el cacao, es necesario aportar en el fortalecimiento de los diferentes eslabones de la cadena productiva. Además, es clave que los avances tecnológicos sean desarrollados con coherencia técnica y equidad social que beneficie a todos los sectores de la sociedad.

La técnica biotecnológica de propagación de última generación, la embriogénesis somática, permite la reducción de costos y altos volúmenes de producción de plantas seleccionadas con características superiores, así mismo, constituye el primer paso para el establecimiento de una plataforma de mejoramiento para el cacao. Haciendo un comparativo con el método convencional de propagación de cacao para Colombia, la injertación, se tiene la experiencia de la Compañía Nacional de Chocolates (CNCH), donde en aproximadamente 1.800m^2 de área de vivero se produce 1.000.000 de plantas al año

(CNCH, 2020). Por los métodos biotecnológicos es posible mediante una infraestructura especializada de laboratorio como una biofábrica, producir en 1.200m^2 al menos 3.000.000 de plantas al año.

Hasta la fecha en Colombia solo el Laboratorio de Fisiología y Cultivo de Tejidos Vegetales ha obtenido resultados prometedores en la propagación de plantas vía ES para los genotipos universales *CCN51*, *TSH565, EET8, ICS1, ICS39, ICS60, ICS95, IMC67* y los genotipos regionales colombianos *CNCh12, CNCh13, CNCh16, CNCh24, CNCh4* (Urrea et al., 2011; Gallego et al., 2016; Henao et al., 2018, 2020, 2021; Osorio et al., 2022). Durante el desarrollo de la tecnología en el grupo de investigación se ha transitado de forma lógica a través de las diferentes etapas de madurez tecnológica (TRL). Urrea et al., (2011) constituye el primer trabajo de investigación básica seguido de Gallego et al., (2016) con los genotipos *ICS39* y *CNCh4* (Universal y Regional). A partir de los resultados positivos se planteó el punto de partida para el avance de la tecnología, transitando a los niveles de madurez Tecnológica TRL1 y TRL2. Seguidamente, Henao et al., (2018) iniciaron con los estudios a escala de laboratorio para probar la tecnología productiva de plántulas en 12 diferentes genotipos de interés agronómico para el país alcanzando con los resultados una TRL3.

Con miras a continuar con el desarrollo de la tecnología y realizar la transición desde la escala de laboratorio a escala industrial (TRL 4 y TRL 5) se planteó que el escalado de la producción de plantas de cacao en una infraestructura tipo biofábrica depende de diferentes parámetros del proceso embriogénico y condiciones de cultivo. Por lo tanto, parámetros como indicadores de producción, desempeño, calidad y económicos determinan la base para el escalado. Para establecer esta línea base del escalado y con miras a hacer más eficiente el proceso se definieron en un primer momento las etapas de producción de plantas *in vitro* por ES para un genotipo élite de interés comercial en Colombia y otros países como Ecuador. Además, se reporta el tiempo mínimo necesario para la propagación, una variable entre otras fundamental a nivel productivo. A este nivel, la calidad genética constituye una característica esencial de los materiales de semilla y representa una de las principales barreras para ingresar en el mercado. En este sentido, con el objetivo de desarrollar un sistema de producción acorde a las exigencias comerciales se describe un sistema de regeneración para plantas de cacao que garantiza estabilidad genética de la producción y, por lo tanto, la calidad del producto.

Definidas las fases del proceso productivo y validación molecular de la estabilidad genética de las plantas obtenidas se continua con nuevos aportes para la gestión eficaz y eficiente del sistema productivo a escala industrial. Se presenta un análisis detallado sobre la producción, planificación y control de la producción comercial a gran escala que involucra un proceso biológico complejo como la ES en una planta leñosa. La identificación de los indicadores para cada una de las fases productivas permite monitorear los procesos y con ello identificar aquellos objetos de mejora o innovaciones incrementales. Finalmente, una vez detallado todo el proceso productivo y sus componentes fue posible definir las variables con mayor impacto en el precio unitario, así mismo, los factores garantes del proceso productivo que permiten identificar cuellos de botella y por lo tanto puntos de mejora, aspectos esenciales en una tecnología productiva en desarrollo.

La línea base para el escalado, resultado del presente trabajo, incorpora nuevos aportes en la producción comercial a gran escala vía ES en cacao. Así mismo, con el empleo de marcadores microsatélites se valida la calidad del producto mediante la estabilidad genética de las plantas obtenidas respecto a las propagadas de forma convencional. Un sistema de control y planificación de la producción establecido a partir de las etapas del proceso productivo que puede incidir en la toma de decisiones gerenciales en la producción a gran escala. Finalmente, una estimación del costo del proceso con la identificación de los factores que influyen en la estructura de costos de forma significativa.

Por lo tanto, desde una perspectiva teórica se generan nuevos conocimientos a los existentes y se crea un esquema de trabajo para la toma de decisiones durante el avance tecnológico del proceso de escalado. Así mismo, desde el punto de vista práctico, todos los resultados obtenidos pueden ser aplicados en otros genotipos de cacao de interés económico para Colombia. Además, los resultados presentados permiten superar la TRL4 y TRL5, en tanto se tiene un producto validado en un entorno de laboratorio y una modelación de la producción y los costos asociados a la tecnología en un escenario productivo industrial como una biofábrica. Los resultados permiten concluir que la tecnología bajo la infraestructura adecuada se puede escalar, siempre y cuando se consideren los parámetros clave identificados en el proceso de desarrollo tecnológico.

Finalmente, la línea base desarrollada ha permitido la integración de los aspectos necesarios para continuar con la etapa de demostración de la tecnología en un ambiente

relevante como el campo en compañía del cacaocultor, como final validador de la misma. La validación permitirá demostrar la factibilidad técnica de la tecnología en condiciones reales, es decir, evaluar el desempeño agronómico de las plantas en condiciones de campo en diferentes zonas edafoclimáticas y validar por parte de los productores de cacao los beneficios y aspectos a mejorar respecto al material vegetal propagado por métodos convencionales, y de esta manera generar la confianza en los cultivadores y actores principales de la cadena productiva del cacao. Una vez superada la TRL 6 se podrá continuar con una escala pre-comercial y evaluación económica de la tecnología, y a largo plazo permitirá ofrecer material vegetal de alta calidad y volúmenes significativos a los productores de cacao, para desarrollar nuevas áreas de cultivo y reemplazar las existentes con genotipos seleccionadas por sus características agronómicas superiores a través del conocimiento y la innovación.

2. Capítulo 1. Cacao (*Theobroma cacao* L.) Somatic Embryogenesis of *CCN51*: From Pilot Scale to Commercial Production

El capítulo 1 fue sometido a Journal of Horticultural Science and Biotechnology con ID 228780151 el pasado 15 de agosto, revista clasificada en Q2 por SJR y los autores se relacionan a continuación:

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2.1 Abstract

The transition of promising technologies for massive production of cacao (*Theobroma cacao* L.) trees from a research scale to commercial scale is often difficult and expensive. As a result, the timeframe estimated for plantlet production is typically underestimated resulting in very long processes in the laboratory that make the production system unfeasible to face to markets. Somatic embryogenesis (SE) is considered the most suitable and integrated technology for the large-scale production of clonal cocoa plants, compared to conventional methods. To date, practical application of ES to produce cocoa genotypes of interest has been limited and for drop-in technology replacements, price is of paramount importance and operating expenditures are a prerequisite for success. Therefore, the objective of this work is to present a production of *CCN51* cacao genotype in a pilot scale in laboratory with an approach to commercial production with a reduction in time production. For this, this work was defining the different production stages of cocoa plantlets by SE: initiation, multiplication, maturation, germination, and acclimation. The minimal time obtained for *CCN51* production was 30 days in initiation, 70 days in multiplication (30 primary somatic embryo, 20 secondary somatic embryo, 20 II secondary somatic embryo), 50 days in maturation, 70 days in germination, and in *ex vitro* culture conditions 30 days for acclimation. The decrease in the time of the production process directly influences the cost of the process and it represents a breakthrough in the development of technology.

Keywords: Commercial-scale propagation, somatic embryo, vegetative propagation, time production.

2.2 Introduction

Producing cacao as the raw material for the chocolate industry has spread in many countries worldwide. There is a significant concentration in tropical countries, led by African countries, among which Ivory Coast, Ghana, Cameroon, and Nigeria stand out with 63.2% of global production. Asia, with Indonesia and Papua New Guinea, has 17.4% of production, and Latin America, with Brazil, Ecuador, Peru, the Dominican Republic and Colombia, contributes to 19.4% of global production (Toma and Săseanu 2020).

In accordance with morphological characteristics and geographic distribution, cacao has traditionally been classified into three major groups: Criollo, Trinitario and Forastero. The latter is also called standard quality cacao and is cultivated the most due to its productivity (1,500 – 1,800 kg/ha). However, due to crossbreeding over time, whether it has been natural or intended, cacao is very variable. Therefore, it is difficult to distinguish between the groups mentioned above based only on phenotypic characteristics. Motamayor et al. (2008), using genetic studies, was able to differentiate cacao into 10 groups: Amelomado, Contamana, Curaray, Guianna, Iquitos, Marañon, Nacional, Nanay, Purús and Criollo, which provide a much more precise classification.

Asexual or vegetative propagation play an important role in faithfully reproducing the desirable characteristics of a tree or group of selected trees. In case of cacao, a perennial species, cross-pollinated and with a considerably long breeding program cycle (15 years), vegetative propagation is traditionally performed by rooting branches and shield budding (Sodré and Gomes 2019). Grafting of canopy is the method used most because of the advantages that can be found in different rootstocks, such as resistance to disease (Ribeiro et al. 2016). However, both techniques have efficiencies or variable multiplication rates. Extensive clonal gardens are also required to produce enough material for propagation and they generally have a considerable cost per obtained plant compared to seeds (Cano 2019).

Multiplication by seeds is an easy, low-cost system for implementation. Nevertheless, due to the genetic variability of propagated trees, this system is not recommended unless the seed's quality has been certified, which are produced through the controlled pollination of the selected genotypes, in which the obtained hybrids have demonstrated high precocity of fruiting and vigorous plant development (Dostert et al. 2011).

As a result of the plant breeding programs at various research centers around the world, there are a considerable number of improved genotypes (Boza et al. 2013; Duval et al. 2017; Phillips-Mora et al. 2009). Nevertheless, one of the largest limiting factors to harnessing this germplasm is the lack of mass cloning methods for the selected genotypes from a financial and agricultural point of view (Gómez 2015). Plant tissue culture methods plays an important role for the mass production of plant material.

This method facilitates the propagation of plants that are difficult to propagate through conventional methods. Clonal production of high-quality plant material for cultivation has generated new opportunities for global business with benefits for producers, farmers, and nursery owners, contributing to rural socioeconomic development. The main advantage of plant tissue culture is to produce high quality, uniform planting material that can be multiplied throughout the year in conditions without diseases in any location and independent of the season or weather. The technological implementation of *in vitro* culture on an industrial scale, on the other hand, requires a high capital, labour, and power investment. Although labour is inexpensive in many developing countries, skilled personnel is limited (Bryan and Chuquista 2016; Pacheco et al. 2016). In addition, electric power and clean water are expensive. Electric power requirements for this technology depend on temperature, the duration of the day and relative humidity. These variables must be controlled during the propagation process.

Somatic embryogenesis (SE) of cacao offers a potential method for propagating clones of interest on a large scale in commercial laboratories used for propagation. Some laboratories may have a production capacity of up to 5,000,000 plants a year in areas of approximately 1,000 m^2 (Acosta 2017; Orellana et al. 2008). Production capacities of just 1,000,000 plants a year in an area of approximately 1,800 m^2 can be achieved in Colombia (CNCH 2020) through conventional vegetative propagation methods, mainly grafting, which

is a manual and scantly industrialized process. Given the lack of efficient protocols for horticultural propagation by grafting, SE is the most promising tool to insure the large-scale dissemination of hybrids.

2.2.1 Somatic embryogenesis for scaling up cacao plantlets production.

ES is considered the next generation method of propagation and presents additional practical advantages compared to micropropagation and conventional methods (Garcia et al. 2018; Guillou et al. 2015; Wickramasuriya and Dunwell 2018). Somatic embryos progress through a developmental pathway that is very similar to their zygotic counterpart (Ikeuchi et al. 2016). Normal development of root apical meristems results in synchrony in the obtained plant's growth with a desirable orthotropic architecture, avoiding problems with plagiotropic growth and the formation of adventitious roots obtained in most cases through propagation via cuttings (Entuni et al. 2018; Goenaga et al. 2015; Tapi et al. 2020). Furthermore, SE maintains the genetic identity of the produced cocoa plants, while conventional grafting may show variability (Ajijah et al. 2016; Fang et al. 2009; Henao et al. 2021; Rodríguez, Wetten, and Wilkinson 2004). For a long time, it was thought that the possibility of genetic modification caused by the rootstock-graft interaction could not exist. However, recent studies have shown that it does occur (Kundariya et al. 2020). This variability can be caused by relationship between macromolecules transported between the graft and rootstock (Kyriacou et al. 2017; Li et al. 2013). In accordance with Wang et al. (2017) and Gaut, et al. (Gaut, Miller, and Seymour 2019), phenotypic changes occur in plants induced by grafting, which suggests underlying regulatory processes to rootstock-graft communication that involve a genetic component known as horizontal gene transfer. This evidence suggests that interactions between grafts and rootstocks can lead to epigenetic changes (Xanthopoulou et al. 2018; Yu et al. 2018), but the prevalence of these changes, their links to phenotypes and possible evolutionary implications remain largely unexplored (Berger, Gallusci, and Teyssier 2018; Gaut et al. 2019).

The possibility of scaling somatic embryo production is one of the most representative advantages of ES, once the technology has been established (Suárez-castellá et al. 2012). In other words, once the embryogenic process has been standardized

and the multiplication coefficients have been defined, it is feasible to move from the research laboratory scale to an industrial scale for mass production of plant material for commercial purposes. In turn, the ES facilitates developing biochemical, physiological, genetic and germplasm conservation studies, among others. For example, for decreasing plant breeding programs times by editing or genetic transformation a regeneration, method such as SE is the obligatory base for regenerating genetically modified plants (Fister et al. 2018). In addition, the opportunity to maintain embryogenic cell lines during prolonged periods in cryogeny allows to perform agronomic performance tests before selecting the cell lines to be propagated (Bajpai et al. 2016). For commercial plant production, especially the *in vitro* phase corresponding to embryo multiplication, SE allows to increase the number of plants by inducing recurrent embryogenesis and using liquid culture media (Park 2014). Therefore, an efficient system of plant production in cacao is needed to meet the demand for biotechnology approaches to this species.

The representative research work with a productive approach was reported by Guillou et al. (2015) on laboratory-scale research, following others (Ajijah and Hartati 2019; Díaz-López et al. 2015; Garate et al. 2017; Kahia et al. 2017; Lázaro et al. 2015). Nestlé launched the Nestlé Cocoa Plan (NCP) in 2009 for large-scale production using solid and liquid media to produce embryos (Guillou et al. 2018). However, the SE response was still highly dependent on genotype and culture conditions, which results in relatively low conversion rates of about 5 to 20%. The process has been evaluated in approximately 140 genotypes that are currently in a germplasm collection propagated by SE in the field and in a nursery in Ecuador, as well as some cryopreserved ones (Guillou et al. 2018). Moreover, they use plants obtained from SE to form orthotropic axes and their subsequent rooting. They have achieved productive efficiencies of over 100 cuttings/year/plant in nursery conditions where, per person, up to 25,000 plants are processed every year (Guillou et al. 2015).

CCN51 genotype constitutes 36% of national cacao production in Ecuador. *CCN51* is a genotype with high yield (Florida et al. 2020), its showed great adaptability to a variety of climates and today it is cultivated in all cacao regions of Ecuador, in some regions of Colombia, Peru, Brazil and some Central American countries such as Guatemala (Jaimez et al. 2022). Also, it has disease resistance to black pod disease caused by *Phytophthora palmivora* and *Phytophthora citrophthora* (Bahia et al. 2015; Barreto et al. 2015) and to

witches' broom (WB) (*Moniliophthora perniciosa*) (Ribeiro et al. 2016) . This cacao is particularly rich in fats, which defines it for its niche market. Therefore, it is a genotype of interest for its mass production by asexual methods, ensuring its agronomic characteristics of interest.

Although much scientific work has been done to develop cacao SE protocols, no data related to production at a precommercial scale for *CCN51* has been reported. Hence, there is a pressing need to successfully scale these processes at the first attempt. In order to compete in the market of plantlets propagated by conventional methods, *in vitro* production must be analyzed to achieve an efficient and effective system to answer to the needs for high quality plant material. The first goal of this work was to define the different production stages of cocoa plantlets according with the steps of somatic embryogenesis development. Second, in a laboratory with commercial approach was defined the minimal time require in the productive process of plantlets by SE in *CCN51* genotype. The decrease in the time of the production process directly influences the cost of the process and the final price per plantlet.

2.3 Materials and Methods

2.3.1 Plant Material

The plant material consisted of closed flower buds of the *CCN51* genotype. The immature closed flower buds were collected from field-grown plants of Yariguíes farms owned by Compañía Nacional de Chocolates in Barrancabermeja - Santander, Colombia. The flowers were transported to Universidad de Antioquia's Laboratory of Plant Physiology in the city of Medellín - Antioquia, Colombia.

2.3.2 Stages for producing *CCN51* cacao plantlets via Somatic Embryogenesis (SE)

Following the guidelines of Egertsdotter et al. (2019) the *in vitro* development stages of SE in a scaling process are initiation, multiplication, maturation, germination, and plantlet formation (Figure 1-2). These stages have been adjusted and adapted to the biological processes implied in the embryogenic development of cacao as described below, taking into account the protocol reported by Fontanel et al. (2002) and the subsequent work based on it, such as: Masseret et al. (2009); Urrea et al. (2011); Gallego et al. (Gallego et al. 2016); Henao et al. (2018); Bustami and Werbrouck (2018); Iracheta, et al. (2019); Kone et al. (2019); Eliane et al. (2019).

2.3.2.1 Initiation

Explant disinfection for *in vitro* introduction and callogenesis are included in this stage. The staminode is defined as a successful explant for *CCN51* and the flower buds are collected [66]. In the field, morphological characteristics of flower buds are considered. Flower bus must be in intermediate development stages, closed, at least 0.3 – 0.5 cm of length between the base of the peduncle and the apex. all flower buds are collected in the afternoon and packaged in a cold DKW salt solution at half its concentration concentration (Driver and Kuniyuki 1984) and then sent to the Laboratory.

First, flower buds with mechanical damage or necrosis and those that open during the disinfection process are excluded. The disinfection process begins before 24 hours have passed since collection (Figure 1-1) and disinfected explants are planted in the INDI culture medium for callogenesis induction (Table 1-1). Data from 10 *in vitro* introduction were randomly selected. In each explant culture 20 - 25 explants were placed per Petri dish. In approximately 2.500 explants contamination by fungi and bacteria (%) was measured 5 days after explants culture, and necrosis was measured after 10 days.

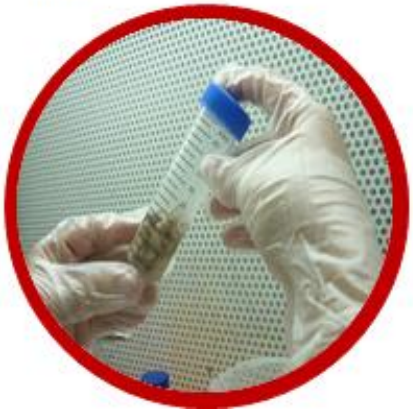
Second, callogenesis was induced in sterile staminodes. These explants were cultivated in INDI culture media to evaluate time in production of calli. The morphological characteristics of calli considered was undifferentiated growth on the filamentous, cream-white staminode in a proportion greater than 70% of the explant. Callogenesis percentage (%) was calculated at 30, 40, 50, and 60 days. Each Petri dish was considered an experimental unit. During induction stage, all the cultures were randomly placed in a growth

chamber and the explants were kept in complete darkness at an average temperature of 26˚C ± 2˚C and 70% relative humidity.

Submerge flower buds in conical tubes with 50 ml of belico fungicide (2 g/L) for 15 minutes with a 360° agitation. Three washes with distilled water are performed.

The explants are moved to conical tubes with sodium hypochlorite at 0.5% for 5 min with a 360° agitation. Three washes with distilled water are subsequently performed.

The explants are moved to new conical tubes with streptomycin at 250 ppm for 10 min with a 360° agitation, renewing the solution every 10 min. Three last washes are performed with distilled water



An antioxidant solution (ascorbid acid at 100 mg/L and citric acid at 100 mg/L) is used for 1 min with a 360° agitation. The flower buds are deposited in a humidity chamber for processing.

Figure 2-1. Flower bud disinfection process for the *CCN51* cacao genotype for *in vitro* introduction

2.3.2.2 Multiplication

The multiplication stage includes the induction of primary somatic embryos (PSE) (expression I), the induction of recurrent embryogenesis or secondary embryogenesis expression II) and a second cycle of proliferation of secondary embryos (SSE) (Expression III). In expression I once the tissues are calli, they are transferred to the expression medium for primary embryos called INDIexp (Table 1-1). The morphological characteristics of embryogenic callus with primary embryos were aggregates of waxy light to dark brown calli with somatic embryos in a globular state of at least 0.5 mm – 3 mm, without walls defined between them and a translucent to off-white color, mostly in a cluster. In *CCN51* an average of 5 – 10 embryos have been obtained from each callus explant (Garcia et al. 2018; A Henao et al. 2018). Different cultivation times were evaluated for primary embryos (30, 40 and 50 days). All primary embryo counts were in the globular development stage.

In expression II, once the PSE have been obtained, they are proceeded to be chopped with a scalpel. Pieces are placed in the recurrent or secondary embryogenesis induction medium called CM2 (Table 1-1). The morphological characteristics of embryos from recurrent embryogenesis were immature cotyledonary embryos with differentiation of the epicotyl and hypocotyl, of at least 2 mm – 5 mm from the base of the radicular pole to the cotyledons, radicular axis with an off-white color. At least 95% of explants respond to the induction of recurrent embryogenesis (Guillou et al. 2015), which considerably increases somatic embryo production, with up to 24.7 embryos per explant (Garcia et al. 2018; A Henao et al. 2018). Different cultivation times were evaluated for primary embryos (10, 20 and 30 days).

Somatic embryos develop asynchronously and, therefore, are harvested from the globular and early cotyledonary stage to prevent embryogenic axes from fusing during development. Subsequently, in expression III the embryos are transferred to the EM2 medium (Table 1-1), where they remain, with a production of 10 embryos per explant. Different cultivation times were evaluated for secondary embryos (6, 14, 20, 28, 35, 42, 50 days). In expression II and III all secondary embryo counts were in cotyledonary development stage. Explants are kept under the same conditions mentioned previously in initiation stage.

