ARTICLE / INVESTIGACIÓN

Regeneration of cocoa (*Theobroma cacao* L.) via somatic embryogenesis: Key aspects in the *in vitro* conversion stage and in the *ex vitro* adaptation of plantlets

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Abstract: Adapting plantlets to *ex vitro* conditions is a decisive step in the micropropagation process via organogenesis or somatic embryogenesis (ES). The percentage of success in this stage determines the quality of the product, an example of which is found in cocoa plantlets regenerated by ES, which require specific conditions to overcome the stress of the new environment. Considering the quality of the *in vitro* plantlets largely determines the survival and growth in *ex vitro* conditions, the effect of two culture media between the embryo maturation stage and the initial stage of conversion to plantlet was evaluated (EM2 - MM6 and EM2 – MF medium), achieving with the latter greater stem height, root length and the number of true leaves. In the final stage of the conversion and growth of the plantlet, the effect of five culture media was evaluated (ENR6, MF, ENR8, EDL, PR), achieving better results in stem height, root length, and the number of true leaves on MF medium. In addition, it was found that the transition of the EM2-MF had a significant development in the presence of the desired pivoting root and fibrous roots. Under nursery conditions, the growth and development of the plantlets was tested through the inoculation of beneficial microorganisms to promote survival. The plantlets that met the minimum morphological parameters for acclimation were planted in a substrate of coconut palm and sand (3:1 v/v) previously selected in the laboratory (BS). The effect of *Pseudomonas* ACC deaminase (PAACd), *Trichoderma asperellum* (Ta) and arbuscular mycorrhiza forming fungus (AMF) and different concentrations of phosphorus (PC) (0%, 50% and 100%) in the Hoagland nutrient solution (1:10) was evaluated. First, for CCN5, 62.5% of survival was obtained with PAACd + AMF. Second, the largest leaf size and survival were obtained with PAACd + Ta for *CNCh12* and *CCN51*; likewise, for *CNCh13*, the best result was obtained with PAACd.

Key words: Cacao, Clonal propagation, Mycorrhiza, Pseudomonas, Trichoderma.

Introduction

Cocoa (*Theobroma cacao* L.) is an economically important crop and the most valuable agricultural product worldwide[1]. It has been grown in the lowlands of tropical regions, South and Central America, West Africa, and Southeast Asia, with social and economic importance. Cocoa powder and cocoa butter are the major cocoa seed products with several common usages, especially in high-demand food industries[2]. Cocoa seeds are rich in phenolic compounds and flavonoids and contain several bioactive compounds, such as procyanidins, anthocyanins, flavone and flavonol glycosides, epicatechin, gallocatechin, epigallocatechin, etc.[3,4]. Cocoa polyphenols have anti-inflammatory, anticarcinogenic, antimicrobial, antiulcer, and immune-modulating properties, and antioxidants with a protective effect against cardiovascular diseases[5–8]. Thus, producing high-quality and uniform cocoa planting materials is crucial to supply industrial demands. This could be achieved through plant tissue culture techniques such as somatic embryogenesis (SE) since conventional methods do not complete the quantities required by the market. In SE a single somatic cell obtained from leaf, flower, or stem explants undergoes several differentiation processes before developing into the whole plant after culture[9]. For cocoa, the protocols for somatic embryogenesis culture have been developed by several researchers[10–12]. However, low acclimation percentages of around 8.3% to 54.57% with different genotypes were reported[13–15].

The acclimation is a transitory phase between the laboratory and the field, whose objective is to take the plantlet from an *in vitro* culture to *ex vitro* conditions[16]. The *ex vitro* adaptation of Vitro plantlets requires time and conditions for the plantlets to acquire the necessary vigor to survive. They usually need several weeks under shade and gradually decreasing air humidity to acclimate to the new requirements and correct some changes in their anatomy and physiology induced by in vitro culture conditions. For plant survival, the most important changes include the development of cuticles, epicuticular waxes, and effective stomatal regulation of transpiration leading to stabilization of water status[17]. From Aguilar *et al.* (1992) and Figueira & Janick, (1995)[1], to

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Jones *et al.* (2022)[20] and Manlé *et al.* (2021)[1], the acclimation of cocoa vitro-plants is a difficult and crucial stage as it happens in most plantlets produced by *in vitro* tissue cultures[21]. Therefore, knowing the factors that affect the survival of vitroplantlets is essential when implementing the production of plant material on a large scale, whose main contribution is the development of an optimal propagation protocol.

On the other hand, it is necessary to have plantlets produced in laboratory commercials that ensure plant material is in good physiological condition to survive the definitive transplant without affecting their establishment and optimal development in the field. In this sense, crucial developmental phases such as the maturation of the somatic embryo, the conversion of the embryos to plantlet, and the acclimation of plants to nursery conditions are essential to ensure physiological vigor characteristics. This last step can be favored using microorganisms as nursery substrate improvers, which are recognized as promoters of root development in cocoa seedlings and their effect on the increase and nutrient absorption capacity[22–26].

Many plants' growth-promoting microorganisms (PGPM) can help in rooting and shoot elongation and can be useful in the acclimation phase. They can protect against the biotic and abiotic stress that occurs *in vitro* propagation, mainly in the acclimation phase, a crucial step for the success of micropropagation[27]. Within these microorganisms, the Trichoderma fungus is considered a soil organism associated with plant roots and is commonly viewed for its potential to control plant diseases in what may be a close association with many typical aspects of endophytic associations with cocoa[28], in addition to being able to behave as a plant growth stimulator[29,30]. Other beneficial microorganisms are mycorrhizal fungi, which are the mutualistic symbiosis between the fungi of the Glomeromycota phylum and the roots of most vascular plants, which are supposed to play a key role in the nutrient cycle of agroforestry systems[31]. In the case of crops with thick roots and few root hairs, such as cocoa, coffee, timber trees, and citrus, they tend to be highly dependent on mycorrhizae[32]. Specifically in cocoa, due to its mycorrhizal dependency, the fertilization practice, mainly nitrogenous and phosphate, must be fully evaluated, considering not only the yield of the crop and the availability of nutrients in the soil but also the composition and behavior of the biota, in order not to inhibit these biological processes or stimulate dependence on external inputs in these systems[33].

Other microorganisms are the bacterias that produce the enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase, being a critical bacterial trait to facilitate plant growth. This enzyme is responsible for the cleavage the plant ethylene precursor, ACC, into ammonia and ketobutyrate. Ethylene is an essential plant hormone, also known as a stress hormone because the induction of a variety of biotic and abiotic stress accelerates its synthesis by lowering ACC levels in plants, ACC deaminase-producing organisms lower ethylene levels, which, when present in high concentrations, can lead to plant growth inhibition or even death[33]. These bacteria are relatively common in soil, having been found in a wide range of environments around the world[33].

