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Effect of the combination of NAA, kinetin and sucrose on the induction of somatic embryogenesis in lulo (*Solanum quitoense* Lam.)

Efecto de la combinación de ANA, kinetina y sacarosa sobre la inducción de embriogénesis somática de lulo (*Solanum quitoense* Lam.)

Hernando Criollo¹, Margarita Perea², Mariano Toribio³, and Johanna Muñoz⁴

ABSTRACT

Lulo is a species of great importance to the fruticulture of Colombia, but has significant phytosanitary problems that require an aggressive breeding program oriented toward the production of genotypes with tolerance to phytopathogens. These programs need to establish highly efficient mass plant propagation protocols, such as somatic embryogenesis. This study focused on research on the somatic embryogenesis of lulo using kinetin, naphthalene acetic acid-NAA (Plant Growth Regulators, PGRs), and different sucrose concentrations in a MS medium. Two lulo varieties, *Solanum quitoense* var. *septentrionale* and *S. quitoense* var. *quitoense*, and two explant types (hypocotyl and cotyledon) were used, incubated in dark conditions at 25±2°C. The highest production percentage of the embryos was obtained when 50 µM of NAA were added to the medium with sucrose (50.0 and 263.1 µM) for the two explant types used. In lulo with spines, the highest percentage of embryonic structures (50%) was observed with cotyledonary leaf explants and 50 µM of NAA; while in the spineless lulo, the embryonic structures were observed in the same type of explant with 50 µM of NAA + 263.1 µM of sucrose (32%).

Key words: embryo culture, recalcitrance, plant growth regulators, tissue culture, plant propagation, fruit.

RESUMEN

El lulo es una especie de gran importancia en la fruticultura colombiana, con grandes problemas fitosanitarios que requieren un agresivo programa de mejoramiento orientado a la producción de variedades tolerantes. Estos programas necesitan establecer protocolos de propagación masiva de plantas de alta eficiencia como la embriogénesis somática. Nuestras investigaciones se orientaron al estudio de la embriogénesis somática de lulo, mediante la interacción de los fitorreguladores kinetina y ácido naftalenacético (ANA), y diferentes concentraciones de sacarosa en medio MS. Se utilizaron dos variedades de lulo (*Solanum quitoense* var. *septentrionale* y *S. quitoense* var. *quitoense*), dos tipos de explantes (hojas cotiledonares e hipocotilos), incubados en condiciones de oscuridad, a 25±2°C. El mayor porcentaje de producción de embriones se obtuvo cuando se adicionaron al medio 50 µM de ANA además de sacarosa (50,0 y 263,1 µM), para los dos tipos de explantes utilizados. En lulo con espinas, el mayor porcentaje de estructuras embrionarias (50%) se observó con hojas cotiledonares y 50 µM de NAA, mientras que en lulo sin espinas estos se observaron en el mismo tipo de explante con 50 µM NAA + 263,1 µM sacarosa (32%).

Palabras clave: cultivo de embriones, recalcitrancia, reguladoras del crecimiento vegetal, cultivo de tejidos, propagación de plantas, fruta.

Introduction

Lulo (*Solanum quitoense* Lam.) originates from the Andes and has great potential for the international markets. Cultivation in Colombia is carried out in marginal coffee regions, in small areas with low technology and with numerous phytosanitary problems. It is a species that is highly susceptible to diseases and pests that cause large losses for producers, which demonstrates the need to create breeding programs with tolerance characteristics, before starting an ambitious exportation program.

The plant breeding programs require efficient methods of plant propagation that allow the mass propagation of selected genotypes. Despite many difficulties, many authors, such as Chandel and Katiyar (2000), Rout *et al.* (2006), George and Debergh (2008), Oláh *et al.* (2009), and Parimalan *et al.* (2011) consider somatic embryogenesis to be the most efficient method, especially when it is necessary to regenerate plants using genetically modified cells, as expressed by Nakagawa *et al.* (2001), and Van Der Vyver (2010).

Rout *et al.* (2006) stressed that the regeneration of plants through somatic embryogenesis has big advantages, such

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as high multiplication rates that can be used in bioreactors, the possibility of encapsulating the embryos and producing artificial seed, cryoconservation, genetic fidelity in the offspring and *in vitro* selection of tolerant plants. Similarly, Campbell *et al.* (2003) considered these processes as valuable complements to the regeneration of genetically transformed plants. However, the principal limitation is related to the genotypic dependence of the somatic embryonic production and its low germination power (Wang *et al.*, 2006), considering that the low germination power is not in all cases a generalized limitation of somatic embryogenesis. Another significant limitation in addition to the genotypic dependence is that only a few species have protocols that are sufficiently refined for mass somatic embryonic multiplication.

The use of somatic embryogenesis and the regeneration of plants have been studied for several cultivated species, with differing results. For example, the research of Wang *et al.* (2006) on the regeneration of recalcitrant cotton genotypes, of Nakagawa *et al.* (2001) and Ren *et al.* (2012) on melon using cotyledonal tissue, and on citrus, Cardoso *et al.* (2012) and Nakano *et al.* (2013), and the cacao, Tan and Furtak (2003) and Quainoo *et al.* (2008); there have been several studies on the somatic embryogenesis of cultivated plants, such as, strawberry (Zhang *et al.*, 2014), onion (Ramakrishnan *et al.*, 2013), carrot (Li *et al.*, 2013), sweet potato (Santa-Maria *et al.*, 2009), chickpea (Mishra *et al.*, 2012), broccoli and cabbage (Qin *et al.*, 2007; Yang *et al.*, 2010; Na *et al.*, 2011), and groundnut (Singh and Hazra, 2009).

