Maturation and germination of somatic embryos of guava var. Cuban Red Dwarf EEA-1840

Maduración y germinación de embriones somáticos de guayabo var. Enana Roja Cubana EEA-1840

Maturação e germinação de embriões somáticos de goiaba var. Anã Vermelha Cubana EEE-1840

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Abstract

Somatic embryogenesis is an alternative for the accelerated propagation of promising guava (*Psidium guajava* L.) materials of agronomic interest. However, low maturation and germination rates of somatic embryos are some of the aspects that limit its application in breeding programs for different purposes. In this sense, the effect of three concentrations (0, 1 and 1.5 $mg.L^{-1}$) of abscisic acid (ABA) on embryo maturation and two concentrations of sucrose (3 and 5 %) and Murashige and Skoog (MS) macronutrients (50 and 100 %) on the germination of somatic embryos of guava var. Cuban Red Dwarf EEA-1840 were studied. After six weeks of culture, ABA had a negative effect on somatic embryo maturation in culture media supplemented with 1 or 1.5 $mg.L^{-1}$, whereas 3.95 to 5.49 times more mature embryos were observed in the absence of ABA. Germination of somatic embryos was significantly improved when the concentration of macronutrient in the culture medium was reduced independently of the sucrose concentration [MS 50 % + 3 % of sucrose (73,3 %) and MS 50 % + 5 % de sucrose (55,0 %)]. It is concluded that the simplification of the culture media with reduction of the macroelements MS and sucrose to standard concentration favors the germination of mature embryos of guava var. Cuban Red Dwarf EEA-1840.

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Resumen

La embriogénesis somática es una alternativa para la propagación acelerada de materiales promisorios de guayaba (*Psidium guajava* L.) de interés agronómico. Sin embargo, las bajas tasas de maduración y germinación de los embriones somáticos son algunos de los aspectos que limitan su aplicación en programas de mejoramiento genético con diferentes propósitos. En este sentido, se estudió el efecto de tres concentraciones (0, 1 y 1.5 $mg.L^{-1}$) de ácido abscísico (ABA) sobre la maduración embrionaria y de dos concentraciones de sacarosa (3 y 5 %) y macronutrientes Murashige y Skoog (MS) (50 y 100 %) sobre la germinación de embriones somáticos de guayaba var. Enana Roja Cubana EEA-1840. Después de seis semanas de cultivo, el ABA tuvo un efecto negativo sobre la maduración de los embriones somáticos en medios de cultivo suplementados con 1 o 1.5 $mg.L^{-1}$, mientras que se observaron de 3.95 a 5.49 veces más embriones maduros en ausencia de ABA. La germinación de embriones somáticos mejoró significativamente cuando se redujo la concentración de macronutrientes en el medio de cultivo, independientemente de la concentración de sacarosa [MS 50 % + 3 % sacarosa (73,3 %) y MS 50 % + 5 % sacarosa (55,0 %)]. Se concluye que la simplificación del medio de cultivo con reducción de los macroelementos MS y sacarosa a la concentración estándar favorece la germinación de embriones maduros de guayaba var. Enana Roja Cubana EEA-1840.

Palabras clave: ácido abscísico, embriogénesis somática, sacarosa, *Psidium guajava* L.

Resumo

A embriogênese somática é uma alternativa para a propagação acelerada de materiais promissores de goiabeira (*Psidium guajava* L.) de interesse agronômico. No entanto, as baixas taxas de maturação e germinação dos embriões somáticos são alguns dos aspectos que limitam sua aplicação em programas de melhoramento para diferentes finalidades. Neste sentido, estudou-se o efeito de três concentrações (0, 1 e 1,5 $mg.L^{-1}$) de ácido abscísico (ABA) na maturação embrionária e de duas concentrações de sacarose (3 e 5 %) e dos macronutrientes Murashige e Skoog (MS) (50 e 100 %) na germinação de embriões somáticos de goiabeira var. Anã Vermelha Cubana EEE-1840. Após seis semanas de cultura, o ABA teve um efeito negativo na maturação de embriões somáticos em meios de cultura suplementados com 1 ou 1,5 $mg.L^{-1}$, enquanto foram observados embriões 3,95 a 5,49 vezes mais maduros na ausência de ABA. A germinação de embriões somáticos foi significativamente melhorada quando a concentração de macronutrientes no meio de cultura foi reduzida, independentemente da concentração de sacarose [MS 50 % + 3 % de sacarose (73,3 %) e MS 50 % + 5 % de sacarose (55,0 %)]. Conclui-se que a simplificação do meio de cultura com redução dos macroelementos MS e sacarose para a concentração padrão favorece a germinação de embriões maduros de goiabeira var. Anã Vermelha Cubana EEE-1840.