According to the above, the objective of this work was to evaluate from the stage of maturation and conversion to plantlet *in vitro* the effect of culture media composition and, in the acclimation phase, the effect of beneficial microorganisms such as Pseudomonas ACC deaminase, the fungus *Trichoderma asperellum* and mycorrhizal fungi on characteristics physiological vigour in plantlets to establish a complete process in the production of plant material on an industrial scale.

Materials and methods

Plant materials and culture conditions

For this research, the experiments included *CCN51*, *CNCh12*, and *CNCh13* genotypes, the primary genotype used in the experiments was *CCN51*, a universal genotype from Ecuador[34]. *CNCh12* and *CNCh13* are Colombian genotypes from the certified clonal garden of the Colombian National Chocolate Company (CNCH). The immature closed flower buds were collected from La Nacional Farm field-grown plants (Támesis, Antioquia-Colombia) and Yariguíes farm (Barrancabermeja, Santander-Colombia). The flower bud's collection and disinfection process were performed following the methodology described by Henao *et al.* (2018)[3]. The explants consisted of staminodes (sterile stamens) for *CCN51*, *CNCh13*, and *CNCh12*. Staminodes were extracted from the basal portion of the flower bud using sterile scalpels and placed on the culture media on the Petri dish. For *CNCh12* was used, the following culture media was according to the stages of the embryogenic process: PCG-SCG-ED-CM2-EM2-MM6, for *CNCh13*, was INDI-INDexp-CM2-EM2-MM6 and for *CCN51* was INDI-IN-Dexp-CM2-EM2-MM6 (Table 1).

All the cultures were randomly placed in a growth chamber in continuous darkness for callogenesis, induction of primary embryo, and induction of secondary embryo stages, at an average temperature of 26˚C ± 2˚C and 70% relative humidity. The mature embryos were cultured in a Petri dish, and later plantlets were cultured in 500 ml vessels. The cultures were placed in a growth chamber under light with a 16-hour photoperiod and a photosynthetic photon flux density (PPFD) of 50 µmol per second, at an average temperature of 26˚C ± 2˚C and 70% relative humidity.

In vitro growth

Embryo maturation

Once the secondary somatic embryos of *CCN51* had been developed, they were transferred for maturating and growth. In this stage, the effect of two different culture media transitions between embryo maturation - plantlet development stage (EM2 – MF and EM2 – MM6) (Table 2) and culture time (30, 60, and 90 days) were evaluated on stem height (cm), root length (cm) and the number of true leaves per plantlet (n). The experiment was laid out in a Completely Randomized Design (CRD) with two factors: culture media (2 levels) and culture time (3 levels). Each treatment with at least 30 experimental units, for a total of n = 524.

Plantlet development

For *CCN51*, the effect of five different plantlet development culture media (ENR6, MF, ENR8, EDL, PR) (Table 2) was evaluated on stem length (cm), root length (cm), number of true leaves (n) after 30 days from the plant out. The experiment was laid out in a CRD with one factor, culture media, with five levels. Each treatment with at least 10 experimental units, for a total of n = 43.

Composition	*CNCh13* and *CCN51*					*CNCh12*		
	Calli Induction	Primary somatic embryo induction	Secondary somatic embryo induction	Embryo maturation	Plantlet development	Calli Induction		Primary somatic embryo induction
	INDI[1]	INDexp[2]	CM2[3]	EM2[4]	MM6[5]	PGC[10]	SCG[11]	ED[12]
Macronutrients	DKW[6]	DKW	MS[14]	MS	½ MS	DKW	Lloyd & McCown[15]	DKW
Micronutrients			DKW	DKW	DKW			
Myoinositol	100	100	100	100	100	100	100	
Nicotinic acid Vit B3	1	1	1	1	1	1	1	
Thiamine Vit B1	2	2	2	2	2	2	10	
Pyridoxine							1	
Glutamine						250		
Glycine	2.18	2.18	2.18					
L-lysine	0.45	0.45	0.45					
L-leucine	0.32	0.32	0.32					
L-arginine	0.43	0.43	0.43					
L-tryptophan	0.51	0.51	0.51					
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)			1					
2,4-dichlorophenoxyacetic acid (2,4-D)	1					1.98	1.98	
1-Naphthaleneacetic acid (NAA)					0.1			
Kinetin (KIN)	0.25						0.3	
Adenine			0.25					
Gibberellic acid (GA_3)					0.02			
Thidiazuron						0.005		
Activated charcoal					1			
Glucose	30	30	30		40	20	20	1
Saccharose				40				20
Gelling	3	3	3	3	4	2.9	2.9	2.9

[35, 4, 6 36, 10, 11, 12 37, 13 38, 14 39, 15 40]

Table 1. Culture media for primary and secondary somatic embryogenesis with CCN51, CNCh13, and CNCh12 genotypes of cacao (T. cacao L.). 1,2,3,5

Ex vitro adaptation

Plantlets of different genotypes with 60 days in MF culture medium were used for the different experiments; these plantlets had stem and root development. The plantlets were removed from culture vessels and washed with running water to eliminate excess gelled culture medium. The plantlets were subsequently transferred to 50 germination trays with 10 cm deep cavities, with a capacity between 67 - 70 g of basal substrate. The tray was covered with a transparent dome for one day, and holes are subsequently made to allow exchanges until 15 days were completed, keeping the substrate hydrated with water as required and a nutrient solution of Hoagland (1:10) every 15 days. After 50 days, the plants were transplanted into 45cm deep bags. Plantlets were kept in growth conditions at the mesh house, relative moisture major to 40%, temperature between 24 - 30°C at day and 16-18 °C at night, 16:8 natural sun photoperiod, and shade of 50%.

Basal substrate (BS)

The basal substrate consisted of a 1:3 (w/w) proportion of washed river sand and ground coconut fiber without enrichment. Once the substrate is prepared, the electrical conductivity of the soil (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 of 5.8 to 7.0[42,43].

Minimum quality characteristics for in vitro plantlets

Plantlets from *in vitro* conditions were classified according to quality characteristics. The minimum quality characteristics of plantlets were evaluated in terms of stem height (cm), root length (cm), number of true leaves (n), length leaf (cm), and width of the leaf (cm) on the survival of the plantlets in the BS.

Beneficial microorganisms' preparation

Pseudomonas ACC desaminasa (PAACd) inoculum

To prepare the inoculum of PAACd, the strains of 5 bacteria preserved in the deep freezer were placed in the TSA (trypticase soy agar) culture medium. They were then grown under stirring for 72 hours in a 50% TSB (trypticase soy broth) liquid medium. After this time, the bacteria were mixed and centrifuged at 3,500 rpm for 50 minutes to eliminate the culture medium. The pellet was resuspended in a 0.03M magnesium sulfate solution with the help of a vortex. Subsequently, this solution was brought to an OD of 0.1-0.15 at 600 nm, which indicates an approximate concentration of 1x108 CFU/mL. 10mL of the bacterial inoculum near the plantlet was added to each well with 69 - 70g.

