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BIOTECHNOLOGY

Effects of glutathione supplementation and carbon source during somatic embryogenesis of *Acca sellowiana* (O.Berg) Burret (Myrtaceae)

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ABSTRACT. The present study intended to investigate the effects of different glutathione (GSH) levels (0, 0.1, 0.5 and 1 mM) on the somatic embryogenesis (SE) induction of *Acca sellowiana*. Besides, we evaluated the effect of different carbon sources (sucrose and maltose) on the somatic embryos conversion. GSH-supplemented treatments resulted in improved SE induction rates (~70%) as compared to the control GSH-free (~35%) after 50 days of culture. The total number of somatic embryos obtained did not differ between treatments, but significant differences were observed for the embryonic stages after 80 days of culture. After 80 days of culture, 0.5 and 1 mM GSH-supplemented treatments showed the largest amount of torpedo-staged somatic embryos. In contrast, treatments supplemented with 0 and 0.1 mM GSH showed equal amounts of somatic embryos at all embryonic stages. These results indicate that GSH accelerates the SE induction process and increases the synchrony of the somatic embryo formation of *A. sellowiana*. The use of maltose for the somatic embryos conversion, as compared to sucrose, did not influence the conversion rate of normal chlorophyllous somatic embryos, but increased the formation of normal achlorophyllous somatic plantlets. This finding can be attributed to the rapid hydrolysis of sucrose, contributing to an enhanced chlorophyll synthesis.

Keywords: *in vitro* culture; micropropagation; somatic embryo conversion; feijoa.

Efeitos da suplementação de glutationa e fontes de carbono durante a embriogênese somática de *Acca sellowiana* (O.Berg) Burret (Myrtaceae)

RESUMO. O presente estudo teve como objetivo investigar o efeito de diferentes níveis de glutationa (GSH) (0, 0,1, 0,5 e 1 mM) na indução da embriogênese somática (ES) de *Acca sellowiana*. Além disso, avaliamos o efeito de diferentes fontes de carbono (sacarose e maltose) na conversão de embriões somáticos em plântulas. Os tratamentos suplementados com GSH resultaram em melhores taxas de indução de ES (~70%) em comparação com o controle isento de GSH (~35%) após 50 dias de cultivo. Após 80 dias as taxas de indução foram iguais. O número total de embriões somáticos obtidos não diferiu entre os tratamentos, mas diferenças expressivas foram observadas nos estágios embrionários. No dia 80 em cultura, os tratamentos suplementados com 0,5 e 1 mM de GSH mostraram a maior porção de embriões somáticos no estádio torpedo. Diferentemente, tratamentos suplementados com 0 e 0,1 mM de GSH mostraram quantidades iguais de embriões somáticos em todos os estágios embrionários. Estes resultados indicam que o GSH acelera o processo de indução do ES e aumenta a sincronia na formação de embriões somáticos de *A. sellowiana*. O uso de maltose no meio de cultura de conversão de embriões somáticos, em comparação com a sacarose, não influenciou a taxa de conversão de embriões somáticos clorofilados normais, mas aumentou a formação de plântulas aclorofiladas normais. Esse resultado pode ser atribuído à rápida hidrólise da sacarose, apresentando translocação de plantas mais eficiente e aumento da osmolaridade do meio de cultura, contribuindo para uma síntese melhorada de clorofila.

Palavras-chave: cultura *in vitro*; micropropagação; conversão de embriões somáticos; feijoa.

Introduction

Acca sellowiana (O. Berg) Burret (Myrtaceae), known as feijoa or pineapple-guava, is a Brazilian native species of the Atlantic Forest, naturally occurring in the States of Santa Catarina and Rio Grande do Sul, with secondary dispersion in Uruguay (Guerra, Cangahuala-Inocente, Dal Vesco, Pescador, & Caprestano, 2013; Cristofolini

et al., 2014). This species is typical of the understory of mature formations of the Mixed Ombrophilous Forest, at altitudes above 1,000 m (Finatto et al. 2011).

The high market value of *A. sellowiana* fruits and flowers makes this species an option for farmers located in high altitude regions. The development of its production would allow the processing of the fruits in products with high commercial value, such as juices, fermented and distilled beverages, ice cream and candied fruits (Vuotto et al., 2000; Ruberto & Tringali, 2004; Beyhan, Elmastaş, & Gedikli, 2010; Weston, 2010). Moreover, this species impacts directly on the recovery of degraded land, riparian forests, permanent preservation areas, legal reserves, and constitution of agro-forest systems (Gomes, Oliveira, Ferreira, & Batista, 2016).

Despite being a native species of Latin America, its cultivation is predominant in countries such as New Zealand, Colombia and Turkey (Guerra et al., 2016), and the increase of cultivation in Brazil is conditioned to the improvement of propagation techniques. Tissue culture techniques, mainly associated to somatic embryogenesis (SE), has the advantage of allowing mass and clonal micropropagation for selected genotypes of this species (Guerra et al., 2016).

SE is an analogous process to zygotic embryogenesis and is based on the totipotency of plant cells to form embryos from somatic cells (Karami & Saidi, 2010). This technique has been extensively studied and improved in the last two decades for *A. sellowiana*; however, somatic embryos maturation and conversion still present low rates (Guerra et al., 2016).

