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INDOL-3-ACETIC ACID IS AN EFFECTIVE AGENT FOR THE INDUCTION AND PROLIFERATION OF CALLUS IN *Theobroma cacao*

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ABSTRACT

***In vitro* propagation protocols for *Theobroma cacao* have focused on the multiplication of genotypes with the use of 2,4-diclorofenoxiacetic acid (2,4-D) and thidiazuron (TDZ) in the initial phase of the process. There are no reports of the use of indole-3-acetic acid (AIA) as a source of auxin. Due to risks for human health and the environment, international bans have been placed on the use of 2,4-D. The objective of this study was to evaluate the capacity of AIA in combination with TDZ as inducers of embryogenic usfrom staminodes and petals of the Creole and Trinitarian genotypes of *T. cacao*. The results showed that the combination of AIA 1.0 mg L^{-1} and TDZ 0.005 mg L^{-1} induced callus in 100 % of explants; the rest of the combinations of AIA and TDZ induced callus in 50 % and 80 % of explants. In conclusion, the use of AIA is useful for the formation of friable callus in *in vitro* cultures of *T. cacao*.**

Keywords: Cocoa genotypes, 2,4-D, somatic embryogenesis, *in vitro* organogenesis.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is cultivated in the humid tropics of several areas of the world (Lanaud et al., 2009). In 2016, cocoa bean production was greater than four million tons worldwide, where Africa lead the production (76.5%), followed by America (16%), and Asia and Oceania (7.5%) (ICCO, 2018).

Several factors can affect cocoa production such as pests, diseases, plantation age and low-yield genotypes (Hernández et al., 2015). The crop has been traditionally propagated through asexual methods such as cuttings, grafting and layering (Alemanno et al., 2008). These methods have some disadvantages like loss of genetic variability or incompatibilities of the rootstock, which have led to the search for propagation alternatives.

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The various techniques of *in vitro* culture are an important alternative in obtaining seedlings with the desired agronomic characters from previously selected genotypes. This is under the fundamental principle that plant tissues have cellular totipotency and thus are able to originate a complete individual under ideal light, temperature, nutrient and relative humidity conditions, and a correct hormonal balance, all of which are regulated for plant development under aseptic conditions (Quainoo and Dwomo, 2012; Ikeuchi et al., 2016).

Morphogenetic pathways of *in vitro* plant regeneration include organogenesis (Huang et al., 2014) and somatic embryogenesis (Fehér et al., 2003). Both can be expressed directly or indirectly, after a callus phase in case of the latter (Quiroz et al., 2006), being a process that depends on factors such as the origin of the explant and the plant genotype, as well as the amino acids, vitamins, and plant growth regulators (PGRs) contained in the culture medium (Urrea et al., 2011; Nic et al., 2015; Henao et al., 2018). PGRs combinations, especially auxin-cytokinin, will act synergistically or antagonistically in promoting callus induction or formation before following the pathway of organogenesis or somatic embryogenesis (Huang et al., 2014; Singh and Sinha, 2017; Iracheta et al., 2019; Jing and Strader, 2019).

In cocoa genotypes, callus induction with plant regeneration potential via somatic embryogenesis has been based on the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a source of auxin and thidiazuron (TDZ) as a cytokinin source from flower buds (Maximova et al., 2002; Urrea et al., 2011; Henao et al., 2018). 2,4-D is included in the World Wildlife Fund (WWF) list of pesticides and is reported as an endocrine disruptor that may influence reproduction, among other processes (IRET, 2023). In consequence, several countries have prohibited or are reviewing the registry of the use of formulations of butyl and isobutyl esters of 2,4-D because of its risk to people and the environment (EPA, 2023).

The use of AIA, as an auxin, has not been reported for the *in vitro* culture of cocoa. Thus, the objective of this study was to evaluate its capacity for the induction and proliferation of callus from petals and staminodes in Creole and Trinitarian genotypes of *T. cacao*, as a substitute for 2,4 D.

