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Investigation on possible occurrence of somaclonal effects in zygotic and embryonic cotton structures based on agronomical traits

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ABSTRACT. Zygotic and embryonic cotton structures were used in *in vitro* procedures to obtain stable plants. Zygotes (24, 48, and 72 h) and immature embryos (10 and 25 days after fertilization) were grown in MS medium supplemented with glucose, indoleacetic acid, kinetin, and activated charcoal. Regenerated seedlings were acclimatized and grown in 10-kg pots for further phenological evaluation, based on agronomic traits and possible occurrence of somaclonal effects. We found that zygotes and immature embryos at 10 days did not develop physiological competence to allow regeneration of plants, based on adopted procedures. Stable plants were achieved with embryos at 25 days, with 68% of regeneration rate. The agronomical traits of these plants were similar to those obtained via seeds. Overall, the maturation cycle of cotton plants was 120 days after acclimation. No somaclonal effect, resulting from *in vitro* procedures was found in regenerated plants, based on agronomical traits evaluated in normal and regenerated plants.

Key words: *Gossypium hirsutum* L.; Zygotes; Immature embryos; Recalcitrance; Regeneration

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an important commodity worldwide and plays a significant role in textile agribusiness. The current contribution of cotton crop exceeds U$20 billion, moving 180 million of persons dependent on fiber and seed markets (Freitas et al., 2014). Despite the economic importance, a strong cotton yield is widely dependent on genotype x environmental factors. The world market of seeds offers a range of robust cultivars with broad environmental adaptation and yield stability, allowing the selection of the best ideotypes to different kind of farmers.

Some environmental factors exert a strong influence on cotton development, emphasizing water and salt stresses and damages caused by pests (Conaty et al., 2012; Echer et al., 2014). Depending on intensity, they may cause serious disturbances in plant physiology, such as nutritional deficiency and hormonal imbalance, leading to abscission of leaves and reproductive structures (Silva et al., 2011). According to Krieg (2005), shedding of young buds and bools in the cotton crop can take up to 65%, when plants are facing on severe biotic or abiotic stress conditions. This rate could be dangerous when dealing with germplasm preservation.

The maintenance and preservation of germplasm are basic strategies for the establishment of a breeding program. In recalcitrant species, the adoption of conservation practices is indispensable for germplasm safety. The current tools of modern biotechnology have contributed markedly to rescue germplasm through several *in vitro* techniques, by using hormonal stimuli. These techniques are based on cell totipotency that refers to the capacity of cell organization to generate a whole plant, without genetic recombination (Neelakandan and Wang, 2012). The regeneration of plants can be accomplished via organogenesis or embryogenesis; in the last case, by somatic or zygotic procedures (Kikuchi et al., 2013).

The *in vitro* crop of zygotic embryos is a valuable tool to rescue germplasm; however, the success rate depends on the complexity of endogenous and exogenous factors, such as culture media, exogenous growth regulators, age of tissues, among others (Polanco and Ruiz, 2001; Umehara et al., 2007; Uma et al., 2011). The procedures that do not involve de-differentiation from explant (callus formation) and subsequent re-differentiation are more likely to provide satisfactory results. However, the methodologies are not suitable for all species. In recalcitrant species, such as cotton, this process may be limited or even unfeasible, hampering the regeneration of interesting accessions (Soares et al., 2014).

Despite large benefits obtained by *in vitro* procedures, clonally propagated plants often exhibited some level of somaclonal, either genetic or epigenetic in origin, displayed among plants derived from cell culture involving the use of somatic plant cells. This is because changes in tissue culture involve disorganized growth at various levels, ranging from systems that lead to disturbing at cell organization. Epigenetic modifications take place in the chromatin allowing cells to maintain distinct characteristics despite containing the same genetic material (Camas et al., 2014). The epigenetic factors emphasize the interaction between genes and the environment, whose modifications occur in an individual's genome that ultimately influence the organism's development and the process of its heredity (Springer, 2013). Such process reflects the adaptation of cells to a different environment, which includes the response to signals that may trigger switches in the developmental program.