Mycorrhizal fungi (AMF) inoculum

For the preparation of the inoculum of AMF, the commercial product Mycorfos® (contained a minimum concentration of 230 spores/gram with *Glomus sp.*, *Acaulospora sp.*, *Scutellospora sp.* and *Entrophospora sp.*) was taken. The product was sieved to 1mm to remove large particles. Between 7-8 g of this product was added to each well and mixed with the substrate.

Trichoderma asperellum (Ta) inoculum

The fungus was grown in PDA medium (potato dex-

Composition	Embryo maturation experiment			Plantlet development experiment			
	EM2[4]	MF[4]	MM6[5]	ENR6[6]	ENR8[7]	EDL[8]	PR[9]
Macronutrients	MS	½ MS	½ MS	½ MS	MS	DKW	DKW
Micronutrients	DKW	DKW	DKW	DKW	DKW		
Myoinositol	100	100	100	100	100	100	50
Nicotinic acid Vit B3	1	1	1	1	1	1	0.5
Thiamine Vit B1	2	2	2	2	2	2	1
Glycine					0.4	0.45	0.45
L-lysine					0.4	0.33	0.33
L-leucine					0.4	0.51	0.51
L-arginine					0.4	0.43	0.43
L-tryptophan					0.2	0.19	0.19
1-Naphthaleneacetic acid (NAA)		0.01	0.1				
Gibberellic acid (GA_3)		0.02	0.02				
Potassium nitrate (KNO_3)					0.3	0.3	
Activated charcoal		1	1				
Glucose		40	40	10	30	20	20
Saccharose	40			5			
Gelling	3	3.8	4	3	3.2	1.8	2

Table 2. Culture media for primary and secondary somatic embryogenesis with CCN51 genotype of cacao (T. cacao L.) in embryo maturation and plantlet development experiments.

[5] (Henao et al., 2018) [35], [4, 6] (Fontal et al., 2002)[36], [7] (Guillou et al., 2018) [41], [8, 9] (Garcia et al., 2018) [11]

trose agar) for five days. Then a spore solution was made in sterile distilled water and inoculated into a bag with rice as substrate. It was incubated at in room temperature with photoperiod 12:12 for 14 days. After this, sterile distilled water was added, it was mixed very well with the rice to obtain the spore suspension, and the solution was filtered in a clean beaker passing through gauze. Then serial dilutions were made, and a spore count was performed in a Neubauer chamber to find the concentration. With this data, the fungus solution to be used was prepared at a concentration of 1x108 spores/mL. 5 mL of the spore suspension of the fungus *T. asperellum* was inoculated per well.

The Agricultural and Environmental Bacteriology Research Group of the Universidad de Antioquia donated all the microorganisms used.

Beneficial microorganisms' experiments

Effects of PAACd and AMF

The genotype used in this experiment was *CCN51* for two months. The effect of PAACd, AMF, and the mixture of both microorganisms and control were evaluated on survival percentage. The experiment was laid out in CRD, the factor type of substrate, with four treatments. Each treatment had 10 experimental units for a total of 40 plantlets.

Effects of PAACd and Ta

The genotypes used in the experiment were *CCN51*, *CNCh12*, and *CNCh13*. The effect of PAACd and *Ta*, the mixture of microorganisms and control, was evaluated on survival percentage and leaf area (cm^2). The experiment was laid out in a CRD with the factor type of substrate with four treatments. Each treatment had 10 experimental units for a total of 40 plantlets.

Effects of AMF and phosphor (P) concentrations

Since a negative effect on mycorrhizal fungi colonization has been described when phosphorus concentrations are greater than 0.02 mg/L., the amount of phosphorus present in BS was quantified. For this, the methodology described by Osorio (2017) was followed, which consists of taking 3 g of an essential dry substrate and transferring them to centrifuge tubes, subsequently adding 30 mL of CaCl2 0.01 M and two drops of toluene for inhibition of microbial activity. The tubes were shaken horizontally for 1 hour, centrifuged at 5000 rpm for 15 minutes, and the supernatant passed through Whatman No. 1 filter paper. The concentration of P was determined by the molybdate blue method by making measurements in a spectrophotometer at a wavelength of 890 nm.

Different concentrations of P in the irrigation solution and AMF quantity (g) were evaluated. In the fertilization process, the Hoagland solution was prepared at 1:10 by modifying the amount of phosphorus as K_2HPO_4 salt at 0%, 50% and 100%. The fertilization was carried out biweekly, adding 5mL per plant for up to 60 days. The plantlets received irrigation with water as needed. The AMF inoculation consisted of applying the commercial formula in different quantities of 0g, 2g, and 3g, which were sieved and mixed with the BS. The effect of AMF quantity (0g, 2g, 3g) and

phosphor concentrations (P) (0%, 50% and 100%) were evaluated on the percentage of mycorrhizal infection and survival percentage. The experiment was laid out in a CRD with nine treatments. Each treatment had 10 experimental units for a total of 90 plantlets.

Statistical analysis

Results were subjected to an analysis of variance (ANOVA), and a comparison test was performed based on residuals to test normality (Shapiro-Wilk test). Additionally, the variance homogeneity test (Levene's test) was performed. Likewise, the comparison of means was carried out through Tukey or Dunnet's test. If they were not normal, the Kruskal-Wallis and Mann-Whitney tests were used. On the other hand, for comparisons between 2 treatments, the t-test was used for parametric data and the Wilcoxon test for non-parametric data. For unequal numbers, an analysis of variance across the generalized linear model (GLM) was performed for some stages with a Poisson variable response distribution. The Pearson Chi-square ($\chi 2$) test was carried out regarding the qualitative data. Dispersion, box, and whisker diagrams were used. A significance of 95% was determined for all comparisons using the R project v3.6.1 software.

Results

In vitro growth

Effects of culture media on embryo maturation

In this experiment, there was a higher average in the number of true leaves per plantlet in the EM2-MF transition. An average of 3.12, 3.12, and 4.10 was obtained at 30, 60, and 90 days, respectively, with significant differences concerning EM2-MM6 (Figure 1 A). For the stem height in the EM2-MF medium, an average of 3.64 cm was obtained at 60 days of cultivation with differences with respect to 2.91 with EM2-MM6 (Figure 1 B). For root length, 4.07 cm was obtained for EM2-MF and 4.4 cm for EM2-MM6, without a difference (Figure 1 C). Consequently, the appropriate medium for somatic embryo maturation was the transition between EM2 and MF medium for the CCN51 genotype with sufficient fibrous and tap root development (Figure 1 S1).