MATERIAL AND METHODS

Plant material

One-hundred forty flower buds of Creole and Trinitarian genotypes of *T. cacao* with a length of 3 to 5 mm were collected from the localities of Actopan and Aparicio, state of Veracruz, Mexico. The plant material was washed with tap water and disinfected by immersion with a household chlorine solution (Cloralex®) diluted to 5 % (v/v) for 20 min, constantly stirring (100 rpm). Subsequently, the material was rinsed twice through immersion in sterile distilled water. The five petals and staminodes of each flower bud were extracted under aseptic conditions in a horizontal laminar flow cabinet. According to the owners of the trees, Creole and Trinitarian genotypes were estimated to be more than 80 and 45 years old, respectively.

Callus induction

To evaluate the callus induction capacity of AIA and to compare it with the results of previous studies of 2,4-D, different combinations of AIA (1.0, 2.0 and 3.0 mg L^{-1}), 2,4-D (1.0 and 2.0 mg L^{-1}) and TDZ (0.005, 0.05 and 0.5 mg L^{-1}) were tested according to Li et al. (1998), Urrea et al. (2011), Henao et al. (2018), Iracheta et al. (2019) and Pola et al. (2019). The treatments are shown in Table 1. Initial tests were previously performed following the protocol of Li et al. (1998) with unfavorable results for the genotypes studied. Regardless of the combination of the phytoregulators studied, a basal culture medium was prepared for the establishment of the assays. The culture medium was formulated from the macronutrients and vitamins of the DKW medium (Driver and Kuniyuki, 1984) and the micronutrients of the MS medium (Murashige and Skoog, 1992), and supplemented with 200 mg L^{-1} of L-glutamine, 100 mg L^{-1} of my-inositol, 20 g L^{-1} sucrose, pH 5.8 and 2.8 g L^{-1} phytagel (Li et al., 1998, Henao et al., 2018). The pH of the medium was adjusted to 5.8 and sterilized at 121 °C/15 pounds/15 min. A volume of 15-20 mL of the culture medium was placed into 100x15 mm Petri dishes.

The cultures were incubated at 25 ± 2 °C in darkness. The percentage of contaminated explants was recorded during the 30 days of the experiment. The percentage of explants that formed callus was calculated and classified according to the appearance of the cell mass into friable callus (++) and compact callus (+) (Ramírez-Mosqueda and Iglesias-Andreu, 2015). The area of the explant from which the callus was generated (basal, middle, or apical zone) was also recorded.

After 35 days of culture, the friable-looking callus were transferred to the previously described basal medium and incubated under the same conditions as the induction stage. Three subcultures were performed every 15 days. At the end of the experiment, the morphogenetic response was evaluated by considering the

Table 1. Effect of concentrations of 2,4-D, AIA and TDZ on callus formation in petals and staminodes of Creole and Trinitarian genotypes of *Theobroma cacao* L.

Genotype	Growth regulator (mg L^{-1})			Petal		Staminode	
				Callus		Callus	
	2,4-D	AIA	TDZ	(%)	Appearance	(%)	Appearance
Trinitarian	0	0	0	0c	-	0a	-
	1	0	0.005	54ab	++	4a	++
	2	0	0.05	44bc	++	70b	++
	0	1	0.005	64b	++	100b	++
	0	2	0.05	80b	++	88b	++
	0	2	0.5	28ac	+	66b	++
	0	3	0.005	84b	++	100b	++
Creole	0	0	0	0ac	-	0a	-
	1	0	0.005	12a	++	10a	++
	2	0	0.05	14a	++	10a	++
	0	1	0.005	56b	+	44b	++
	0	2	0.05	74b	++	78b	++
	0	2	0.5	28c	++	64b	++
	0	3	0.005	40ac	+	46b	+

Different letters between columns are significantly different ($P< 0.05$). Callus appearance: friable (++), compact (+) and without callus formation (-).

percentage of callus that developed into at least some of the phases of organogenesis (formation of roots or shoots) or somatic embryogenesis (globular, heart-shaped, torpedo or cotyledon).

Experimental design and data analysis

A completely randomized factorial design was used. Each genotype of *T. cacao* (Creole and Trinitarian) was represented by two types of explants (petal and staminode) and seven treatments (Table 1), with five replicates per treatment. Each replica consisted of five explants (either petal or staminodes) per Petri dish with culture medium. Data analysis were executed using a generalized linear model with a Poisson distribution at a 95% confidence interval in R version 4.0.2. Models with $P < 0.05$ were considered significant.