In the Solanaceae family, despite being considered a recalcitrant species for somatic embryogenesis, successful protocols have been produced by Kaparakis and Alderson (2002), Kantharajah and Golegaonkar (2004), Mir *et al.* (2008), and Ribeiro *et al.* (2009) for *Solanum melongena*; JayaSree *et al.* (2001), Sánchez-Enciso *et al.* (2005), Vargas *et al.* (2005), and Motallebi-Azar *et al.* (2013) for *Solanum tuberosum*; Kintzios *et al.* (2000), Bodhipadma and Leung (2002), Bárány *et al.* (2005), Koleva-Gudeva *et al.* (2007), Kaparakis and Alderson (2008), and Solís-Ramos *et al.* (2010) for *Capsicum annuum*; Chandel and Katiyar (2000) and Vinoth *et al.* (2014) for *Solanum lycopersicum*; Correia and Canhoto (2009) and Arahana *et al.* (2010) for *Cyphomandra betacea*; Dhavala *et al.* (2009) for *Solanum trilobatum*; Zapata-Castillo *et al.* (2007) for *Capsicum chinense*. However, there have been no reports for somatic embryogenesis in lulo (*S. quitoense*) and the attempts carried out by Segovia (2002) using a cultivation medium appropriate for *C. betacea* failed.

The present study aimed to evaluate the effect of different hormone combinations, explants and incubation conditions on the induction of somatic embryogenesis of two botanical varieties of lulo, *S. quitoense* var. *septentrionale* (with spines) and *S. quitoense* var. *quitoense* (without spines).

Materials and methods

This study was carried out in the Plant Tissue Laboratory of the Biology Program, Faculty of Science, Universidad Nacional de Colombia, Bogota and in the Plant Tissue Laboratory of the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA-Spain).

The donor plants were obtained by germinating the seeds extracted of fruits of one lulo plant with spines and one lulo plant without spines, sterilized using soap, sterile water and 15 min in 1.5% sodium hypochlorite; followed by five washings with sterile water and transfer to a MS culture medium (Murashige and Skoog, 1962) in petri dishes and germination trays.

After 30 d, hypocotyl and cotyledonary leaves were taken as explants from the lulo with spines and the lulo without spines and planted in a MS medium supplemented with different combinations of PGRs of NAA (19.8, 31.9 and 50.0 μM) as factor A, kinetin (0 and 13.9 μM) as factor B and different sucrose concentrations (14.6, 58.4 and 263.1 μM) as factor C. The explants were kept in dark conditions at $25\pm 2^\circ\text{C}$. At 30 d, the plants were evaluated and transferred to a MS medium without PGRs, with a 16/8 h photoperiod.

Each botanical variety and each of the experiments were subjected to an unrestrictive random experimental design with a factorial arrangement, with 10 repetitions with four explants each.

A descriptive analysis was carried out on the characteristics of the produced calluses and the presence of roots and calluses was determined for each treatment. The average size of the formed calluses was determined by quantifying each callus using a scale of 1 to 5, determining the average for each experimental unit and transforming them to a percentage with 5 being 100% and 1 the absence of calluses; the embryos and embryo like structures formed in each experimental unit were counted. The experimental data were subjected to ANOVA and a Tukey mean comparison test for simple effects and interactions with $P\leq 0.05$.

Results

Effect of the concentrations of NAA, kinetin and sucrose on the growth of the explants of lulo with spines

The use of cotyledonary leaves from lulos with spines determined the interactions: NAA*kinetin and NAA*sucrose, in the formation of roots, and NAA*kinetin and Kinetin*sucrose, in the formation of calluses. The NAA*kinetin interaction was observed in the somatic embryo induction. As simple effects, the three factors significantly influenced the formation of the roots; with kinetin in the formation of the calluses; and NAA and kinetin in the induction of embryos and embryo like structures after 30 d of incubation.

Root induction progressively decreased when the NAA concentration increased from 19.8 and 50.0 μM (Fig. 1A). Kinetin exerted a strong influence in the low NAA doses (19.8 μM), with statistical differences between the highest percentage of rooted explants without the addition of kinetin and that observed with kinetin (13.9 μM); with 31.9 μM of NAA, there was also a significant reduction in the percentage of rooted explants with kinetin applications; however, with the highest NAA dose (50.0 μM), the effect of the kinetin application was not significant.

Sucrose concentration behaved differently; with a low concentration of NAA, the largest rooting was observed with 58.4 μM sucrose, while with the average dose of NAA, the highest rooting was obtained with 58.4 and 263.1 μM sucrose; with the highest concentration of ANA, the effect of the sugar concentration was not significant (Fig. 1B).

With the low and high NAA concentrations, kinetin did not present a significant effect on the percentage of

cotyledonary leaves with formed calluses but, in the medium dose (31.9 μM), the explants that grew in the absence of kinetin presented a higher induction of calluses than those obtained in the medium with kinetin (Fig. 2A). In the kinetin*sucrose interaction, the influence of the sucrose concentration was not significant in the medium without kinetin, while, in the medium with kinetin, the medium sucrose concentration (58.4 μM) induced a higher production of explants with calluses (Fig. 2B).