In plant regeneration obtained by *in vitro* procedures, epigenetic variations have been reported during cell differentiation and de-differentiation (Miguel and Marum, 2011; Yang et al., 2013). The processes of de-differentiation and re-differentiation may involve both qualitative

and quantitative changes in the genome, and different DNA sequences may be amplified or deleted during these changes (Lee and Phillips, 1988). Somaclonal variation, therefore, can arise from somatic mutations already present in the donor plant (Karp, 1994; Bairu et al., 2011) and undesired effects of adaptive adjustments may compromise the objectives for which the plant cells or tissues were cropped. Therefore, it is necessary to follow up the phenotype of regenerated plants, at least the main agronomic traits, to discard previously atypical plants that can further compromise the genetic uniformity of matrix cultivar. Among factors that determine the frequency of variation during crop tissue procedures, the propagation methods, genotype, and growth regulators are more reported (Uma et al., 2011; Miguel and Marum, 2011; Yang et al., 2013). To investigate possible somaclonal variations arising from zygotic structures and immature embryos grown *in vitro*, this study was carried out, based on comparisons of agronomic traits in stock and regenerated cotton plants.

MATERIAL AND METHODS

In vitro procedures

Seeds of cotton cv. BRS 8H were grown in pots (10 L) containing fertilized soil (20:60:20, v/v/v, NPK) and daily watered, in a greenhouse. At blooming, young and fertilized bolls were collected in five growth stages and disinfected (0.5% sodium hypochlorite). Zygotes (24, 48, and 72 h) and immature embryos (10 and 25 days after fertilization) were aseptically excised and subsequently inoculated into bottles (250 mL) containing MS medium (Murashige and Skoog, 1962), pH 5.7, supplemented with glucose (3%), indoleacetic acid (IAA, 0.1 mg/L), Kinetin (Kin, 0.5 mg/L) and activated charcoal (1.0 g/L) (Carvalho et al., 2003). Fifteen structures/bottle were used for zygote treatments, whereas five were used to immature embryos. Assays were performed in five replicates for each treatment.

The samples were incubated in a growth chamber (Biofoco, Brazil), in the dark (25 ± 2°C) until the appearance of radicle (about 30 days). Then, seedlings were transferred to glass tubes (30 mL) containing hormone-free MS medium, and incubated in the growth chamber (25 ± 2°C, 16-h light and light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$), for 15 days.

Seedling showing secondary leaves and roots were transferred to polypropylene pots (100 mL) containing sterile vermiculite and substrate (1:1, v/v) (Plantmax, Brazil), and incubated in the growth chamber (25 ± 2°C, 16-h light and light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days. Then, they were acclimatized in the greenhouse (28 ± 2°C, 12:12-h light/dark).

Assay in the greenhouse

To investigate possible somaclonal variation in plants (cv. BRS 8H) due to *in vitro* procedures, regenerated plants (Population 1) were evaluated phenotypically based on agronomic traits, compared to plants grown by seeds (Population 2). Plants from Population 1 (30 days of acclimatization) were transferred to pots (10 L) containing fertilized soil (20:40:20, v/v/v, NPK) and daily watered. To try to conciliate the phenological age of both populations, seeds of Population 2 were sown 30 days before acclimatization of Population 1. Plants were cropped in the same conditions as Population 1. Watering was performed by sprinkler irrigation keeping the pot capacity. Each population was composed of 17 individuals and plants were kept free of insects and weeds. The ranges of temperature and relative humidity recorded

during the assay in the greenhouse were 32-35°C and 58-66%.

The follows traits were evaluated: the plant height, the number of bolls, the weight of mature bolls, cycle, and oil content. All data were recorded individually in each plant of two populations. The oil content was estimated in seeds (20 g), by nuclear magnetic resonance from each plant. The calibration curve conditions were previously established, and oil contents were estimated from the total mass of plant seeds. Five repetitions were taken from each plant. The considerations were inferred from averages of each population.

RESULTS AND DISCUSSION

Regeneration of zygotes and immature embryos

Based on the procedures adopted in this study, we found that tissues from zygotes and immature embryos collected at 24 h to a 10-day interval did not get physiological competencies to respond to hormonal stimuli after 30 days of cropping. Only embryos collected with 25 days generated viable seedlings. An oxidative process occurred similarly in zygote treatments despite the addition of 1.0 g/L activated charcoal (Figure 1A). This element is quite important in growth medium because it reduces oxidation of tissues and also promotes absorption of exudates released by explants (George, 1996). We concluded that the concentration used in this assays was not enough to avoid oxidation in tissues. According to Pollock and Jensen (1964), the pattern of early cell divisions in cotton zygote is irregular and *in vitro* regeneration of these structures becomes limited due to intrinsic conditions of explant, such as cells still undifferentiated, absence of endosperm and the reserves of zygote that decrease during early cell division (4 to 5 days after acclimation).