RESULTS

***In vitro* establishment**

The results revealed that 22.5% of the explants of petals and staminodes of the Creole genotype developed fungal and/or bacterial growth, while only 2.3% of the explants of the Trinitarian genotype were contaminated during the initial phase of callus development.

Callus induction

Callus developed by the third day of the assay establishment. Significant differences were found between the experimental factors: genotype, type of explant and PGRs ($P < 0.001$). AIA treatments were more efficient in generating friable callus for both genotypes (Table 1). 100% of the staminodes of the Trinitarian genotype with 1 and 3 mg L^{-1} of AIA in combination with 0.005 mg L^{-1} of TDZ produced friable callus of crystalline appearance (Table 1) (Fig. 1b). For the Creole genotype, treatment with 2 mg L^{-1} of AIA in combination with 0.05 mg L^{-1} of TDZ was the most efficient at producing callus in 74% of the petals and 78% of the staminodes (Fig. 1a). Only 70% of the staminodes of the Trinitarian genotype responded favorably to the formation of friable callus with 2 mg L^{-1} of 2,4-D and 0.05 mg L^{-1} of TDZ, while both explants of the Creole genotype generated low percentages (10 and 14%) of friable callus (Table 1) (Fig. 1e and 1f). Treatment with 3 mg L^{-1} of AIA and 0.005 mg L^{-1} of TDZ induced the formation of compact callus from petals and staminodes of the Creole genotype (Fig. 1, c, and d).

Callus development by explant area

Regarding the total number of staminodes that generated callus in both genotypes, 80%