The addition of kinetin to the cultivation medium reduced the induction of somatic embryos when it interacted with NAA; with 19.8 μM of NAA, with or without kinetin, embryos did not form, while, with 31.9 and 50.0 μM of NAA, embryos were observed in 50 and 56% of the explants, respectively, with significant differences with respect to these NAA treatments supplemented with 13.9 μM of kinetin (Fig. 3).

The presence of embryonic structures was characterized by the initial formation of white callus zones in the basal regions of the cotyledonary leaves, calluses of gelatinous textures and granular appearance; the aberrant embryo always started with an enlarged base structure making up the caulinar apex and giving rise to the cotyledonary leaves with a radical system immersed in the callus (Fig. 4).

When the explants were hypocotyls, interactions were observed in the formation of roots between NAA and kinetin, NAA and sucrose and kinetin*sucrose; during the development of embryonic structures, interactions were seen between NAA with kinetin and sucrose and between kinetin and sucrose. The three factors influenced the formation of roots, the sucrose induced calluses and NAA and sucrose induced embryos.

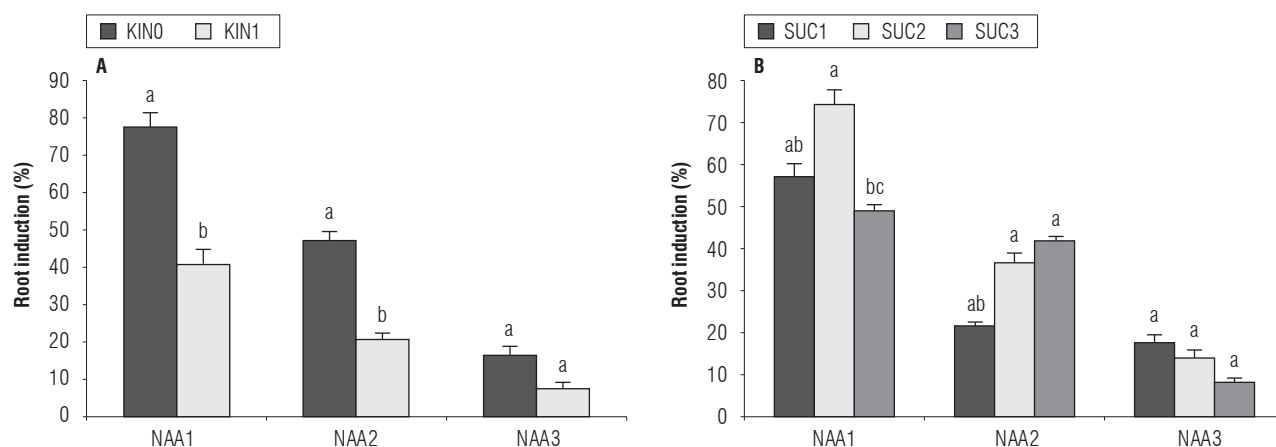


FIGURE 1. Effect of different concentrations of NAA, Kinetin (KIN) and sucrose (SUC) on the percentage of root induction in cotyledonary leaves. A, NAA*kinetin interaction; B, NAA*sucrose interaction. NAA1, 19.8 μM ; NAA2, 31.9 μM ; NAA3, 50.0 μM ; KINO, 0 μM ; KIN1, 13.9 μM ; SUC1, 14.6 μM ; SUC2, 58.4 μM ; SUC3, 263.1 μM . Means with different letters indicate significant differences according to Tukey test ($P \leq 0.05$) ($n = 4$).

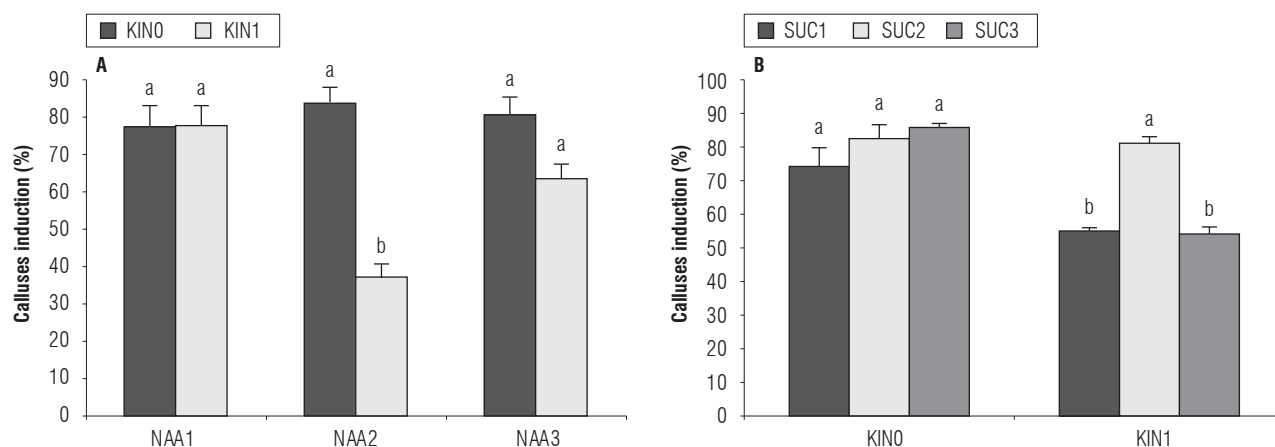


FIGURE 2. Effect of the different concentrations of NAA, kinetin (KIN) and sucrose (SUC) on the percentage of cotyledonary leaves with callus induction. A, NAA*kinetin interaction; B, kinetin*sucrose interaction. NAA1, 19.8 μM ; NAA2, 31.9 μM ; NAA3, 50.0 μM ; KINO, 0 μM ; KIN1, 13.9 μM ; SUC1, 14.6 μM ; SUC2, 58.4 μM ; SUC3, 263.1 μM . Means with different letters indicate significant differences according to Tukey test ($P \leq 0.05$) ($n = 4$).