Effects of culture media on the development of plantlet

At this stage, the average number of leaves per plantlet was 4.77 obtained in the ENR8 medium, 4.55 in the MF medium, and 4,26 in the ENR6 medium without a difference (Figure 2 A). Likewise, the highest values of height stem were 4.42 cm in the MF medium and 4.03 cm in the ENR8 medium (Figure 2 B), and finally, the most excellent root length of 5.67 cm was obtained in the MF medium, followed by 4.31 cm in ENR8 (Figure 2 C). Therefore, the adequate development of the plantlets was achieved in general in the MF medium, with results consistent with the experiment detailed in the maturation assay.

Ex vitro adaptation

BS on survival plantlets

During the evaluation of the BS substrate's effect on the *ex vitro* adaptation process, it was possible to verify that the conductivity of the substrate is critical in the process since only the addition of organic matter (MO) can significantly alter this parameter. In previous experiments, using irrigation solutions such as full and half concentration MS and DKW salts or including MO in the essential substrate, it was found that the electrical conductivity increased more than twice the ideal for cocoa (Table 3). Due to this condition, the plants stopped their growth and development; on the contrary, the mortality was 100%. Likewise, in previous trials, it was found that the corrections in the conductivity of the BS substrate supplemented with different plant growth-promoting components like AMF and OM had a positive effect on survival. In addition, the formation of the white root is observed, like a characteristic of healthy roots for cacao (Table 1 S1, Figure 2 S1).

Minimum morphological quality characteristics

Identifying the plantlet's minimum morphological quality characteristics for survival during the adaptation process was possible. *CCN51* plantlets must have at least: 3.98 cm of height stem, five true leaves with 3.58 cm of length and 1.76 of width, and prominent radical development of 6.71 cm root length with both primary pivotal roots and secondary roots (Table 4).

Effect of PAACd and AMF mix on plantlet's survival

In the effects of PAACd and AMF on the survival of plantlets, no association was found between the different treatments evaluated and the survival. The highest survival percentage of 62.5% was obtained with the substrate supplemented with PAACd + AMF, the same result was obtained with only PAACd (Figure 3B).

Effects of PAACd and Ta on plant survival and leaf area

For the leaf area, an average of 20.41 cm^2 was obtained in the substrate supplement with PAACd + Ta for the *CNCh12* genotype, with a significative difference from the control. The same result was for *CCN51*, with an average of 37.65 with PAACd + Ta but without significant differences between PAACd and control. For the *CNCh13* genotype, an average of 14.64 was obtained with PAACd, with a significative difference from the control (Table 5). In the survival percentage, 83% was obtained with PAACd + Ta for *CNCh12*, 67% was obtained with PAACd for *CNCh13*, and 67% was obtained with PAACd and Ta for *CCN51* but without significative differences (Figure 3 S1).

Effects of AMF and P concentrations on survival and mycorrhizal infection

The phosphorus absorbance measurements recorded from known concentrations allowed the drawing of a standard calibration curve (Figure 4 S1), on which the phosphorus concentration in the substrate was determined. For the BS sample, an absorbance of 0.1139 was obtained, which corresponds to a concentration of 0.2651 mg/L of P.

The percentage of mycorrhizal infection in the treatments with 2g and 3g was significantly different from to control, with an average of 74% of condition for three phosphor concentrations (Figure 5). Survival of 100% of the plantlets was observed in the treatments of 2g AMF + 50% P and 3g AMF + 50% P, results that reveal the importance of phosphorus for the process of adaptation of cocoa plantlets to the *CCN51* genotype (Figure 5 S1).

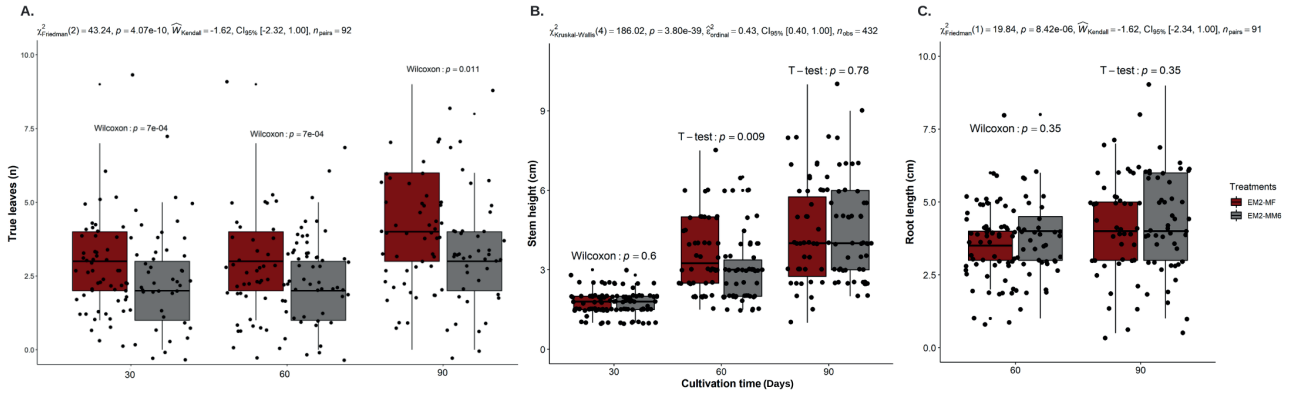

Figure 1. In the maturation stage of somatic embryogenesis development for the CCN51 genotype. Effects of culture media transition EM2-AMF and EM2-MM6 on (A) number of leaves (n) per plantlet at 30, 60, and 90 days of time cultivation, (B) height of stem (cm) at 30, 60 and 90 days of time cultivation and (C) root length (cm) at 60, and 90 days of time cultivation.

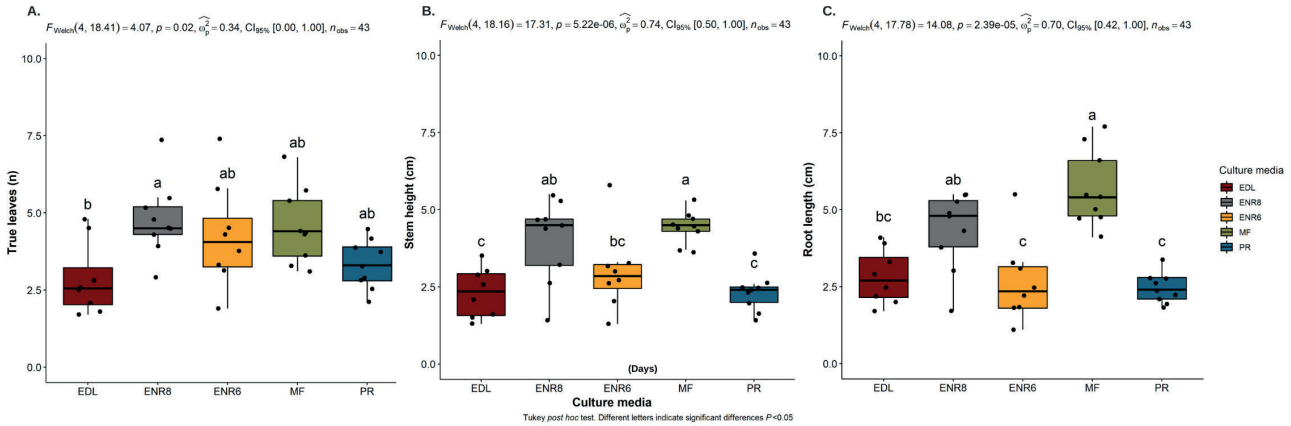

Figure 2. In the germination stage of somatic embryogenesis development for CCN51 genotype. Effects of culture media (ENR6, AMF, ENR8, EDL, PR) on (A) number of true leaves (n), (B) height stem (cm), (C) root length (cm).

