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Effect of osmoregulatory on the secondary somatic embryogenesis of cocoa (*Theobroma cacao* L.)

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ABSTRACT

Cocoa (*Theobroma cacao* L.) is the raw material for the worldwide chocolate industry. To ensure high quality and efficient field production, the industry relies on a uniformity of elite cocoa plants, which can be achieved through vegetative propagation methods like somatic embryogenesis. However, the low yield rate in the multiplication and regeneration of cocoa plants remains a challenge for basic research. To obtain a greater number of secondary somatic embryos (SSE) in cocoa, the effect of three osmoregulators on the disc cells of primary somatic embryos (PSE) of cocoa was studied. PSE was induced from cocoa staminodes of genotype I52. Epicotyls were selected from PSE at the torpedo and cotyledon stages, segmented into 3 mm discs, and placed in a secondary callus growth medium. The explants were transferred to embryo development medium 3, where they were exposed to the osmoregulatory polyethylene glycol (PEG), mannitol, and sorbitol at the following concentrations: 0%, 1%, 3%, and 5%. D-Mannitol at 3% and D-Sorbitol at 1% increased the number of SSE at the torpedo, globular, and cotyledon stage per explant. The culture medium with 1% PEG significantly increased the formation of SSE in the globular and cotyledonal stages. The results presented a positive effect of the osmoregulators on the formation of cocoa SSE.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a tropical species native to the Amazon Basin of great economic importance to produce cocoa beans, the basis of the global chocolate industry [1,2]. Annual cocoa production represents more than US$10 billion in income for numerous farming families around the world, producing approximately 4.5 million tons of cocoa per year [3]. Peru ranks ninth among the world's largest cocoa producers and third in the Americas, with approximately 160,000 tons of cocoa per year in the past 2 years [4]. Peru is among the world's leading producers and suppliers of fine aroma and organic cocoa [5]. National production is led by the San Martin region with 60,000 tons of cocoa, accounting for 38% of the country's total production [6].

The demand for chocolate increases annually, but it is estimated that there will be a shortage of raw materials due to the spread of diseases that can lead to losses from 20% to the entire production of cocoa [7]. In this regard, traditional propagation methodologies have been developed, such as grafting and rooting of cocoa plant cuttings with the best agronomic characteristics such as resistance to diseases, higher production, and seed quality. These methodologies have certain disadvantages associated with the low rate of propagation and the undesirable bushy growth pattern [8]. Alternatives such as tissue culture, through somatic embryogenesis, allow the clonal propagation of superior cocoa genotypes with the same genetic backgrounds. This biotechnological tool was employed in a wide variety of cocoa genotypes [9,10].

Somatic embryogenesis relies on the totipotency of plant somatic cells to express cellular reprogramming [11], which has the potential to largely produce numerous individuals from a single explant and this is applied in different species and genotypes [12]. This morphogenetic pathway is developed by different biochemical and molecular events that encompass the stages of induction, expression, maturation, and germination [13,14]. Likewise, through somatic embryo induction,

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high propagation rates of material of genotypes of interest can be obtained while maintaining genetic stability, allowing its application for mass propagation of species with agronomic potential, conservation of genetic resources [15], application in genetic transformation [16,17] and gene editing [18].

Primary somatic embryos (PSE) formed from initial explants can be used for the formation of secondary somatic embryos (SSE) [19,20]. When opposed to primary somatic embryogenesis, secondary somatic embryogenesis has the advantage of the formation of a higher number of somatic embryos and repeatability [14,21]. In addition, numerous cycles of secondary embryogenesis can maintain the embryonic stage for long periods [22].

The osmotic potential of the culture medium can affect the development and percentage of somatic embryo formation [23]. As a solution, high molecular mass osmotic agents such as polyethylene glycol (PEG) and polyols (D-Mannitol and D-Sorbitol), which could reduce the osmotic potential in the culture medium, have been used. Thus, the application of these osmotic agents has increased the rate of somatic embryo multiplication in different species [24-26].

Therefore, the objective of the research was to determine the effect of three osmoregulatory (PEG, D-Mannitol, and D-Sorbitol) on the production of cocoa SSE of a highly productive elite genotype.