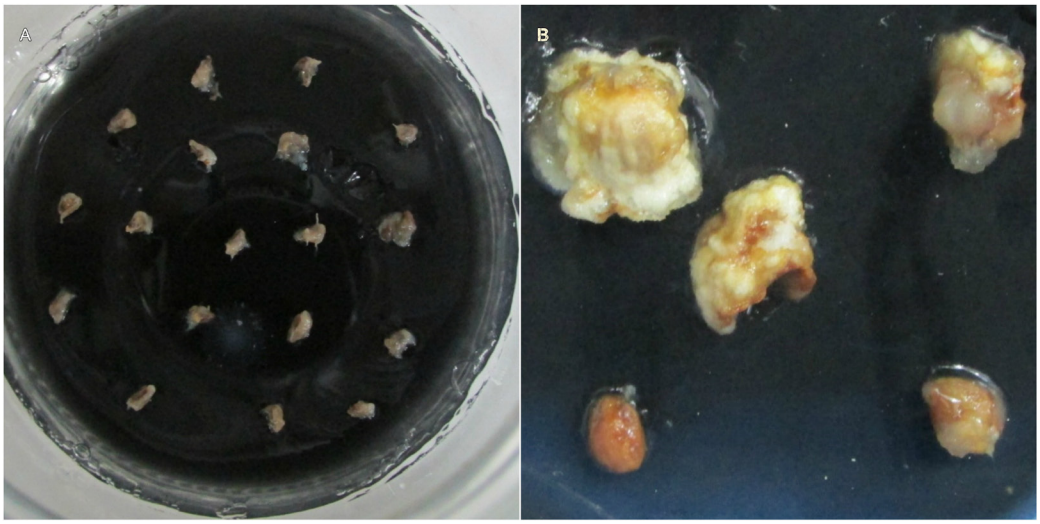

Figure 1. A. Zygotes with 72 h showing oxidation after 30 days of the crop. **B.** Callogenesis and oxidation of immature embryos 10 days after fertilization.

Shen et al. (2011) report that rescue of zygotes or immature embryos depends widely on components of culture medium to promote morphogenesis of undifferentiated cells (callus). In recalcitrant species, such cotton, regeneration is a difficult process in some genotypes due to natural inability to acquire morphogenic competence, during differentiation procedures. The stimulus depends primarily on adjustments in hormonal balance and successive subcultures of calli. Therefore, we understand that the procedures adopted here for zygotes and immature embryos require better adjustments to get success on the rescue of these tissues.

Regarding immature embryos, viable seedlings were achieved only with embryos excised 25 days after fertilization (Figure 2), with a regeneration rate of 68% after acclimatization of seedlings (Table 1). This rate was quite satisfactory and benefited due to the autotrophic capacity of embryos to respond better to components of culture media. The 10 days after fertilization embryos were unviable and did not show embryogenic calli (Figure 1B).

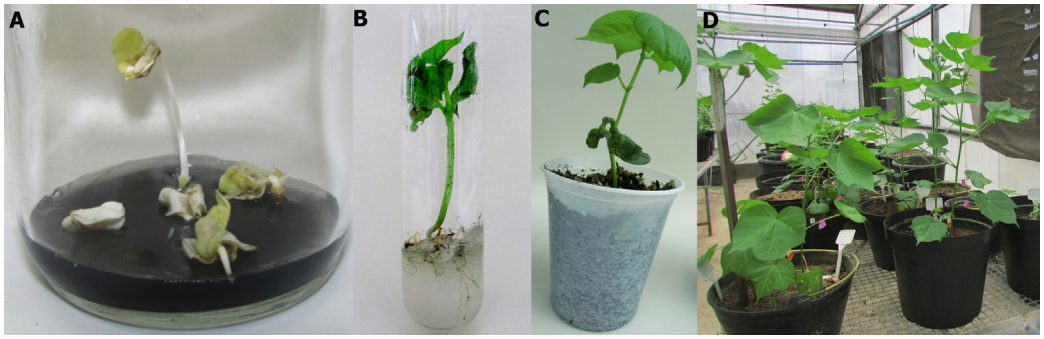

Figure 2. Plants regenerated from immature embryos 25 days after fertilization. **A.** Seedlings showing radicle and hypocotyl developed after 20 days of cropping in MS medium supplemented with 0.1 mg/L IAA + 0.5 mg/L Kin + 1.0 g/L activated charcoal; **B.** seedlings grown in MS medium without growth regulators; **C.** plants acclimatized in sterile substrate; **D.** plants regenerated and acclimatized in the greenhouse.

Table 1. Regeneration rates of zygotes and immature embryos from cotton genotype (cv. BRS 8H) cropped *in vitro*.

