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Research Paper

Evaluation of the potential of regeneration of different Colombian and commercial genotypes of cocoa (*Theobroma cacao* L.) *via* somatic embryogenesis

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ABSTRACT

This paper aims to establish efficient protocols for the *in vitro* propagation of different cocoa genotypes (*T.cacao*), the potential of regeneration *via* somatic embryogenesis in the Colombian genotypes *SYS12, SYS13, SYS16, SYS24, SYS4* and commercial/universal genotypes *CCN51, TSH565, EET8, ICS1, ICS39, ICS60, ICS95* was evaluated. The effects of a different culture media, kind of explant and culture time on the induction of embryogenic structures (PE), somatic globular embryos (GE) and cotyledonary somatic embryos (CE) were studied in addition to the effect of the culture time on the average number of secondary somatic embryos (SSE) and the culture medium over the SSE conversion. Finally, different substrates on the hardening of the stage of seedlings was evaluated. In this study a regeneration protocol *via* a high frequency SSE for para *CCN51*, *TSH565*, *SYS4* y *SYS13* is described in addition to the development induction up to a cotyledonary stage of the genotype *SYS24* and the induction of proembryogenic masses and globular embryos for *SYS12* y *SYS16*. For the commercial genotypes *ICS60* reached the development up to cotyledonary stage but it was not possible to reach a successful conversion and for *ICS1*, *EET8*, *ICS95, ICS39* we reached the induction of globular embryos that quickly experienced a dedifferentiation process. It is expected that the high frequency found in the embryos formation allows a contribution to the reduction of production costs and thus promote the massive utilization of superior cocoa genotypes propagated *via* somatic embryogenesis.

Abridgment

Primary somatic embryogenesis (SE), Secondary somatic embryogenesis (SSE), Primary callus induction medium (PCG), Induction medium (INDI), Secondary callus induction medium (SCG), Expression medium (INDexp), Multiplication medium (CM2), Maturation medium (MM6), Maturation medium (ED), Murashige and Skoog (MS) Murashige and Skoog (1962), Driver and Kuniyuki, (1984) and Tulecke and McGranahan, (1985) (DKW), (Lloyd and McCown, 1981) (McCown's), Vitaminas Gamborg's (B5) Gamborg et al. (1966), Tidiazuron (TDZ), 2,4-Dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichlorophenoxyacetic acid (2, 4, 5-T), 1-Naphthaleneacetic acid (NAA), Gibberellic acid (GA_3), Santander Yariguíes Selection (SYS), Colombian National Chocolate Company (CNCH), somatic globular embryos (GE), cotyledonary somatic embryos (CE), proembryogenic structures (PE).

1. Introduction

Theobroma cacao L. (2n = 20) is a neotropical plant native to Central and South America regions and was domesticated in Central America. Among the 26 species of the genus, *T. cacao* is the only plant cultivated on a large scale to produce chocolate (Richardson et al., 2015). Despite the significant increase in global demand for cocoa, its production has not followed the same trend and therefore there is a global deficit in the supply that was 150,000 tons in 2015–2016 (ICCO, 2017). Global climate change, pest infestation and diseases occur plus the reduction of productivity of the plants, due to aging of the crops, have caused instability in the global production. The constant production of *T. cocoa* is important in production countries to maintain stability of export and to ensure the continuity of the supply of industrial raw materials for the growing industry of chocolate. Cocoa plays an important economic role in Colombia, however, currently the average productivity reaches 350 kg/ha/year, which is much lower than the

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average yield of plants propagated by seeds which reach up to 1.800 kg/ha/year.

The use of low quality materials, the lack of modernization in crops, pests and the increase in the occurance of diseases due to the advanced age of the plants are the major contributors to low productivity in 2017 (FEDECACAO). In order to increase the levels of production at a national level, it is suggested that the renewal of 80,000 ha and the generation of 50,000 ha of new crops until 2021 be implemented; in this context, the implementation of efficient methods in the massive multiplication of plant material is required.

In general, the use of sexual seed and grafting are the most commonly used methods of propagation. Given that *T. cocoa* reproduces naturally by cross pollination, sowing materials from seeds generally exhibit a highly heterogeneous genetic background and the agronomic yield of crops is therefore highly variable. The propagated materials by cloning through graft materials do not have high rates of multiplication and the bushy growth pattern is more pronounced, which is considered an undesirable feature. In this sense, the modern techniques of propagation and massive multiplication such as somatic embryogenesis can improve the production of plant material from *T. cacao*.

Somatic embryogenesis is a method of *in vitro* regeneration for plants, which after standardized is highly efficient, achieving a high rate of multiplication. The *in vitro* regeneration of seedlings through somatic embryos has been developed successfully in different plant species, *e.g. Arabidopsis* (Wójcikowska and Gaj, 2016), *Zea mays* (Garrocho-Villegas et al., 2016), *Bixa orellana* (Monteiro et al., 2016), *Capsicum annuum* (Neftali, 2016), *Coffea arabica* (Loyola-Vargas et al., 2016), *Agave* sp. (Rodríguez, 2016), *Cocos nucifera* (Sáenz-Carbonell et al., 2016), *Pinus* sp. (Lelu-Walter et al., 2016), *Zingiber officinale* (Shajahan et al., 2016), *Musa paradisiaca* (Escobedo-Gracia et al., 2016), and *Jatropha curcas* (Kumar et al., 2016) and others.