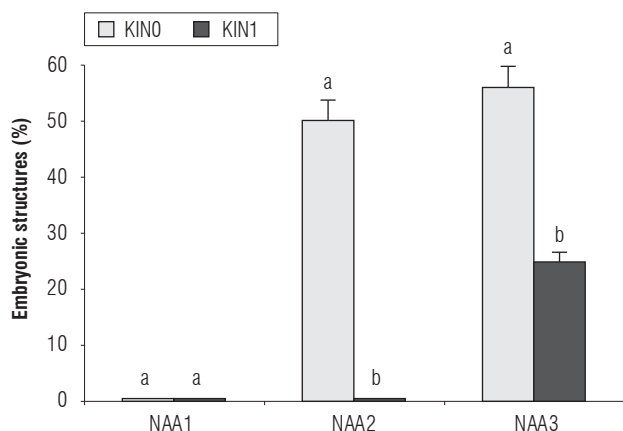


FIGURE 3. Effect of the addition of kinetin (KIN) on the action of the different concentrations of NAA (in a MS medium), in the emergence of embryonic structures on the explants of cotyledonary leaves of lulo with spines. NAA1, 19.8 μM ; NAA2, 31.9 μM ; NAA3, 50.0 μM ; KINO, 0 μM ; KIN1, 13.9 μM . Means with different letters indicate significant differences according to Tukey test ($P \leq 0.05$) ($n = 4$).

Roots did not present with the use of kinetin, especially with the medium (31.9 μM) and high NAA dose (50.0 μM), while a reduction in rooting was seen with sucrose (263.1 μM) and NAA (19.8 μM) and with sucrose (14.6 μM) and NAA (58.4 μM). The interaction between kinetin and sucrose demonstrated an inhibitory effect on the roots with high concentrations of sucrose and kinetin but with a minor effect when the regulator was not applied.

The formation of calluses in the hypocotyls with the application of PGRs was high and there were only statistical differences between the percentages of explants with calluses, seen in the low (77.9%) and the medium (79.3%) sucrose concentrations, and the percentages of hypocotyls

with callus formation, seen in the high concentration of sucrose (52.6%).

Looking for the appearance of embryonic structures evaluated after 30 d of incubation, the higher percentages of explants with embryos (25%) were achieved without kinetin and a medium dose of NAA. The same dose presented a higher percentage of embryos with the medium and high sucrose doses. Similarly, with the cotyledonary leaf explants, the hypocotyl embryos originated in white, bright calluses that had a granular, gelatinous appearance and friable consistency with wide, round bases that surrounded the caulinar apex, submerged in the callus body (Fig. 5).

Effect of NAA, kinetin, and sucrose on the growth of explants of lulo without spines

The results obtained with lulo without spines were very similar to those obtained from lulo with spines. The rooting of the cotyledonary leaves was influenced by the three studied factors and the NAA*sucrose interaction. There were no significant differences between the factors for the formation of the calluses. The induction of embryonic structures was determined by the effect of NAA, kinetin, sucrose and by the NAA*kinetin, NAA*sucrose and kinetin*sucrose interactions.

The percentage of rooted explants was higher without the addition of kinetin. The sucrose concentrations did not affect the rooting percentages with the NAA dose but, with the medium and high doses, the higher concentrations of sucrose significantly reduced the rooting. With the low and medium NAA doses, the inclusion of kinetin reduced the formation of embryonic structures but, with the high dose,