2. MATERIAL AND METHODS

2.1. Vegetal Material

Immature flower buds were obtained from the I52 genotype, which is known for producing the highest quality chocolate among the cocoa collection at the Instituto de Investigación para el Desarrollo Sustentable de Ceja de Selva (INDES-CES) in Amazonas, Peru. Flower buds 6–8 mm long, immature in appearance, were obtained from healthy plants with vigorous growth. The flower buds were transported to the Laboratorio de Fisiología y Biotecnología Vegetal at the INDES-CES of the Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas (UNTRM-A).

2.2. Indirect Primary Somatic Embryogenesis

The immature flower buds were soaked in a 1% calcium hypochlorite solution for 25 min and then rinsed 3 times with sterile distilled

Table 1: Composition of culture media for cocoa somatic embryogenesis.

Component	Culture media for cocoa somatic embryogenesis				
	PCG	SCG	ED4	ED3	EDL
DKW macronutrient solution A (mL/L)	100	-	100	100	100
DKW macronutrient solution B (mL/L)	100	-	100	100	100
DKW micronutrient solution (mL/L)	10	-	10	10	10
DKW vitamin solution (mL/L)	1	-	1	1	1
DKW amino acid solution (mL/L)	-	-	-	-	1
WPM salt (g/L)	-	2.3	-	-	-
Gamborg's vitamin solution (mL/L)	-	1	-	-	-
Glucose (g/L)	20	20	-	-	20
Sucrose (g/L)	-	-	40	30	-
Glutamine (mg/L)	250	-	-	-	-
Myoinositol (mg/L)	200	-	-	-	-
2,4-D (mg/L)	2	2	-	-	-
TDZ (µg/L)	5	-	-	-	-
BAP (mg/L)	-	0.05	-	-	-
Potassium nitrate (g/L)	-	-	-	-	0.3
Phytagel (g/L)	2	2.2	2	2	1.8

WPM: Woody plant medium, TDZ: Thidiazuron, BAP: 6-benzylaminopurine, 2,4-D: 2,4-dichlorophenoxyacetic, PCG: Primary callus growth, ED4: Embryo development 4, SCG: Secondary callus growth.

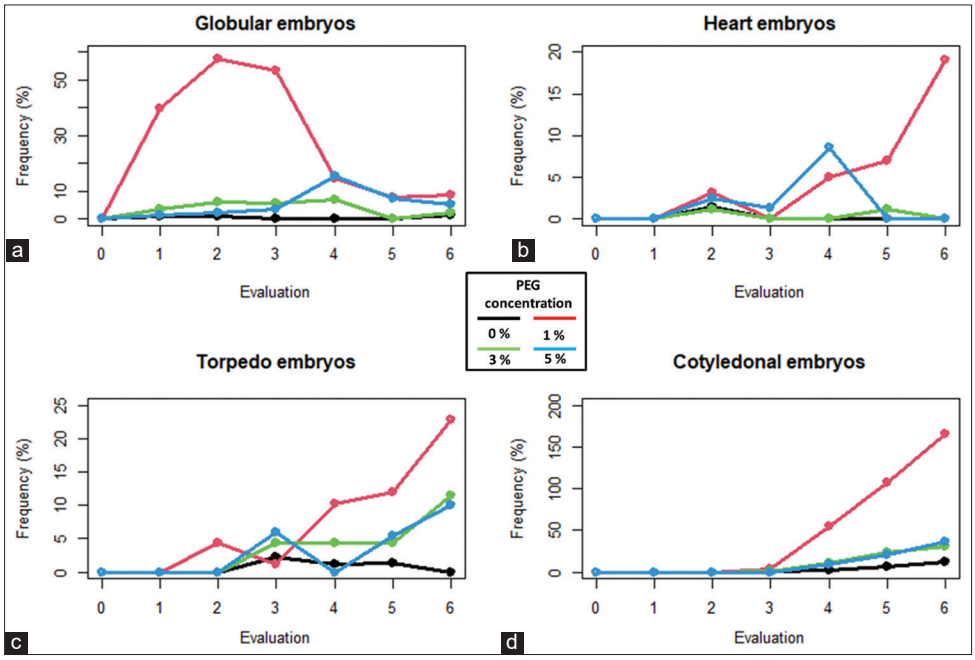

Figure 1: Frequency of formation of secondary somatic embryos with polyethylene glycol 6000 in six different periods of time. Somatic embryos in stage: Globular (a), heart (b), torpedo (c) and cotyledonal (d).