Substrate	EC (mS/cm).	pH	EC (Tap water)	pH (Tap water)
BS in sowing	0.44	6.21	0.12	6.97
BS 30 days after sowing	0.27	6.35	0.14	6.33
BS + MO in sowing	2.13	6.01	0.09	6.67
BS + MO 30 days after sowing	2.34	5.95	0.08	6.28
BS + ½ MS	2.89	4.31		
BS + ½ DKW	3.28	4.21		

Table 3. Conductivity and pH results in basal substrate supplemented with organic matter and irrigation with ½ de MS and DKW macronutrients and micronutrients.

Discussion

Cacao somatic embryogenesis process is divided into six well-defined steps induction, expression, multiplication, maturation, germination, and plant conversion[44]. All these steps are mediated by a complex regulatory network where genetic and epigenetic mechanisms regulate different genes at the transcriptional level[45]. In this work, the acclimation process is addressed from the stage of embryo maturation. Conceptually, the developmental stages of the somatic and zygotic embryo are divided into two main metabolic stages; the first is a morphogenetic stage that is characterized by cell division and the onset of cell differentiation; the second is a metabolic stage or maturation phase that is characterized by biochemical activities, which involves the accumulation of significant storage products and the preparation for desiccation, dormancy, and germination/conversion[46,47]. In this last phase, somatic embryos achieve both morphological and physiological maturity, guaranteeing satisfactory post-embryonic performance. Therefore, the conversion potential is programmed during embryo maturation[48].

Embryo maturation

Better characteristics were obtained in the plants from embryo maturation in the transition between EM2-MF medium. The EM2 medium first reported by Fontanel *et al.* (2002)[36] has been effective for the maturation different researchers have often modified it according to the genotype of interest. On the other hand, the MF medium is a modification of the MM6 medium that was first reported by Fontanel *et al.* (2002)[36] and later modified by Henao *et al.* (2018)[35]. However, the MF medium has not been as widely used as the ED medium initially reported by (37), a medium without growth regulators for the maturation process. Both MF and MM6 medium have NAA and GA_3, a combination of growth

Stem height (cm)													
Survival	**Length**	**Min**	**Max**	**Median**	**Mean**	**ipr**	**Mad**	**sd**	**se**	**ci**	**range**	**cv**	**Var**
Survive	33	1,7	6,0	4	3,98	2,6	2,07	1,42	0,24	0,50	4,3	0,35	2,03
Demise	101	1,0	8,5	3	3,34	2,0	1,48	1,66	0,16	0,32	7,5	0,49	2,73
Root length (cm)													
Survival	**Length**	**Min**	**Max**	**Median**	**Mean**	**ipr**	**Mad**	**sd**	**se**	**ci**	**range**	**cv**	**Var**
Survive	33	1,5	14,0	6,2	6,71	3,0	1,77	2,79	0,48	0,99	12,5	0,41	7,82
Demise	101	0,3	15,2	4,5	5,02	3,2	2,22	2,53	0,25	0,50	14,9	0,50	6,44
Number of true leaves (n)													
Survival	**Length**	**Min**	**Max**	**Median**	**Mean**	**ipr**	**Mad**	**sd**	**se**	**ci**	**range**	**cv**	**Var**
Survive	33	1	12	5	5,57	3	1,48	2,46	0,42	0,87	11	0,44	6,06
Demise	101	1	8	3	2,87	2	1,48	1,45	0,45	0,28	7	0,50	2,12
Length leaf (cm)													
Survival	**Length**	**Min**	**Max**	**Median**	**Mean**	**ipr**	**Mad**	**sd**	**se**	**ci**	**range**	**cv**	**Var**
Survive	33	1,3	5,2	3,5	3,58	1,4	0,74	0,95	0,16	0,33	3,9	0,26	0,90
Demise	101	0,4	5,0	2,3	2,50	1,0	0,88	0,86	0,08	0,17	4,6	0,34	0,75
Width leaf (cm)													
Survival	**Length**	**Min**	**Max**	**Median**	**Mean**	**ipr**	**Mad**	**sd**	**se**	**ci**	**range**	**cv**	**Var**
Survive	33	0,4	8	1,6	1,76	0,6	0,44	1,20	0,20	0,42	7,6	0,68	1,44
Demise	101	0,2	7	1,0	1,03	0,5	0,44	0,71	0,07	0,14	6,8	0,69	0,51

Table 4. Central tendency measures for minimum morphological quality characteristics of CCN51 in vitro-plantlets to continue ex vitro adaptation.