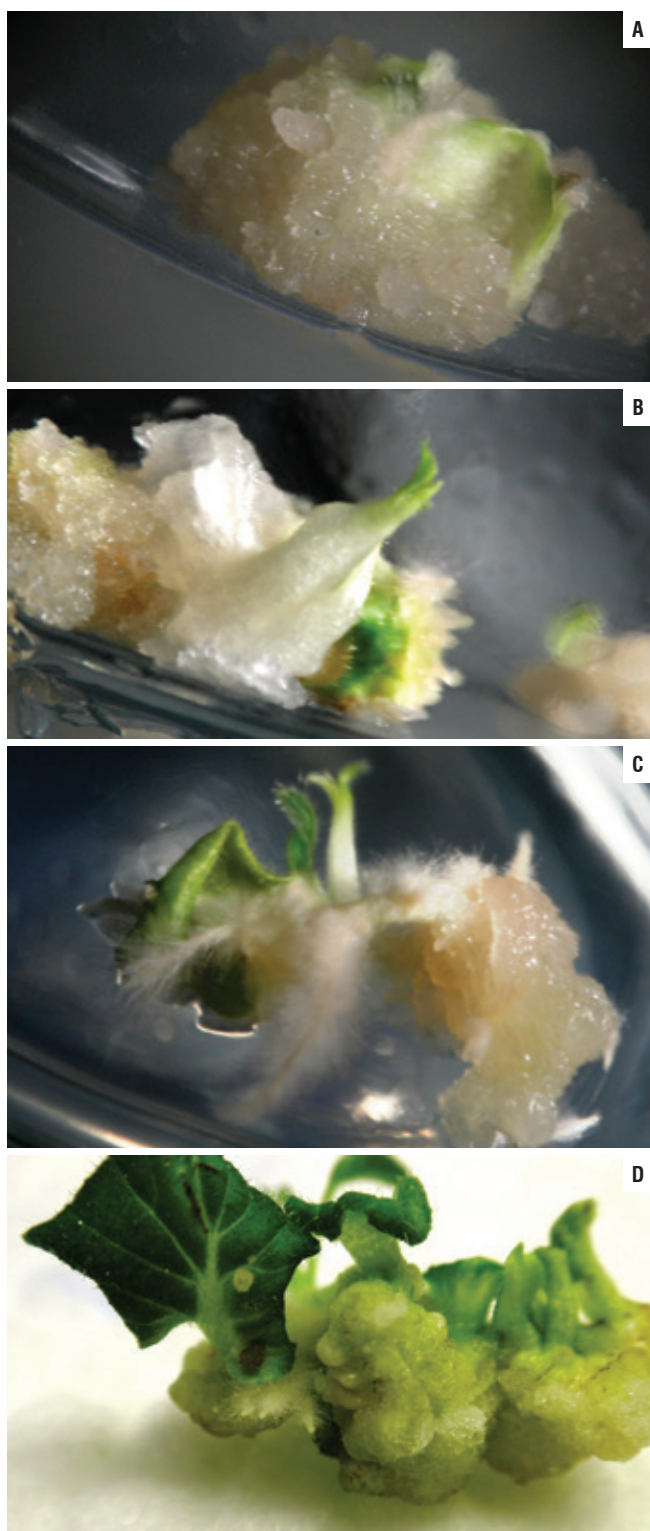


FIGURE 4. Induction of somatic embryogenesis from cotyledonary leaves of lulo with spines. A, embryogenic callus obtained on medium with 31.9 μM of NAA and 58.4 μM of sucrose showing a granular and gelatinous morphology; B, somatic embryos developing roots on medium with 31.9 μM of NAA, 13.9 μM of kinetin and 263.1 μM of sucrose; C, a somatic embryo differentiating from embryogenic callus on medium with 50 μM of NAA and 58.4 μM of sucrose; D, embryonic tissue subcultivated onto medium supplemented with low levels of PGRs.