Regeneration of *T. cocoa* plants through somatic embryogenesis has been achieved in DKW basal medium supplemented with different growth regulators (PGR), thidiazuron (TDZ) (Li et al., 1998), 2,4-dichlorophenoxyacetic acid (2,4-D) (Maximova et al., 2002) and kinetin (KIN) (Fontanel et al., 2002). However, the success percentages in the somatic embryo formation are still low (25%) and is limited to a few clones, Sca 6 being the most widely reported (Garcia et al., 2016). Although the TDZ has been reported as effective for inducing somatic embryogenesis in *T. cocoa*, the cost of this growth regulator is high, making itunsuitable for most commercial laboratories in developing countries.

On the other hand, the need to implement breeding programs for this crop is known, and that one of the key requirements in different genetic transformation methods used so far, is having a system for plant regeneration from the transformed tissues. Somatic embryogenesis is the most reliable since the process of formation of embryos generally involves a set of cells, which increases the probability that regenerated plants in the selection medium are true transformants.

As described above, it is clear that the availability of an efficient *in vitro* regeneration system is crucial for the strengthening of the first link of the cocoa production chain in the short and medium term. To contribute to this achievement, the aim of this research was to evaluate the potential of *in vitro* regeneration *via* somatic embryogenesis of 12 cocoa regional and universal clones with the protocols described by Li et al. (1998) and Fontanel et al. (2002) with modifications.

2. Materials and methods

2.1. Plant material and explant preparation

For this research, the experiment design included 12 genotypes in total, 7 universal/commercial genotypes *CCN51, TSH565, TSE8, ICS1, ICS39, ICS60, ICS95* and 5 Colombian genotypes from the Colombian National Chocolate Company (CNCH) called Santander Yariguíes Selection (SYS), which where denominated *SYS12, SYS13, SYS16, SYS24* and *SYS4*. The plant material was collected at the farms La Nacional (Tamesis, Antioquia-Colombia) and the Farm Yariguíes (Barrancabermeja, Santander-Colombia) of the CNCH. The explants consisted of staminodes (sterile stamens) and petals of the immature flower buds of each one of the genotypes indicated. The flower buds (4–5 mm) were collected between 7 and 9 in the morning, once cut they were submerged in a previously sterilized solution and served in conical tubes of 50 mL cold, half concentrated solution of basal salts DKW. This collected material was kept cold until arrival at the Laboratory of Physiology and Plant tissue culture of the University of Antioquia, Colombia. The flower buds were superficially sterilized by immersion in a 0.5% (v/v) sodium hypochlorite solution for 5 min, under constant agitation; then they were washed with sterile distilled water (3 times), and changed to new sterile conical tubes, subsequently to be submerged in a 250 ppm streptomycin solution for 20 min under constant agitation. Finally, the staminodes and the petals were extracted from the basal portion of the flower button and were planted in petri dishes (60 × 15 mm) containing the corresponding culture medium.

2.2. Media and culture conditions

The disinfected explants were sown in the following sequence of culture media: PCG-SCG-ED and INDI-INDexp-MM6 (Li et al., 1998; Fontanel et al., 2002) with amendments and supplements of 20–40 gL^{-1} glucose, 2–3.5 gL^{-1} Gellex, and specific growth regulators, which are detailed below. The pH of the media was adjusted to 5.8 with 1 M NaOH or 1 M HCl before being sterilized at 121 °C for 20 min.

2.3. Induction of proembryogenic structures (PE) and formation of primary somatic embryos (SE)

For the induction of embryogenic callus, the disinfected petals and staminodes were sown in the PCG medium (macronutrients, micronutrients and vitamins DKW, (250 mgL^{-1}) L-glutamine, (2 mgL^{-1}) 2,4-D, (0.005 mgL^{-1}) TDZ and in the INDI medium (DKW macronutrients, micronutrients and vitamins), (1 mgL^{-1}) 2,4-D, (0.25 mgL^{-1}) Kinetin, mixture of amino acids per Urrea et al. (2011). The cultures were maintained in these media for 30, 40, 50 and 60 days, and then were transferred to the expression media. Twenty-five explants were put in every Petri dish, and each treatment consisted of 10 Petri dishes. The experiment was repeated 5 times.

For the expression stage of the SE, tissues with callus were transferred to the SCG media (McCown's salts), supplemented with 1 mgL-1 B5, (2 mgL^{-1}) 2,4-D, (0.3 mgL^{-1}) KIN and coconut water (5%) and INDexp media (DKW macronutrients, micronutrients and vitamins). The effect of the culture medium, type of explant and culture time on the formation of the embryos were calculated with the average number of proembryogenic structures, somatic globular embryos (GE) and cotyledonary somatic embryos (CE) formed in each explant at 15, 25 and 35 days after being transferred to the expression media. Twenty-five explants were put in every Petri dish, and each treatment consisted of 10 Petri dishes. The experiment was repeated 5 times. With both cultures, induction as expression wasmaintained at 28 °C ± 2 °C under dark conditions during the proposed cultivation times.

2.4. Proliferation and conversion of the somatic embryos

For the induction of secondary somatic embryos (SSE), the primary embryos in cotyledonary state (1–4 mm) from the varieties *TSH565, CCN51, SYS4* and *SYS13* were segmented and cultured in the callus multiplication medium (CM2) (MS macronutrients, micronutrients and DKW vitamins, 1 mg L^{-1} 2, 4, 5-T, 0.25 mgL^{-1} adenine and the amino acids mixture according to Urrea et al.) (2011). To evaluate culture time on the formation of SSE, the average number of embryos at different stages was calculated; for this analysis, we chose the CE at 10, 15, 20, 25, 30, 35 and 40 days after being transferred to the CM2 medium. For this