Treatment	Sample (N)	Regeneration	
		N	Rate (%)
Z 24 h	75	0	0
Z 48 h	75	0	0
Z 72 h	75	0	0
IE 10 days	25	0	0
IE 25 days	25	17	68

Z = zygotes; IE = immature embryos.

Phenology of plants in the greenhouse

The seventeen plants obtained from embryos 25 days after fertilization (Population 1) were grown in the greenhouse, adopting the same management as to Population 2 (cv. BRS 8H grown by seeds). We found that both populations had normal growth during the life cycle and no significant difference was found in plants, at all (Figure 2D). The average cycle of Population 1 was 125 days after anthesis. Whereas acclimatization lasted 30 days, we consider that both populations had close phenological cycles (Population 2 = 158 days). The

agronomic traits registered in both populations are in Table 2. The values found individually in plants were too close, so that the means were quite similar in both populations, suggesting no occurrence of any somaclonal variations in regenerated plants, based on the traits evaluated.

Table 2. Agronomic traits registered individually in cotton plants obtained via *in vitro* cropping and by seeds.

Pop 1	PH	NB	WB	C	O	Pop 2	PH	NB	WB	C	O
R1	101	15	5.7	124	19.8	S1	98	12	6.2	157	20.3
R2	99	12	5.9	124	19.9	S2	98	12	5.6	157	19.9
R3	106	15	6.1	125	21.1	S3	102	11	5.9	156	21.1
R4	98	12	6.2	126	21.2	S4	99	12	6.4	158	21.3
R5	98	12	5.9	126	21.0	S5	106	15	5.7	159	19.8
R6	101	13	5.5	124	21.2	S6	104	15	6.5	159	19.3
R7	105	12	5.9	124	20.3	S7	98	13	5.9	158	20.3
R8	98	13	6.3	124	20.6	S8	103	14	6.3	158	20,2
R9	96	14	6.2	125	20.5	S9	99	15	6.1	156	21.0
R10	99	15	6.5	125	20.3	S10	99	15	5.9	156	21.1
R11	101	12	5.9	125	19.9	S11	103	13	6.0	157	20.9
R12	102	12	6.1	126	19.9	S12	104	14	6.2	157	20.8
R13	104	13	6.1	124	19.8	S13	101	12	6.5	158	21.2
R14	101	14	6.3	126	20.6	S14	105	12	5.8	158	21.0
R15	101	14	6.2	126	21.0	S15	99	14	6.0	159	19.9
R16	99	15	5.9	125	21.1	S16	100	11	6.2	158	21.0
R17	98	14	5.9	125	19.9	S17	99	11	6.1	158	19.9
Mean	100	13	6.0	125	20.5	Mean	101	13	6.1	158	20.5

PH = plant height (cm); NB = number of bolls; WB = weight of mature bolls (g); C = cycle (days); O = oil content in seeds (%). Pop 1 - plants obtained from regenerated embryos via *in vitro* cropping; Pop 2 - plants obtained from seeds.

Somaclonal variation in commercial species generated by *in vitro* crop has been widely reported in the literature as well as their use to plant breeding, as a way of generating variability (Miguel and Marum, 2011; Yang et al., 2013; Springer, 2013). In immature organs, the effects of somaclonal variation are unpredictable considering that the cell complex is still in the intensive process of differentiation. In recalcitrant species, the understanding of the morphogenic response *in vitro* requires knowledge of the function of genes directly involved in cell competence route, to assist the adoption of strategies to improve the morphogenesis (Kikuchi et al., 2013; Zhu et al., 2014; Zheng and Perry, 2014). The genetic programs that control the embryo development in zygotic and somatic embryogenesis display several similar routes, although the mechanisms determining the induction phase of these two processes are quite different. The development of zygotic embryo begins with the formation of the zygote following fertilization, while somatic cells acquire embryogenic competence as a result of different chemical and physical stimuli (Karami et al., 2009). According to Skirvin et al. (1994), the inductions generated by somatic embryogenesis procedures can reveal alterations between cells, tissues, and organs thereby creating variation within cultures, or among the somaclones, that may be physically different from matrix plants by which the culture was derived. Maintaining genetic integrity of matrix plants is the most crucial aspect in plant propagation, and components of growth media are important factors that can promote somaclonal variation, especially the type and availability of growth regulators.

CONCLUSIONS

Based on media components used in this study, especially growth regulators, no alterations were found in regenerated plants from 25 days after fertilization, meaning that the

genetic integrity of cv. BRS 8H was maintained, at least, in traits evaluated. However, it is worth noting that despite the importance of uniformity of regenerated plants, variations arising from asexual propagation could be an interesting alternative for further use in crop breeding depending on the advantage that the trait brings to the program.

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