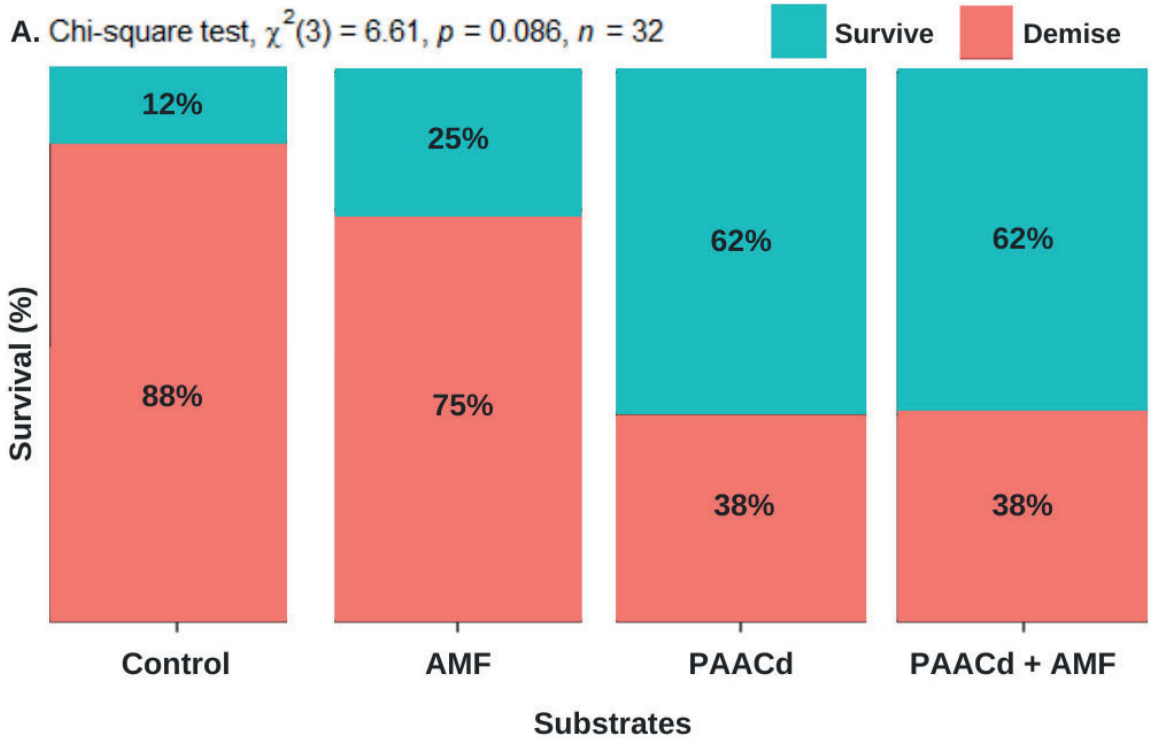

Figure 3. Effects of Pseudomonas ACC desaminase (PAACd), mycorrhizal fungi (AMF), and mix of Pseudomonas ACC desaminase (PAACd), mycorrhizal fungi (AMF) and control.

regulators suitable for different cocoa genotypes, such as *CCN51*.

Plantlet development

In the maturation of the embryo and the conversion to plantlet, the MF medium had the best results in the number of leaves and length of stem and root. The results obtained with the MF medium were like the results with the ENR8 medium. The ENR8 medium does not have the growth regulators NAA-GA_3; instead amino acids. The continuation of embryo development and conversion in the MF medium result from the effect of the NAA and GA3, activated carbon and other culture medium components, such as high concentrations of macroelements, especially nitrogen as NH_4NO_3 and high carbon concentrations like glucose. NAA and GA_3 have also been used by Iracheta *et al.* (2019)[50] to facilitate cotyledonary embryo development plus 3.7 µM of abscisic acid. Various authors have developed somatic

Substrate	Leaf area (cm^2)		
	CNCh12	*CNCh13*	*CCN51*
PAACd + Ta	20,41 a	7,45 ab	37,65 a
Ta	10,90 ab	9,45 ab	9,88 b
PAACd	10,88 ab	14,64 a	14,70 ab
Control	6,16 b	4,87 b	14,92 ab

Tukey post hoc test. The values followed by the same letter within a column indicate that they are not significantly different ($p < 0.05$).

Table 5. Effects of Pseudomonas ACC deaminase (PAACd) and Trichoderma asperellum (Ta) on leaf area of regenerated plantlets for CNCh12, CNCh13, and CCN51 genotype.

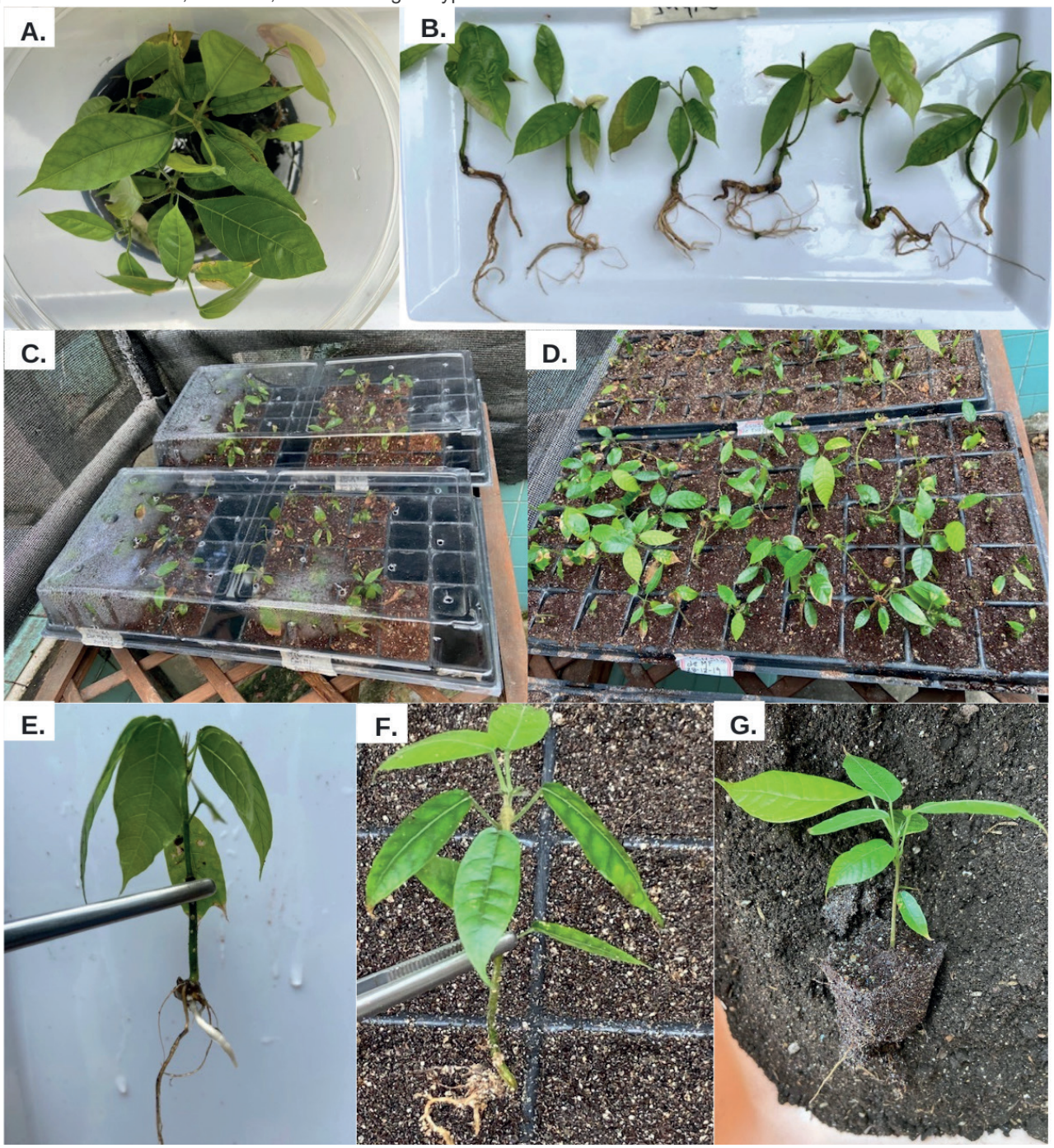

Figure 4. In vitro plantlets of CCN51 in (A) EM2-AMF transition media during maturation stage; (B) Conversion to plantlet in MF culture media; (C) Transparent dome used for the first day of ex vitro adaptation process and holes for 15 days; (D) Experiment of beneficial microorganism Pseudomonas ACC desaminase (PAACd), mycorrhizal fungi (AMF) and Trichoderma asperellum (Ta) on survival and leaf area; Plantlets with pivotal and fibrous roots of (E) CNCh12 and (F) CNCh13; Cocoa seedling with adequate state of development and vigor with at least one month of ex vitro adaptation process ready to be transplanted from the bag (40cm h x 15 cm d).