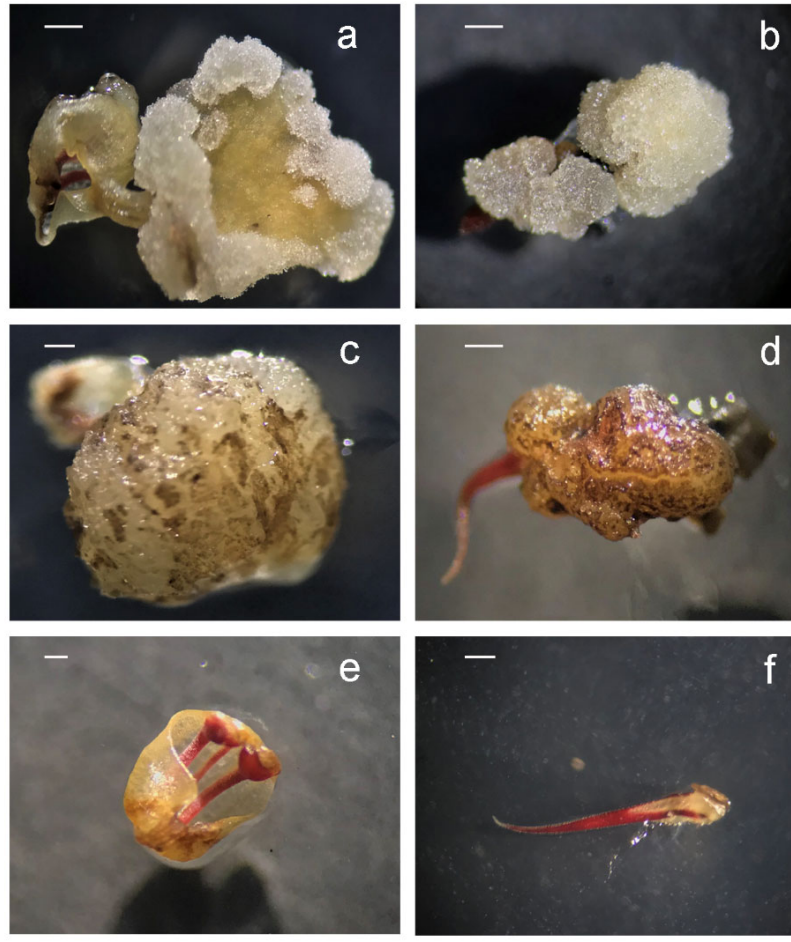

Fig. 1. Effect of the combination of plant growth regulators on embryogenic callus formation in two *Theobroma cacao* genotypes at 35 days of culture: panels *a* and *b* show the friable callus of a petal and a staminode with 2 and 1 mg L^{-1} of AIA and 0.05 and 0.005 mg L^{-1} of TDZ, respectively; panels *c* and *d* show a compact petal and staminode callus with 1 and 3 mg L^{-1} of AIA and 0.05 and 0.005 mg L^{-1} TDZ, respectively; panels *e* and *f* show a petal and a staminode explant without callus formation with 1 and 2 mg L^{-1} of 2,4-D and 0.005 and 0.05 mg L^{-1} TDZ. Scale: 0.5 cm.

corresponded to those generated from the tissue of the basal zone of the explant, and 65% to those generated from the tissue of the petal explants (Table 2) (Fig. 2c and 2d). Between 10 and 20 % of callus were developed from the middle zone of the petal explants (Table 2) (Fig. 2e). The tissue of the middle and apical zones of the staminode explants were not good callus generators (Fig. 2f and 2h).

Morphogenetic responses

Somatic embryogenesis. Proembryonic structures (phase one) and characteristic structures of phase two of somatic embryogenesis originated seven days after subculture, corresponding to pale yellow globular embryos, in callus generated from petals of both genotypes (Fig. 3a and 3e). These responses were induced to a greater degree in the Trinitarian genotype with the combination of AIA (1 and 3 mg L^{-1}) and TDZ (0.005 mg L^{-1}) (Table 3).

At eight weeks, a process of oxidation of the cells began in the callus that developed globular embryos. At this point, elongated crystalline hyaline structures appeared (Fig. 3d), but only in callus originated from petals of both genotypes. Pale yellow, heart-shaped structures with a crystalline appearance (Fig. 3f), characteristic of phase three of somatic embryogenesis, developed in calluses originating from petals of the Trinitarian genotype at nine weeks.

Table 2. Callus induction zone in explants of petals and staminodes of Creole and Trinitarian genotypes of *Theobroma cacao*.

Genotype	Growth zone	Explant	
		Percentage of the total explants that generated callus	
		Petal	Staminode
Trinitarian	Basal	65.54	82.43
	Middle	19.77	9.46
	Apical	14.69	8.11
Creole	Basal	64.75	93.68
	Middle	23.74	6.32
	Apical	11.51	3.68