Palavras-chave: ácido abscísico, embriogénese somática, sacarose, *Psidium guajava* L.

Introduction

The introduction of new crop varieties requires the accelerated multiplication of these genotypes, and *in vitro* culture techniques are one of the alternatives to achieve this goal. Guava (*Psidium guajava* L.) is considered the most prized fruit of the *Psidium* genus and one of the most valuable in the tropics, being the Cuban Red Dwarf EEA-1840 an elite variety for its notable stability (González *et al*., 2023), characterized by being a material of low growth, very branched and that can reach yields above 100 $t.ha^{-1}$ of fresh fruit (Ramos *et al*., 2023).

Somatic embryogenesis is a promising method for vegetative propagation of a large number of Myrtaceae species, due to the bipolar nature of the embryo and the ease with which the entire production process can be automated. In addition, the process allows to get high multiplication coefficients in short time periods based on the principles of microbial kinetics, added to the possibility of encapsulating the embryos and obtaining artificial seeds (Guan *et al*., 2016). This biotechnological technique is an alternative for the accelerated field introduction of new varieties and cultivars.

The ability of totipotent cells to properly proliferate and mature is greatly influenced by physicochemical aspects of *in vitro* culture, including the balance of plant growth regulators (Gao *et al*., 2021) and mean osmotic pressure (do Nascimento *et al*., 2021).

Within the process of somatic embryogenesis, the final steps are germination and conversion into plants. The first signs of germination of somatic embryos are elongation of the hypocotyl, appearance of green color in the cotyledons and elongation of the radicle (Alemano *et al*., 1997; Canhoto *et al*., 1999). Conversion into plants is defined by some authors as the development of the shoot with emission of the first pair of true leaves (Alemano *et al*., 1997); while others define it as the survival of the propagule under *ex vitro* environmental conditions (Stuart and Strickland, 1984).

Although most reports on somatic embryogenesis of woody species do not give much information on these processes, it is known that low rates of plant regeneration are caused by problems in the germination and conversion of somatic embryos (Rezende *et al*., 2011).

In some cases, it has been indicated the need to give several recultures in the germination medium, to improve the germination of somatic embryos due to the developmental asynchrony of these structures (Cruz *et al*., 1990). Gómez (1998), points out that partial or total drying of the somatic embryo increases its subsequent germination and growth, normalizing and synchronizing germination; besides, it facilitates the simultaneous growth of roots and shoot.

Somatic embryos of Myrtaceae species have been reported to germinate well on simple media, usually at half salts and without hormones (Rai *et al*., 2008) and the synchronization in germination is improved when somatic embryos are recultured on medium with a sucrose content of 2 % (Canhoto *et al*., 1999). Sucrose is the most common source of carbohydrate in culture medium (Kaur *et al*., 2022).

On the other hand, gibberellic acid as well as coconut water, enhanced germination of somatic embryos in *Myrciaria cauliflora* (Litz, 1984a), Eugenia sp. (Litz, 1984b) and *Myrtus communis* (Canhoto *et al*., 1999). Somatic embryos do not enter dormancy, but treatment of somatic embryos with exogenous ABA can induce a dormancy state similar to that of zygotic embryos. (Rai *et al*., 2008). ABA has been recognized as a factor in promoting normal development and maturation of somatic embryos (Cipriano *et al*., 2018).

The objective of this research was to evaluate the effect of ABA on embryo maturation, and sucrose and mineral salts on germination of somatic embryos of guava (*Psidium guajava* L.). var. Cuban Red Dwarf EEA-1840.

Materials and methods

This research was carried out in the facilities of the Biotechnology Laboratory "Profa. Silvia León de Sierralta" of the Faculty of Agronomy of the University of Zulia.

Plant material

For the somatic embryo maturation assay, embryogenic callus of guava variety "Cuban Red Dwarf EEA-1840" with embryos at the globular stage was used as plant material (figure 1a). For the somatic embryo germination assay, mature somatic embryos at the cotyledonary stage were used (figure 1b). Previously, the induction of somatic embryogenesis and embryo multiplication were carried out following the protocol described by Vilchez-Perozo *et al*. (2002).