Table 2-1. Culture media for production via SE of cacao genotype *CCN51.*

	Initiation	Multiplication		Maturation		Germination	
Composition	**INDI[1]**	**INDIexp[2]**	**CM2[3]**	**EM2[4]**	**EM2**	**MM6[5]**	**MM6**
Macronutrients	DKW[6]	DKW	MS[7]	MS	MS	½ MS	½ MS
Micronutrients	DKW	DKW	DKW	DKW	DKW	DKW	DKW
Myo-inositol	100	100	100	100	100	100	100
Nicotinic acid	1	1	1	1	1	1	1
Thiamine	2	2	2	2	2	2	2
Glycine	0.19	0.19	2	2	2	2	2
L-lysine	0.45	0.45					
L-leucine	0.32	0.32					
L-arginine	0.43	0.43					
L-tryptophan	0.51	0.51					
2,4,5-Trichlorophenoxyacetic acid(2,4,5-T)			1				
2,4-Dichlorophenoxyacetic acid (2,4-D)	1						
Naphthaleneacetic acid (NAA)						0.01	0.01
Kinetin (KIN)	0,25						
Adenine			0.25				
Gibberellic acid (GA_3)						0.02	0.02
Activated carbon						1	1
Glucose	30	30	60			40	40
Sucrose				40	40		
Gellex™	3	3	3	3	3	4,1	4,1
PH	5.8	5.8	5.8	5.8	5.8	5.8	5.8

[1,2] Fontanel et al. (Fontanel et al. 2002) culture medium with aminoacids reported by Traore (Traore 2000) and glycine by Henao et al. (A Henao et al. 2018); Fontanel et al. (Fontanel et al. 2002) culture medium with sucrose concentration reported by Garcia et al. (Garcia et al. 2018).

2.3.2.3 Maturation

Once the multiplication phase stage has ended, immature cotyledonary embryos are selected and transferred again to the EM2. The morphological characteristics of cotyledonary embryos from recurrent embryogenesis were somatic embryos with a defined polarity, differentiation between the epicotyl and hypocotyl, with an axis of at least 5 mm – 10 mm from the base of the radicular pole to the apex, an off-white axis, radicular axis with brown basal grooves and different shades in the cotyledons (transparent, ivory, pink and greenish). In this stage different cultivation times were evaluated for embryo development (50, 60 and 70 days), with renovation of culture media every 30 days. In this stage, cultures were placed in a growth chamber under light with a 16:12 photoperiod and a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} per second, at an average temperature of 26˚C ± 2˚C and 70% relative humidity.

2.3.2.4 Germination

Mature cotyledonary embryos from the previous stage with normal cotyledons are transferred to the MM6 medium where they convert into plantlets. The morphological characteristics of mature cotyledonary were somatic embryos with a defined polarity, differentiation between the epicotyl and hypocotyl, with an axis of at least 10 mm – 20 mm from the base of the radicular pole to the apex, an off-white axis and different shades in the cotyledons (transparent, ivory, pink and greenish). In this stage, 500 ml containers (magenta) are used to allow conversion to plantlets at height and the expansion of true leaves. In this stage different cultivation times were evaluated for embryo development (40, 50, 60, 70, 80 and 90 days) with change of culture medium every 30 days. In this stage, placed was maintained in the same conditions of the maturation stage.

2.3.2.5 Acclimation

Plantlets are transferred to greenhouse and later to nursery conditions for *ex vitro* adaptation. Plantlets with a stem length of at least 3 cm, at least 1 distinguishable internode, prominent radical development of at least 5 – 10 cm width and both primary pivotal roots and secondary roots, and at least 1 – 4 true leaves were selected. They were removed from culture vessels and washed with running water to eliminate excess gelled culture medium. The plantlets were subsequently transferred to germination trays with 10 cm deep cavities in which a substrate prepared with a 1:1 proportion of washed river sand and a coconut palm substrate (p/p) without enrichment in greenhouse conditions. The tray is covered with a transparent dome for 1 day and holes are subsequently made to allow exchanges until 15 days are completed, keeping the substrate hydrated with a nutrient solution of Hoagland (1:10) every two days. After 15 days the plants are transplanted into 45cm deep bags with oil palm substrate in nursery conditions. After 15 days have passed, fertilization with a slow-release fertilizer, such as Basacote™ with a dosage of 2 g per plant and the plants remain in the nursery for 4 months. During greenhouse and nursery time plantlets are kept in the growth conditions inherent to the cacao culture shaded with a 70% HDPE shade structure, a temperature of between 24 - 30 ºC during the day and 16 - 18 ºC during the night, 12:12 photoperiod.

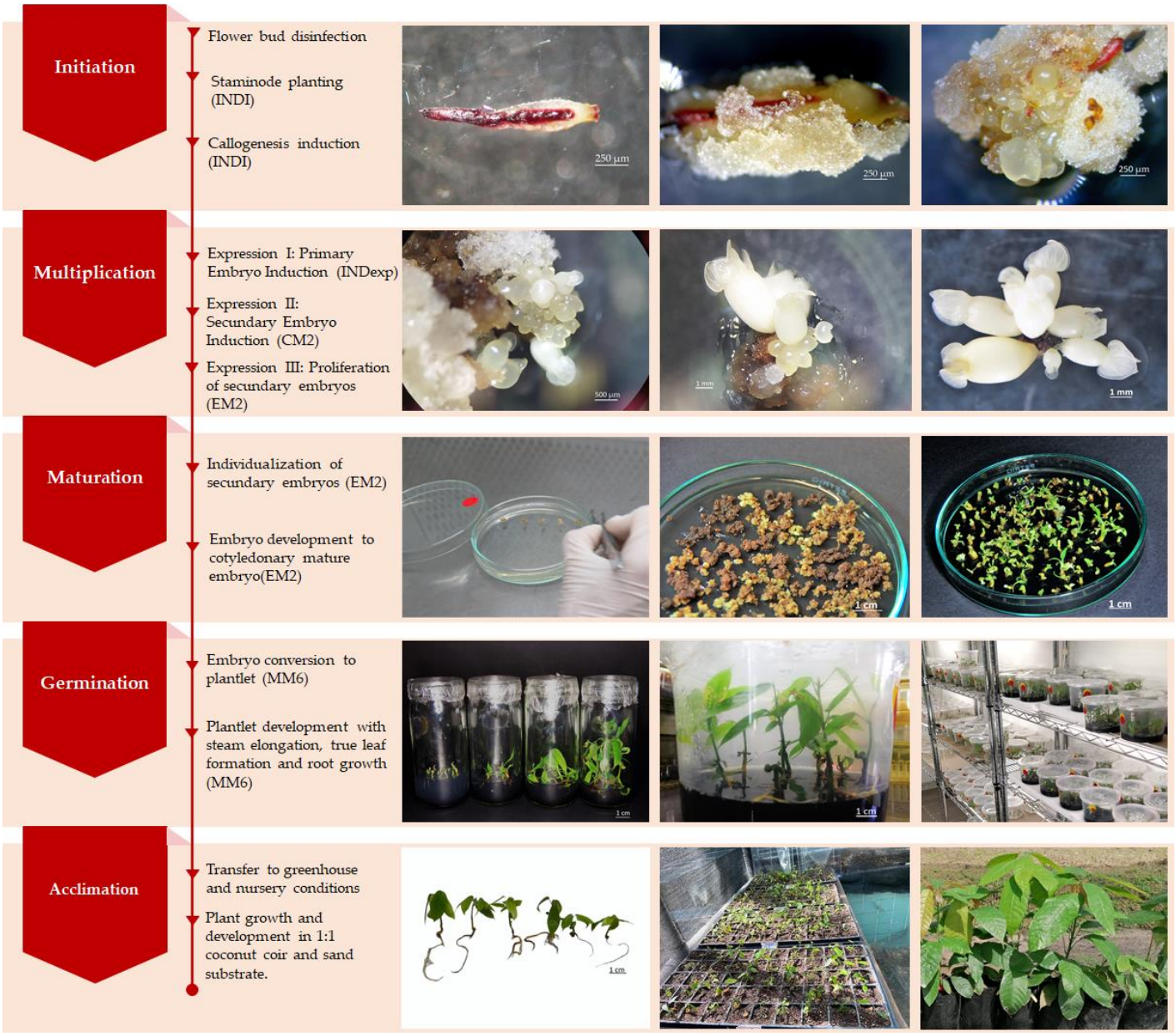


Figure 2-2 Flow chart of the plantlets production stages and somatic embryogenesis stages in cacao genotype *CCN51* at a research scale.

2.4 Results

2.4.1 Iniciation

The reported disinfection protocol is adequate and allows to obtain high disinfection percentages and has a minimal effect on the mortality of the explants. Explant disinfection was obtained between 75 - 100% (Figure 1-3 A) and necrosis of explants after *in vitro* introduction were minimum between 0 – 20% (Figure 3 B). In addition, it has been estimated that 90,85% of explants will achieve a satisfactory callogenesis at 30 days, without significative difference with 40, 50 and 60 days (Figure 1-3 C).

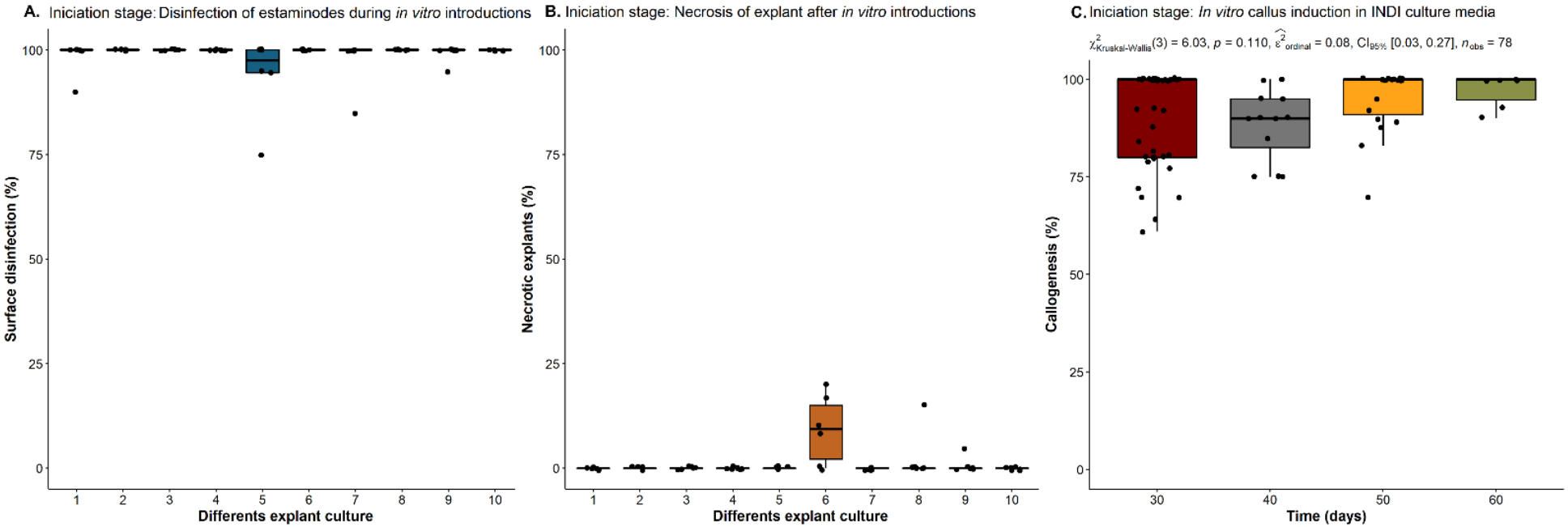


Figure 2-3 Initiation stage of production cacao plantlets of CCN51 genotype. A. disinfection, B. Necrosis and C. Time of callogenesis induction.

2.4.2 Multiplication

In expression I the best response to primary embryogenesis induction in the INDIexp culture media was at 30 days after cultivation with significative difference by 40 and 50 days with an average of 8,5 globular primary embryos are harvested for each explant (Figure 1-4 A). Subsequently, for expression II in the CM2 culture media an average of 20 cotyledonary secondary embryogenic is formed by explant at 20 days and 30 days, without significative difference between theirs (Figure 1-4 B). Expression III comes next, new expression of repetitive embryogenesis in EM2 culture medium, in which 20 cotyledonary secondary embryogenic is formed by explant at 20 days, without significative difference with 35 days (Figure 1-4 C).

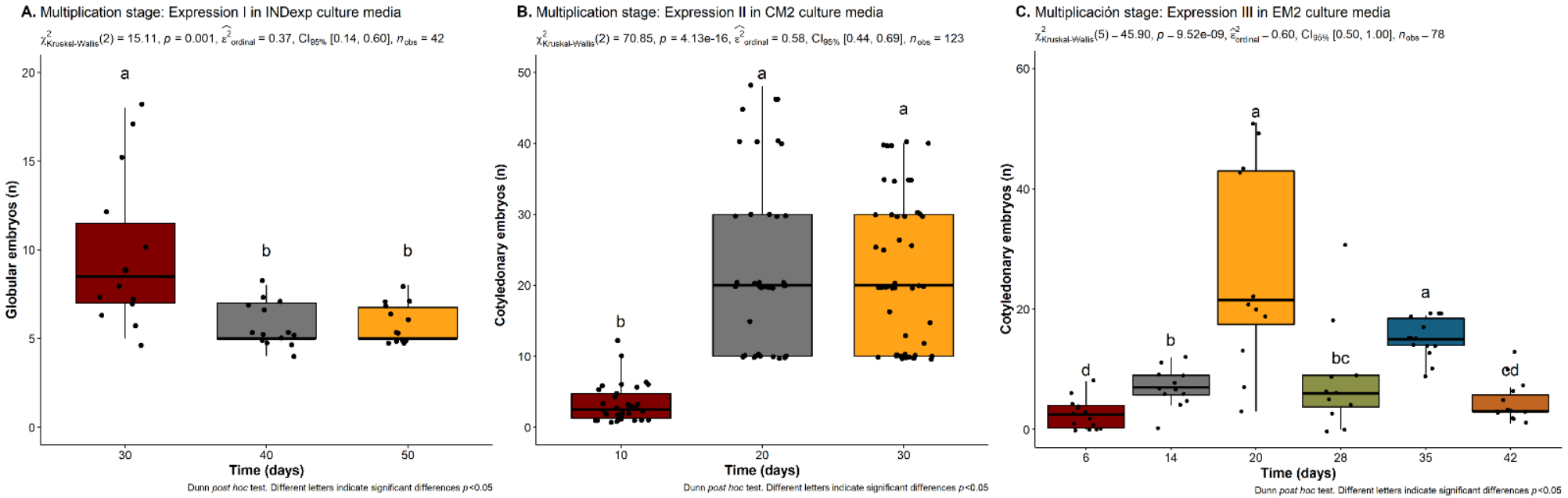


Figure 2-4. Time of multiplication stage of production cacao plantlets. A. In expression I number of globular PSE, B. In expression II number of cotyledonary secondary somatic embryos and C. In expression III number of cotyledonary secondary somatic embryos.

2.4.3 Maturation and Germination

The maturation stage begins in the same EM2 medium, where 50 days is adequate for embryo maturation and an average of 20,3 late cotyledon embryos was obtained, with significative differences with 60 and 70 days (Figure 1-5 A). At this point, even though immature embryos continue their development process, recurrent embryogenesis occurs once again.

Subsequently, the germination stage, in which conversion and development of plantlet occurs, significative differences was observed between time. The plantlets require between 70 and 90 days for producing an average of 2,9 – 4,1 true leaves (Figure 1-5 B), with a significant difference between 40, 50, 60 days. In addition, to reach 4,7 cm in length of the root was necessary 70 days with significant difference (Figure 1-5 C).

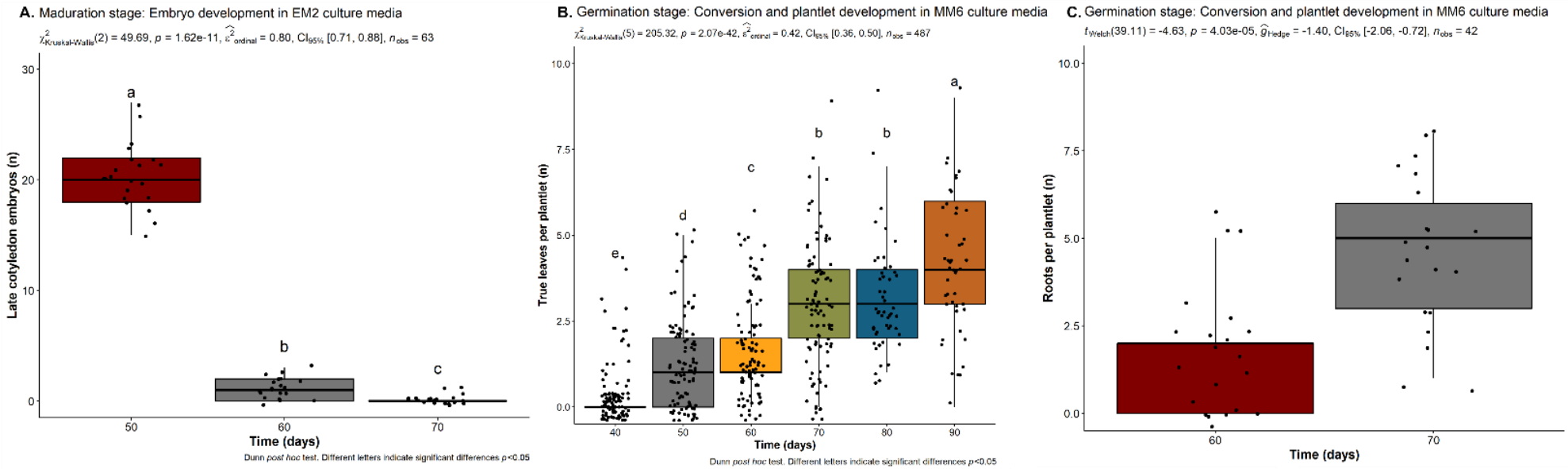


Figure 2-5 Time of maturation and germination stage of production cacao plantlets. A. Number of late cotyledon secondary somatic embryos, B. Number of true leaves of plantlet from secondary somatic embryos C. Number of roots of plantlets.

2.4.4 Acclimation

Cocoa plantlets achieve complete adaptation to *ex vitro* conditions in a greenhouse in a tray covered with a transparent dome for 1 day and holes are subsequently made to al-low exchanges until 15 days. Additional 15 days are required where the plants are transplanted into 45cm deep bags with oil palm substrate in nursery conditions. In total, 30 days are necessary for the complete adaptation of the plants under the procedures detailed in the methodology, with survival greater than 80% (Data not shown) similar results obtained by Kahia et al. (2017). For the plants to reach adequate growth to be taken to the field, they require another 4 months with management under nursery conditions. During this process it has been shown that the electrical conductivity of the soil and substrate (EC) must be verified, since for cocoa it must be kept at an optimal level of (<1 mS/cm). Likewise, the Ph must be verified, which must be within the optimal range (5.1 to 7.0) (Barrezueta 2018; Vargas et al. 2019).

2.5 Discussion

In the induction stage, the flower bud disinfection protocol allows for high disinfection, low necrosis and a significant callogenesis response in staminode-type explants in the case of *CCN51*. The percentages of disinfection of close to 100%, with necrosis below 20% and callogenesis of 90.85%, exceed the percentages reported by Echenique and Calle (2020), where a disinfection of 64% was obtained for staminodes, 36% for necrosis and 35% for callogenesis with the use of sodium hypochlorite. However, as reported by other authors (Iracheta et al. 2019; Osorio et al. 2022), the exposure of tissues to fungicide improves the disinfection process but with tissue oxidation. One of the components that affect the disability *in vitro* is the high phenol content and phenol oxidase compounds. Oxidation of this compound is an inhibiting factor for multiplication and tissue regeneration (Pancaningtyas 2021b). During the disinfection process, antioxidant compounds such as

ascorbic acid and citric acid are also used, which is a way to inhibit the production of phenolic compounds or reduce their accumulation in tissue culture media.

In the induction stage, 90,85% of explants will achieve a satisfactory callogenesis at 30 days, this findings are in accordance with Ajijah and Hartati (2019), who obtained callus formation at 30 days. In the same way, Daouda et al. (2019) managed to obtain in 28 days a callogenesis between 70-80% in staminode-type explants, similar to Eliane et al. (2019) with 28 days of callus induction but with petal explant. Despite the fact that Iracheta et al. (2019) reports in a shorter period of time than that reported in this work of 21 days in the induction of callus, they only achieved 87%. Kone et al. (2021) reports higher values between 80 - 100% callogenesis with 28 days of culture but with culture media modified in inorganic salts like K2SO4 and MgSO4 according to the genotype.

In the multiplication stage that includes the formation of PSE, SSE a second round of production of SSE, higher yields were obtained in the present work like previous works. While Ajijah and Hartati (2019) at 30 days obtained an average of 1,5 primary embryos per explant, in this study 8,5 were obtained in 20 days. Iracheta et al. (2019) who reported 1,7 primary embryo by explant in 42 days and Kone et al. (2021) reports the highest amount of PSE per explant of 19.8 - 23.5 but in a time of 84 days.

In the formation of PSE Ajijah and Hartati (2019) at 45 days an average of 8 secondary embryos per explant was obtained, in this work an average of 20 secondary embryos per explant by 20 days was obtained, in addition, at the following 20 days on the same explants manages to obtain again an average of 20 embryos per explant. In the same sense, Eliane et al. (2019) obtained a greater number of embryos, with an average of 6.2 primary embryos in a long period of time of 84 days with K2SO4 and MgSO4 modified concentrations.

Daouda et al. (2019) obtained SSE in 60 days without making a distinction between the induction time of primary and secondary embryos. On the contrary, in the present work, an adequate formation of PSE after 20 days was obtained, at 20 days for forming PSE and another 20 days for developing secondary embryos in the cotyledonary state. For the

maturation of the SSE, Daouda et al. (2019) reports 28 days, while in the present work, the maturation of the embryo takes 50 days with a change of medium at 30 days. The difference in times may be the result of the effect of genotype and the composition of the culture medium. Similar results were obtained by Adu-Gyamfi and Wetten (2020). Callus induction took 30 days, followed by PSE formation in 30 days. However, in the maturation of the embryo it reports 45 days while in the present work it was 50 days. In germination coincides with 60-70 days.

Pancaningtyas (2021b) reports shorter times in some stages of embryogenic development, in the induction of callogenesis 30 days, in the formation of PSE 60 days, in the formation of SSE 60 days, in the development or maturation of the embryos 30 days and for germination until embryo in mature cotyledonary stage were 21 days. For the present work, it coincides with the induction of callogenesis of 30 days, however, in the induction of PSE the time was less than 30 days and in the induction of SSE it was 20 days. In the maturation of the embryo to the mature cotyledonary stage, 50 days were obtained, a result very similar to Pancaningtyas (2021b) of 51 days, however, in this work the conversion of the embryo to a plantlet and its subsequent development is not contemplated. The difference in results can obviously be the effect of the genotype, in addition, the maturation culture medium was supplemented with adenine (0.025 mgL-1), a compound that in the present investigation is used only in the PSE induction stage. Similarly, Garcia et al. (2018) reports the complete production process until acclimation, but without embryo production data. The author reported callus induction takes 14 days, a figure less than that obtained in this work of 30 days. To produce PSE, the research reports 14 days and again another 14 days for SSE induction, values less than 20 days for both PSE and SSE induction reported in the present work. In the maturation of the embryo, 44 days are reported while we report 50 days. In the germination they report 90 days, a data higher than 70 days reported in the present work. In acclimation under greenhouse conditions, the authors report 40 days while we report 30 days. In total, Garcia et al. (2018) obtains greenhouse-adapted plants at 7.1 months, while in the present work a total time of 8.2 months is reported for *CCN51*, an important advance for a genotype of interest.

The differences found between the results in the different works cited may be the effect of the culture media, the culture conditions and genotype (Garcia et al. 2016; Pancaningtyas 2021a). In the first instance, there is enough valid information to date to indicate that growth regulators for callogenesis induction such as 2,4-D, 2,4,5-T in combination with KIN are generally effective for different genotypes. To date, 2,4,5-T (1-4 mgL-1) is the most widely used and effective growth regulator to break recalcitrance in different cocoa genotypes (Kone et al. 2019). In addition Kone et al. (2021) reported potassium sulphate (4705 mgL-1 K_2SO_4) and magnesium sulphate (1805 mgL-1 $MgSO_4$) are the concentrations of the most suitable mineral salts to overcome recalcitrance of cocoa genotypes (Alemanno, Berthouly, and Michaux 1996; Tan and Furtek 2003). To induce the formation of PSE in previously formed embryogenic callus, it is necessary to remove hormonal stress and leave only the effect of nutrients, amino acids, and vitamins (Ramasamy et al. 2022). Next, for the induction of SSE, another pulse of 2,4,5-T or 2,4-D is necessary, a growth regulator that allows greater efficiencies to be obtained and allows another series of SSE to be obtained again when subculturing.