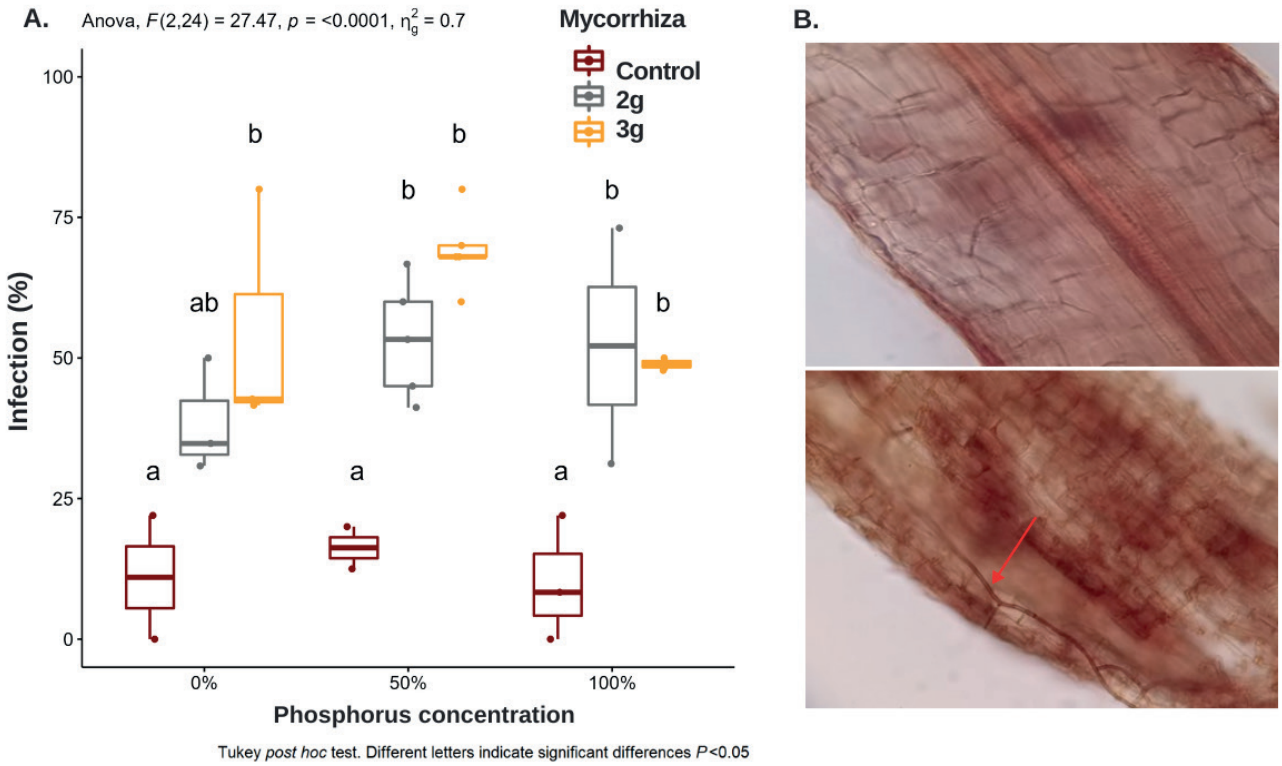

Figure 5. Effects of AMF (0g, 2g y 3g) and different concentrations of phosphorus (0%, 50% and 100%) in the Hoagland nutrient solution (1:10) on the percentage of infection. B. Above: the root of control plant without structures of mycorrhizal fungi. Under: plant root treated with 2g of AMF and Hoagland's solution with 50% P with hyphae traversing the root.

embryos in the culture medium with MS salts supplemented with NAA and GA_3[51]. In addition, GA_3 has also been used to elongate and produce embryos and plantlets in other species[52]. Furthermore, activated carbon can significantly affect embryo development because it can absorb substances, such as toxic metabolites and phenolic exudates, which inhibit the process, preventing correct maturation[53].

The biggest bottleneck in the embryogenic process is presented in the maturation and germination stage. Genotype plays a dominant role in somatic embryogenesis response and in the conversion rate of embryos 54. The embryo-to-plant conversion rate reached 20 to 40% with a very strong genotype dependency and high batch-to-batch variability[10,44,55].

Beneficial microorganisms

The inoculation with beneficial microorganisms improves the characteristics of the SE cocoa plants in the evaluated genotypes. With mycorrhizal fungi, better physiological characteristics were observed in the roots of the plantlets of the *CCN51*, *CNCh12* and *CNCh13* genotypes. Respect mycorrhizal fungi; they are known as a biostimulant and are anchored on their potential to increase plant nutrient uptake, improve plant resilience to drought, and reduce pesticides and inorganic fertilizers[33]. For abiotic stresses, the mechanisms of adaptation of mycorrhizal fungi to these stresses are generally linked to increased hydromineral nutrition, ion selectivity, gene regulation, production of osmolytes, an extension of the root absorbing area, and the synthesis of phytohormones and antioxidants. These benefits are influenced by its ability to colonize its host plant, a phenomenon that depends on the fungal genotype, the soil characteristics and the plant genotype[30].

For the *CCN51* genotype, using mycorrhizal fungi allows a better characteristic in root development was observed. This has been evidenced in other plants but directly in seeds with four selected microorganisms (*Pseudomonas chlororaphis* MA342, *P. fluorescens* CHA0, *Clonostachys pityriasis* IK726d11 and *Trichoderma harzianum* T22), generating a decrease in seedling mortality and stimulation of establishment[56]. Directly in cocoa plants at the greenhouse level, it was shown that inoculation with a mixture of microorganisms such as *Trichoderma sp.*, *Candida utilis* and *Pseudomonas putida* had better behavior in these plants compared to the control[57]. Also, in cocoa with *Trichoderma spp.* was possible to obtain a beneficial association, and the association could be a first step in developing of biocontrol strategies against diseases[58]. In another study, colonization of cocoa seedlings by the *Trichoderma hamatum* isolate DIS 219b was shown to enhance seedling growth, alter gene expression, and delay the onset of cocoa drought response in leaves at the molecular physiological and phenotypic level[58]. With the *Pseudomonas* ACC deaminase promoting plant growth from seed has been observed, specifically in the production of tomato roots[59]. Likewise, in cocoa, *Pseudomonas* ACC deaminase significantly affected on plant height, number of leaves, stem diameter, wet weight and dry weight of roots, number of roots, and root volume[60]. In the present study, different responses with each genotype were obtained; however, an improvement in survival was observed in the acclimation process for *CNCh12* y *CNCh13*, as well as in the *CCN51* genotype.