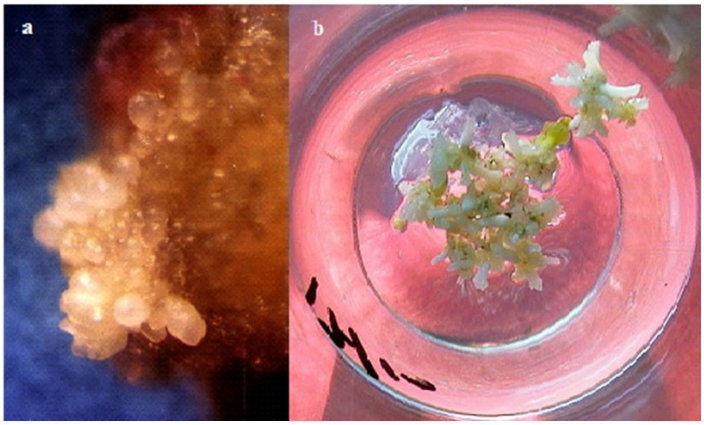

Figure 1. Plant material used in maturation and germination of somatic embryos of guava var. "Cuban Red Dwarf EEA-1840". (a) Embryogenic callus with embryos at the globular stage. (b) Mature somatic embryos at the cotyledonary stage (marked with an arrow).

Effect of abscisic acid concentration on the maturation of guava somatic embryo

To evaluate the effect of ABA on the maturation of guava somatic embryos, three concentrations were evaluated: 0, 1 and 1.5 $mg.L^{-1}$. The culture medium used was MS (Murashige and Skoog, 1962) with half the macronutrients, 3 % sucrose and pH was adjusted to 5.8 with NaCl or 0.5 M HCl. The culture media were gelled with 4 $g.L^{-1}$ of Agargel (Sigma-Aldrich®). A randomized experimental design was used with ten replicates per treatment. Each replicate consisted of a 120 mL glass bottle with 25 mL of culture medium and 25 mg of embryogenic callus with approximately fifty globular-stage embryos.

After six weeks of culture, the number of white-opaque somatic embryos at torpedo-elongate and cotyledonary stages was evaluated. The use of the white-opaque appearance of somatic embryos as a criterion of somatic embryo maturation has been indicated in several species (Corredoira *et al*., 2003); (Perán-Quesada *et al*., 2004).

Effect of sucrose concentration and MS macronutrients on the germination of guava somatic embryos

To evaluate the effect of sucrose and MS macronutrients concentration on the germination of guava somatic embryos, a factorial experiment 2 x 2 was established, testing two concentrations of sucrose (3 and 5 %) and two concentrations of MS macronutrients (50 and 100 %), table 1. In addition, culture media were supplemented with 0.25 $mg.L^{-1}$ N^6-benzylaminopurine (6-BAP) and 0.01 $mg.L^{-1}$ DI-31 (a brassinosteroid analog $C_{27}H_{42}O_5$, equivalent to Biobras 16), following the protocol of Gómez *et al*. (2005). All culture media were gelled with 4 $g.L^{-1}$ Agargel (Sigma-Aldrich®) and pH was adjusted to 5.8 with NaCl or 0.5 M HCl.

Table 1. Treatments resulting from the combination of the study factors sucrose concentration and macronutrients of the culture medium MS (Murashige Skoog, 1962), on the germination of somatic embryos of guava var. Cuban Red Dwarf EEA-1840.

Treatments	Sucrose (%)	MS macronutrients (%)
1	3	50
2	5	50
3	3	100
4	5	100

Twelve replicates were used per treatment. Each replicate consisted of a 120 mL capacity glass bottle with 25 mL of culture medium, described above, in which five mature embryos at the cotyledonary stage were cultured. The torpedo-cotyledonary and cotyledonary stages have been reported as the most suitable ones for initiating the germination phase (Rai *et al*., 2008; Oliveira *et al*., 2022).

After eight weeks of culture, the germination percentage was evaluated per replicate and calculated as follows: total germinated embryos divided by total embryos seeded and this ratio multiplied by one hundred. Somatic embryos were considered to be germinated when they changed from white-opaque to green colour and presented development of the caulinar axis, cotyledonary leaves and root axis (Gómez *et al*., 2005).

Culture conditions

The embryo maturation and germination phases were carried out under continuous white fluorescent light with an irradiance level of 200 $\mu mol^{-1}.m^{-2}.s^{-1}$, temperature of 26 ± 1°C and average relative humidity of 46 %.