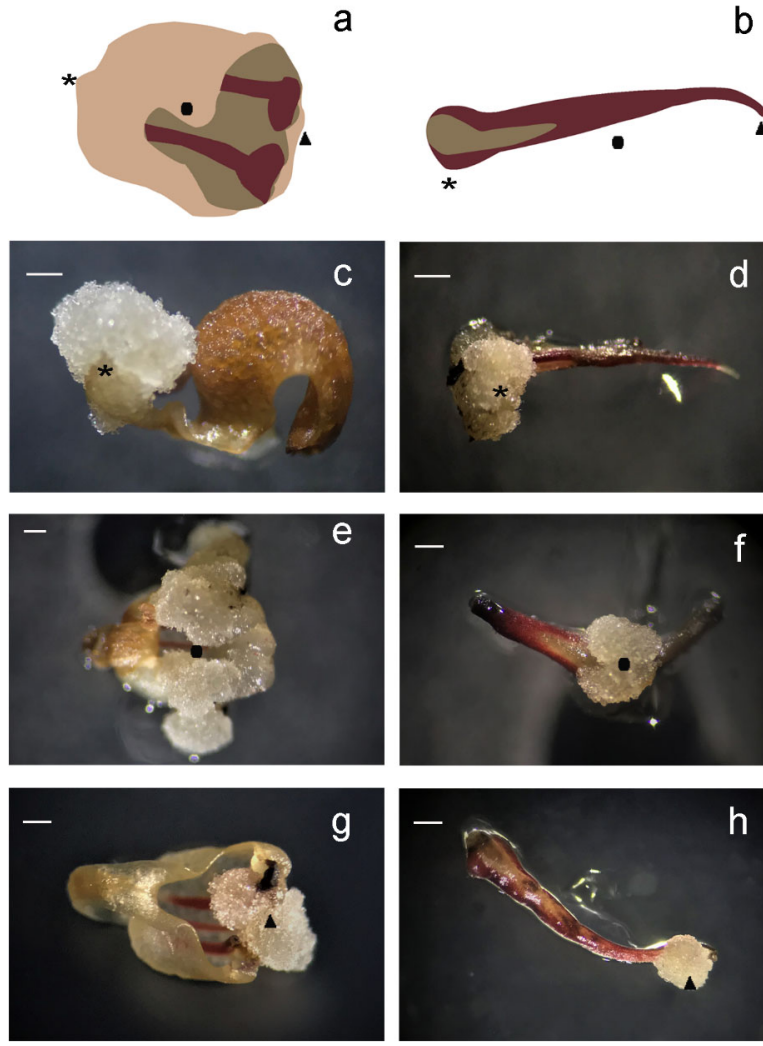

Fig. 2. Callus growth zone in floral explants of the Creole and Trinitarian genotypes of *Theobroma cacao*. Classification of the zone in the explant of petal (a) and staminode (b) - basal zone (■), middle zone (•) and apical zone (▲). Development of a callus at seven days of culture in the basal zone of the petal (c) and the staminode (d); at nine days of culture in the middle zone of the petal (e) and the staminode (f); and at four days of culture at the apical zone of the petal (g) and the staminode (h). Scale: 0.5 cm.

Table 3. Somatic embryos (SE) developed in callus from floral explants of Creole and Trinitarian genotypes of *Theobroma cacao*, under the effect of different concentrations of 2,4-D, IAA and TDZ.

Genotype/Explant	2,4-D (mg L^{-1})	AIA (mg L^{-1})	TDZ (mg L^{-1})	No. of SE/explant
Trinitarian / Petal	0	0	0	0a
	1	0	0.005	6a
	2	0	0.05	11a
	0	1	0.005	26b
	0	2	0.05	19a
	0	2	0.5	4a
	0	3	0.005	28b
Trinitarian / Staminode	0	0	0	0a
	1	0	0.005	2a
	2	0	0.05	7a
	0	1	0.005	5a
	0	2	0.05	8a
	0	2	0.5	10a
	0	3	0.005	12a
Creole / Petal	0	0	0	0a
	1	0	0.005	3a
	2	0	0.05	2a
	0	1	0.005	8a
	0	2	0.05	14a
	0	2	0.5	4a
	0	3	0.005	7a
Creole / Staminode	0	0	0	0a
	1	0	0.005	0a
	2	0	0.05	2a
	0	1	0.005	0a
	0	2	0.05	0a
	0	2	0.5	4a
	0	3	0.005	0a

SE = Somatic Embryos. Different letters between columns are significantly different ($P < 0.05$).

Rhizogenesis. After seven days of subculture, the development of roots in staminode callus of both genotypes began in two of the treatments with 2,4-D (1 and 2 mg L^{-1}) and TDZ (0.005 and 0.05 mg L^{-1}) (Table 3) (Fig. 3b). Only treatments with 2 mg L^{-1} of AIA and 0.5 mg L^{-1} of TDZ generated roots from petal callus of the Creole genotype. A low percentage (4 and 10 %) of callus generated roots. Roots generated from staminodes of both genotypes with 2,4-D and AIA (2 mg L^{-1}) and TDZ (0.05 and 0.5 mg L^{-1}) developed root hairs (Fig. 3b and 3c) at 14 days.

DISCUSSION

Callus induction in *T. cacao* using TDZ alone (Li et al., 1998) or in combination with 2,4-D (Henao et al., 2018; Iracheta et al., 2019) has been previously reported. In this study, the combination of both PGRs promoted poor callus formation in both genotypes; in contrast, all treatments with AIA+TDZ induced callus. AIA is the main auxin in plants and regulates growth and development processes such as cell division and elongation and tissue differentiation, among other processes (Aloni et al., 2006). TDZ increases the biosynthesis of endogenous auxins in the explant and specifically, it increases the synthesis, transport and signaling of AIA (Huetteman and Preece, 1993; Govindaraj, 2018).