water in a laminar flow chamber. Cuts of 1/3 length from the base of the flower were made to extract the staminodes. Staminodes were transferred to Petri dishes containing culture media and sealed with parafilm to avoid contamination, plates were incubated in total darkness at a temperature of 25 ± 2°C. The composition of the culture media was based on DKW medium [27], which included DKW 10× macronutrient stock solutions A and B, DKW 100× micronutrient solution, DKW 1000× vitamin solution and DKW 1000× amino acid solution. In addition, the culture media were supplemented with salts from woody plant medium [28], Gamborg's vitamin solution [29], and growth regulators such as thidiazuron, 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid. The culture medium used for the staminodes culture initially consisted of primary callus growth medium followed by secondary callus growth medium (SCG), the Embryo development 4 (ED4) medium, and subcultured 6 times in the Embryo development 3 (ED3) medium, with intervals of 14 days between each medium [14]. Incubation was carried out in the absence of light at 25 ± 2°C. At the end of the process, the somatic embryos

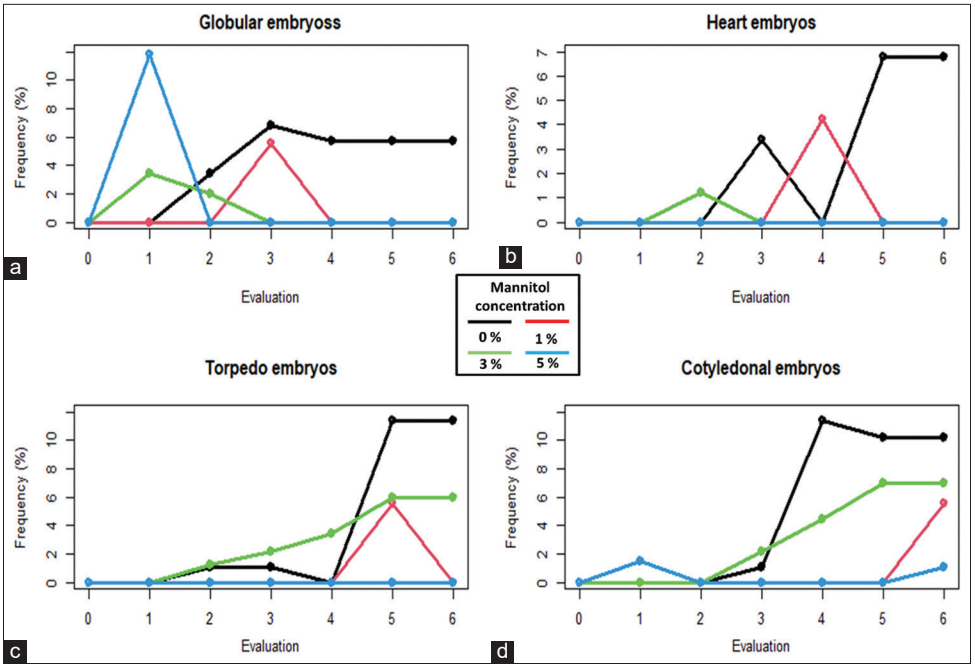

Figure 2: Frequency of the formation of secondary somatic embryos with D-mannitol in six different periods of time. Somatic embryos in stage: Globular (a), heart (b), torpedo (c) and cotyledonal (d).