In the maturation of the embryo it has been observed that plant growth regulators are not required (Henao et al. 2018; Ramasamy et al. 2022). However, Pancaningtyas (2021a) recommends a treatment combinations of adenine 0.0125 mgL-1 + ascorbic acid 100 mgL-1. In the germination of the embryo, the low concentration of salts favours the process and, in some genotypes, growth regulators such as ANA and GA_3 are required (Bustami and Werbrouck 2018). In a second instance, it has been proven that temperature is essential for the induction of all phases of somatic embryogenesis in cocoa. The temperature between 25 ºC - 27ºC is suitable for the process (Garcia et al. 2018), for *CCN51* it is appropriate 26ºC. In addition, it has been found that, depending on the genotype, the embryogenic process can occur even in temperature ranges of 32ºC - 35ºC, but with 40ºC and 45ºC being lethal for explants (Ajijah, Darwati, and Ibrahim 2021; Ajijah, Syafaruddin, and Inoue 2020).

In the germination stage where the conversion to plantlet takes place continues to be the bottleneck in the process of embryogenic development, the conversion percentages are in a range of 20 to 40% depending on the genotype and for the present work it

constitutes the longest stage of at least 60 days. This stage is where the greatest number of embryo losses due to malformation occurs, according to Garcia et al. (2019, 2021) has been almost identified 873 differentially methylated genes in the CpG context between zygotic embryos, normal and abnormal somatic embryos, with important roles in development, programmed cell death, oxidative stress, and hypoxia induction, which can help to explain the morphological abnormalities of somatic embryos. Likewise, it has been identified the role of ethylene and its precursor 1-aminocyclopropane-1-carboxylate in several biological processes, such as hypoxia induction, cell differentiation and cell polarity, that could be associated to the development of abnormal somatic embryos.

In this context, according to Fang et al. (2014) and Minyaka et al. (2017) the progress in embryological development inhibition of somatic embryo of cacao would be due, in addition to the insufficiency or toxicity of certain compounds used, to a strong secretion of ethylene and polyphenols by the explants of certain genotypes. Polyphenols by their oxidation act as inhibitors of metabolic or antagonistic reactions of growth substances in cacao (Gallego et al. 2016). Alemanno et al. (1996) and Boutchouang et al. (2016) conducted on cacao flowers showed that they would synthesize a significant quantity of phenolic compounds. Indeed, these compounds intervene in the defence of plants (Kouassi et al. 2017; Minyaka et al. 2017). When the plant is subjected to mechanical injury, simple phenols are synthesized, and the peroxidase activity characteristic of the lignifying tissues is stimulated. Phenolic secretions and other exudates in plant tissue culture systems inhibit the development of the callogenic explant in embryos (Kouassi et al. 2017). The work of Fajardo et al. (2022) conducted on cacao flowers showed that the most represented metabolites were alkaloids, which were mainly found in flowers and calli. In the latter, flavonoids were most abundant.

Finally, according to Garcia et al. (2018), the acclimation process requires 90 days for the *CCN51* genotype, however, in the present study, 30 days are reported for the process. Likewise, Masseret et al. (2009) reports a longer period of acclimatization under greenhouse conditions of 60 days. Next, some authors reports around 6 months later (Entuni et al. 2018; Masseret et al. 2009), the tree development is compatible with field plantation, while in the present work 4 months are reported under nursery conditions.

Emphasis is placed on the importance of maintaining relative humidity above 80% during the first days of adaptation. Likewise, the plantlets are kept for the first 7–14 days under low light intensity until a new leaf develops (Bustami and Werbrouck 2018; Entuni et al. 2018; Goenaga et al. 2015; Tapi et al. 2020).

From a commercial perspective, somatic embryogenesis continues to be a viable option for the propagation of cacao on an industrial scale. In the multiplication stage each cluster has an average ability to multiply about 3 to 5 times that of the initial cluster with suitable parameters (Pancaningtyas 2021b). Despite some losses due to the production of abnormal embryos it is theoretically and practically possible to produce more than 4,000 plants of secondary embryos derived from a single flower in about a year (Sodré and Gomes 2019). The development of this technology to date has produced plantlets with a conversion rate of 65% and relatively low percentage of somaclonal variation (Adu-Gyamfi et al. 2016; Ajijah et al. 2016; Fang et al. 2009). In profits, cacao genotypes propagated from SE culture exhibited true to type reproductive characteristics similar with conventional grafted clones (Entuni et al. 2021). Furthermore, the bean qualities of elite cacao genotype propagated from SE has been validated and no abnormalities were found in the morphological characteristics of beans in different genotypes, therefore, the bean quality is not affected after the SE culture process (Entuni et al. 2020).

Further development of SE protocol modified by various commercial companies has been outlined in various international patent documents (Henao-Ramírez and Urrea-Trujillo 2020; Wickramasuriya and Dunwell 2018). Despite continuous progress, there still exists a high degree of genotype-to-genotype variation and so protocol customization is necessary (Garcia et al. 2016; Jones et al. 2022). This study set up an improved protocol for *CCN51* propagation compared to previous works in terms of time and quantity of embryo production for cacao.

2.6 Conclusion

SE technology is suggested as the most convenient technology for producing clonal *T. cacao* plants on a large scale as a complement to traditional methods. This methodology is

very useful in plant multiplication programs for elite genotypes like *CCN51*. However, its practical application for producing genotypes of interest has been limited to date. From the results obtained, it can be stated that for *CCN51*: (1) the use of 2,4-D + KIN is suitable for callus induction and the formation of PSE; (2) the use of 2,4,5-T for the greater amount formation of SSE; (4) growth regulators are not required for the maturation of the embryo; (5) ANA + GA3 is required for conversion of the embryo into a plantlet with subsequent elongation and growth. All the previous conditions with a strict control of 26ºC in culture and dark in initiation and multiplication stage allows to obtain suitable times of production of plantlets for the production on a larger scale. For a ligneous species, such as *T. cacao*, the time factor and frequency of productive lots is a particularly important consideration, since it requires an average of 8,2 months to complete the entire cycle for *CCN51*. Future optimizations should be performed to increase multiplication coefficients and higher rates of conversion from embryo to plantlet. Likewise, the evaluation in open field conditions of the agronomic performance of the propagated plants to provide the cocoa producer with all the information associated with the productivity of the crop.

Statistical Analysis

The statistical analyses were performed using the programming language called R (version 4.0.3). The linear model of data was analysed by means of an ANOVA or a univariate t-Student distribution, which a reliability level of 95%. Non-parametric methods (Kruskal-Wallis or Wilcoxon) were performed when the residuals obtained by the model did not fulfil statistical assumptions (normality and homoscedasticity), using a 95% reliability level. The graphical analysis was performed by means of box plots created in the same programming language.

Author Contributions

Conceptualization, writing—original draft preparation, review and editing, investigation, and formal analysis: Ana María Henao Ramírez. Review, editing and formal analysis: Aura Inés Urrea Trujillo. Resources and data curation: Diana María Cano Hajduk. Editing and formal

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Capítulo 2. Determination of Genetic Stability in Cacao Plants (*Theobroma cacao* L.) Derived from Somatic Embryogenesis using Microsatellite Molecular Markers (SSR)

El capítulo 2 fue publicado en una revista internacional *International Journal of Fruit Science* en el 2021, revista clasificada en Q3 por SJR y la cita se detalla a continuación:

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2.8 Abstract

The clonal propagation of *T. cacao* by somatic embryogenesis (SE) is a promising approach to multiply elite genotypes. Assessing clonal fidelity in plants regenerated from somatic embryos is the first step towards ensuring genetic uniformity in the mass production of planting material. This study assessed the genetic stability of cacao plantlets propagated by SE and conventional grafting for genotypes *CCN51* and *TSH565* using 13 SSR. The leaves of *in vitro* plantlets (IVL) were collected from 6-month-old plants and leaves of field plants (FPL) were selected from 3-year-old trees. The 13 analysed loci revealed 25 alleles in genotype *CCN51* and 24 alleles in genotype *TSH565*. The highest PIC value was observed for all SSR, only mTcCIR8 and mTcUNICAMP09 were intermediate, with PIC values of less than 0.250. IVL and FPL populations were genetically equal. According to the results, no differences in allelic composition were observed between FPL and IVL in each genotype. This indicates that plants propagated by SE did not show perceptible detriment to their genome with the used SSR. In addition, Jaccard's coefficient showed more than a 91% similarity for *TSH565* and 92% for *CCN51*. The UPGMA and PCA showed that the populations tended to group within two genotypes. The SSR results obtained do not exclude the occurrence of other changes in the nuclear genome. However, considering the morphological stability of *in vitro* propagated plants, the results indicate that the protocol used is suitable and efficient for large scale, true-to-type propagation of genotypes *CCN51* and *TSH565* for commercial purposes.

Keywords: Allele composition, *CCN51*, Clonal fidelity, DNA markers, Somaclonal variation, *TSH565*.

2.9 Introduction

Cacao (*Theobroma cacao* L.) is a neotropical tree fruit species distributed in tropical regions as cocoa crops, mainly in Africa and South America, where its beans are used to produce chocolate. Cacao is currently a commodity in global markets, and its most recent attractive

capacity for public awareness is the health benefits acquired through consumption (Ajijah, et al. 2016).

Despite cacao's high growth in demand and use, there are currently no highly efficient systems to produce plant material for planting. When cacao is propagated clonally by cuttings or grafts, the result in terms of volume and production rate is low, in addition to an undesirable bushy growth pattern. In this sense, according to Thondaiman et al. (2013), using plant tissue culture technology and molecular biology is required to accelerate cacao breeding programs.

Seeking to produce sufficient plant material for planting, in the last three to four decades, using large-scale propagation tools has become a serious approach to many economically important plant species like cocoa. For this reason, *in vitro* micro-propagation techniques using somatic embryogenesis have shown several potential advantages over conventional propagation methods (Nandhakumar, et al. 2017). Somatic embryogenesis propagation methods offer a greater capacity for the rapid multiplication of elite genotypes and genetically similar plants. However, that is not always the case. There is a risk of producing so many mutant regenerants that the approach's economic feasibility is compromised (Etienne and Bertrand, 2016). Maintaining the true-to-type nature of *in vitro* propagated plants in commercial and marketing processes is crucial for upholding certain agronomic and horticultural traits when using elite genotypes (Alizadeh, et al. 2015).

Nevertheless, over long-term periods, plant micropropagation technology has a phenotypic and genetic variation of propagated plants known as somaclonal variation (Larkin and Scowcroft 1981). This epigenetic phenomenon can remain quite stable for many generations (Kaeppler, et al. 2000). Nowadays, the phenomenon of somaclonal variation still threatens the commercial viability of somatic embryogenesis technology (Butiuc-Keul, et al. 2016). This variation implies genomic changes (large-scale deletions and gross changes in chromosome structure/number and directed and undirected point mutations) and epigenetic changes (histone acetylation, DNA methylation, chromatin remodelling, etc.) (Wang and Wang 2012). In different phases of the *in vitro* process, the differentiation and multiplication phase involving callus production is considered the most

unreliable for clonal propagation. Meanwhile, plantlets regenerated through enhanced axillary branching or direct somatic embryogenesis have been reported to be genetically uniform. However, the possibility of somaclonal variations cannot be ruled out completely (Krishna, et al. 2016).

As reported by Rodríguez et al. (2010), the scarcity and stochastic nature of genetic mutations represent a significant challenge for scientists seeking to characterise de novo mutation frequency at specific loci. Therefore, evaluating the genetic fidelity of plants multiplied *in vitro* has been a central theme in research. In molecular biology, molecular markers have been used to elucidate punctual changes in genetic terms and establish relationships through phylogenetic analysis. DNA-based molecular markers have served as an important tool to assess the genetic homogeneity of micro-propagated plants (Chittora, et al. 2015). Something similar occurs with DNA methylation level, which is strongly related to somatic embryogenesis. Locus-specific modulations can alter genes, and their use mainly depends on the objective of research and type of tissue (Fehér, 2015). In general, the following molecular techniques have been used to assess the genetic fidelity of many micro-propagated plants species: Restriction fragment length polymorphism (RFLP) (Devarumath, et al. 2002), Methylation sensitive amplification polymorphism (MSAP) (Ghosh, et al. 2017), Random Amplified Polymorphic DNA (RAPD) (Patil and Bhalsing 2015; El-Mahrouk, et al. 2016; Singh, et al. 2016; Devi, et al. 2017; Moharana, et al. 2018), Amplified fragment length polymorphism (AFLP) (Coste, et al. 2015; Maki, et al. 2015; Scherer, et al. 2015; Onay, et al. 2016) and Inter simple sequence repeat (ISSR) (Lata, et al. 2016; Teeluck, et al. 2016; Viehmannova, et al. 2016; Alansi, et al. 2017).

However, microsatellites, also known as simple sequence repeats (SSR) short tandem repeats (STR), or simple sequence length polymorphisms (SSLP), have shown to be very important in genetic fidelity assessment due to some desirable characteristics, such as high reproducibility, co-dominant inheritance nature, the enormous extent of allelic diversity, high abundance in organisms, and strong discriminatory power (Solanke, et al. 2017).

SSR markers have previously been used to detect somaclonal variation chromosomes, and locus loss represents an additional source of genetic change - another advantage of this technique (Goyali, et al. 2015; Chhajer and Kalia 2016; Bandupriya, et al. 2017; Bradaï, et al. 2019). During PCR, reproducibility in the relative amplification efficiencies of alternate alleles may allow detecting allele loss or the presence of a new allele within a mutation chimera, even if they are present in relatively few cells (Solanke, et al. 2017). Since parental and mutant allele amplifications are performed in the same reaction and with the same primers, directly comparing the parental genotypes' allele peak profiles with that of the chimeric mutant may even allow a semi-quantitative assessment of mutant abundance.

The somaclonal variation continues to be presented as a phenomenon that threatens the commercial viability of somatic embryogenesis reproduction technology for cacao. Therefore, it is necessary to validate if the reproduction method allows maintaining the genetic characteristics of cacao populations to apply it as a mass reproduction method and overcome these uncertainties - either through molecular markers or markers sensitive to epigenetic methylation changes.

Seeking to consider genetic integrity as an essential quality feature of cacao plants produced by somatic embryogenesis technology, this study's main goal was to determine the genetic stability of two important cacao genotypes, *CCN51* and *TSH565*, regenerated via somatic embryogenesis through SSR. The former, *CCN51*, is planted extensively and exhibits many attractive agronomic traits, such as high productivity and disease resistance. This cacao is particularly rich in fats, which defines it for its niche market. For example, *CCN51* constitutes 36% of national cacao production in Ecuador (Vega and Beillard 2016). Furthermore, genotype *TSH565* is planted widely in several countries, mainly Brazil, due to its productivity, disease resistance, and traditional, fine-flavoured bean quality (Bastos, et al. 2019).

The research considers two populations: a population of *in vitro* plantlets (IVL) derived from somatic embryogenesis and a population of field plants (FPL) derived from grafting, which is the primary conventional multiplication method for cacao plants in

Colombia. This work is the first report on genetic uniformity for the studied genotypes. The advances in somatic embryo production provide the basis for continuing breeding programs for the widely used *CCN51* and *TSH565* genotypes and developing their genetic transformation.

2.10 Materials and Methods

2.10.1 Plant Material Propagated by Grafting in the Field

Plants were collected in the field for cacao genotypes *TSH565* and *CCN51* from the clonal garden in Yariguíes farm, owned by Compañía Nacional de Chocolates (CNCH) (Barrancabermeja, Santander, Colombia). Each tree with 10 years old from the clonal garden was identified and labelled with tree marking tapes with individual and project information. After a careful visual inspection, at least 10 young leaves in perfect phytosanitary conditions were taken from a total of 20 cacao trees propagated by grafting for each genotype between March and April.

The young leaves were packed in tissue wipers, grouped in resealable plastic bags, and placed in coolers with dry ice to ensure the lowest possible temperature for transportation back to the Plant Physiology lab at the Universidad de Antioquia (Medellín, Antioquia, Colombia) for long-term storage at -80°C until processing (Thermo Scientific). To ensure analysis, cacao leaves were collected in the D stage (Fig. 2-1). This developmental leaf stage is identified 25 days after emergence, when elongation ceases and leaves accumulate chlorophyll, displaying a light green colour (Fig. 2-1 a). Henao et al. (2018a) suggest that the D stage of development in field leaves was efficient for extracting high-quality genomic DNA

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2.10.2 Plant Material Propagated by Somatic Embryogenesis

The *CCN51* and *TSH565* genotypes were propagated *in vitro* through somatic embryogenesis between 2019-2020. From previously marked clonal garden trees, floral buds were immediately collected and stored in sterile basal DKW salts on the ice during transportation to the Plant Physiology Laboratory. Flower buds were sterilised following the protocol reported by Urrea et al. (2011). Staminodes and petals were extracted from the basal portion of the flower bud, and different phases of SE (induction, expression, maturation, conversion) were induced according to Henao et al. (2018b) protocol (Fig. 2-b-c). A pool of at least 40 cacao plantlets propagated by somatic embryogenesis was selected from each genotype for DNA extraction.

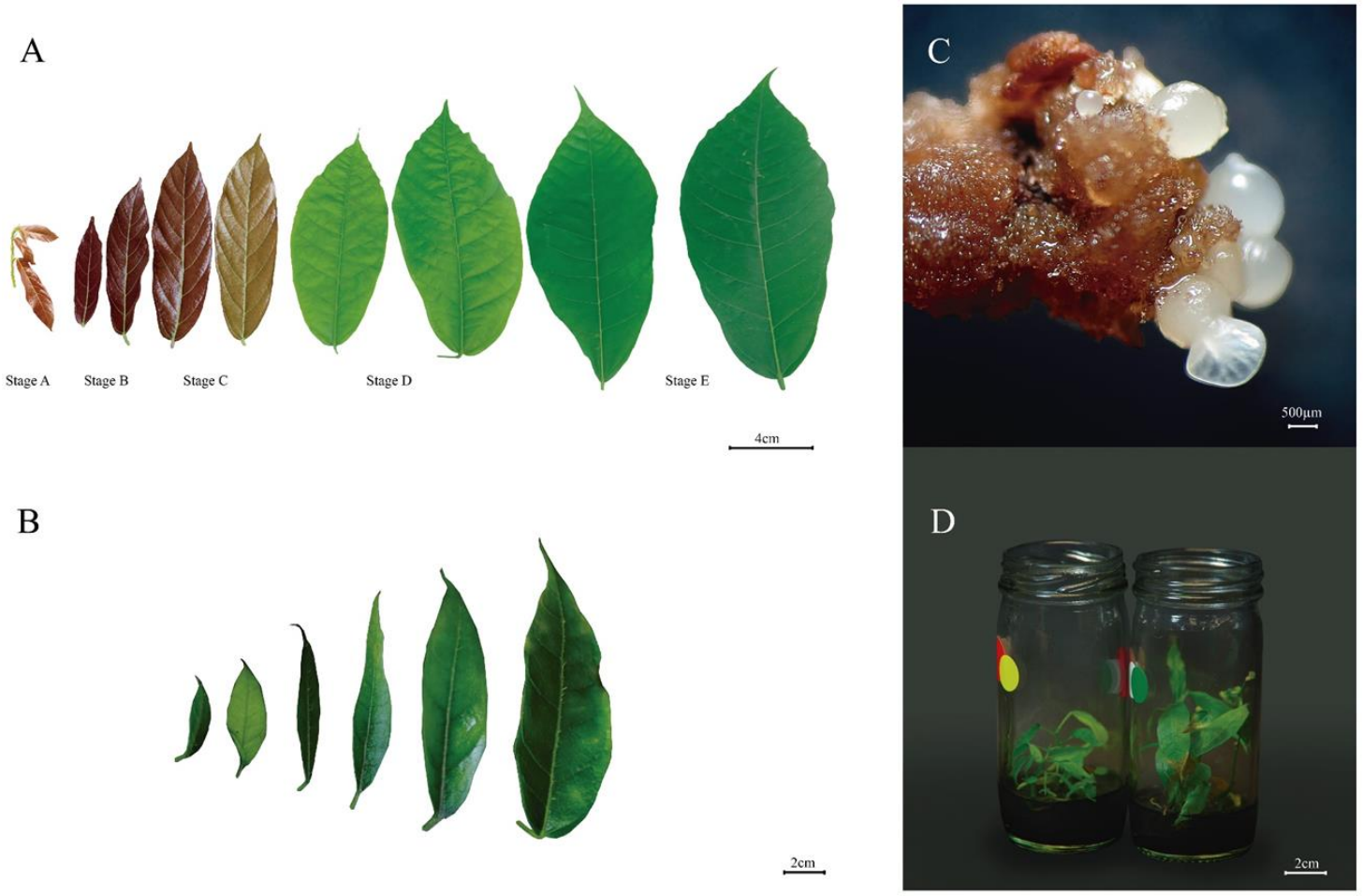

Figure 0-1 Cacao field plant leaves (FPL) and *in vitro* plantlet leaves (IVL) for extracting genomic DNA. A) *CCN51* genotype FPL developmental stages. B) *CCN51* IVL. C) TSH565 cotyledonary and globular somatic embryos. D) TSH565 *in vitro* plantlets (left) and *CCN51in vitro* plantlets (right) (90 days) days).

2.10.3 DNA Extraction

Total DNA extraction was achieved from *in vitro* grown plantlets (IVL) with three months in a MM6 medium, showing leaves from 2-5 cm length (Fig. 2-1 d). Leaves were selected and removed from the plant under sterile conditions in a laminar flow chamber. Likewise, for field plant leaves (FPL), 1cm x 1cm portions were cut with a scalpel, avoiding the removal of midrib tissue. This was performed in equally sterile conditions. The leaves were cut and immediately transferred to 2 ml tubes in liquid nitrogen and stored at -80°C until processing. The protocol reported by Henao et al. (2018a) was used to extract DNA from the FPL and IVL. The Power Plant® Pro DNA Isolation Kit MoBio (Qiagen) modified with an additional sorbitol buffer wash was successfully used to avoid mucilage, typical of cacao leaves. Mucilage is an abundance of polyphenols and polysaccharides. In summary, the sorbitol buffer was added to 0.1 g of macerated leaf tissue in Eppendorf tubes. The tubes were then placed in a cellular disruptor device (BeadBug™) for 5 minutes at 3,000 rpm. They were then heated to 65°C. Finally, samples were centrifuged for 10 minutes at 5,000 rpm, and the supernatant was ultimately discarded. This procedure was repeated twice, continuing with the other steps of the Power Plant® Pro DNA Isolation Kit MoBio protocol (Cat. no. 13400-50). DNA extraction experiments were performed using twenty biological replicates of FPL and IVL for each genotype. At least ten technical replicates were performed.

The integrity and concentration of obtained DNA were determined using NanoDrop™ (Thermo Scientific) spectrophotometer and later verified on 0.8% agarose gel stained with ethidium bromide (EB) (0.5 mg/ml) and visualised under a UV lamp. DNA quantity and purity were evaluated by measuring the A260/A280 and A260/A230 absorbance ratios.