The use of AIA for callus induction in *T. cacao* has not been previously reported. Thus, these results are useful for its recommendation as a substitute for 2,4-D, especially considering the

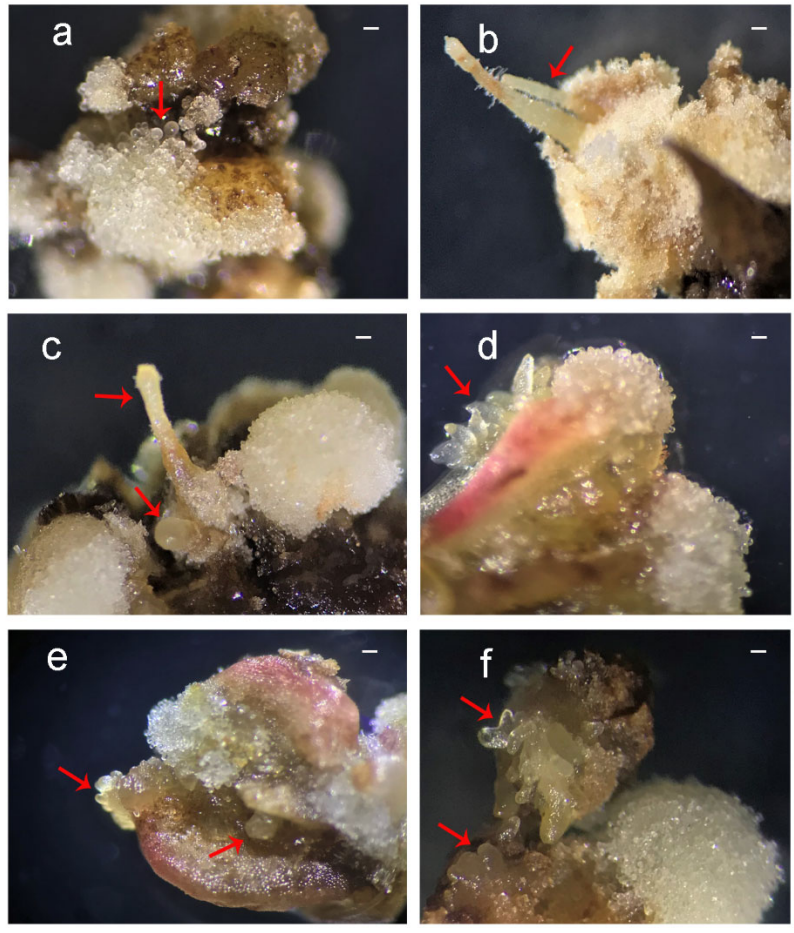

Fig. 3. Morphogenetic responses of floral explants in the induction of callogenesis and proliferation in the Creole and Trinitarian genotypes of *T. cacao*. Panel *a* shows a petal with proembryonic structures (PE); panel *b* shows staminode with rhizogenesis (RG); panel *c* shows a staminode with a globular somatic embryo (GSE) and RG; panel *d* shows transparent elongated hyaline structures (EHA) in petal; panel *e* shows a petal with an GSE group; and panel *f* shows a somatic cream-colored and transparent heart-shaped embryos. Scale: 2 mm.

international restrictions to which it is subject to because of its risk to human health and its environmental impact. The addition of AIA to the culture medium alone or in combination with other PGR has been studied in other species, such as *Aloe vera*, with favorable results in callus induction (Matos and Sánchez, 2011). In such study, 1.0 mg L^{-1} of AIA and 0.1 mg L^{-1} of benzylaminopurine (BA) induced callus formation in up to 95% of explants. Conversely, Pacheco et al. (2003) obtained low percentages of callus (8.0 and 27.5%) from anthers in *Solanum iopetalum* using combinations of 1.0 mg L^{-1} AIA + 1.0 mg L^{-1} BA and 1.0 mg L^{-1} AIA + 3.0 mg L^{-1} BA. Thus, auxin-cytokinin combinations are shown to act synergistically or antagonistically in promoting callus induction dependent on the explant (Aloni et al., 2006; Huang et al., 2014; Singh and Sinha, 2017; Iracheta et al., 2019). In their physiological development plant explants carry concentrations of endogenous phytohormones. In consequence, depending on the species, there is a positive or a negative interaction in the *in vitro* response when different types and concentrations of PGR are added to the culture medium (Nic et al., 2015; Singh and Sinha, 2017; Henao et al., 2018).