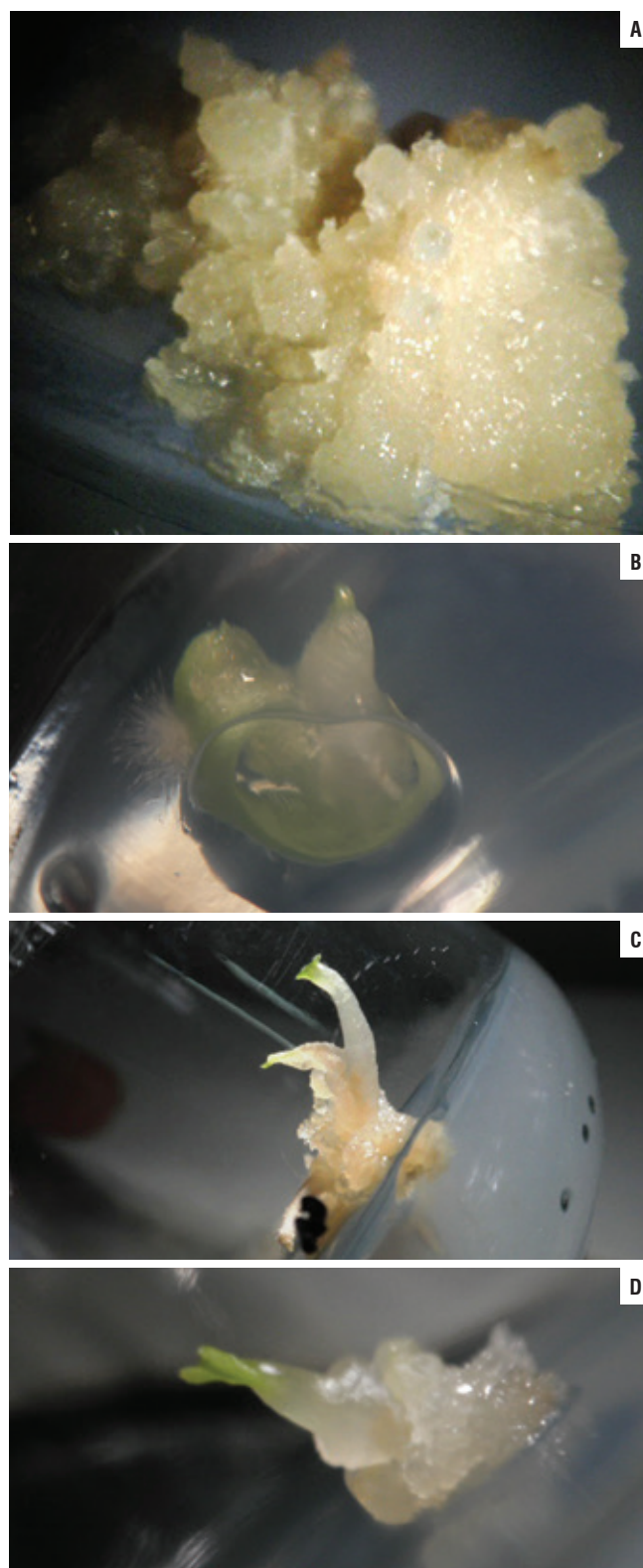


FIGURE 5. Induction of somatic embryogenesis from hypocotyls of lulo with spines. A, embryogenic callus obtained on medium with 31.9 μM of NAA and 50 μM of sucrose; B and C, somatic embryos obtained on medium with 31.9 μM of NAA and 10 μM of sucrose; D, somatic embryo induced on medium with 31.9 μM of NAA and 263.1 μM of sucrose.

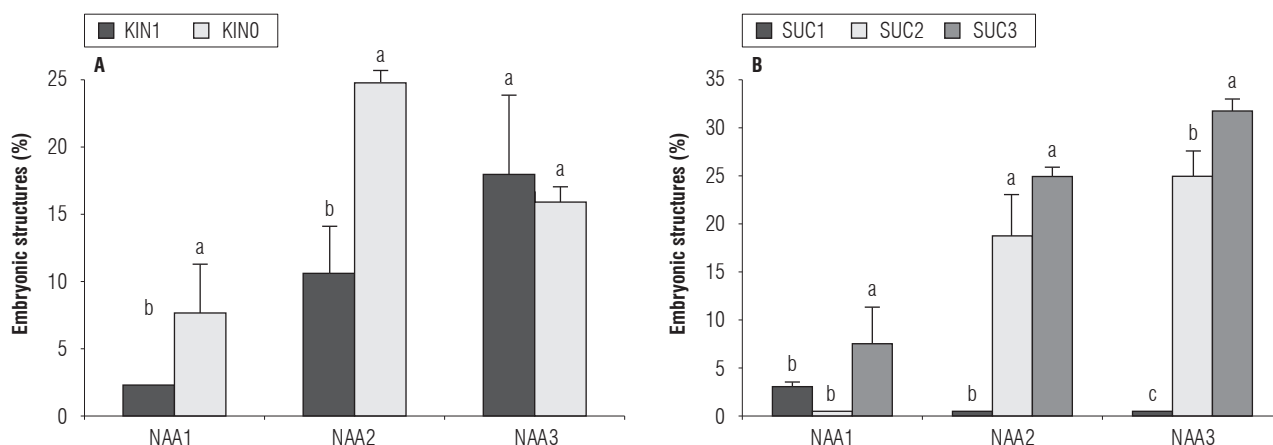


FIGURE 6. Effect of the different concentrations of NAA, kinetin (KIN) and sucrose (SUC), on the frequency of embryonic structure induction in cotyledonary leaves of lulo without spines. A, NAA*kinetin interaction; B, NAA*sucrose interaction. NAA1, 1.98 μM ; NAA2, 31.9 μM ; NAA3, 50.0 μM ; KINO, 0 μM ; KIN1, 13.9 μM ; SUC1, 14.6 μM ; SUC2, 58.4 μM ; SUC3, 263.1 μM . Means with different letters indicate significant differences according to Tukey test ($P \leq 0.05$) ($n = 4$).

the addition of kinetin did not affect the percentage of explants with embryos. Similarly, the positive effect of the higher concentrations of sucrose on the appearance of embryos was obtained without kinetin in the medium (Fig. 6).

The formation of roots was higher (58.7%) with the hypocotyls of lulo without spines as explants with the lowest NAA concentration (19.8 μM), as compared with the medium (31.9 μM) (34.17%) and high doses (50.0 μM) (24.89%), which did not differ. Similarly, a kinetin and sucrose interaction was observed, presenting a higher inhibitory effect of the sucrose without kinetin in the medium. In the formation of calluses in the explants, marked effects were seen from NAA and kinetin, without an interaction between them. The higher percentages of hypocotyls with

the presence of calluses were observed with NAA (19.8 and 31.9 μM) and without kinetin in the medium.

Each of the studied factors (NAA, kinetin and sucrose) significantly affected the percentage of somatic embryos that appeared in the medium with interactions between NAA and kinetin and between kinetin and sucrose. In all the NAA concentrations, the addition of kinetin significantly reduced the presence of embryos (Fig. 7A). With the 31.9 and 50 μM of NAA concentrations, the higher percentages of somatic embryos were obtained (22.5 and 17.0%, respectively). The kinetin*sucrose interaction demonstrated a very significant effect on the sucrose when kinetin was not applied, with higher percentages of somatic embryos with 50.1 and 263.1 μM of sucrose in the MS medium (Fig. 7B).