2.10.4 SSR Analysis

“Thirteen SSR were chosen according to their high polymorphic information content (PIC) show in previous *T. cacao* genetic diversity analyses”: mTcCIR6, mTcCIR8, mTcCIR11, mTcCIR15, mTcCIR25, mTcCIR26, mTcCIR33, mTcUNICAMP01, mTcUNICAMP02, mTcUNICAMP05, mTcUNICAMP09, mTcUNICAMP16, and mTcUNICAMP17 (Lanaud, et

al. 1999; Irish, et al. 2010; Santos, et al. 2012) (Table 2-1). The primers were synthesised by Macrogen (Macrogen Humanizing Genomics, Seoul, Korea). For each SSR marker, the forward oligonucleotide (F) was labelled with a different fluorescent dye (6-FAM, HEX, and TAMRA): mTcCIR15, mTcCIR25, mTcUNICAMP05, mTcUNICAMP09 were labelled with 6-FAM (blue); mTcCIR6, mTcCIR26, mTcCIR33, mTcUNICAMP01, mTcUNICAMP16 were labelled with HEX (green); mTcCIR8, mTcCIR11, mTcUNICAMP02, mTcUNICAMP17 used TAMRA (Red).

Table 0-1 Theoretical characteristics for the thirteen microsatellite markers (SSR) used for the assessment of genetic fidelity experiments

	Primer sequence	**PIC**	**Size range (bp)**	**Reference**
mTcCIR6	F:TTCCCTCTAAACTACCCTAAAT R:TAAAGCAAAGCAATCTAACATA	0,96	222–247	Irish et al., (2010; Lanaud et al., (1999)
mTcCIR8	F:CTAGTTTCCCATTTACCA R:TCCTCAGCATTTTCTTTC	0,92	288–304	
mTcCIR11	F:TTTGGTGATTATTAGCAG R:GATTCGATTTGATGTGAG	0,74	288–317	
mTcCIR15	F:CAGCCGCCTCTTGTTAG R:TATTTGGGATTCTTGATG	0,87	232–256	
mTcCIR25	F:CTTCGTAGTGAATGTAGGAG R:TTAGGTAGGTAGGGTTATCT	0,84	124–170	
mTcCIR26	F:GCATTCATCAATACATTC R:GCACTCAAAGTTCATACTAC	0,69	282–307	
mTcCIR33	F:TGGGTTGAAGATTTGGT R:CAACAATGAAAATAGGCA	0,73	264–346	
mTcUNICAMP01	F: TCATGCAAAGCAAAGTGAAG R:ACGGGAAACTCATCATTACA	0,27	190-208	Santos et al., (2012)
mTcUNICAMP02	F: GGTCCTCCAAGCTGAGTAACA R:CTCCCTATTTGCATCGCATT	0,69	260-290	
mTcUNICAMP05	F:AGCTGTTTATGATTCACATCC R:GAAGCAGCAATTGTAACCAC	0,45	233-242	
mTcUNICAMP09	F:TTCGGCAGTTCGATCTATGA R:ATCCACCGTAAGCCTTTCCT	0,59	148-190	
mTcUNICAMP16	F:CAGGAAGGATACTTCTTAAAGG R:AGTAGAGTCGAGTGGCTTGA	0,24	244-254	
mTcUNICAMP17	F:CTGCACAGCTTCATGGATCTC R:TGATGATCAGGTGGTTTCTCA	0,21	296-300	

Each SSR was tested and amplified with the previously reported annealing temperature until its standardisation (Table 2-1). As explained by Don et al. (1991), a thermal touchdown amplification profile was used to set the exact banding temperature, reducing unspecific artefacts during replication. First was 5min at 94°C, melting 40s at 94°C and annealing 40s at primer temperature. Second were ten cycles with a decrease of 1 °C per cycle until it

reached melting temperature and 40s at 72°C for extending. This was followed by using the standard annealing temperature of each primer 20 more times. Lastly, there was a final 10min extension at 72°C, reaching 30 replication cycles. The enzyme Taq DNA Polymerase of Thermo Scientific (1 U/µl) was used. The PCR stock solution (20 µl) included 1.35 µl of DNA template (10 ng/µl), 1X PCR Thermo buffer, 2 mM dNTPs, 2.5 pmol each of forward (labelled) and reverse primers, 2 mM MgCl2, 0.5 ng/ml BSA (Bovine Serum Albumin), 0.1 µl Taq DNA polymerase and sterile water to complete the total volume of the reaction. Amplifications were made in a thermal cycler (LTCG Labocon 48-101). The amplified products were checked by 3% agarose gel electrophoresis after staining with ethidium bromide (0.5 mg/ml). Allele sizes were estimated by comparison with a DNA standard length marker (Gene ruler 1kb DNA Ladder® Bioline). Control samples were included to provide the correct approach to allele size SSR, and the PCR was repeated to ensure PCR reproducibility (6-10 replicates for SSR). Fragment analyses were performed in an automated ABI3730XL sequencer by the company, Macrogen (Macrogen Humanizing Genomics, Seoul, Korea).

2.10.5 Data Analysis

Allelic peak sizes were identified in Geneious Prime software 2018.2.1 (https://www.geneious.com) using the microsatellite plugin. The polymorphism information content (PIC) was calculated using the formula established by Botstein et al. (1980). Genetic diversity parameters like the number of alleles (Na) and observed heterozygosity (Ho) were computed for each marker using GenAlEx (Peakall and Smouse, 2006, 2012). Pairwise similarity matrices between the FPL and IVL plants were generated by Jaccard's coefficient of similarity using the R Project Software (Team 2013). Likewise, based on the genetic similarity matrix, a cluster analysis was performed using *Poppr* library of R Project Software (Kamvar, et al. 2014) with the Nei distances (Nei, 1972) by the unweighted pair group method with arithmetic average (UPGMA). In parallel, a principal component analysis (PCA) was also performed with the "princomp ()" function.

2.11 Results

2.11.1 DNA Extraction

The total extractions were obtained using Power Plant® Pro DNA Isolation Kit MoBio modified with an additional double Sorbitol buffer wash. Enough high-quality DNA was obtained (Fig. 2-2). A higher concentration of DNA was obtained from field plant leaves than from *in vitro* plant leaves. For every 0.1g of tissue, an average of 121.96 – 129.43 ng/µL for FPL and 58.46 – 60.75 ng/µL for IVL was obtained for both genotypes (Table 2-2). The absorbance ratio values at 260/230 were below 2 (≤2), indicating some DNA contamination by carbohydrates, salts, or organic solvents; however, it was sufficient and useful for using SSR markers.

The annealing temperatures of previous studies were used for SSR amplification, but they were not successful. Therefore, each SSR annealing temperature was standardized individually: mTcCIR6 and mTcCIR8 (46º); mTcCIR11 (38º); mTcCIR25 and mTcCIR26 (40º); mTcCIR33 (45º); mTcUNICAMP01 (47º); mTcUNICAMP02 (62º); mTcUNICAMP05 (55º); mTcUNICAMP09 (61º); mTcCIR15 (54º); mTcUNICAMP16 (59º); mTcUNICAMP17 (53º) (Data not shown). Product fragments of SSR amplification were verified using agarose gel (3%) separation electrophoresis for both FPL/IVL of *TSH565* and *CCN51* (Fig. 2).

2.11.2 Genetic Structure

The use of microsatellites mTcCIR6, mTcCIR8, mTcCIR11, mTcCIR15, mTcCIR26, mTcCIR33, mTcUNICAMP02, mTcUNICAMP05, mTcUNICAMP09, and mTcUNICAMP16 was presented in 10 individuals for both IVL/FLP of *TSH565* and IVL/FLP of *CCN51.* Microsatellites mTcCIR25, mTcUNICAMP17, and mTcUNICAMP01 were obtained for 6 individuals of each treatment by genotype (Table 2-3).

Among the 13 analysed loci, 25 alleles were found in *CCN51* and 24 in genotype *TSH565*. According to the detected loci, no differences were observed between FPL and IVL populations in each genotype, indicating genetic fidelity between the individuals resulting from propagation by somatic embryogenesis. Through the approach used with the SSR, no fixed alleles, or perceptible detriment were observed (Fig. 2-4). The mTcUNICAMP01 locus of *TSH565* had a 193/193 and 193/209 allelic composition in FLP/IVL, respectively. The allelic variation of parental field plants remains in plants derived from somatic embryogenesis (Fig. 3). A particular change was found at the mTcUNICAMP02 locus for the two genotypes studied in the allelic composition in the FLP population. In the IVL population of *TSH565,* the allelic composition was 277/295 for all individuals. For *CCN51,* the allelic composition was 269/295 for all individuals. However, the 290/306 loci were only found in the FPL populations in both genotypes and were not found in the IVL population. It should be noted that this change only occurred in 2 individuals.

The polymorphic information content (PIC) of the 13 SSR ranged from 0.305 - 0.997. Botstein et al. (1980) reported that the PIC index can evaluate the level of gene variation. When PIC>0.5, the locus is of high diversity when PIC<0.25, the locus is of low diversity, and the locus is of intermediate diversity when the PIC is between 0.25 and 0.5. Among these 13 SSR, 11 of them (mTcCIR6, mTcUNICAMP05, mTcCIR33, mTcUNICAMP16, mTcUNICAMP02, mTcCIR25, mTcUNICAMP01, mTcUNICAMP17, mTcCIR15, mTcCIR26, mTcCIR11) were high polymorphic loci with PIC values of over 0.500. Only mTcCIR8 and mTcUNICAMP09 were intermediate; none were low polymorphic loci with PIC values less than 0.250 (Table 2-3).

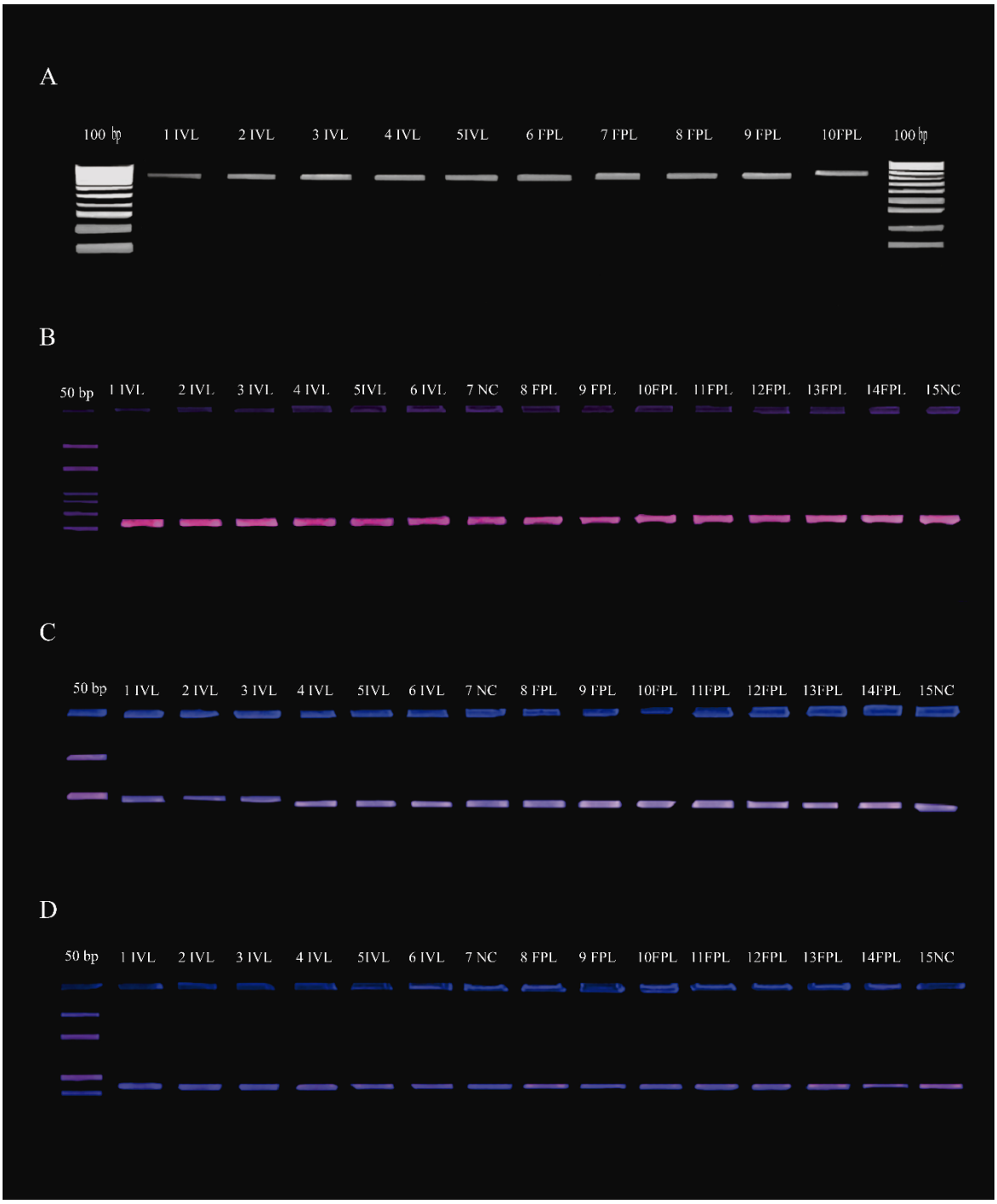

Figure 0-2. A) 0.8% agarose gel stained with ethidium bromide (EB) with DNA samples of *CCN51*. Lanes 1 -5 *in vitro* somatic embryogenesis (IVL) and 6-10 *ex vitro* field graft plants (FPL). M: 1 kb molecular marker (HyperLadder® Bioline). Amplification of different SSR in agarose gel (3%) separation PCR products for FPL and IVL from *CCN51*: lanes 1-6 IVL, 8-14 FPL, 7, and 15 negative controls (NC). B) mTcCIR25. C) mTcCIR15. D) mTcUniCamp9. M: 50bp molecular marker (HyperLadder® Bioline).

Genetic parameters (Na) and (Ho) were also determined for the applied SSR (Table 2-4). It was concluded that 10 and 9 polymorphic loci were presented for the *CCN51* and *TSH565* genotypes, respectively. Likewise, high heterozygosis was observed for both genotypes. A low homozygosis was observed for *CCN51,* present in loci mTcCIR25, mTcCIR26 and mTcUNICAMP09, and *TSH565* in markers mTcCIR6, mTcCIR8, mTcCIR25, and mTcUNICAMP17 (Table 2-4).

Table 0-2 Quantity and quality (OD260/A280-OD260/A230) of DNA isolated from field plants leaves (FPL) and *in vitro* leaves plantlets (IVL) of TSH565 and *CCN51*.

Genotype	***Source tissue***	***DNA concentration (ng/µL)***	***Absorbance ratio*** *OD260/A280*	*OD260/A230*
CCN51	FPL	129,43± 79,61	1,84± 0,03	1,85± 0,24
	IVL	60,75± 8,31	1,92± 0,03	1,88± 0,14
TSH565	FPL	121,96± 35,18	1,92± 0,09	1,18± 0,29
	IVL	58,46± 8,68	1,91± 0,05	2,37± 0,60

Data are means ±SE (n = 10).

Table 0-3 Description and polymorphism information content (PIC) values and allele sizes established of 13 SSR of T. cacao derived of *in vitro* somatic embryogenesis (IVL) and *ex vitro* field grafts plants (FPL) from genotype TSH565 and *CCN51.*

Locus	**PIC**	***CCN51* Genotype**				***TSH565* Genotype**			
		IVL		**FPL**		**IVL**		**FPL**	
		Allele size (bp)	**Number of samples**	**Allele size (bp)**	**Number of samples**	**Allele size (bp)**	**Number of samples**	**Allele size (bp)**	**Number of samples**
mTcCIR6	0,555	241/249	10	241/249	10	231/231	10	231/231	10
mTcCIR8	0,305	290/306	10	290/306	10	290/290	10	290/290	10
mTcCIR11	0,897	304/316	10	304/316	10	316/316	10	316/316	10
mTcCIR15	0,897	235/235	10	235/235	10	235/235	10	235/235	10
mTcCIR25	0,984	136/148	6	136/148	6	136/148	6	136/148	6
mTcCIR26	0,995	298/304	10	298/304	10	284/304	10	284/304	10
mTcCIR33	0,555	292/346	10	292/346	10	274/346	10	274/346	10
mTcUNICAMP01	0,968	193/211	6	193/211	6	193/193	4	193/193	3
						193/209	2	193/209	3
mTcUNICAMP02	0,997	269/295	10	269/295	9	277/295	10	277/295	9
				290/306	1			290/306	1
mTcUNICAMP05	0,555	233/235	10	233/235	10	233/239	10	233/239	10
mTcUNICAMP09	0,375	197/197	10	197/197	10	187/187	10	187/187	10

mTcUNICAMP16	0,897	248/248	10	248/248	10	238/248	10	238/248	10
mTcUNICAMP17	0,995	296/300	6	296/300	6	294/300	6	294/300	6

Jaccard's similarity coefficient (Jaccard, 1908) generated from SSR marker profiles indicated that all the IVL plants and FPL plants were genetically similar and could be grouped under one major cluster, showing more than 91% similarity for *TSH565* and 93% for *CCN51*. The cluster analysis using the similarity dendrogram (Fig. 2-5) shows the differentiation pattern between individuals based on the 13 loci studied in the genotypes. This result coincides with the results obtained for allelic composition. This explains the short genetic distance between individuals when comparing the same genotypes and treatments. The clusters that occur within *TSH565* are determined by the alleles or polymorphisms of mTcUNICAMP01 and mTcUNICAMP02. Likewise, this clade drastically separates the two analysed genotypes, *CCN51,* and *TSH565*. A Principal Component Analysis (PCA) was performed to confirm and better understand relationships between FPL and IVL populations, showing a similar cluster structure to the UPGMA method (Fig. 2-4). Principal component 1 explained 95.49% of the variation, and principal component 2 explained 2.71%. The PCA plots reveals two groups that correspond to genotypes *CCN51* and *TSH565*. Since most individuals per genotype have the same genetic profile, all samples overlap in only 5 individuals (Supplemental Fig 2-1). The PCA grouping pattern was very similar to the cluster revealed by UPGMA, with some FPL population individuals being different in the two studied genotypes, an additional result that corroborates the genetic uniformity of IVL plants. The genetic relationship between the individuals in each genotype and each population is probably due to their gene pool variation's stability. This similarity demonstrates how useful plant regeneration through somatic embryogenesis is for carrying out *T. cacao* multiplication processes from the field's parental material.

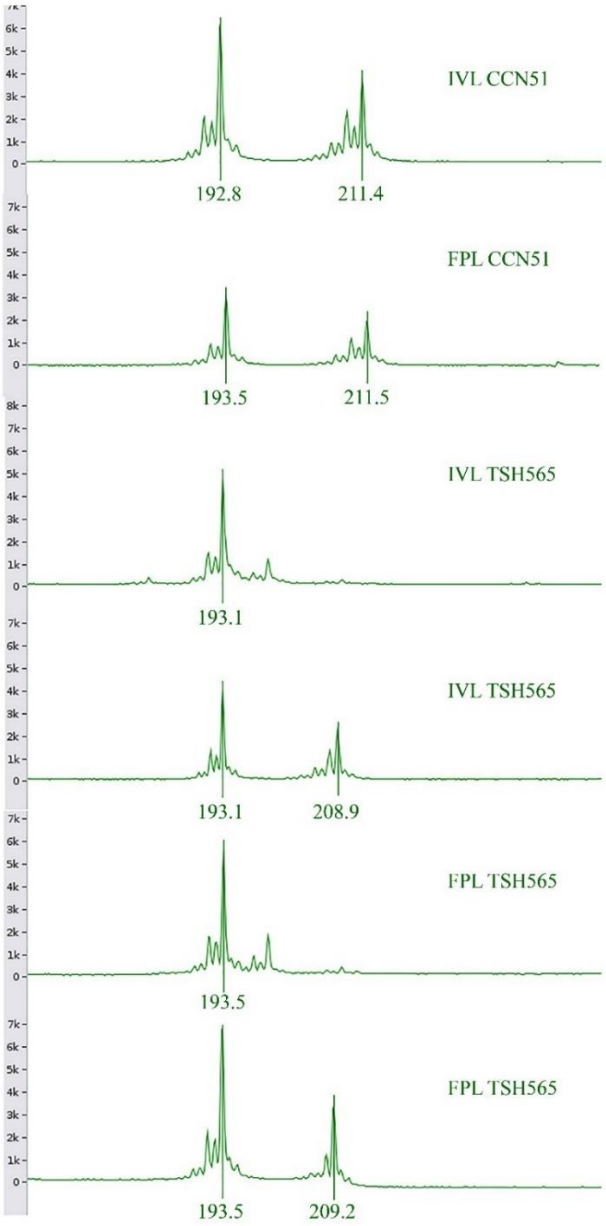

Figure 0-3 Allele peak profiles of mTcUniCamp1 locus, *TSH565* genotype has an allelic composition of 193/193 and 193/209 for both FLP and IVL. *CCN51* genotype has an allelic composition of 193/211 for both FLP and IVL.

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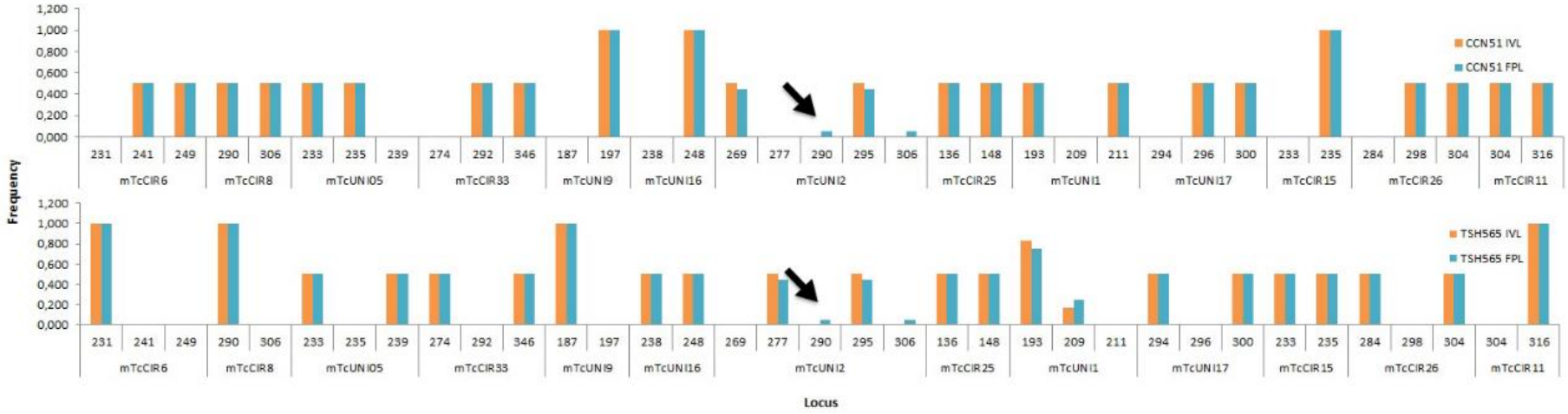

Figure 0-4 Allele frequency comparison. Field plant leaves (FPL) and *in vitro* plantlet leaves (IVL) treatments in *T. cacao CCN51* and *TSH565* genotypes for loci: mTcCIR6, mTcCIR8, mTcCIR11, mTcCIR15, mTcCIR25, mTcCIR26, mTcCIR33, mTcUNICAMP01, mTcUNICAMP02, mTcUNICAMP05, mTcUNICAMP09, mTcUNICAMP16 and mTcUNICAMP17.

Table 0-4 Details and genetic parameters of the 13 SSR used for evaluation of genetic stability of *CCN51* and *TSH565* genotypes of *T.cacao*. Allelic parameters: (Na) number of alleles and (Ho) observed heterozygosity.