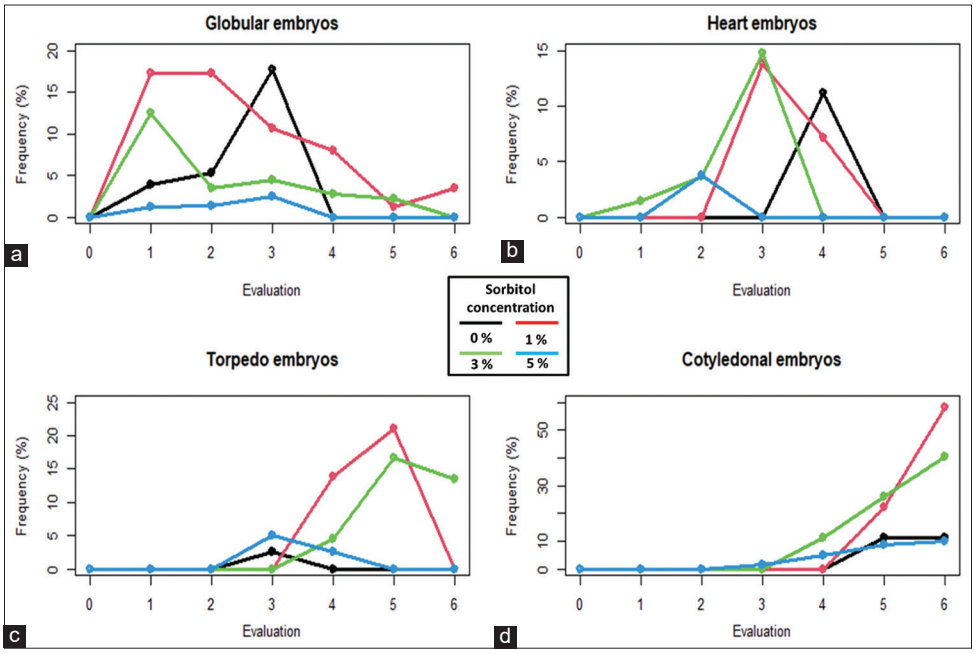

Figure 3: Frequency of the formation of secondary somatic embryos with sorbitol at six different periods of time. Somatic embryos in stage; globular (a), heart (b), torpedo (c) and cotyledonal (d).

reached maturity. The preparation of all culture media used is indicated in Table 1.

2.3. Indirect Secondary Somatic Embryogenesis

Primary embryo epicotyls were collected in the torpedo and cotyledonal stages of 10–40 mg and transverse cuts of 2 mm were made before seeding in a Petri dish containing SCG medium. The explants were transferred to Petri dishes with ED4 medium containing the osmoregulators PEG 6000, D-Mannitol or D-Sorbitol at concentrations of 0%, 1%, 3% and 5%. The explants were then subcultured in ED3 medium containing the same concentrations of the respective osmoregulators. Subculture in this latter medium was performed 6 times (at 14-day intervals) until the somatic embryos reached maturity. The entire process of indirect secondary somatic embryogenesis was carried out with incubation conditions in total darkness, at 25 ± 2°C. Finally, the embryos formed were transferred to Embryo germination and conversion (EDL) medium [Table 1].

2.4. Statistical Analysis

The experiment was established by four independent replicates composed of three experimental units (each experimental unit represented a Petri dish with 25 embryo segments with induced secondary embryogenesis) per treatment. To determine the formation of secondary embryos in each treatment, the frequency of callus and embryo formation was recorded in six subcultures in the ED3 medium. The effect of the osmoregulators was evaluated on six occasions, the first five evaluations were carried out every 7 days after sowing (DAS) and the sixth evaluation was carried out at 63 das. All generated data were subjected to an analysis of variance (ANOVA) using the generalized linear model (GLM) and means comparison by treatment using the Tukey test ($P \leq 0.05$), and were analyzed with the R software (version 4.1.1).

3. RESULTS

3.1. The Effect of Osmotic Regulation

The highest number of SSE in cocoa was obtained with PEG 6000 at 1%. 39.8% of SSE was recorded in the first evaluation, as well as 53.5% at 14 DAS and 57.7% at 21 DAS, dropping to less than 20% from 35 DAS [Figure 1a]. The formation of heart embryos from globular embryos reached the highest frequency at 63 DAS with 19% [Figure 1b]. The evolution of the embryos from the heart state to the torpedo state reached 22.8% at 63 DAS [Figure 1c]. Embryos in the cotyledonary stage reached 165.4% at 63 DAS [Figure 1d].

The addition of D-Mannitol at a concentration of 5% was notable with the generation of 11.8% globular embryos on the 7th day. However, this type of embryos disappeared at 14 DAS and, in general, this was the treatment with the lowest frequency of embryos [Figure 2a]. The highest frequency of formation of heart stage embryos was obtained in the absence of D-Mannitol with a maximum frequency of 6.8% at 49 DAS [Figure 2b].