Statistical analysis

Data processing was carried out using Statistix® version 8.0 analytical software (Analytical Software, Tallahassee, Florida, USA, 2003). Prior to statistical analysis, data obtained in each experiment were subjected to check the assumption of a normal distribution to the Shapiro-Wilk test a significance level of $P \leq 0.05$ (Sokal and Rohlf, 2013). Subsequently, analysis of variance (ANOVA) was executed in order to determine the effect of the study factors with a significance level of $P \leq 0.05$. In those cases where the effect of the study factor and/or its interaction was statistically significant ($P \leq 0.05$), mean comparison was performed using the Tukey's test.

Results and discussion

Effect of abscisic acid on the maturation of guava somatic embryos

The effect of different ABA concentrations on the maturation of somatic embryos of guava var. "Cuban Red Dwart EEA-1840" table 2 shows. ANOVA revealed the existence of significant differences ($P \leq 0.05$) among the ABA concentrations tested (0, 1 and 1.5 $mg.L^{-1}$). In general, higher somatic embryo production was observed at the elongated torpedo (27.4) and cotyledonary (12.1) stages on ABA-free maturation medium. In *Olea europea*, maturation of somatic embryos on growth regulator-free medium has been reported (Mazri *et al*., 2020). A significant decrease in somatic embryo production at

both developmental stages was observed as the ABA concentration increased. This behavior was probably due to the fact that evaluated concentrations of ABA could be high for this species, affecting the hormonal balance and triggering an inhibitory effect on the embryo maturation process. A series of investigations evidenced a similar behavior in the maturation of somatic embryos of *Juglans regia* (Vahdati *et al*., 2008) and *Coffea arabica* var. Colombia (Riviello-Cogco *et al*., 2021), where ABA concentrations above 2 mg.L^{-1} reduced the maturation and growth of somatic embryos.

Table 2. Effect of abscisic acid (ABA) on the maturation of guava somatic embryos var. Cuban Red Dwarf EEA-1840.

Concentration of ABA (mg.L^{-1})	Number of embryos per culture	
	Torpedo-elongated	Cotyledonary
0	27.4 a	12.1 a
1	8.1 b	1.9 b
1.5	5.5 b	1.7 b
CV: 19.6	SE:1.95	SE: 1.5

Different letters indicate significant differences obtained by Tukey's mean comparison test (P≤0.05).

Additionally, in all treatments the characteristic asynchrony of the culture was observed, with a predominance of somatic embryos at the globular-heart stage, which were impossible to quantify. The differentiation of somatic embryos into white-opaque torpedo-cotyledonary and cotyledonary stages was observed, being an indication of the accumulation of reserve substances, necessary for their subsequent germination (Vahdati *et al*., 2008), which was especially evident in the ABA-free culture medium. Maturation is a critical stage between embryo development and germination and, for the achievement of maximum conversion of somatic embryos to seedlings, it is necessary to use well matured embryos (Perán-Quesada *et al*., 2004).

Effect of sucrose and MS macronutrients on germination of guava somatic embryos

After 12 days of culture, the first morphological changes in the germination process of somatic embryos were visualized; e.g., changes of cotyledon coloration and epicotyl development (figure 2a), which gradually continued until the eight week of evaluation.

Most embryos showed normal germination, although several types of anomalies could be observed, such as incomplete germination (figure 2b), which can be caused either by a deficient accumulation of reserve substances (LEA proteins) during the maturation of somatic embryos. Rong *et al.* (2023) determined in *Vitis vinifera* L. that the morphological differences in these phenotypically abnormal embryos were likely associated with endogenous phythormones levels, how indoleacetic acid and ABA. On the other hand, Perán-Quesada *et al*. (2004) indicate that a high concentration of sucrose in the medium or the continuous culture in relatively high level of osmotic pressure can be a source of abnormal germination in somatic embryos. This has also been previously reported in this guava variety (Gómez *et al*., 2005). Secondary embryogenesis was also eventually observed at the base of some embryos (figure 2c), with new embryo formation at different stages of development during the evaluation period.