SSR Locus	Na				Ho			
	CCN51		*TSH565*		*CCN51*		*TSH565*	
	IVL	FPL	IVL	FPL	IVL	FPL	IVL	FPL
mTcCIR6	2,000	2,000	1,000	1,000	1,000	1,000	0,000	0,000
mTcCIR8	2,000	2,000	1,000	1,000	1,000	1,000	0,000	0,000
mTcCIR11	2,000	2,000	2,000	2,000	1,000	1,000	1,000	1,000
mTcCIR15	2,000	2,000	2,000	2,000	1,000	1,000	1,000	1,000
mTcCIR25	1,000	1,000	1,000	1,000	0,000	0,000	0,000	0,000
mTcCIR26	1,000	1,000	2,000	2,000	0,000	0,000	1,000	1,000
mTcCIR33	2,000	2,000	2,000	2,000	1,000	1,000	1,000	1,000
mTcUNICAMP01	2,000	2,000	3,000	3,000	1,000	1,000	0,333	0,500
mTcUNICAMP02	2,000	4,000	2,000	4,000	1,000	1,000	1,000	1,000
mTcUNICAMP05	2,000	2,000	2,000	2,000	1,000	1,000	1,000	1,000
mTcUNICAMP09	1,000	1,000	2,000	2,000	0,000	0,000	1,000	1,000
mTcUNICAMP16	2,000	2,000	2,000	2,000	1,000	1,000	1,000	1,000
mTcUNICAMP17	2,000	2,000	1,000	1,000	1,000	1,000	0,000	0,000

2.12 Discussion

Producing true-to-type plants through *in vitro* propagation is one of the most important criteria for any successful protocol for economically important crop plants. Plant tissue culture techniques cause genetic instability due to variations in ploidy level, point mutations, or translocations, leading to somaclonal variation, which occurs by using various growth regulators in the medium (Largia, et al. 2015). Plants regenerated as explants using axillary buds, and meristematic tissue has shown the lowest genetic variation between 0 – 10 % (Vinoth and Ravindhran, 2016; Sherif, et al. 2018). However, many authors reported that plants regenerated through somatic embryogenesis exhibit genetic uniformity and integrity (Rai, et al. 2012), some reports substantiate the presence of genetic modification in plants derived from somatic embryos (Viehmannova, et al. 2016). The callus phase during indirect somatic embryogenesis can be an important source of somaclonal variation. In this study, we have regenerated plants through somatic embryogenesis with intermittent callus phases, which increases the chance of genetic change among regenerants. A disorganised growth phase in tissue culture, using growth regulators, the number and

duration of a subculture, stress, and genotype are all factors that enhance somaclonal variation (Bairu, et al. 2011). Due to the above, evaluating the genetic stability of *in vitro*-raised plants is a requirement for applying biotechnology to micro-propagate true-to-type clones. For this purpose, molecular markers could be effectively engaged to assess the genetic homogeneity and true-to-type nature of *in vitro*-regenerated plants.

This study reported a remarkable genetic uniformity *in vitro* plantlets when compared to the genetic composition of plants propagated by the conventional grafting method in genotypes *CCN51* and *TSH565*. Among the IVL and FLP populations for each genotype, no differences were found in the genetic composition of the 13 analysed loci, indicating that there were no stochastic variations caused by the propagation method. The conservation of allelic composition between the two populations shows that no somaclonal variations appeared. This result coincides with other studies on genetic polymorphism in soma-clones by SSR in *T. cacao* (Rodríguez, et al. 2004; Fang, et al. 2009; Ajijah, et al. 2016).

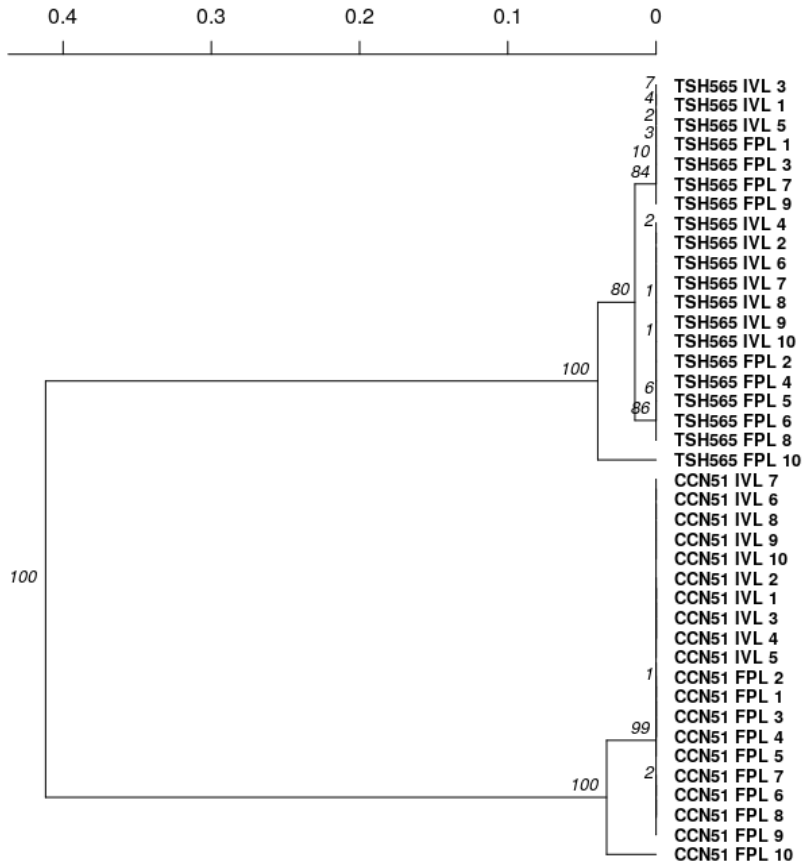

Figure 0-5 Hierarchical grouping between populations of *CCN51* and *TSH565* genotypes using the Nei distances (Nei 1972) by UPGMA method.

The high genetic fidelity displayed by the regenerated plants regarding the source of explants shows no significant variation induced by the applied regeneration protocol. The minor changes observed in the genetic similarity of 91% for *TSH565* and 92% for *CCN51* using 13 SSR alleles are from the presence, in the allelic composition changes, of the FPL population of the mTcUNICAMP02 locus in both genotypes and the change in allelic composition in the mTcUNICAMP01 locus in *TSH565* in both the FLP and IVL populations. Therefore, it cannot be concluded that somaclonal changes have occurred in regenerants, which is in line with previous studies' results. Rodríguez et al. (2004) used fifteen SSR alleles at heterozygous loci in *T. cacao* and somatic embryogenesis cacao regenerants. They observed 31% putative chimeric mutants for slippage mutation or allele loss across two loci. In addition, Fang et al. (2009) used eighteen SSR to screen a population of primary

somatic embryos and secondary somatic embryos. They observed 38.1% polymorphic profiles in the primary somatic embryos, while the frequency was 23.3% in secondary embryos. Ajijah et al. (2016), using nineteen SSR markers, observed a 97 – 100% level of similarity among regenerated plantlets. The differences with the previous studies may be the result of the genotypes used in each study. For example, Rodríguez et al. (2004) and Rodríguez et al. (2010) used LCTEEN 37/1, LCTEEN 162 / S-1010, SC3, and SIAL93, and Ajijah et al. (2016) used Cimanggu 2.

Likewise, the frequency of somaclonal variation differences may have occurred because of genotype differences or the PGR's used for inducing somatic embryogenesis in cacao. Fang et al. (2009) and Rodríguez et al. (2004) used TDZ in primary embryo induction media, while Ajijah et al. (2016) uses 2,4-D and KIN, but only for inducing primary embryogenesis. This study 2,4-D was used during the induction of primary embryogenesis with 2,4,5-T in the induction of successive embryogenesis. Some studies suggest that different PGR's can induce morphological abnormalities during *T. cacao* somatic embryogenesis because of its multi-dimensional function and chemical nature, leading to genetic modification (Garcia, et al. 2019). More significant abnormalities have been observed using TDZ (Rodríguez, et al. 2004; Rodríguez, et al. 2010) compared to 2,4-D and 2,4,5-T (Henao, et al. 2018b). In *T. cacao, the* somaclonal variation could result from genetic changes (mutation) (Rodríguez, et al. 2004; Fang, et al. 2009; Ajijah, et al. 2016) or epigenetic modifications (Rodríguez, et al. 2010; Adu-Gyamfi, et al. 2016; Pila, et al. 2017). These reports claim that morphological, cytological, and molecular variations may be generated *in vitro* for several factors, such as the genotypes and protocols used for *in vitro* culture. Consequently, more studies are required to elucidate the genetic and epigenetic changes according to each stressful stimulus that leads to the embryogenic process, such as the type of PGR and culture conditions.

Therefore, the percentage of similarity for the regenerant pool was higher using SSR (>90%). According to Ajijah, et al. (2016), the genetic stability obtained from the somatic embryogenesis method used in this work presents adequate values to be used as a method for the clonal propagation of cacao. This result supports the conclusion that the regenerated *CCN51* and *TSH565* plants produced via somatic embryogenesis had no variations with

respect to field plants. Similar results, where a genetic homogeneity of close to 100%, have been obtained from other species derived from somatic embryogenesis, both directly and indirectly, from *Bacopa monnieri* (Largia, et al. 2015), *Citrullus lanatus* (Vinoth and Ravindhran 2016), *Abutilon indicum* L. (Seth, et al. 2017), *Hibiscus sabdariffa* L (Konar, et al. 2018), *Cucumis melo* L. (Raji, et al. 2018) and *Anoectochilus elatus* Lindl (Sherif, et al. 2018).

2.13 Conclusion

In conclusion, this study validates the propagation protocol via somatic embryogenesis to produce plants from genotypes of interest, such as *CCN51* and *TSH565,* for commercial cultivation purposes. Furthermore, it confirms that molecular markers like SSR are reliable, robust, and quick tools that require very little plant material and relatively low-cost inputs with good results in terms of information for analysing somaclonal variation in somatic embryogenesis cacao regenerants. As perspectives, it is proposed to continue with the evaluation of the agronomic performance of the plants regenerated by ES under field conditions where the environmental and genetic factors are fully expressed. In this way, identify possible variants under conditions and finely determine the technical viability of the plants propagated by ES.

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Capítulo 3. Indicator framework for large-scale cacao (*Theobroma cacao* L.) *in vitro* plant production planning and controlling.

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2.15 Abstract

Somatic embryogenesis (SE) is considered the most suitable and integrated biotechnology for the large-scale production of clonal cacao plants, compared to conventional methods. Hence, scale-up of relevant technologies must be interfaced with effective and efficient management of productive process at industrial scale like biofactories. Production facilities like biofactories serve to transform plant resources into products like plantlets. This technology constitutes an important innovative variant since it allows to obtain high multiplication coefficients in short periods of time. Currently, there is no reference to carry out an adequate planning of the entire production process, for this reason it is not used to maximum production capacity of the facilities and there is a high level of uncertainty. With the aid of production planning and controlling (PPC) systems, inputs can be planned to achieve a determined output of products. Therefore, the objective of this work is to propose a production planning and controlling (PPC) system for SE cacao plantlet multiplication through the pilot large-scale. This paper presents input and output information considering main indicators of the production process like materials, labour, quality, and performance. Emphasis is placed on technical information on the production process of 100.000 plantlets in batch from *CCN51* genotype. Through the analysis of the model, challenges, and requirements for PPC system have been defined as a basis for future works and it will have allowed managed successfully the productive process.

Keywords: Commercial-scale propagation, cost of production, indicators, somatic embryo, vegetative propagation.

2.16 Introduction

Production planning and control (PPC) is the brain and the nervous system of the production program and is responsible for ensuring the availability all materials, part of assembly at the right time, at the right place, and in right quantities in order to enable the progress of operations according to the predetermined schedules at the minimum possible costs (Akmal et al. 2018). Nevertheless, this function forms part of the production system, and hence we must know more about the production system. PPC works with procurement, manufacturing, and program management to develop plans to execute customer requirements. Production planning is dynamic in nature and always remains in a fluid state, as plans may have to be changed according to changes in circumstances (Belhadi, Touriki and Fezazi 2018).

Input-output models are usually used to simplify real systems. The simplified model can be used to investigate the behaviour of the system in specific situations and to solve a research question. An input-output model consists of two basic elements: a system and input/output streams (Günther and Velten 2014). No general guideline exists for developing an input-output model (Moreau and Massard 2017). To describe and investigate the behaviour of a system, Günther and Velten (2014) defined the following general four steps:

(1) System analysis;

(2) Modelling;

(3) Simulation;

(4) Validation.

In step one, system analysis, all required input information to describe the behaviour of the considered system is collected. Based on the collected information from step one, a model (e.g., analytical, numerical, probabilistically) is modelled in step two. The model describes the relationship between the input and output information from the system. In step three, the model is transferred into a simulation. Based on the information collected in step one, the first experiments are carried out to present the real system. In step four, validation, the simulation results are tested in the real system to solve the research questions.

In step one of system analysis is essential the selection of indicators. An indicator is used to monitor an issue or condition and demonstrate a desired outcome. Indicators are varied and depend on the type of systems they are monitoring, with different levels of complexity (Schreiber et al. 2020). Indicators can be defined to describe the status of a system. The selection of the right indicators is a process that needs scientific and technical knowledge about how the system works and what information are required (Bila 2016).

An indicator does not mean the same as an indication or goal, an indicator is generally quantifiable, by aggregating different and multiple data. The resulting information is synthesised and is used to measure goals achievement. Indicators should be based on criteria and present some characteristics, such as specific, measurable, understandable, relevant and realistic, reliable, etc (Joung et al. 2013).

In Input-output model for conventional PPC systems production facilities serve to transform raw materials into products, usually with the goal of achieving a designated output (in terms of quantity and quality) at minimum cost (Stark, Seliger, and Bonvoisin

2017). PPC systems have two main functions for this transformation process. First, with the aid of PPC systems, raw material input, labour, and costs are planned to achieve a determined output of products. Secondly, PPC systems control the order management from order acceptance to delivery of the products. For both functions, the data bill of materials (BOM) and production plans are necessary (Gronau 2014). BOM is a formal list of raw materials, which presents material inputs required to produce a specific product. Work plans describe the required production steps to produce a specific product. The description contains information on dependencies to other production steps (subsequent-steps and simultaneously steps); required workstations; time between two subsequent-steps; production times divided into waiting time, setup time, processing time, and clearing time; and required raw materials (Trost, Claus, and Herrmann 2017).

In plant production through ES, we must solve the research question aimed at identifying the anticipated real cost of production in the biofactory facilities. The first step is to develop a system analysis, which uses the input and output model, and which describes the behaviour of the system's production from a PCC perspective in a biofactory. A search for indicators related to biofactories is carried out, where an overview of applicable indicators and/or guides for the selection of applicable indicators for PPC processes in plant biotechnology are not presented. To fill this gap, this paper presents information for current PPC systems in plant production by SE. An input-output model has been developed and through analysis of challenges and requirements for plant biotechnology PPC systems have been defined as a basis for future work and analyses.

2.17 Methodology

In the analysis system each part of the ES cacao plant production at biofactory was detailed. The required input and output information to describe the behaviour of the considered system was collected with the direction staff belong to the biofactory of Universidad de Antioquia and researchers with significative experience in ES cacao production (Gallego et al. 2016; Henao-Ramírez and Urrea-Trujillo 2020; Henao et al. 2021; Henao, De-La-Hoz, et al. 2018; Henao, Salazar, and Urrea 2018; Osorio et al. 2022). Production plans and data bill of materials (BOM) for 100.000 batch plant production were realized according to Gronau, (2014) (Supplemental 1 and 2). From BOM and production plan following Joung et al., (2013) conceptual proposals were selected the indicators based in the next criteria and characteristics:

(1) Specific: An indicator should be detailed and specific as possible, precisely formulated in order to measure only the desired output;

(2) Measurable: In a clearly and concrete way the indicator defines the measurement type, allowing data collection to be consistent and comparable. It can be simply and easily measured by quantitative or qualitative means;

(3) Understandable: An indicator should be easily interpreted, and one should know exactly what the output of an indicator demonstrates, in order to act accordingly to the needs;

(4) Relevant and realistic: An indicator must be relevant, fitting the measuring purpose and underlying the pointed issue, directly related to meaningful and purposeful aspects; and realistic considering that the needed data to calculate the indicators should be collected only through available resources, not being the collection too difficult or too expensive.

(5) Reliable: It must give a reliable picture of what is measuring, which does not mean the same as being precise, but rather that it contains trusted and accurate information.

(6) Timely manner: The data collection, calculation, and evaluation must be done in a timely manner, providing a structure that allows a meaningful progress monitoring. In other words, it should be perfectly stated the desired frequency for calculating the indicator, to better track the outcome results;

(7) Long term-oriented: It must ensure their future use, and reflect the development and adoption of organizational, process or product changes.

2.18 Results and discussion

It is the result of more than ten years of research, development of knowledge and experience in the production of elite plants from biotechnological techniques, for the improvement of agro-industrial production similar to others biotechnologies in South America (Galian et al. 2019). In the analysis system of the SE cacao plant production was identified different productions stages. SE as a productive approach is a complex process involving different biological mechanisms in each one of its stages: initiation, multiplication, maturation, germination, similar to reported by Egertsdotter et al., (2019). Briefly, the initiation stage includes the introduction to *in vitro* conditions (laboratory) and the induction phase. In the introduction phase, the plant material from the donor or parental plants is taken from field conditions to the laboratory for a disinfection process and obtaining sterile material. In the induction phase, plant material in controlled conditions is required a culture medium that promotes cellular differentiation and the formation of embryogenic cells (Kouassi et al. 2017). Once the embryogenic potential is

induced, indirectly proembryonic masses (PEM) are formed (Daouda et al. 2019). At this point, the SE allows unlimited multiplication of the original plant material (Fehér 2019). Cacao SE could be developed directly on previously somatic embryos in a process called secondary or recurrent somatic embryogenesis producing more embryos in multiplication stage (Henao-Ramírez and Urrea-Trujillo 2020). The change of culture conditions in embryogenic tissues allows developing the somatic embryo through the maturation stage and germination stage (Ajijah et al. 2016; Bustami and Werbrouck 2018; Garcia et al. 2018). In the conversion phase, the embryo transitions to a plantlet with the extension of the first leaves, elongation of the stem and formation of primary and secondary roots. Then, the plantlets continue its development until it forms at least -14 leaves, a stem, and roots with a length greater than 3-10 cm. Subsequently, the obtained plantlets can be transferred to *ex vitro* conditions for hardening or acclimatization. The seedlings adapt to the new environmental conditions in the greenhouse with a specific substrate, lighting, and irrigation adequate. Later, seedlings are transferred to a new substrate and when they reach adequate growth, they are taken to the nursery for their subsequent transfer to the field (Figure 3-1.). The productive approach is similar to other success process of plant *in vitro* propagation Al-Aizari et al., (2020); Dhiman et al., (2021); Sriskanda et al., (2021); Vyas et al., (2021).

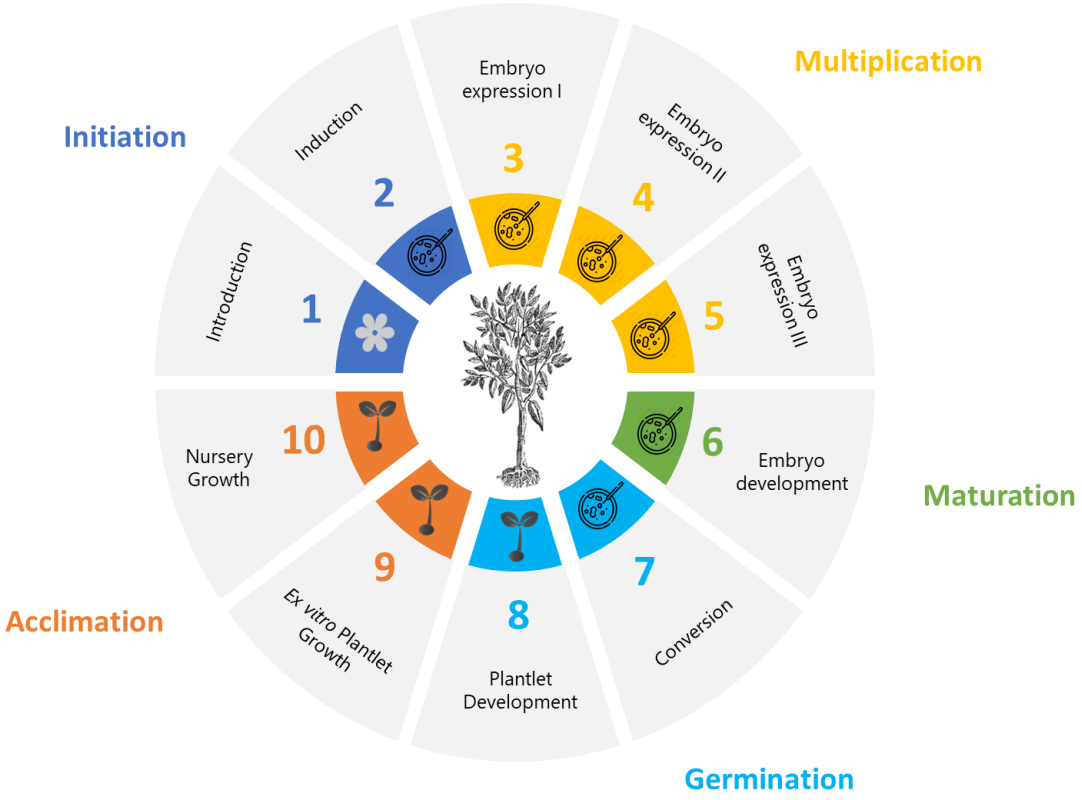

Figure 0-1 Flow chart of the plantlets production stages via somatic embryogenesis for different cacao genotypes.

Different informational elements on controls are considered for the productive process of cacao plantlets. This allows to measure the degree of objective attainment desired to be achieved in each stage of the process. The following has been taken into account when creating indicators in each stage: (1) Plant production requires a close relationship between the stages of SE and labour operations; (2) Labour operations must be executed in an inviolable sequential manner from the initiation stage to acclimation (3) The type of tissue obtained at the end of a phase of the initial material for the subsequent phase; (4) Labour operations are completely manual and are the basis for calculating the work (amount of plant material by vessel being processed by work day per person).

Based on BOM (Supplemental 1) and production plan description (Supplemental 2) for each stage: initiation, development, maturation, germination, and acclimation of ES cacao plant production and about the needed requirements to create useful input-output model, figure 3-2 shows an overview of basic model for PPC systems. The indicators for PPC systems for ES plant production were categorised into five groups: materials, costs, labour, quality, and performance (Figure 3-3) similar to Chen, (2016).

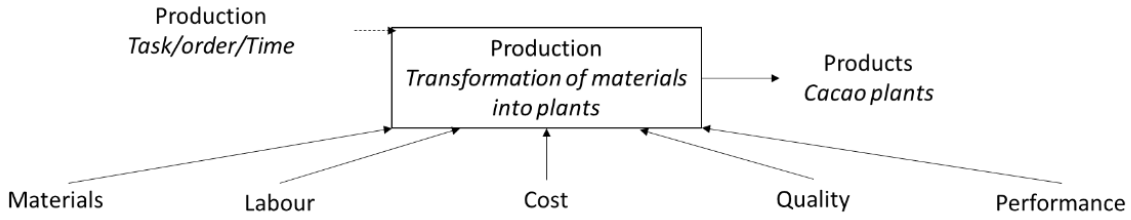

Figure 0-2 Input-output model for somatic embryogenesis (SE) cacao plant production planning and controlling (PPC) system.