The absence of D-Mannitol in the culture medium allowed higher frequencies of embryo formation with 11.4% in the torpedo stage at 49 DAS [Figure 2c] and 11.4% in the cotyledonary stage at 35 DAS [Figure 2d]. From the last cotyledonary stage, it is possible to generate complete plants.

The 1% sorbitol allowed the largest number of embryos compared to the other concentrations and the absence of this osmoregulator. Embryos in the globular, heart, torpedo and cotyledonary stages

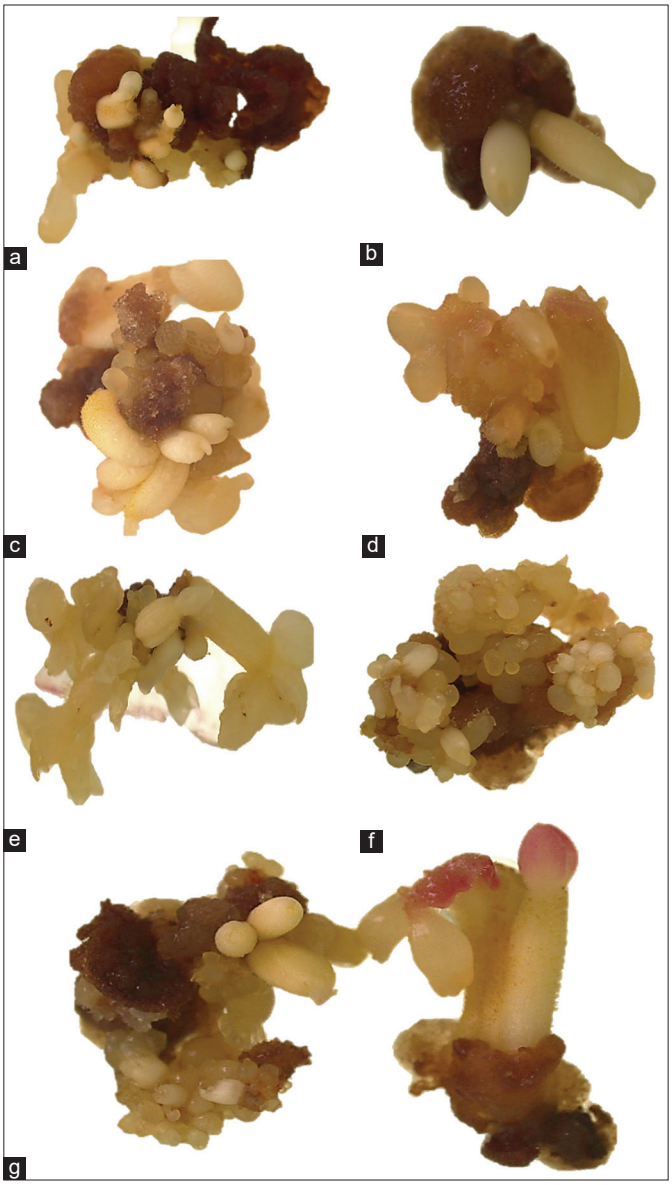

Figure 4: Somatic embryos developed in a medium free of osmoregulators (a). Embryos in the heart and torpedo stage developed in a medium with 3% D-Mannitol (b). Development of embryos in the torpedo state with 1% D-Sorbitol (c). Embryos in cotyledonary stage with 1% D-Sorbitol (d). Somatic embryos predominantly in the cotyledonary state with polyethylene glycol (PEG) 6000 at 1% in the medium (e). Pro-duction of embryos with PEG 6000 in the medium, in which three stages are observed: globular, heart and torpedo (f). Embryos in mature cotyledonary stage developed in medium with 1% PEG 6000 (g).

reached frequencies of 17.2% in the first 7 days [Figure 3a], 14.7% at 21 DAS [Figure 3b], 21% at 49 DAS [Figure 3c], and 57.9% frequency of embryo formation at 63 DAS [Figure 3d], respectively.

3.2. Number of Somatic Embryos by Development Stage

Callus formation in the SCG medium occurred in the first 14 days, obtaining a good percentage of embryos in the torpedo and cotyledonal stage, which gave rise to globular embryos from the 7th day after their

Table 2: Effect of osmoregulators and their concentration on the number of secondary somatic embryos in cocoa by degree of development.