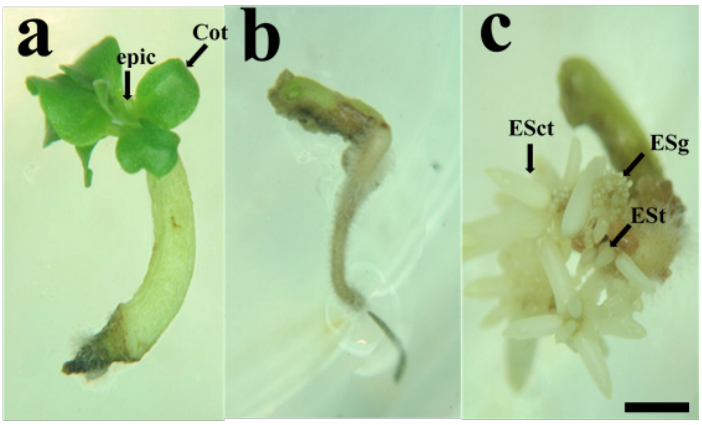

Figure 2. General aspects of germination of somatic embryos of guava var. "Cuban Red Dwarf EEA-1840". (a) Beginning of embryo germination, note the cotyledon (Cot) color change and the beginning of epicotyl (epic) development. (b) Abnormal somatic embryo germination. (c) Presence of secondary embryogenesis at the base of the germinating somatic embryos, with development of embryos at the globular (ESg), torpedo (ESt) and cotyledonary (ESct) stages. Black bar = 3 mm.

The results obtained revealed significant differences (P ≤ 0.05) among the treatments tested (figure 3), with higher germination rates achieved in culture medium with MS macronutrients at half strength and 3 % or 5 % sucrose.

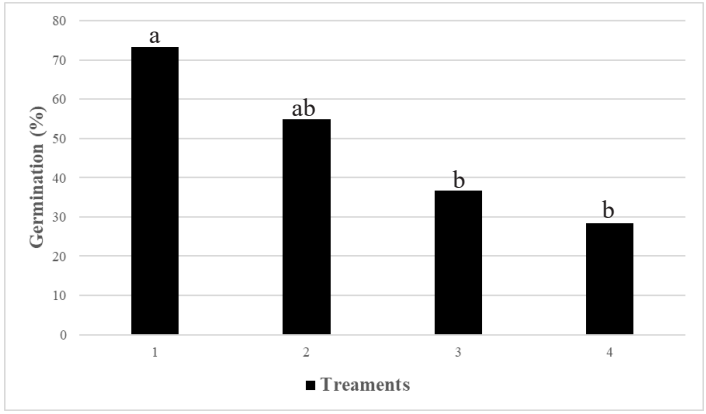

Figure 3. Effect of interaction the concentration of macronutrients in the MS medium and sucrose on the germination of somatic embryos of guava var. "Cuban Red Dwarf EEA-1840". 1: MS 50 % + 3 % sucrose; 2: MS 50 % + 5 % sucrose; 3: MS 100 % + 3 % sucrose; 4: MS 100 % + 5 % sucrose. Values with different letters differed statistically (P ≤ 0.05) for Tukey's mean comparison test. MS: Murashige and Skoog (1962) culture medium.

In general, higher germination percentages were reached with the lowest concentrations evaluated of MS macronutrients.

This could be explained by the fact that the concentration of mineral elements and sucrose are determining factors in the availability of water in the culture medium, as they increase its osmolarity (Kubeš *et al*., 2014; do Nascimento *et al*., 2021), decreasing water potential and thus hindering the free movement of water from the culture medium to the somatic embryos. In this regard, George *et al.* (2008)

points out that cells maintained in an environment with low osmotic potential lose water and decrease their water potential, altering their morphogenenic capacity. This could explain the lower germination values at 3 and 5 % of sucrose with 100 % of the MS macronutrients. In this sense, Shohael *et al*. (2013) point out that high concentrations of nutritional elements in the culture medium could have an adverse effect on the germination of somatic embryos, possibly due to their toxicity; although this effect is closely related to the absorption of elements and will also depend on the level of the nutritional element in the plant tissue.

Rai *et al*. (2007 and 2008) reported that the germination percentage of guava somatic embryos decreased as sucrose concentration increased above 3 %. This behavior has been reported to be caused by dormancy of somatic embryos, where the resumption of growth depends on a specific treatment or condition, in addition to the presence of water (Gray, 1987). On the other hand, Choi and Jeong (2002) also reported an inhibition of germination of somatic embryos in peanut (*Arachis hypogaea* L.) due to high sucrose concentrations in the culture medium and pointed out that the induction of dormancy in somatic embryos was related to the accumulation of ABA as sucrose concentration increases and with it a high osmotic stress.

Conclusions

ABA concentrations tested have negative effects on the maturation of somatic embryos of guava var. Cuban Red Draft EEA-1840. Seventy-three per cent germination was obtained in culture media with MS macronutrients at half strength and 3 % sucrose. The germination percentage tends to decrease as the concentration of macronutrients in MS medium increases.

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