2.18.1 Materials

The total amount of materials that are used to produce and package the plantlets produced is detailed in BOM (Supplemental 1). This includes raw materials, materials that are needed for the manufacturing process but are not part of the final product and materials for packaging purposes. The group of indicators materials considers the predictable material consumption from production specifically the supplies required for preparing culture media and the consumables of the process' operation like chemical products, gelling agent, culture vessels, glassware, culture tools, laboratory tools and

other various articles that are necessary for the process (Prakash, Hoque, and Brinks 2004).

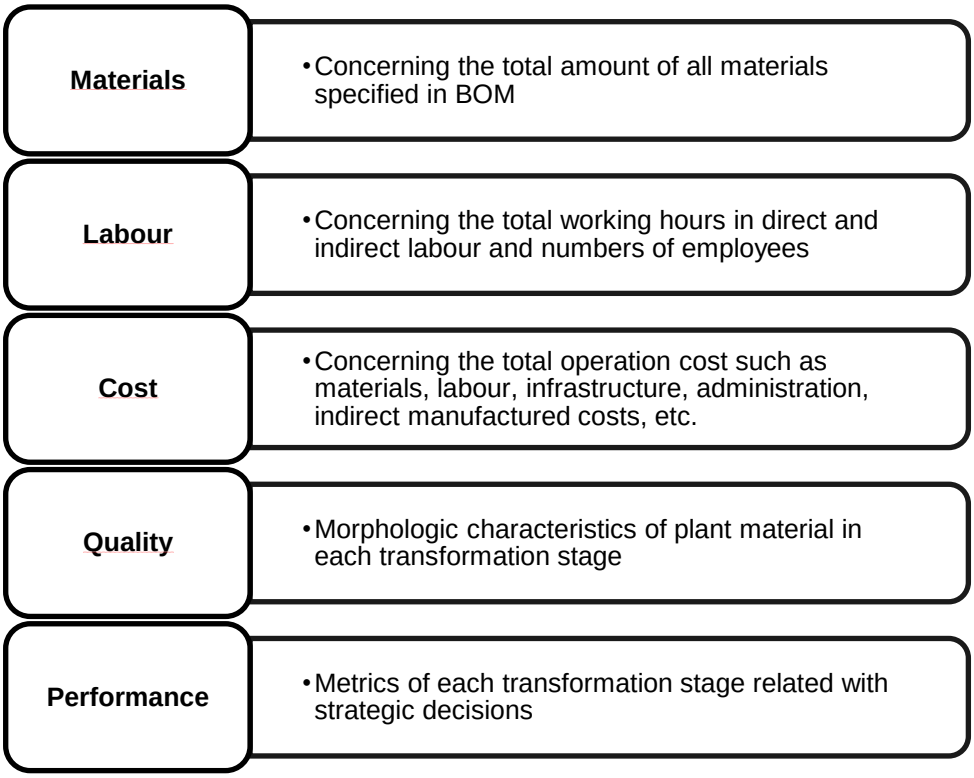

Figure 0-3 Indicator categorization structure containing five main groups: materials, costs, labour, quality, and performance for ES plant PPC system.

2.18.2 Labour

The group of indicators labour considers the predictable worktime of employees in the production facility. The total time of working hours in the production defines the total time of employees working in production to produce plantlets. The total number of employees in the production gives the number of employees working in production. For Colombia, a potentially working time per month of 166 hours was obtained (Supplemental 2). Labour was defined in two ways, direct and indirect labour, in direct labour there are considered functions in laminar flow chamber in all production stages like: disinfection of

explants, disinfection of tools and containers, bowl protection removal, vessels opening, cutting of the explants, culture medium replacement, selection of keys with embryos, cut to separate the embryos, selection by size of embryos, material discard, closing of the reseeded container, container labelling, fill tracking lists, etc. In indirect labour there are people who perform operational and technical functions and work individually in purchase of reagents, preparation of transport boxes for plant material from the field, repackaging reagents, storage, weighing of reagents, preparation of culture medium, autoclaving of culture media and materials, dispensing of culture media, washing of glassware and tools, etc.

In accordance with Cervelli and Senaratna (1995) supervisory positions must also be considered, which include people in charge for generally supervising direct work. For example, a supervisor must calculate the number of vessels and culture media required every day and every week and manage chemical products, media stock, equipment, and the maintenance of the respective laboratory stations for working. They are responsible for ensuring all explants begin production without any contamination, on time and in the right amounts. Besides, they are responsible for maintaining productivity and, most importantly, managing the group of direct workers and deciding whether crop production must be continued, harvested, or discarded at their discretion.

The strategy implemented to monitor the work carried out by an operator is using productivity norms or standards (W), which are obtained from the average time it takes for the personnel to carry out a certain activity. The staff is expected to develop a volume of processes in each time. In the following table it is presented and abstract of the activity, and the unit of measure (Supplemental 2).

2.18.3 Cost

The group of indicators costs considers the incurred and predictable costs from production. The total economic value is the sum of material of culture medium materials, direct and indirect labour of employee including wages, and benefits, indirect cost of manufacturing like transportation and laboratory tools and operation expense. Operation expenses are cash payments for facilities, energy, services purchased, depreciation of construction, installations and equipment (refrigerator, oven, autoclave, scale, pH meter, magnetic stirrer, stereomicroscope, water distillation unit, lighted shelves, timers, air conditioning, orbital shakers, etc.) and administration (Tomar et al. 2010). Services purchased includes payments to self-employed persons, temporary placement agencies and other organisations providing services (e.g., maintaining a machine). Employee wages and benefits are the total payroll, including employee salaries, employee taxes, levies, and unemployment funds and total benefits like pensions, insurance, health, and safety. The costs will be presented in the next chapter with the productive simulation scenario.

2.18.4 Quality

Quality indicators are tangible and quantifiable measurement instruments that allow evaluating the quality of each one of the process' stages, considering both input and output factors. The ideal morphological characteristics of flower buds, primary embryogenic calli, globular embryos, repetitive somatic embryos and plantlets are specified for each stage. In addition, indicators, such as microbial contamination, necrosis and abnormality are also specified (Table 3-1).

Table 0-1 Quality indicators for producing cacao plantlets in a biofactory (initiation, multiplication, maturation, germination, and acclimation stages).

Stage	Input	Output
Initiation	**Flower Buds** Morphological characteristics of flower buds: Flower buds in intermediate development stages, closed, at least 0.3 – 0.5 cm of length between the base of the peduncle and the apex. Flower buds with mechanical damage or necrosis and those that open during the disinfection process are excluded.	**Calli** Morphological characteristics of calli: Undifferentiated growth on the filamentous, cream-white staminode in a proportion greater than 70% of the explant.
Multiplication	**Calli** Morphological characteristics of calli: Undifferentiated growth on the filamentous, cream-white staminode in a proportion greater than 70% of the explant. INDexp-CM2 transition. Morphological characteristics of embryogenic calli with primary embryos: Aggregates of waxy light to dark brown calli with somatic embryos in a globular state of at least 0.5 mm – 3 mm, without walls defined between them and a translucent to off-white color, mostly in a cluster. Transition between CM2-EM2. Morphological characteristics of embryos from repetitive embryogenesis: Somatic embryos is an immature cotyledonary state, with differentiation of the epicotyl and hypocotyl, of at least 2 mm – 5 mm from the base of the radicular pole to the cotyledons, radicular axis with an off-white color. Embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos with numerous trichomes on their axes, embryos without apical or radicular development.	**Immature Embryos** Morphological characteristics of embryos from repetitive embryogenesis: Somatic embryos in an immature cotyledonary state, with differentiation of the epicotyl and hypocotyl, of at least 2 mm - 5 mm from the base of the radicular pole to the cotyledons, radicular axis with an off-white color. Embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos without apical or radicular development, number of contaminated embryos, number of necrotic embryos.
Maturation	**Immature Embryos** Morphological characteristics of cotyledonary embryos from repetitive embryogenesis: Somatic embryos with a defined polarity, differentiation between the epicotyl and hypocotyl, with an axis of at least 5 mm – 10 mm from the base of the radicular pole to the apex, an off-white axis, radicular axis with brown basal grooves and different shades in the cotyledons (transparent, ivory, pink and greenish).	**Mature Embryos** Morphological characteristics of mature cotyledonary embryos from repetitive embryogenesis: Somatic embryos with a defined polarity, differentiation between the epicotyl and hypocotyl, with an axis of at least 10 mm – 20 mm from the base of the radicular pole to the

	Embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos with curved or coiled hypocotyls, embryos with numerous trichomes on their axes, embryos without apical or radicular development.	apex, an off-white axis and different shades in the cotyledons (transparent, ivory, pink and greenish). Embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos with curved or coiled hypocotyls, embryos without apical or radicular development.
Germination	**Mature Embryos** Morphological characteristics of mature cotyledonary embryos from repetitive embryogenesis: Somatic embryos with a defined polarity, differentiation between the epicotyl and hypocotyl, with an axis of at least 10 mm - 20 mm from the base of the radicular pole to the apex, an off-white axis and different shades in the cotyledons (transparent, ivory, pink and greenish). Embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos with curved or coiled hypocotyls and embryos without apical or radicular development. MF-MF transition: Morphological characteristics of plantlets: a height of between 1 cm - 1.5 cm, radical development of at least 1 – 2 cm with primary roots, photosynthetically active cotyledons and the formation of 1 - 2 true leaves. Mature embryos with abnormalities: monocotyledonous embryos, multicotyledonous embryos, embryonic axis fusion, embryos with curved or coiled hypocotyls, embryos with numerous trichomes on their axes, embryos without apical or radicular development.	**Plantlets** Morphological characteristics of complete plantlets: with a stem length of at least 3 cm, at least 1 distinguishable internode, prominent radical development of at least 5 – 10 cm, and at least 1 – 4 true leaves. Plantlets with abnormalities: tuberous roots, roots without the presence of pivotal roots, bifurcated stems, curved or twisted roots.
Acclimation	**Plantlets** Morphological characteristics of complete plantlets: with a stem length of at least 3 cm, at least 1 distinguishable internode, prominent radical development of at least 5 - 10 cm, and at least 1 – 4 true leaves.	**Hardened Plantlets** Morphological characteristics of complete plantlets: with a stem length of at least 8 cm, at least 2 - 3 distinguishable internodes, prominent radical development of at least 20 cm, and at least 6 – 10 true leaves.

2.18.5 Performance

Performance indicators allow qualitatively and quantitatively demonstrating progress in each stage expressed as percentages defined in accordance with the productive biofactory-scale works (Alvarez 2014; Sánchez 2018) and empirical experience (Table 3-2). It was defined three group of main indicators: disinfection, biological response and, multiplication coefficients.

Percentages of disinfection: The production process requires a high level of safety, and which can be jeopardized by involving contaminated material, contamination of the culture medium, or contamination caused by poor washing of containers. Contamination processes are too risky for the process, since they can lead to the complete loss of a productive batch.

Percentage of biological response: It is estimated that only a percentage of the plant material introduced in the process generates a callus, which develops embryos, and these develop into seedlings, without malformations. According with quality indicators (Table 3-1).

Multiplication coefficients: In the multiplication stages of the production process, there is three phases in which several embryos are obtained from one callus and previous embryos. This process is strategic, since it manages to exponentially increase the formation of embryos that later become seedlings. However, it is important to note that the asynchronous development of the embryos leads to a continuous revision of the tissues at different moments in time during expression I, II and III in the multiplication stage.

For each stage was defined percentages of disinfection, survival, microbial contamination, callogenesis, calli forming embryos, primary globular somatic embryos, primary cotyledonary somatic embryos, calli with recurrent embryogenesis, different types and amounts of existing abnormalities, cotyledonary embryos with secondary roots, photosynthetically active cotyledonary embryos, normally developed *in vitro* plantlets, normally developed *ex vitro* plantlets, etc. (Table 3-2).

Table 0-2 Performance indicators for producing cacao plantlets in a biofactory (initiation, multiplication, maturation, germination, and acclimatization stages).

Initiation		**Multiplication**			**Maturation**	**Germination**		**Acclimatization**
Introduction	**Induction**	**Expression I**	**Expression II**	**Expression III**		**Conversion**	**Plantlet development**	
≥ 90% disinfection	≥ 95% callogenesis	5 embryos per explant	5 embryos per explant	10 embryos per explant	10 embryos per explant	≥ 70% mature cotyledonary embryos with normal steam	≥ 50% vitroplantlets with normal steam	≥ 60% plantlets with normal steam
≥ 90% survival	≤ 5% contamination	≤ 5% contamination	≤ 5% contamination	≤ 5% contamination	≥ 70% cotyledonary embryos	≥ 70% cotyledonary mature embryos with secondary roots	≥ 50% plantlets with secondary roots	≥ 80% plantlets with secondary roots
	≤ 50% embriogenic calli	≤ 10% primary somatic embryos (globular and cotyledonary)	≥ 90% repetitive embryogenesis	≥ 90% repetitive embryogenesis	≥ 70% cotyledonary embryos with secondary roots	≥ 70% photosynthetic mature cotyledonary embryos	≥ 70% vitroplantlets with true leaves	≥ 80% plantlets with true leaves
			≥ 70% globular and cotyledonary secundary somatic embryos	≥ 70% globular and cotyledonary secundary somatic embryos	≥ 70% photosynthetic cotyledonary embryos	≤ 5% contamination	≤ 5% contamination	≤ 60% survival
			≤ 30% abnormalities	≤ 30% abnormalities	≤ 30% abnormalities	≤ 30% abnormalities		

In addition to the indicators, Figure 3-2 contains the information stream production Task/Order/Time. Through this information, it is possible to normalise the indicators. The input indicators for ‘Costs’ and ‘Materials’ can be expressed as material consumption or costs per production order, production tasks, product output, and time (Stevenson, Hendry, and Kingsman 2005). The normalised indicator can be used to increase the efficiency of the production through, e.g. monitoring production, comparison of equal production orders, and for benchmarking processes (Pechmann and Zarte 2017). Also, the group of indicators product considers the product output from production. The weight or volume of products is the outcome of products, which are produced in production with the input materials.

In this sense, the Task/Order/Time refers to the level of efficiency of the production process, it is the ratio of flowers that enter the process, with respect to the seedlings that leave it. In the efficiency of the production process, it is sought to reduce the number of explants such as cacao flowers that enter the production process, and that the number of plants that leave it is greater.

2.19 Conclusion

Production planning and controlling (PPC) plays an important role in modern production enterprises. Current production management systems consider resources such as material, labour and production capacity and their respective costs, but sometimes neglect the role of quality and performance for cost savings. The PPC system has been identified, an analytical modelling of the operation of the productive propagation was carried out through ES plant cacao production, in which the main and support activities were identified, with the respective consumption, until the final product is obtained. With the data achieved from the model, the simulation was carried out, in which the indicators that are considered of greater relevance are monitored, and that tell us about the production process. This work constitutes the first step forward for an approximation of costs closer to reality.

Supplementary Materials

Supplementary 1. Bata bill of materials. Sheet 1 Inventory, Sheet 2 Culture media. Supplementary 2. Production plan. Sheet 1. Batch of Plants to be obtained, Sheet 2. Stages of production, Sheet 3. Direct and Indirect labour, Sheet 4. Culture medium and tools, Sheet 5. Assumptions.

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Conflicts of Interest

The authors declare no conflict of interest.

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Capitulo 4. Cost Analysis of Cacao (*Theobroma cacao* L.) Plant Propagation through the Somatic Embryogenesis Method

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2.21 Abstract

In vitro cacao (*Theobroma cacao* L.) production via somatic embryogenesis (SE) is being implemented to mass propagate clonal plant material with the donor material's prominent characteristics. Though it is an advanced technology, it is considered expensive compared to other propagation techniques. This work focused on identifying the key financial feasibility factors for the SE productive process. The process's costs were estimated, identifying factors that influence each laboratory's standardized ES process. A Monte Carlo Simulation (MCS) was performed to evaluate different variables upon increasing productive scale in a biofactory (commercial scale production). The projected lot volume was 100,000 plantlets in solid media, considering the process flow from *in vitro* introduction to acclimation. A biofactory operational model was projected, establishing time and operator movements, identifying direct and indirect costs in this way. Costs were defined by the standardized or integral method, with estimated and budgeted calculations, in order to set the cost per plantlet. The identified cost components were culture medium (CM), indirect manufacturing costs (IMC), labour (direct and indirect) and operating expenses. Labour had the greatest share of the costs, at 53%, followed by operating expenses, at 30%, CM, at 12%, and IMC, at 5%. The MCS helped define that the variables with the highest impact on unit price were the embryos' response in the germination-acclimation stage and the proliferation coefficient during the maturation stage. This projection yielded a figure of US $0.73 per plantlet. However, strategies to reduce this cost have been proposed. These strategies are mainly conducive to optimizing labour and implementing practices that increase the multiplication rate.

Keywords: Plant Tissue culture, Cost analysis, Large-scale production, Biofactory, Monte Carlo Simulation (MCS).

2.22 Introduction

Cacao (*Theobroma cacao* L.) production as a raw material for the chocolate industry has grown in many countries around the world. A concentration can be observed in tropical countries, led by African countries, such as Ivory Coast, Ghana, Cameroon and Nigeria, with 63.2% global production [1]. The continent of Asia, in Indonesia and Papua New Guinea, holds 17.4% of all production, and Latin America, in the countries of Brazil, Ecuador, Peru, the Dominican Republic, and Colombia, contributes 19.4% of the global output [2,3]. Colombia is the ninth-largest producer worldwide, with a minimum participation of 1.3% despite having significant production potential [4]. Colombia's geographic characteristics, such as its topography, climate, hydrography, and relative humidity, are ideal for developing this crop. These conditions can be found in a significant percentage of the territory [5]. Production is currently performed in 29 of the country's 32 departments, in which Santander, Antioquia, Arauca, Huila, and Tolima stand out, representing 70% of national production [6].

Developing mass propagation systems that facilitate plants' high availability to establish new crops and renew existing old crops is required to increase national production competitively. Asexual or vegetative propagation plays an essential role in reliably reproducing desirable characteristics, and, in the case of vegetative propagation, it is traditionally performed through grafting and root cuttings. However, both techniques have low production levels, the reason why extensive clonal gardens are required to produce enough material, limiting automation and scaling. Moreover, due to genetic improvement programs performed in several research centres around the world for cacao, there are a considerable amount of improved genotypes [7–10]. Nevertheless, one

of the largest limiting factors to harnessing this germplasm is the lack of mass cloning methods for the selected plants that are financially and agriculturally efficient.

In this sense, developing *in vitro* plant tissue culture techniques allows obtaining plants from one same tissue by taking advantage of plant cells' totipotence [11]. The main advantage of *in vitro* cultures as a propagation method is the uniform production of plants that conserve the original plant's characteristics. Besides, the processes are amenable to being scale up to an industrial level [12]. In addition, it allows mass propagating elite plant material in any season, independent of natural environmental conditions, conserving the plants' genetic potential [13]. There is a possibility of obtaining pathogen-free plants (fungi, bacteria, viruses) [14]. This technology facilitates optimizing the use of controlled environmental and nutritional factors, optimizing culture areas with large numbers of plants per unit of area, and conserving genotypes selected for prolonged periods. Furthermore, genetically modified plants can be obtained using *in vitro* regeneration technology, shortening plant breeding program times.

There are various *in vitro* asexual propagation methods. Among them is multiplication via SE, which, in accordance with Mendéz et al. 2019 [15], is defined as a process in which a bipolar structure with radial and apical axes similar to a zygotic embryo is developed from a somatic cell without a vascular connection to the original tissue and is capable of growing and creating typical plants. Studies on SE in cacao have been performed for over 40 years [16]. Esan 1977 [17] was the first to report the formation of somatic embryos, and, since then, numerous studies have been performed to optimize the number of embryos formed by explant [18–21]. Subsequently, the work performed by Maximova et al. 2002 [22] and Fontanel et al. 2002 [23] stand out, in which secondary embryogenesis was proven to allow obtaining a larger number of regenerants synchronously. In the last five years, the work performed by Garcia et al. 2018 [24] has

stood out, which reports efficiently obtaining plants via direct SE and in both liquid media and solid media.

On the other hand, it has been demonstrated that cacao plants resulting from this propagation system have an agronomic behaviour without significant differences concerning plants propagated by conventional methods, such as grafting [25–27]. Companies, such as Nestlé, have currently standardized the process to establish their crops in countries such as Ecuador, Indonesia, Puerto Rico, Brazil, Ghana, and Ivory Coast [28]. There are no reports in Colombia of large-scale cacao plant material production by biotechnological methods, such as SE. SE propagation results have been obtained in solid media for universal genotypes *CCN51, TSH565, EET8, ICS1, ICS39, ICS60, ICS95* and *IMC67* [29] and regional genotypes *CNCh12, CNCh13, CNCh16, CNCh24 and CNCh4* [30,31].

The financial analysis's importance to ensure the feasibility of the production process via SE has been mentioned in various studies. It is currently recognized that one of its most challenging aspects is reducing the process's cost [32–34]. There has been increased interest in the problems related to large-scale plant production in the last two decades [35–38], as well as in reducing commercial production costs. However, the real details of calculating costs of producing plantlets by SE are seldom discussed or reported [12,39–41]. A series of cost reduction strategies have been developed to overcome this limitation in the entire production process, but the cost would largely depend on the effectiveness and efficiency of the SE, which depends on the cacao genotype of interest. It usually has been argued that high propagated material costs, specifically by SE, can be justified by increasing the crop's productivity and uniformity [42]. Even though this is plausible, it will not always be achieved until they can prove it at the corresponding

production scale and know how much of a return on investment can be obtained and, even more importantly, when it will be obtained [38].

The time factor and frequency of productive lots are particularly important considerations for a ligneous species like cacao since it requires a long time to finish the entire cycle. In addition, the species is recalcitrant to *in vitro* cultures and has different responses depending on the genotype [43]. Various researchers have estimated the cost of producing plants propagated via SE for ligneous species and have observed that approximately over 50% of the total cost per plantlet corresponds to labour, clarifying that they are studies performed in developed economies, such as the United States [44–46]. Labour is highly competitive in countries like Colombia, circling US $229 as of October 2020 [47], representing an opportunity to develop these technologies.

Nowadays, risk management is an integral part of evaluating any project. Therefore, techniques, such as the Monte Carlo Simulation (MCS), a computerized mathematical approach that allows taking risk into account in quantitative analyses and decision-making, become relevant [48]. The study considers repeating a process that generates many random samples linked to specific variables of interest *n* times. Professionals from fields as disparate as finance, project management, energy, manufacturing, engineering, research and development, insurance, oil and gas, transportation, and the environment using this technique [49]. The MCS offers the responsible person or organization the option to make decisions from a series of possible results, as well as the probability that they will occur according to the measures taken. It shows extreme possibilities, the results of taking the riskiest and most conservative estimates, and all the possible consequences of intermediate decisions, which are

pertinent for this case study because production on an industrial scale is being discussed.

In this context, and with the Laboratory of Plant Physiology and Tissue Culture of Universidad de Antioquia's experience with SE in cacao, this study's objective is to analyze the productive process of cacao plantlets by SE from a financial perspective. The process's costs were estimated to identify each standardized stage in the laboratory, and a projection of the production process was performed on an industrial production scale like biofactory (commercial laboratories used for propagation). The analysis allows planning a pilot for biofactory installations, defining and estimating the factors that influence the cost per obtained plant the most and the least through an MCS. This information is useful for identifying low-cost strategies to apply during technological development.