This was reiterated in this study, where the induction of callus in *T. cacao* was more efficient from staminodes of both genotypes with the addition of AIA + TDZ. While the petal explants of both genotypes were more efficient in the generation of characteristic structures of somatic embryogenesis, especially the petals of the Trinitarian genotype that generated globular

and heart-shaped structures (phase 2 and 3). At the same time, the staminodes of both genotypes where 2.4 D + TDZ was added and the petals of the Creole genotype where AIA + TDZ was added generated roots with absorbent hairs. In this regard, Iracheta et al. (2019) and Koné et al. (2019) mention that petals in cocoa genotypes express greater regenerative response, while Li et al. (1998) differ in reporting better results from staminodes.

Atta et al. (2009) and Sugimoto et al. (2010) found that callus contain organized structures and gene expressions like those of root meristems, and thus a greater probability of these structures is expected to generate roots. In the present study, however, this did not happen in callus generated from petal explants of the Trinitarian genotype, nor in most callus induced with AIA. This may indicate that the generation of roots was due to the interaction of 2,4-D with the staminode explant, regardless of the genotype of *T. cacao*, which agrees with Li et al. (1998).

The obtained results confirm that the differences observed are attributed to the physiological characteristics of the cocoa genotype like recalcitrance, resulting in less or more plasticity of cells to differentiate (Maximova et al., 2002; Alemanno et al., 2008). These differences may also be attributed to the characteristics of the explant. According to Urrea et al. (2011), the petals differ from the staminodes because they are found on the exterior of the flower bud of the cocoa plant by being in the central zone, which is an area with a greater presence of meristematic cells. This could explain why the staminodes of both genotypes respond better to callus induction. On the other hand, the callus mostly originated from the basal zone or the growth zone of these structures, where there is greater proliferation and cell growth and thus greater auxins synthesis according to Iwase et al. (2011) and Govindaraj (2018).

According to Kouassi et al. (2017), despite somatic embryo production and seedling regeneration having been achieved in many cocoa genotypes, efficiency remains low. Therefore, there are still obstacles for the practical use of *in vitro* culture for clonal propagation of *T. cacao* because of the inability to induce somatic embryogenesis from most elite cocoa genotypes, among other factors. This was clearly evidenced in this study since the callus of the Trinitarian genotype, obtained in combination of IAA and TDZ, generated globular embryos and in turn presented a state of oxidative stress. In this sense, it has been indicated that during the *in vitro* establishment stage, many of the explants begin a darkening phase due to the release of exudates into the culture medium. Exudates are a complex mixture of phenolic substances capable of oxidizing various components without restrictions, which can lead to the destruction of cells, resulting in more severe damage during the initial stages of the culture. However, it has been observed that the problem can stop and disappear when the explant begins its growth, as a response to habituation, by ceasing to respond to the oxidative stimulus (Azofeita, 2009), which would explain the results obtained with the formulation of growth regulators used.

Therefore, it is essential to continue generating information towards the optimization of each stage of the procedure, including callus generation, which is an intermediate stage of utmost importance for indirect morphogenesis.

CONCLUSIONS

AIA in combination with TDZ is efficient at inducing the generation and proliferation of a callus in *T. cacao* from staminodes and petals of Creole and Trinitarian genotypes, with higher values to those previously reported for 2,4 D. In addition, two AIA and TDZ combinations had the ability to form globular and heart-shaped somatic embryos, especially from Trinitarian cocoa petals. This provides sufficient bases to recommend it as a substitute for clonal *in vitro* propagation protocols of *T. cacao*.

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Author's contribution

All of the authors contributed in all stages of this research (literature review, definition of methods, discussion of results and approving final version of the manuscript).

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