Osmoregulators concentration (%)			Embryogenic callus	Number of somatic embryos per explant			
PEG 6000	D-Mannitol	D-Sorbitol		Globular	Heart	Torpedo	Cotyledonal
0	0	0	2.00^{d}	0.55^{b}	0.23^{c}	0.45^{d}	3.63^{c}
1	0	0	22.65^{a}	30.33^{a}	5.70^{a}	8.40^{a}	55.2^{a}
3	0	0	4.68^{c}	3.98^{b}	0.35^{c}	4.13^{c}	10.80^{b}
5	0	0	5.00^{c}	5.78^{b}	2.05^{a}	3.58^{c}	11.03^{b}
0	1	0	1.18^{d}	0.92^{b}	0.70^{c}	0.92^{d}	0.92^{b}
0	3	0	4.18^{c}	0.90^{b}	0.20^{c}	3.13^{c}	3.40^{c}
0	5	0	0.35^{d}	1.95^{b}	0.00^{d}	0.00^{e}	0.45 ^{d}
0	0	1	4.83^{c}	9.57^{b}	3.48^{b}	5.80^{b}	13.38^{b}
0	0	3	9.33^{b}	4.23^{b}	3.28^{b}	5.75^{b}	12.90^{b}
0	0	5	3.50^{c}	0.82^{b}	0.63^{c}	1.25^{d}	4.15^{c}

Mean values in the column with diferent letters are signifcantly diferent ($P \leq 0.05$).

introduction in the ED3 medium. The degree of development of globular somatic embryos varied between treatments. The total number of resulting somatic embryos was affected by a significant interaction of each osmoregulator and its concentration. Data showed that in the presence of PEG 6000 at a concentration of 1%, a greater development of globular and cotyledonal somatic embryos was produced, with means of 30.33 and 55.2, respectively [Table 2].

Treatment without the addition of osmoregulators showed a low formation of SSE [Figure 4a] compared to the other treatments. The addition of 3% D-Mannitol to the culture medium allowed the number of SSE in the torpedo stage to increase to 3.13 [Figure 4b]. The osmoregulator D-Sorbitol at 1% increased the number of somatic embryos in all stages, but the highest number was observed in the globular and cotyledonal stages [Figure 4c and d]. The osmoregulator that allowed the greatest formation of somatic embryos was PEG 6000 at 1%, with somatic embryos in a higher frequency of globular and cotyledonal stages [Figure 4e and g].

4. DISCUSSION

Plant somatic cells have a high embryogenic capacity, but for this, it is necessary to induce cell reprogramming *in vitro* and obtain PSE. The develop embryogenic cells that continue to divide and allow the formation of SSE. From these embryogenic cells found in the outermost layers of developing embryos, new meristematic centers are formed [30]. The response to secondary embryogenesis can vary between species and even between cultivars, due to the presence of genotype dependence [10,21].

The induction of primary and SSE is a function of the supplementation of growth regulators and abiotic (osmotic) stress factors to which the ex-plants are exposed. These factors influence the initiation of signal expression leading to genetic reprogramming related to callus formation before somatic embryo induction [31]. The incubation of the explants under osmotic stress factors such as PEG 6000, D-Sorbitol, and D-Mannitol at low concentrations causes a greater accumulation of proline as an indicator of stress in the embryogenic cells. The accumulation of endogenous proline exerts a stimulating effect on somatic embryogenesis mainly through the cell wall glycoprotein that is related to morpho-regulatory functions [32,33].

Embryonic asynchrony is the main disadvantage of somatic embryogenesis when mass propagation of cocoa plants is intended [34,35]. To date, no research has been reported on the induction of secondary embryogenesis of cocoa primary embryo cells using osmoregulators. However, some studies evaluated the embryogenic response of cocoa explants, and showed that the expression time of secondary embryos is shorter than obtaining primary embryos and, apparently, there are no significant differences in terms of the response of globular explants and cotyledons [10,36].