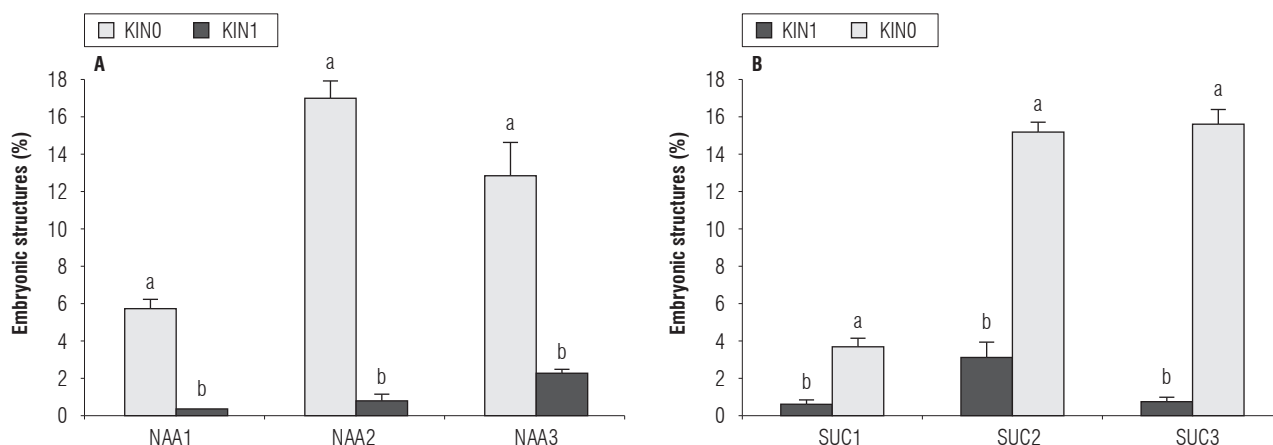


FIGURE 7. Effect of different concentrations of NAA, kinetin (KIN), and sucrose (SUC) on the frequency of hypocotyls of lulos without spines that presented somatic embryo induction. A, NAA*kinetin interaction; B, sucrose*kinetin interaction. NAA1, 1.98 μM ; NAA2, 31.9 μM ; NAA3, 50.0 μM ; KINO, 0 μM ; KIN1, 13.9 μM ; SUC1, 14.6 μM ; SUC2, 58.4 μM ; SUC3, 263.1 μM . Means with different letters indicate significant differences according to Tukey test ($P \leq 0.05$) ($n = 4$).

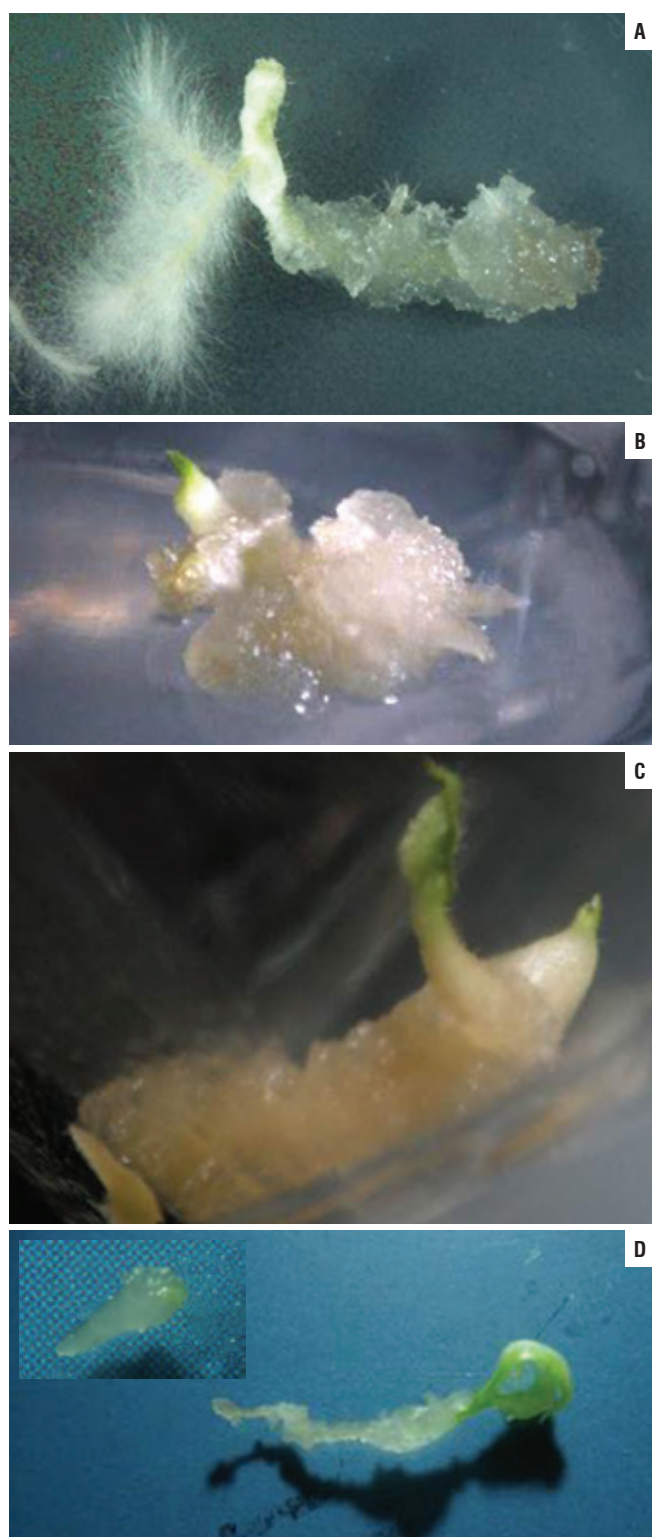


FIGURE 8. Induction of somatic embryogenesis from hypocotyl of lulo without spines. A, embryogenic callus differentiating roots on medium with 19.8 μM of NAA and 50 μM of sucrose; B and C, embryogenic calluses differentiating somatic embryos on medium with 31.9 μM of NAA, 261.3 μM of sucrose and 50 μM of NAA, 50 μM of sucrose, respectively; D, somatic embryo at the torpedo stage of development (upper-left corner square) and germinating embryo isolated from embryogenic callus on medium with 31.9 μM of NAA and 261.3 μM of sucrose.