2.23 Methodology

2.23.1 Location

The study was carried on the cacao plants' productive process via standardized SE at the Laboratory of Plant Physiology and Tissue Culture of Universidad de Antioquia, located in the city of Medellín, in the department of Antioquia - Colombia. The work team has a significant track record researching this topic, from 2008 until now [13,16,30,31,50,51]. The infrastructure of the Universidad de Antioquia biofactory was used to project costs on an industrial scale, located in the municipality of Carmen de Viboral in the department of Antioquia, Colombia. Universidad de Antioquia's biofactory is the only installation in Colombia specialized for *in vitro* plant material. It has a strategic location 20 minutes from José María Córdoba international airport. It is a 1,227 m^2 building capable of

producing up to 7 million plants each year via organogenesis and SE. The installations allow automating and scaling propagation protocols, have an architectural design that harnesses sunlight efficiently all day in growth chambers, a natural water source in accordance with the regional environmental authority and its own water purification system. They also have a modular design that facilitates expanding specific areas, an automated 276 m^2 greenhouse for plantlet acclimation, and 1,101 m^2 of manual operation greenhouses. All these structural characteristics allow efficiently using sunlight and water at a low cost. Other authors have reported these factors to be significant within process costs [52].

2.23.2 Stages of Cacao Plant Production via SE

The productive concepts proposed by Egertsdotter et al. 2019 [38] for producing ligneous plants were adapted in order to define the stages of cacao plant production by SE.

2.23.3 Cost Components

The systems engineering methodology for *in vitro* tissue cultures proposed by Chen 2016 [53] was used to identify and select the factors that influence the production process. Three main components were included in the cost structure: direct and indirect labour, the cost of materials and supplies required for plant propagation, which extends to some indirect manufacturing costs, and, lastly, operating expenses, which include the infrastructure, depreciation, and administrative expenses required to launch the business. All parameters and their composition are listed in detail in supplement 1.

Assumptions

The following premises were considered to project cacao propagation costs:

- Losses due to contamination and necrosis depend on the quality of the initial tissue and the planting process.
- Multiplication coefficients and percentage of explants' response depend on controlling subculture times and environmental factors, such as light, temperature, and relative humidity.
- Work performed by employees is calculated under-skilled labour's operational efficiencies with at least one year of experience handling explants in each stage.
- Labour standards can change depending on the hired technical personnel's training time and experience.
- Thirty percent of the total is regarded to cover the biofactory's administrative expenses, in which operating expenses and utility are considered.
- The practical exercise was performed to produce 100,000 plants, which is equal to 1.4% of the biofactory's installed capacity.
- The performed analyses are a projection of data obtained in the laboratory over various years. They allow establishing a baseline of work by simulating productive factors.

The analysis was performed based on the total plants to be produced by lot to begin identifying cost components, considering the number of explants, vessels, and quantity of culture media for each process stage through the following variables and formulas listed in table 4-1.

Table 4-1 Variables and formulas for calculating the number of explants, number of vessels and quantity of culture media by SE productive stage

Productive Stage	Phase	Period	Loss Rate	Multiplication Rate	% Explant Response	No. of Explants per Stage	No. of Vessels	Quantity of Media
Initiation	Introduction	t_{15}	L_0	1	B_0	$Ni=N_0/((1-L_0)*B_0)$	$V_0=Ni/C_0$	V_0*R_0
	Induction	t_{45}	L_1	1	B_1	$N_0=N_1/((1-L_1)*B_1)$	$V_1=N_0/C_1$	V_1*R_1
Multiplication	Expression I	t_{75}	L_2	M_1	B_2	$N_1=N_2/((1-L_2)*B_2*M_1)$	$V_2=N_1/C_1$	V_2*R_2
	Expression II	t_{95}	L_3	M_2	B_3	$N_2=N_3/((1-L_3)*B_3*M_2)$	$V_3=N_2/C_2$	V_3*R_3
	Expression III	t_{115}	L_4	M_3	B_4	$N_3=N_4/((1-L_4)*B_4*M_3)$	$V_4=N_3/C_2$	V_4*R_4
Maturation	Maturation	t_{135}	L_5	M_4	B_5	$N_4=N_5/((1-L_5)*B_5*M_4)$	$V_5=N_4/C_2$	V_5*R_5
Germination	Conversion	t_{155}	L_6	1	B_6	$N_5=N_6/((1-L_6)*B_6)$	$V_6=N_5/C_2$	V_6*R_6
	Plantlet Development	t_{215}	L_7	1	B_7	$N_6=N_7/((1-L_7)*B_7)$	$V_7=N_6/C_3$	V_7*R_7
Acclimation		t_{335}	L_8	1	B_8	$N_7=N_8/((1-L_8)*B_8)$	$V_8=N_7/C_4$	V_8*R_8

t (Time): The period each stage of the productive process requires, measured in days; L (Loss rate): Measured as a percentage, this is each productive stage's losses due to contamination or necrosis; M (Multiplication rate): The multiplication coefficient of plant material during the multiplication and maturation stages. It is produced at different moments during embryo development. Five times the initial number was obtained in M1 and M2 and it doubled in M3 and M4 (ten); B (Explant response): Measured as a percentage, it is equal to the rate of explant response in each stage; N (No. of explants per stage): This is the number of explants processed in each productive stage. The model projects labour requirements and materials in accordance with the desired plants at the end of the process. For this reason, N8 was calculated as the amount of cacao plants desired at the end of the process, N8 = 100,000. It is added, for the calculation, that Ni (initial) represents the flower buds entering the process, which have 5 staminodes, for which reason it increases by 5 from Ni to N0; V (No. of Vessels): This is the number of vessels required in each productive stage. The calculation is performed based on the number of processed explants and between each type of vessel's capacity; C (Vessel Capacity): This refers to the number of explants in accordance with the type of vessel used in each stage. The vessels are conical tubes, Petri dishes, magenta vessels and germination

trays. The assigned variables are: C0 is a conical tube with a capacity for 350 flower buds, C1 is a Petri dish with a capacity for 25 calli, C2 is a Petri dish with a capacity for 49 embryos, C3 is a magenta vessel with a capacity for 20 plantlets, and C4 represents trays with a capacity for 50 plantlets; R (Culture medium): Equal to the volume of culture media required by type of vessel in a 50 ml conical tube, 30 ml Petri dish, 150 ml magenta vessel and 100 g germination trays; Q (Quantity of Media): This is the result of the number of vessels required in each productive stage by the necessary media volume per vessel.

2.23.4 Culture Medium (CM)

Each productive stage has different culture media formulations in accordance with the developmental stage of SE to be induced: induction - INDI, multiplication (expression I - INDIexp, expression II - CM2, expression III - EM2), maturation EM2, germination - MM6 (Table 4-1 S1: Composition culture medium), and the cost may differ (Sheet 4 S2: Culture medium). The culture medium is calculated with the formula (1) (Table 4-2). On the other hand, it is important to keep in mind that most culture media supplies are imported, and costs are affected by the Representative Market Rate (RMR).

Table 4-2. Formulas and variables for calculating the components of the productive process' cost.

Culture Medium (CM)	**Indirect Manufacturing Costs (IMC)**	**Direct Labour**	**Indirect Labour**	**Operating Expenses**	**Total Cost (TC)**
(1) MC= (V * C) * (P1*I1+P_2*I_2+… +Pi*Ii)	(2) CIF = MH / U + CT + DWV	(3) W_t= (V/Wc + Wa) * (SMLMV + FP)	(4) W_t= (Ta * D * MOI) * (SMLMV + FP) * (r)	(5) GO: (MC + CIF + MOD + MOI) *(1/(1-%Admon) – 1)	(6) CT = MC + CIF + MOD + MOI + GO
- P (Price): Each input's price. - I (Input): The inputs required to produce one liter of culture medium. - V (No. of Vessels) - C (Vessel Capacity)	- MH (Materials and Tools): The materials and tools that get partially or fully worn out in producing the productive lot. - U (Uses): The number of times a partially worn-out material or tool is used. - CT (Transportation cost): The cost of transporting the plant material to the greenhouse. - DWV (Daily staffing): Daily staffing required for the productive lot's production.	- V (No. of Vessels): This is the number of vessels required in each productive stage. - Wc (LCF work standard): The days an employee requires to process the volume of vessels in a workday. - Wa (support process work standard): The days an employee requires to provide support in each productive stage, measured in days. - MOD (Direct labour): The labour required for total production. The obtained result is given in workdays multiplied by cost. - SMLMV (Current Legal Monthly Minimum Wage): The minimum wage established by the Colombian national government. - FP (Social benefit factor): The additional payment due to employee benefits, legal and extralegal benefits, subsidies, and all additional payments enforced by the Colombian national government for employees.	- Ta (Accumulated time): The time of each productive stage is calculated in months from when the induction phase begins until it ends with acclimation. - D (% Time allocation): Refers to the supervisor's time. - MOI (Indirect labour): The labour required for supervision. - SMLMV (Current Legal Monthly Minimum Wage) - FP (Social benefit factor)	- CM (Culture medium) IMC (Indirect Manufacturing Costs) - MOD (Direct labour) - MOI (Indirect labour) - %ADMON: The percentage of administration designated by the biofactory's management.	- CM (Culture medium) - IMC (Indirect Manufacturing Costs) - MOD (Direct labour) - MOI (Indirect labour) - GO (Operating Expenses)

2.23.5 Indirect Manufacturing Costs (IMC)

Indirect manufacturing costs are part of the production but cannot be allocated to a production plant or lot because they also have a staggering behavior. Indirect manufacturing costs are calculated using formula (2) (Table 4-2).

2.23.6 Labour

Direct labour includes the hours required to collect the plant material used as explants (flower buds), which extends to packaging and shipping them. It also includes work in the laminar flow cabinet (LFC) and work performed in support activities, such as preparing, dispensing, and replacing culture media; preparing and disposing of materials; washing vessels, monitoring, and selecting.

There is also indirect labour provided by a supervisor with functions related to coordinating direct work, calculating the number of necessary dishes and media, managing chemical products and media stock, equipment, and maintenance for the critical areas. Moreover, they are responsible for ensuring all explants begin production without contamination, on time, and in the correct amounts. They are responsible for maintaining productivity and, most importantly, they will decide whether crop production must be continued, harvested, or discarded at their discretion. The supervisor must coordinate decisions on what product, how many units, and when and where to ship with the administrative department during packaging and shipping. Direct labour is calculated with the formula (3), and indirect labour is calculated using formula (4) (Table 4-2).

2.23.7 Operating Expenses

The biofactory's operating expenses include management, sales, equipment depreciation (caster racks, laminar flow, pH meter, balance, magnetic stirrer, autoclave, distillation unit, minor Equips, mist chamber, fridge, etc.), leasing and public utilities (water, light, Internet, telephone), among others. A historical average of the years the biofactory has operated was considered to calculate operating expenses. Operating expenses are calculated using formula (5) (Table 4-2).

Finally, the total costs associated with production are calculated following formula (6) (Table 4-2).

2.23.8 Monte Carlo Simulation (MCS)

The RMR, loss percentage, percentage of explants with responses, multiplication coefficient, and standard productivity variables are defined in each of the production stages and phases of SE as variables of interest to establish their impact on costs when modifications are made to the productive process. Each one of the variables is assigned a triangular distribution, which is traditionally used for cost analyses. Three model estimation points are defined, placing the laboratory results as the central value, and a 10% variation range is established above and below each variable. A triangle-shaped distribution takes form due to the above, where the highest probability is around the middle.

A great number of simulations can be performed using the MCS analysis, modifying the established ranges for each variable. Having all variables in a similar range allows identifying the most strategic variables on which the team's work should focus to obtain the best results. In this study, the MCS was performed with @risk 8.0.1 [54] software, with 100,000 iterations for each variable and 100 simulations of the same process. The response objective was cost per plant.

2.24 Results

2.24.1 Stages of Plant Production via SE

The production process for the *in vitro* propagation of cacao plants by SE was identified to have five stages: initiation, multiplication, maturation, germination, and acclimation (Figure 4-1). Various processes, such as the explant disinfection process, called introduction, and callogenesis induction, are included in the initiation stage. In the multiplication stage, undifferentiated tissues are transferred to the primary embryo expression medium with a first multiplication coefficient of 5 for the phase called expression I. An average of 5 embryos are produced for each callus. Once primary somatic embryos are obtained, they are divided using a blade. The pieces are placed in the repetitive or secondary embryogenesis

induction media with a second multiplication coefficient of 5. This phase is called expression II. Therefore, somatic embryos develop asynchronously and are harvested from the globular and early cotyledonary stage to prevent embryonic axes from fusing during development. The embryos are subsequently transferred to a hormone-free medium where repetitive embryogenesis occurs again, with a multiplication coefficient of 20. This is called the expression III phase. Up until this point, both the initiation and multiplication stages are performed in a dark room at an average temperature of 26 – 27 ± 2 °C. In the maturation phase, somatic embryos develop with clear differentiation between their apical and radicular meristematic poles. Embryo maturation is set off by a change in the culture medium's composition, where the source of carbon and growth regulators are vital components. Subsequently, the germination stage is composed of the conversion phase to plantlets and their development in terms of increased stem length, secondary root formation, and leaf formation. When they reach a certain height, they are transferred to 500 ml culture vessels. Plantlets are kept under natural lighting in growth rooms during the maturation and germination stages. Finally, plantlets are transferred to greenhouse conditions for growth and elongation during the acclimation phase. Plantlets that are 3 - 5 cm high, with 1 - 3 leaves and both primary and secondary roots, are selected, removed from their culture vessels, and washed with top water to eliminate culture medium surpluses. They are then transferred to 50 alveoli germination trays with a substrate made of a mixture of sand and unenriched basic coconut coir. The tray is hermetically sealed for one day, and holes are subsequently opened to allow gas exchange, keeping the substrate hydrated with a Hoagland solution [55]. After this time, the tray is opened and kept in the ideal growth conditions for cacao in this stage, with a 50% shade percentage, the temperature of 24 – 30 ºC, and relative humidity over 60%.

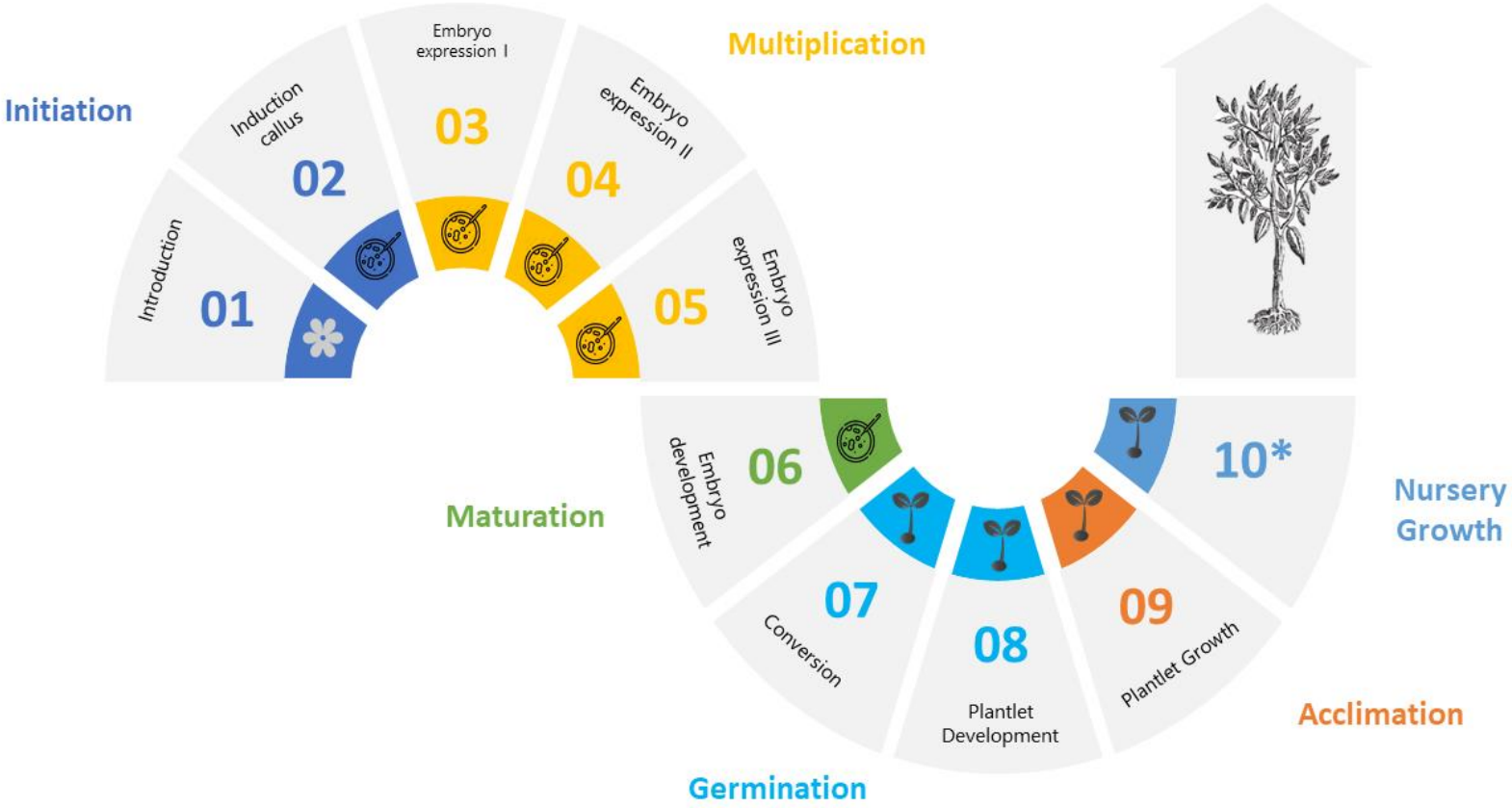

Figure 4-1 Stages of cacao plant production via somatic embryogenesis (SE).

* Plantlets growth in greenhouses is not considered in this study.

2.24.2 Cost Components

It was determined that N_i 4190 flower buds are required during plant material introduction, and N_0 9429 staminodes must be processed to induce callogenesis in order to produce a batch of 100,000 plantlets (Sheet 2 S2: Stages of production). For the multiplication stage, $N_1 + N_2 + N_3$, a total of 27,827 embryos were processed. 101,189 embryos were processed in the N_4 maturation stage, and 506,803 plantlets were processed in germination stages N_5, N_6, and N_7 (Table 3).

Table 4-3. Total number of explants processed for each productive stage.

SE productive stages	Formula	Calculation Details	No. of Total Explants
Initiation	$N_i=N_0/((1-L_0)*B_0)$	9.427/((1-10%)*50%) =	4.190 (Flower buds)
	$N_0=N_1/((1-L_1)*B_1)$	8.776/((1-2%)*95%) =	9.427 (Staminodes)
Multiplication	$N_1=N_2/((1-L_2)*B_2*m_2)$	4.300/((1-2%)*10%*500%) =	8.776 (Primary embryos)
	$N_2=N_3/((1-L_3)*B_3*m_3)$	14.751/((1-2%)*70%*500%) =	4.300 (Secondary embryos)
	$N_3=N_4/((1-L_4)*B_4*m_4)$	101.189/((1-2%)*70%*1000%) =	14.751 (Secondary embryos)
Maturation	$N_4=N_5/((1-L_5)*B_5*m_5)$	694.155/((1-2%)*70%*1000%) =	101.189 (Cotyledonary embryos)
Germination	$N_5=N_6/((1-L_6)*B_6)$	340.136/((1-2%)*50%) =	694.155 (Plantlets)
	$N_6=N_7/((1-L_7)*B_7)$	166.667/((1-2%)*50%) =	340.136 (Plantlets)
Acclimation	$N_7=N_8/((1-L_8)*B_8)$	100.000/((1-0%)*60%) =	166.667 (Plantlets)
	$N_8=$		100.000 (Plantlets)

Considering the following amounts and effective work times is required for labour payments in Colombia, as presented in the Substantive Labour Code (Sheet 3 S2: Direct and indirect labour). The base salary in Colombia is US $250.8, transportation allowance US $29.4, health US $21.3, pension US $30.1, occupational risks US $1.3, severance payments US $22.6, premium US $23.3, losses US $23.3, interest on severance pay US $2.8, vacation

US $10.5 and endowment US $12.5. The sum of the total cost of a salary in Colombia is US $428. To calculate effective work time, it was decided that, of the year's 365 days, 52 are Sundays, 18 are holidays, 15 are vacation days, and an average of 3 are taken off due to incapacity. Therefore, there are 277 effective workdays a year and 23 effective workdays a month. In accordance with the above, taking a salary into account with the social benefit factor and effective monthly workdays, this yields US $18.53/effective workday.

Once the daily cost of labour was identified, the time personnel used to perform work in each process's phases was calculated. Three main activities were identified: (1) collecting flower buds from the genotypes of interest in the field, (2) processing material in an LFC, and (3) support processes, among which are preparing, dispensing, and replacing culture media, preparing materials, reviewing to discard material, cleaning functions and vessel and tool disinfection (Table 4).

Table 4-4. Personnel days allotted to propagation activities and support processes in each one of somatic embryogenic phases

Work days	**Collection in the Field**	***In vitro* Introduction**	**Induction**	**Expression I**	**Expression II**	**Expression III**	**Maturation**	**Conversion**	**Plantlet Development**	**Plantlet Growth**	**Total Days**
In the Field	28										28
In LFC		6	4	1	4	13	86	96	47	28	285
In Support Processes		23	24	27	17	21	54	555	612	216	1.547

A total of 28 days, or 168 hours of work (approximately), were observed to be required to collect flower buds in the field. Personnel working in the LFC involves 285 days or 1,710 hours to execute all the productive process' stages, and support personnel requires 1,547 days of work or 9,286 hours. Therefore, the production process requires 334 days from flows bud introduction to plantlet attainment, and 7 employees are required to execute the process.

The cost of personnel was obtained by multiplying the 1861 required days for batch production by the cost of one effective monthly workday at US $18.53/day, for a total of US $34,500. Supervision does not need to be project-exclusive once standardized processes are achieved, for which reason a 25%-time allotment was established during the productive year. A monthly price with a social benefit factor of US $1,284 was obtained for the supervisor, with a process duration of 12 months. Therefore, the total cost of indirect labour was US $3,852.

It was observed that the germination stage contributes significantly to the total cost for culture media since it requires more culture media than other stages (Sheet 4 S2: Culture medium). The amount goes from 30 ml in a Petri dish to 150 ml in a higher-volume vessel (Table 5). For IMC, transportation resulted in US $543, personnel endowment US $282, and materials and tools US $3,049, which adds up to US $3,874. It was previously clarified those operational expenses are calculated as 30% of total production costs. Therefore, they correspond to US $21,895 (Sheet 5 S2: IMC). Using this estimation methodology, the total cost of US $72,986 was obtained for a productive batch with 100,000 plants, with a cost per plantlet of US $0.73/unit.

Table 4-5 Culture media costs in each stage of via somatic embryogenesis (SE) production.

Stage	Phase	Amount (USD)	Unit	Liters Required	Lot Cost in USD
Initiation	Introduction	$ 1,74	cost/liter	0,47	$ 0,84
	Induction	$ 1,74	cost/liter	11,31	$ 19,74
Multiplication	Expression I	$ 1,83	cost/liter	10,53	$ 19,22

	Expression II	$	1,73	cost/liter	2,63	$	4,55
	Expression II	$	1,61	cost/liter	9,03	$	14,52
Maturation	Embryo development	$	1,61	cost/liter	61,95	$	99,59
Germination	Conversion	$	2,05	cost/liter	2.124,96	$	4.361,81
	Plantlet Development	$	2,05	cost/liter	2.551,02	$	5.236,33
Acclimation	Plantlet Growth	$	0,01	cost/kilogram	11.666,67	$	133,33

2.24.3 Monte Carlo Simulation Analysis (MCS)

The most influential component of cost was identified to be direct labour after structuring costs, which represents 53% of the total cost. The cost of culture media was 12% of the total, IMC represented 5%, and operating expenses, including administrative expenses and infrastructure, were 30% (Figure 4-2).