The use of PEG has been reported in the induction of somatic embryos in *Carica papaya* L. Golden THB variety, exerting a positive effect by inducing 17.58 somatic embryos per callus using a concentration of 40 g/L of PEG, higher than those treatments without the addition of the osmoregulator. Likewise, the use of this osmoregulator positively influences the maturation of somatic embryos. At higher concentrations of the osmoregulator, there was a negative effect on the number of total somatic embryos, with differences of up to 31.85% in relation to the control [26]. This effect has been confirmed in the present study. At concentrations of 30 and 50 g/L of PEG 6000 in the medium, a reduction was observed in somatic embryos in the cotyledonary stage was observed. In a similar study in *Phoenix dactylifera* L. by increasing PEG concentrations in the culture medium, the growth rate of embryogenic callus and somatic embryos decreased, showing that the effect of this osmoregulator varies depending on embryonic development and the hormonal content of the culture medium [37].

The induction of somatic embryos has been optimized in *Cajanus cajan* L. by adding osmoregulators in the culture medium. Specifically, by exposing the embryogenic callus to dehydration stress imposed by PEG and osmotic stress created by mannitol, producing high-frequency somatic embryos, with 83 somatic embryos in the globular stage and 38 somatic embryos in the heart stage when using 4% PEG; while using 0.6 M mannitol, 87 somatic embryos in the globular stage and 28 somatic embryos in the heart stage [32].

The addition of the osmoregulator mannitol in the culture medium allowed the increase of the embryogenic rate in celery (*Apium graveolens*). By adding 3% and 4% mannitol, cell lysis is avoided and an increase in the number of somatic embryos obtained per explant is evidenced with the formation of 39.5% and 47.4% of somatic embryos, respectively. It is clear that the osmotic potential is an important factor for somatic embryogenesis, but not the only one, since the effect of mannitol could be manifested after a short exposure period (2–3 days), suggesting an embryogenic induction mechanism [38].

Negative effects have also been reported with the addition of mannitol in culture medium, as observed in studies carried out on *Dioscorea*

spp., where it was determined that the use of different osmotic agents affects the *in vitro* survival of the species *Dioscorea alata*. In this sense, the use of mannitol in the culture medium of this species is not recommended, as it causes the death of all explants [39]. This fact was corroborated in our study, since the formation of embryos obtained was relatively low, with respect to the medium in the absence of this osmoregulator. Mannitol had a negative effect on *Eucalyptus globulus* cotyledons, inhibiting callus and somatic embryos by adding 36.44 g/L of mannitol to the culture medium [40]. This could indicate that the addition of mannitol in culture medium does not have a positive effect on the somatic embryogenesis of woody species. Likewise, mannitol can reduce the water absorption capacity of the culture medium, generating a reduction in water potential, much more than other osmoregulators such as sorbitol [41].

Sorbitol has the ability to generate less severe osmotic stress than other osmoregulators and is used as an energy source for explants [39]. For this reason, there are many investigations using sorbitol in somatic embryogenesis, except for cocoa, in which no investigations have been reported. In the case of avocado (*Persea americana* L.) good results were obtained using sorbitol, superior to those that can be obtained with PEG, with a maximum of 58.2% regeneration in medium with 2.5% sorbitol [42]. These results are very close to those obtained in our study, since an average frequency of 57.9% of cotyledonary embryos was reached at 63 days of induction of SSE.

The use of sorbitol has allowed to induce higher rates of SSE in *Elaeis guineensis*; by adding 0.2 M sorbitol, 22 somatic embryos per primary embryo have been obtained [24]. It has also been reported the increase in the formation of somatic embryos in *Swietenia mahagoni* with the addition of 20 and 40 g/L of sorbitol combined with 6 BAP to the culture medium, for which it was determined that sorbitol is one of the osmotic agents that influence the obtaining of somatic embryos and that the response to it can vary according to concentrations and genotypes [43].

5. CONCLUSION

In this work, the effect of the osmoregulators PEG 6000, D-Mannitol, and D-Sorbitol in the increase of SSE of cocoa was determined. The greatest response was achieved by adding PEG 6000 1% and D-Sorbitol 1% to the ED3 culture medium, obtaining a greater number of somatic embryos that completed the cotyledonal stage. The greatest formation of embryos was achieved with PEG 6000 1%, with 30.33 SSE in the globular stage and 55.2 SSE in the cotyledonal stage per explant.

6. ACKNOWLEDGMENTS

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7. AUTHORS’ CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All datasets were generated and analyzed in the present study.

11. PUBLISHER’S NOTE

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