Furthermore, an improvement in plant height, number of leaves, and root length were observed. The results obtained for *CCN51* agree with other reports[61–63]. Chavez-Jalk *et al.* (2022)[62] used *T. harzianum* with a 100% colonization of the root hairs and trichomes on stems, and (65) used strains of Trichoderma and arbuscular mycorrhizal fungi ob-

taining a higher yield of cocoa beans. This could be because Trichoderma has different auxin production mechanisms that, when entering into symbiosis with the root, improve the agronomic characteristics of the cocoa plant in such a way that when developing a greater amount of root, this has greater ease of absorption of the nutrients available in the soil; in addition, Trichoderma together with the microorganisms creates associations that help increase the rhizosphere of the soil, degrading the organic matter in less time and allowing the plants to extract the nutrients with a greater degree of assimilation[66,67]. On the other hand, the positive effects on plantlet development occur through the reduction of ethylene, a hormone that negatively affects root growth under biotic and abiotic stress[68,69].

Phosphate is the second essential nutrient required by plants, and its bioavailability is associated with increased plant growth[70]. Only a low percentage of this amount of P (15–30%) can be taken by plants, while the remaining part is converted into insoluble complexes[71]. Therefore, increasing P-use efficiency is a major challenge in intensive agricultural production systems. In this regard, using rock phosphate as substrates in P solubilization by microorganisms is a promising strategy[72]. This strategy aims to reduce P adsorption and precipitation by promoting P sources with a low solubility instead of soluble P sources[73]. The result showed that the survival of plantlets was more significant in the treatments with 2g and 3g of mycorrhiza and 50% phosphorus. Therefore, for the *CCN51* genotype, AMF can make an alternative to reduce phosphorus loss by improving P-use efficiency, plant health, and growth[74].

The concentration of P in the basal substrate was 0.2651 mg/L, and the concentration of P in the Hoagland solution negatively affected the plantlets' survival (Data not shown). However, when the concentration of P in the Hoagland solution was modified to half of KH_2PO_4 and the substrate was supplemented with mycorrhizae, the effect on plant growth and survival was positive. As described by Osorio (2012)[75], for correct AMF colonization in plant roots, the substrate must have a phosphorus (P) concentration of 0.02 mg/L, and low concentrations of 0.001-0.005 mg/L do not allow a response to the inoculation of AMF, while very high concentrations above 0.2 mg/L inhibit the effectiveness of the fungus. According to Quiñones *et al.* (2012)[76] , the mechanism of the dose-effect on mycorrhizae is not precise. Still, it seems to depend on the interaction of the fungus and the plant species since different species of mycorrhizae are tolerant to high concentrations. The results of this study show that cocoa seedlings obtained by somatic embryogenesis inoculated with mycorrhizae were favored in terms of survival by phosphorus concentrations higher than 0.02 mg/L in the substrate, even requiring an additional supply of phosphorus in irrigation.

Based on the results obtained, it is proposed one possibility for the future is to carry out tests where plant defenses are reinforced by inoculating competent plant growth-promoting microorganisms from the *in vitro* phase to the *ex vitro* phase through a process called digitization[77]. These could act as biostimulants or biocontrol agents and help deal with biotic and abiotic stressors[78–80]. Finally, work on rhizosphere bacteria and fungi has already shown potential in managing various agricultural problems. Their use in the form of biofertilizers and biopesticides has resulted in a lesser reliance on synthetic agrochemicals[81].

Conclusions

In *ex vitro* adaptation processes of cocoa plantlets obtained by SE, the adequate physiological development of plantlets is essential in embryo maturation and conversion to plantlet. In the present work, it was possible to address critical factors affecting the growth of cocoa plantlets from ES *in vitro* to *ex vitro* transition. It was possible to establish efficient culture media for embryo maturation, plantlet conversion and development, as well as some parameters that affect development in *ex vitro* conditions. The culture of the embryos in the cotyledonary state in the EM2 and MF media, and with subsequent subculture in the MF medium, allows plants with prominent root development, stem elongation and leaf formation to continue to the greenhouse adaptation phase. The use of mycorrhizae in the *ex vitro* adaptation stage significantly improved the survival percentages, thus advancing the production process. It was found that, although the concentrations in the substrate were greater than 0.2 mg/L, a concentration reported as high and that can affect mycorrhizal infection, in the case of cocoa plantlets and the mycorrhizae used, there was no affectation in the infection for the mycorrhizae.

For this reason, it is recommended that for each genotype, it should be established how much phosphorus in the substrate and the irrigation solution affect mycorrhizal infection and plant growth. These results will help optimize beneficial microorganisms' use in SE plantlets production and the subsequent physiological performance of plants under nursery and open field conditions. Also, it is a significant starting point to carry out in other cacao genotypes, achieving increasing survival and contributing to the establishment of complete production protocols via ES.

Supplementary Materials

The following are available online at www.revistabionatura.com/xxx/s1, Figure 1 S1. Association of culture media transition EM2-AMF and EM2-MM6 (Secondary embryogenesis-maturation) and the fibrous root and tap root percentage in *CCN51* genotype plantlets, Table 1 S1 Effect of BS (Basal substrate) supplemented with MZ and OM on plant survival of *CCN51* genotype, Figure 2 S1. Effects of mycorrhizae (MZ) and organic matter (MO) increased in the percentage of BS substrate on white roots in *CCN51* genotype. Green: Survival, Pink: Demise. Figure 3 S1. Effects of Pseudomonas ACC deaminase (PAACd) and *Trichoderma asperellum* (Ta) supplemented in the substrate on survival (%). A. Effects on CNCh12 genotype. B. Effects CNCh13 genotype. C. Effects CCN51 genotype. Figure 4 S1. Figure 4. Standard calibration curve of phosphorus (P) measurement in the basal substrate (BS). Figure 5 S1. Figure 5. Effects of AMF (0g, 2g y 3g) and different concentrations of phosphorus (0%, 50% and 100%) in the Hoagland nutrient solution (1:10) on the percentage of survival.

Author Contributions

Conceptualization, Ana María Henao Ramírez and Aura Inés Urrea Trujillo; methodology and software, Julian David Morales Muñoz, Ruth Tatiana Hernández Hernández; validation and formal analysis, Juliam David Morales Muñoz, 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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