The somatic embryos, as in the lulo with spines, originated from calluses with a friable appearance that were white to creamy and had a granular appearance. The embryos that germinated in the induction medium presented basal widening that culminated in the apex where the emergence of cotyledons started and, in the distal part, the radical grew through the calluses without achieving complete independence during the initial development (Fig. 8). In all the cases in which the calluses were subcultures in a medium without regulators, problems of phenolization or growth detention were seen.

Discussion

Solanaceae are considered recalcitrant species in studies related to somatic embryogenesis, although, currently, important species of this family have been cloned through embryonic induction, such as eggplant (*S. melongena*) (Huda and Bari, 2007; Mir *et al.* 2008; Ribeiro *et al.* 2009); tomato (*S. lycopersicum*) (Kaparakis and Alderson, 2002; Vinoth *et al.*, 2014), the potato (*S. tuberosum*) (JayaSree *et al.*, 2001; Sánchez-Enciso *et al.*, 2005; Vargas *et al.*, 2005; Sharma *et al.*, 2008) and pepper (*C. annuum*) (Kintzios *et al.*, 2001; Bodhipadma and Leung, 2002; Bárány *et al.*, 2005; Koleva-Gudeva *et al.*, 2007; Solís-Ramos *et al.*, 2010). Despite the importance of lulo (*S. quitoense*) as a fruit species of high nutritional value, there have been no successful reports on its cloning with somatic embryogenesis; only studies have been carried out by Segovia (2002), who tried without success to regenerate lulo with embryogenic methods.

Levels of NAA, kinetin and sucrose determined the induction rates of roots, callus and somatic embryos in both explants (cotyledonary leaves and hypocotyls) confirming that the addition of kinetin inhibited the rooting action of small NAA doses and reduced the percentage of explants with calluses, which agrees with the findings of Van Staden *et al.* (2008), who confirmed that high cytokinin concentrations (0.5-10.0 mg L^{-1}) prevented radical growth and the rooting promoting effects of auxins, but is contrary to the report of Thornton *et al.* (2013), who stated that organogenic processes must be supplemented with auxins and cytokinins.

The appearance of somatic embryos in the induction medium confirmed the recalcitrant characteristic of lulo and the presence of only a few embryonic cells in the differentiated cell medium. These observations agree with Santos and Feveireiro (2002), who stated that cells with an embryogenic capacity can coexist in the same callus with

cells with no embryogenic capacity. According to González *et al.* (2005), embryogenic cells originate in meristematic cells and from their division in specific meristematic tissues, as confirmed by permanent embryonic cells.

Similarly, a positive effect was observed from the sucrose on the appearance of embryogenic structures, possibly due to the fact that, in addition to being an energy source for the process, sucrose acts as an osmotic stress factor that improves the efficiency of somatic embryogenesis (Daigny *et al.*, 1996; Iraqi and Tremblay, 2001; Jheng *et al.*, 2006). Hong *et al.* (2008) reported that the water stress originating from high sucrose concentrations induces the accumulation of protein and carbohydrates in the cells, which favors the induction of embryogenic cells and inhibits the early germination of embryos in *S. melongena*. This finding is contrary to the observations of the present study, which confirmed the recalcitrant characteristic of lulo, with embryos appearing sporadically in the calluses that grew in the induction medium without generating the continuation of the recurrent process; moreover, the lack of the embryogenic cell proliferation necessary for the formation of PEMs, despite the high hormone concentrations present in the medium, allowed for the appearance of fewer embryos in the cultured explants.

When the regulators were eliminated from the medium, the calluses experienced necrosis without generating new embryonic structures or other organogenesis processes, as occurs in other species. It is possible that, as observed by Arahana *et al.* (2010) in studies on the somatic embryogenesis of *S. betacea*, calluses with embryogenic potential do not form embryos due to a reduction in the cellular maturation due to a lack of sucrose at suitable concentrations, which allows for the induction of nutrient accumulation in embryogenic cells.

Conclusions

Lulo can be considered a recalcitrant species for the induction of somatic embryogenesis that does not respond suitably to protocols that are successful for other Solanaceae species. In dark conditions at $25\pm 2^{\circ}\text{C}$ with a MS medium and young lulo explants, with or without spines (hypocotyls and cotyledonary leaves), it was possible, for the first time, to obtain germinated embryos in the induction medium with additions of NAA (31.9 and 50 μM) and sucrose (50.0 and 263.1 μM), 30 d after sowing, that are easily reproduced in a MS medium.

The elimination of PGRs from the cultivation medium caused a reduction in growth and necrosis in the calluses, completely eliminating the possibility of obtaining a recurrent embryogenic process.

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