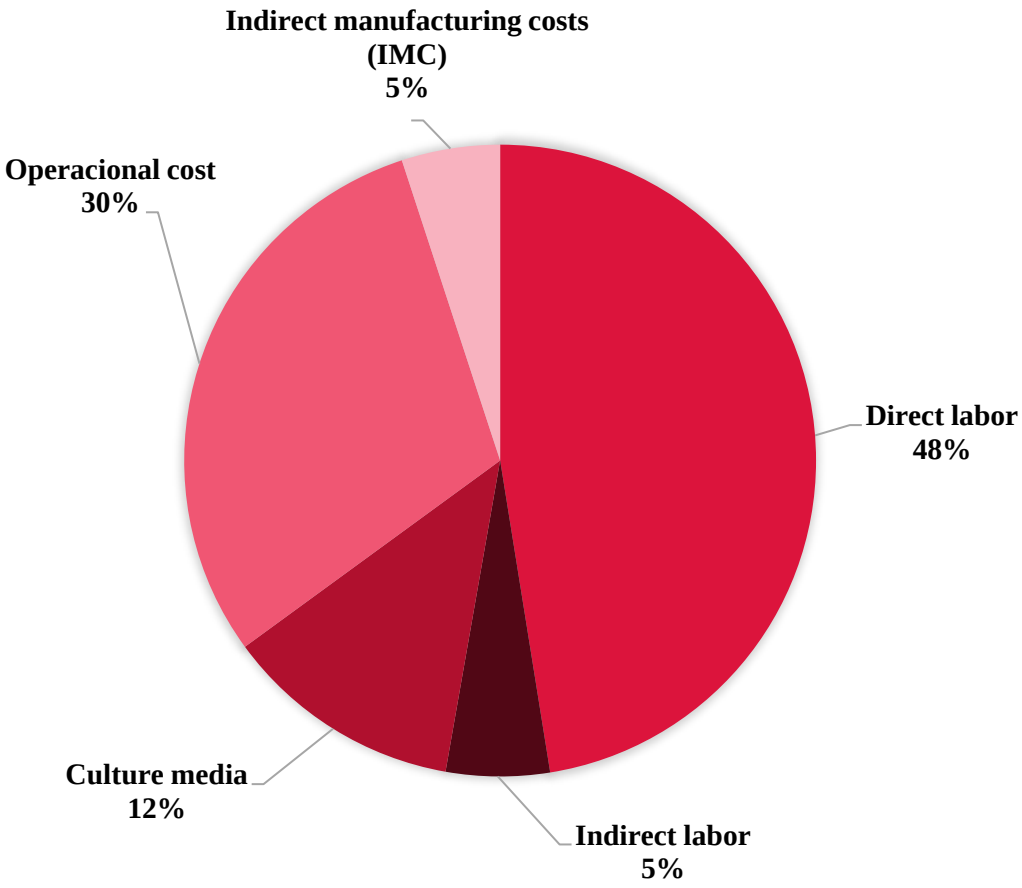

Figure 4-2 Components of production cost share in the total cost of producing cacao plantlets.

Plants propagated by SE. Figure 4-3 shows that the cost of plants per unit can be inferred between USD $0.6835 and USD $0.7786, with an average USD $0.7290 (Sheet 1 S2: Total cost), due to the production process's cost structure. If strict control is maintained over the variables while executing the productive lot as they have been established in this study, the average price per plantlet has been proposed to be USD $0.7290, with a 95% reliability. However, as one may observe, there is a certain asymmetry towards the right,

which indicates that the process could increase in cost. That is to say, the cost may have deviated over the average, or over USD $0.7290 after moving the lot.

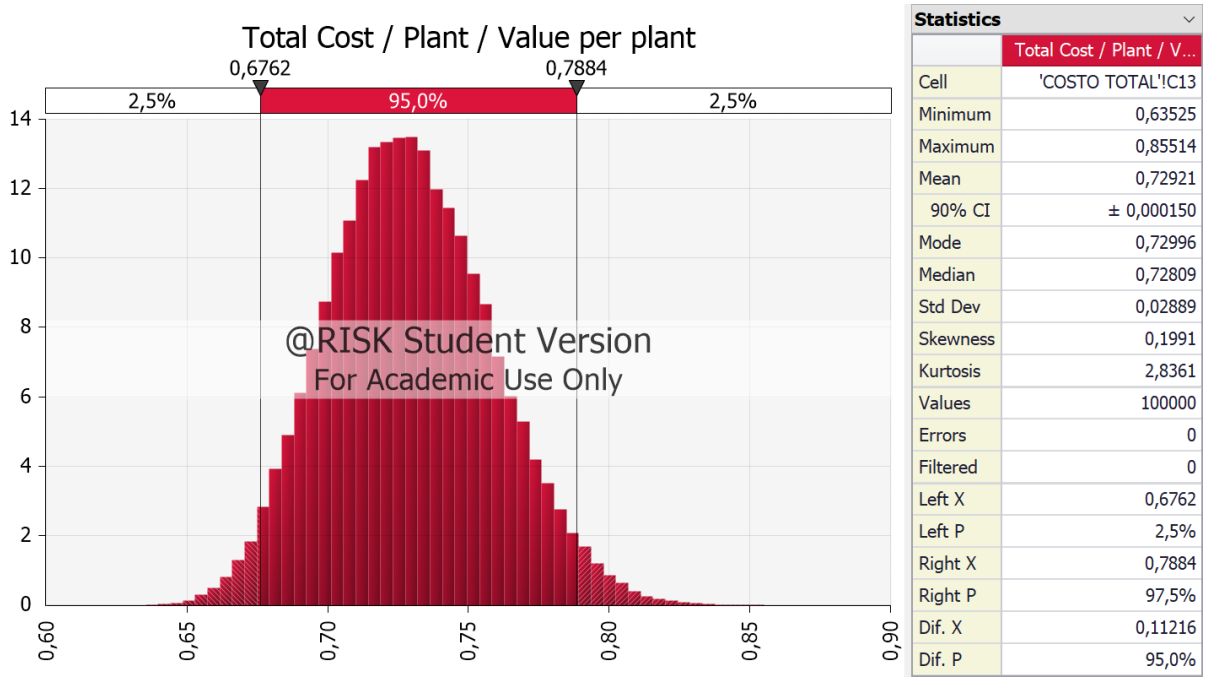

Figure 4-3 Frequency distribution for the cost of producing one cacao plantlet by somatic embryogenesis.

On the other hand, a regression was performed on the correlation coefficients of each one of the cost model's variables to identify which ones influence the variable response, which is to say cost the most. This analysis showed that the most significant variables are in the productive process' last stages, corresponding to germination and acclimation, followed by the maturation stage. It was specifically found that the plantlet growth (-0.69) and plantlet development (-0.60) phases, as well as the plantlet conversion phase (0.35) had more significant effects on cost, with 95% reliability. Therefore, when these variables, which are expressed as the percentage of explants' response, rise above 60%, 50%, and 50%, respectively, the variable of cost per plantlet decreases. During the embryos' development phase, both the multiplication coefficient (-0.07) and percentage of explants' response (-0.07) tend to decrease cost per plantlet as their prices increase. They

are currently at 10% and 70%, respectively. Besides, it was observed that the RMR (0.15) has a positive effect on cost. Cost per plantlet will increase as RMR increases (Figure 4-4).

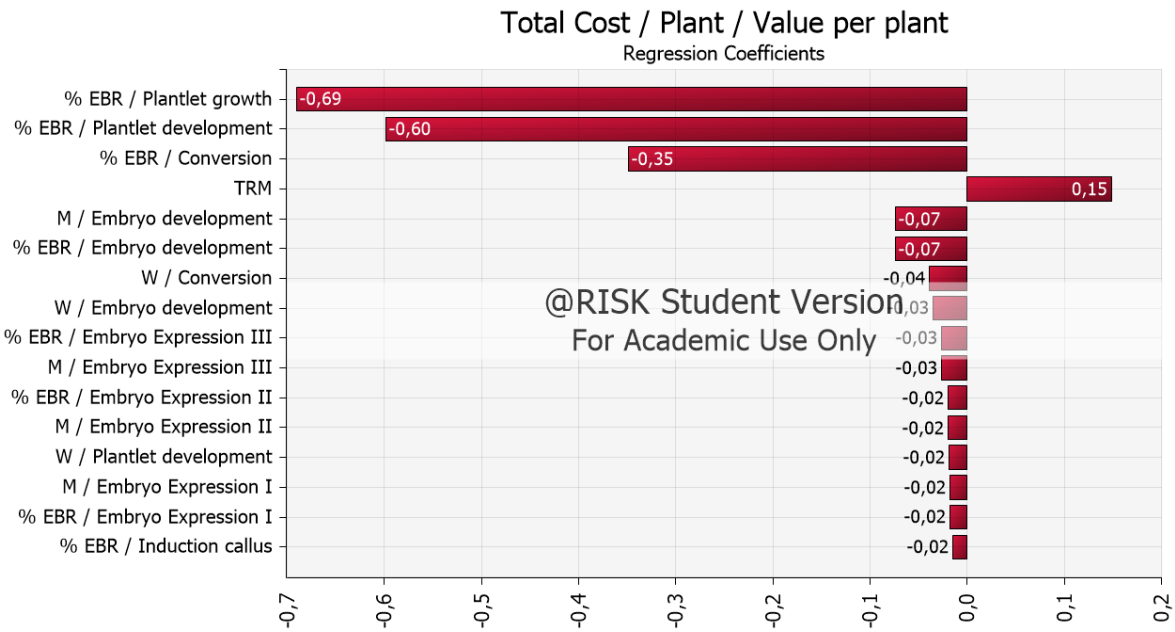

Figure 4-4 Regression coefficients for the variables associated with the productive process of cacao plants propagated by somatic embryogenesis over the production cost per plant. Variables: % EBR (% explants with response), RMR (Representative Market Rate), M (Multiplication rate), W (Labour standard).

2.25 Discussion

In Colombia there are approximately 176,000 cultivated hectares that benefit 52,000 families in 422 municipalities of 30 departments, with biannual growth of 4% in planted area. It is established that in the short term, the establishment of approximately 7000 hectares biannually is required, as well as some 10,000 hectares for renovation. Having an average amount of 1,100 plants per hectare, with 5% additional plants to replace those that die at the time of being transplanted, it is estimated that the potential demand is 19.6 million plants biannually for 2021 [56]. There are growing of demand for high-quality, high-performance,

and pathogen-free planting material in the last two decades, increasing demand for agricultural, forest, and horticultural products. Within this trend, cultivating plant tissue *in vitro* has become an important, commercially feasible tool for generating high-quality, high-performance, and pathogen-free planting material regardless of climate fluctuations. Most notably, SE's potential as a propagation method has not only been described in the last years for cacao [24,57,58] but also other plant species of interest [59–61]. Various authors agree that the main factor limiting SE's deployment on a commercial scale is the plants' relatively high cost compared to those cultivated in greenhouses [62]. Therefore, financial cost analyses for these processes are vital, and even more, so is examining strategies to reduce the cost without affecting production efficiency and quality [41].

In the analysis performed for cacao, the component that contributed most to cost was direct labour, at 53%. This result coincides with what other authors reported for plants obtained by SE other than cacao. For example, for labour, Cervelli and Senaratna (1995) [63] reported a 70% contribution to cost and Chu (1995) [64] reported a 63.8% contribution. However, other authors recommend decreasing direct labour's percentage of contribution as much as possible, by between 30 and 40% [36,52]. In accordance with these results, it is evident that cacao plantlet production via SE is a labour-intensive system, and labour is the main production cost component. Maintaining high labour efficiency is essential to make the process feasible. Therefore, one of the methodological strategies to achieve this objective is based on optimizing personnel's actions in LFCs. For example, unnecessary existing times can be eliminated. Despite having adequate efficiencies in processing material, such as the quantity of transfer operations per day, this study has estimated 6,000 explants/ workday for the initiation stage, a work standard similar to that which Ahloowalia & Savangikar (2004) [36] reported for this same operation, at 5,000 explants/ workday. On the other hand, in accordance with Suárez-Castellá (2006) [65], productivity of 4,595 explants/ workday can be achieved by providing training on handling and processing tissue. This figure is evidently higher than the work standards for the cacao multiplication stage, which oscillates at 1,176 explants/ workday on average. However, lack of skilled labour is always the main problem in countries such as Colombia. Implementing training programs is an

alternative to promote operating personnel's work capacity and overcome this limiting factor.

The second component that contributes most to cost is operating expenses, at 30% of the total cost. This percentage is very close to the ranges reported when propagating other species, such as *Phalaenopsis sp.,* where operating expenses were 32.7% [66]. However, lower figures are reported for *Paulownia tomentosa* production, at 19% [45] and *Saccharum officinarum,* 24.4% [67]. It must be clarified that both are productive processes performed by organogenesis. In this respect, it is essential to indicate that one of the highest infrastructure costs is electricity associated with air conditioning systems used to regulate culture room temperatures. According to Tomar (2010) [52], this factor can consume 85% of the electricity in *in vitro* propagation installations. Therefore, if the process does not require exact control over temperature, it is recommended facilities avoid using this resource if the area's climate conditions allow it. Another aspect of electricity consumption is associated with expenses during the water distillation process, which is the main component of culture media. Distilled, double-distilled or deionized water is generally used in *in vitro* tissue cultures. However, on a productive scale, according to Sahu (2013) [68], using alternative water sources is recommended to reduce the cost of sterilizing tap water in an autoclave, as long as the water has proper conductivity, pH, is free of heavy metal or pollutants [69].

The third most important component within the cost structure is culture medium, which corresponds to 12% of the total cost and can also be improved to reduce costs. Most plant tissue culture media's main components are mineral salts and sugars as sources of carbon and water. Other components can include organic supplements, growth regulators, and gelling agents [70]. According to Prakash et al., (2004) [71], the chemical reagents of culture media cost less than 15% of the total production cost of the vitroplant. In some cases, the cost can lower down to 5%. Of culture media components, gelling agents, such as agar, contribute to 70% of the total cost, followed by sources of carbon and, finally, growth regulators, which minimally influence the cost of production, since they are reasonably inexpensive due to their minimal concentration within culture media [12]. Sources of carbon, such as glucose, fructose, and maltose, are commonly used. However, they are reagents

with high purity levels, which makes them expensive. Sucrose is the most widely used carbon source in the *in vitro* propagation of plants. Table sugar has also been used as a strategy to reduce culture media costs. Regular sugar has a high enough quality for micropropagation, and using it reduces media culture costs between 78 and 87% [41]. In Colombia, the cost of local sugar is USD $0.86 / kg compared to USD $8.13 / kg for imported sucrose. Another strategy for decreasing costs is associated with the mineral formulation. Many companies sell media prepared as a liquid or powder. However, the benefits and disadvantages of formulations prepared in laboratories must be considered with respect to commercially prepared media. Although prefabricated media save time, their relative cost is high. Pre-packaged media preparations are usually useful when the required amounts are small - generally on a scale for research - and they have less errors. For large-scale use, it is much more cost-effective to prepare media by combining the basic ingredients. Nevertheless, if errors occur, losses are more significant depending on the size of the lot. Moreover, the time the hired technician takes preparing the formulations must be considered, for which reason it is recommended for stock solution concentrations to be up to 1,000X.

Indirect manufacturing costs are included in the fourth component, with 5% corresponding to transportation, culture vessels, materials, supplies, tools, etc. Some authors recommend presterilized, reusable, plastic Petri dishes because the material is more inexpensive than glass [71]. However, our organization encourages minimizing its ecological impact by substituting single use supplies or tools for lasting and reusable materials due to environmental social responsibility policies. Furthermore, in accordance with Datta (2017) [41], using supplies, such as aluminium, is not recommended for wrapping tools before sterilization. Instead, sterilizable stainless steel containers can be used in autoclaves as substitutes with significantly long useful lives. Also, PVC or polyethylene plastic film is used as lids in common practice and for large-scale production. These lids have been replaced with stainless steel or polypropylene screw caps sterilizable in an autoclave.

The sensitivity analysis showed that the most significant variables on cost per plantlet are in the productive process' last stages, corresponding to plantlet germination and acclimation. This specifically applies to the percentage's response during the plantlet conversion, development, and growth stages. These results are consistent given the accumulated time, effort, and energy invested up to this point in the productive process. Following Von Aderkas et al. (2016) [72], practices performed during the entire production process via SE, especially managing embryogenic structures and somatic embryos in cotyledonary states, significantly affect both performance and the embryo's quality and conversion, as well as production costs. One of the most crucial stages is when plantlets are transferred from *in vitro* to *ex vitro* conditions, since approximately 8 months have transpired in the process' previous stages. It is well-known that plantlet survival can be improved by optimizing somatic embryos' maturation and germination conditions in cotyledonary states. These conditions can be optimized by, for example, making adjustments to components, such as abscisic acid and gibberellic acid and modifying the culture medium's water potential and nitrogen source, among others [73–75]. In this stage of the process, the production cost can also be reduced if time in hardening facilities is shortened without reducing plantlet survival rates. Plants require relatively less time to harden if natural light is previously used during plantlet development [41] and if microorganisms that promote growth, such as fungi and bacteria, are used [76].

The variables associated with the maturation stage, which is to say the development phase of embryos, such as multiplication coefficient and percentage of explants' response, were also significant on cost in the sensitivity analysis. Under other authors and the obtained results, improving embryo multiplication rate is one of the most efficient ways to reduce costs [66]. Some of the alternatives for improving the multiplication rate in the case of SE include selecting the most suitable development stage of primary embryos or primary embryonic tissues as a source for producing repetitive embryos. That is to say, the more control there is over the exact point in which tissues answer to recurring embryo formation, the more embryos will be produced. The same applies to using substances to promote embryo formation, such as demethylating agents like 5-Azacytidine (5-azaC). In this sense, in terms of cacao, it has been demonstrated that decreases in embryogenic potential seem

to be related to DNA methylation [77]. In addition, environmental conditions like microclimate, at least while controlling temperature between 27 and 28 °C and total darkness in the culture room, are definitive in the cacao multiplication stage. In this context, all these stages add up cumulatively, contributing to reducing production costs.

A price per plantlet of USD $0.73 was obtained based on the analysis performed on the cacao production process, with a final sales price to customers of USD $0.88 and grafted of USD $1.16. Upon reviewing the market prices of traditionally grafted cacao plants, an average USD $0.71 was obtained for Colombia and, in countries like Ecuador, USD $0.70. Also, the cost in Colombia is above the prices offered in Latin America. For example, cacao plantlets propagated by SE in a biofactory in Brazil cost USD $0.70 [78]. Despite there being a significant difference with respect to plantlets propagated by the traditional method, there is a significant opportunity to be considered by developing the technology on a biofactory scale with obvious cost reduction objectives. In this sense, to validate technology for cacao plant production by SE and for it to be a cost-effective product on the national market, the factors that contribute most to cost, such as percentage of explants' response, must continue to be optimized, and other options that do not compromise quality must be considered. Adopting flawed low-cost strategies can make the production process prone to failure since it must be highlighted that this is a productive process inherent to a biological process. These processes may vary significantly after changing some culture conditions. Low-cost techniques will only be successful if they scrupulously comply with tissue culture's primary requirements, thus maintaining plantlet quality. According to Datta (2017) [41], procedure standardization is more critical for ensuring cultivated plants' quality than procedure sophistication. It can be said that continuing to develop a low-cost productive process signifies an advanced generation technology, improving the process’ efficiency and use of resources. Therefore, different strategies can be intelligently adopted, such as simplifying various technological operations to reduce specialized facility costs, such as a biofactory’s costs.

2.26 Conclusion

A calculation matrix that estimates production costs by defining the number of plants required by productive batch and estimating cost developing volume was created in this study. Labour costs contributed to 53% of the total cost, followed by operating expenses at 30%, culture media at 12%, and IMC at 5%. The proposed costing and analysis approach can be adapted and applied to other cultures propagated by SE.

Percentage of explants' response variable in the germination and acclimation stages are considered to affect cost per unit most significantly for cacao plantlet production by SE, as well as the multiplication coefficient during the maturation stage.

The applicability of the MCS was proven, showing that it is possible to consider different strategies for reducing costs. In this sense, deciding what productivity indicator should be used to consider these scenarios is practiced nowadays in the industry. It represents an opportunity for developing technology and business. Finally, it is important to mention that the presented cost reduction strategies must be tested on a short-term pilot batch to validate the production’s performance.

Supplementary Materials

The following are available online at www.revistabionatura.com/xxx/s1, Table S1: Composition culture medium, Sheet 1 S2: Total cost, Sheet 2 S2: Stages of production, Sheet 3 S2: Direct and indirect labour, Sheet 4 S2: Culture medium, Sheet 5 S2: IMC, Sheet 6 S2: 6. Assumptions.

Author Contributions

Conceptualization, Ana María Henao Ramírez and Aura Inés Urrea Trujillo; methodology and software, Hernando David Palacio Hajduck and Ana María Henao Ramírez; validation and formal analysis, Ana María Henao Ramírez; investigation, resources, data curation, writing—original draft preparation, Ana María Henao Ramírez; writing—review

and editing and supervision, Aura Inés Urrea Trujillo. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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6. Conclusion

SE technology is suggested as the most convenient technology for producing clonal *T. cacao* plants on a large scale as a complement to traditional methods. This methodology is very useful in plant multiplication programs for elite genotypes like *CCN51*. However, its practical application for producing genotypes of interest has been limited to date. From the results obtained, it can be stated that for *CCN51*: (1) the use of 2,4-D + KIN is suitable for callus induction and the formation of PSE; (2) the use of 2,4,5-T for the greater amount formation of SSE; (4) growth regulators are not required for the maturation of the embryo; (5) ANA + GA3 is required for conversion of the embryo into a plantlet with subsequent elongation and growth. All the previous conditions with a strict control of 26ºC in culture and dark in initiation and multiplication stage allows to obtain suitable times of production of plantlets for the production on a larger scale. For a ligneous species, such as *T. cacao*, the time factor and frequency of productive lots is a particularly important consideration, since it requires an average of 8,2 months to complete the entire cycle for *CCN51*. Future optimizations should be performed to increase multiplication coefficients and higher rates of conversion from embryo to plantlet. Likewise, the evaluation in open field conditions of the agronomic performance of the propagated plants to provide the cocoa producer with all the information associated with the productivity of the crop.

In conclusion, this study validates the propagation protocol via somatic embryogenesis to produce plants from genotypes of interest, such as *CCN51,* for commercial cultivation purposes. Furthermore, it confirms that molecular markers like SSR are reliable, robust, and quick tools that require very little plant material and relatively low-cost inputs

Production planning and controlling (PPC) plays an important role in modern production enterprises. Current production management systems consider resources such as material, labour and production capacity and their respective costs, but sometimes neglect the role of quality and performance for cost savings. The PPC system has been identified, an analytical modelling of the operation of the productive propagation was carried out through ES plant cacao production, in which the main and support activities were identified, with the respective consumption, until the final product is obtained. With the data achieved from the model, the simulation was carried out, in which the indicators that are considered of greater relevance are monitored, and that tell us about the production process. This work constitutes the first step forward for an approximation of costs closer to reality.

A calculation matrix that estimates production costs by defining the number of plants required by productive batch and estimating cost developing volume was created in this study. Labour costs contributed to 53% of the total cost, followed by operating expenses at 30%, culture media at 12%, and IMC at 5%. The proposed costing and analysis approach can be adapted and applied to other cultures propagated by SE.

Percentage of explants' response variable in the germination and acclimation stages are considered to affect cost per unit most significantly for cacao plantlet production by SE, as well as the multiplication coefficient during the maturation stage.

The applicability of the MCS was proven, showing that it is possible to consider different strategies for reducing costs. In this sense, deciding what productivity indicator should be used to consider these scenarios is practiced nowadays in the industry. It represents an opportunity for developing technology and business. Finally, it is important to mention that the presented cost reduction strategies must be tested on a short-term pilot batch to validate the production’s performance.