The γ-glutamylcysteinylglycine or glutathione (GSH-reduced form) is a thiol tripeptide involved in the process of cell division and differentiation, with effect on the induction and control of SE in several plant species (Belmonte, Donald, Reid, Yeung, & Stasolla, 2005; Vieira et al., 2012; Fraga et al., 2016). GSH supplemented to culture medium during the early stages of SE creates a reducing environment, enhancing cell proliferation and the development of immature somatic embryos (Stasolla, 2010). In *Araucaria angustifolia*, GSH supplementation in the culture medium increased the somatic embryos formation in the initial phase of development, related to increased nitric oxide emission (Vieira et al., 2012). Similarly, GSH supplementation also increased the number of somatic embryos of *Podocarpus lambertii* obtained during maturation stage, besides improving morphological features (Fraga et al., 2016).

Somatic embryo development is a complex multi-step process, which demands high energy (Konrádová, Lipavská, Albrechtová, & Vreugdenhil, 2002; Dinakar, Djilianov, & Bartels, 2012). During early stages, establishment and growth of embryo structures prevails, while later stages, such as maturation, are characterized by deposition of storage compounds (Konrádová et al., 2002). Thus, the transition phases during SE is accompanied by changes in carbohydrate metabolism (Iraqi & Tremblay, 2001). In *A. angustifolia*, the use of maltose as a carbohydrate source promoted the embryonic tissues differentiation, which was attributed to its slow hydrolysis (Steiner, Vieira, Maldonado, & Guerra, 2005).

In this context, the aim of the present study was to evaluate the effect of different GSH concentrations on the SE induction in *A. sellowiana* and to compare different carbon sources (sucrose and maltose) supplementation during the step of somatic embryos conversion to plantlets.

Material and methods

Plant material

Seeds of *A. sellowiana* (accession 101) were collected from fruits of the germplasm collection of the Research and Extension Agency of Santa Catarina State (Epagri), São Joaquim, south Brazil (latitude 28º 17' 39", longitude 49º 55' 56", altitude 1415 m). The fruits were transported in plastic boxes and the seeds were extracted and disinfested in laminar flow chamber with ethanol (70%) for 1 min and sodium hypochlorite (2%) for 20 min, as described by Guerra, Dal Vesco, Ducroquet, Nodari, and Reis (2001).

Somatic embryogenesis induction

Mature zygotic embryos were excised and incubated in a solution with 2,4-dichlorophenoxyacetic acid (200 μM) for 1 hour, according to Fraga et al. (2012), and then inoculated into a Petri dish containing 25 mL of culture medium consisting of LPm macro and microsalts (von Arnold & Eriksson, 1981) supplemented with Morel vitamins (Morel & Wetmore, 1951), maltose (30 g L^{-1}) and glutamic acid (1.35 g L^{-1}). In this step, different concentrations of GSH (0, 0.1, 0.5 and 1 mM) were tested, consisting of 4 treatments. The pH of culture medium was adjusted to 5.8, gelled with Phytagel® (2 g L^{-1}) and autoclaved at 121°C for 15 min. Cultures were maintained in the absence of light at 25 ± 2°C.

The experimental design was completely randomized, consisting of 4 treatments and 6

replicates, and each sample unit corresponded to 5 zygotic embryos. The percentage of SE induction after 50 and 80 days in culture as well as the number of somatic embryos obtained at each embryonic stage after 80 days in culture were evaluated. The number of responsive and non-responsive explants was submitted to chi-square analysis at 5% probability. These results are presented as induction percentage throughout the manuscript. For the number of somatic embryos at different developmental stages, analysis of variance was performed followed by SNK means separation test at 5% of probability.

Somatic embryos conversion

Torpedo- and pre-cotyledonary-staged somatic embryos were selected, isolated and inoculated in Petri dishes containing 25 mL of conversion culture medium. This culture medium was composed by LPm macro- and micro-salts, supplemented with Morel vitamins, glutamic acid (1.35 g L^{-1}), gibberellic acid (1 µM), fluridone (0.05 µM), 6-benzylaminopurine (0.5 µM) and activated charcoal (1.5 g L^{-1}). In this step, two different carbon sources were tested: sucrose or maltose (30 g L^{-1}). The pH of culture medium was adjusted to 5.8, gelled with Phytagel® (2 g L^{-1}) and autoclaved at 121°C for 15 min. Cultures were maintained in a growth room at 25 ± 2°C and 16 hour photoperiod, with a light intensity of 40-50 μmol m^2 s^{-1} provided by white LED lamps (GreenPower TLED W; Philips™).

The experimental design was completely randomized blocks, with 2 treatments and 8 replicates, with each sample unit consisting of 30 somatic embryos. The number of somatic embryos converted to normal chlorophyllous, normal achlorophyllous and abnormal plantlets after 4 weeks in culture was evaluated. We considered normal plantlets those with two cotyledons, radicle protrusion and presence of chlorophyll. Abnormal somatic plantlets were those which showed only one cotyledon, fused cotyledons, more than two cotyledons, and/or no radicle protrusion. Data were submitted to analysis of variance followed by SNK means separation test at 5% of probability.

Results and discussion

Effects of different GSH concentrations on SE induction

GSH-supplemented treatments resulted in improved SE induction rates (~70%) as compared to the control GSH-free (~35%) after 50 days in culture, with no statistical difference between GSH-supplemented treatments. After 80 days in culture there was no statistical difference regarding the induction rate (Table 1).

Table 1. Somatic embryos induction rate of *A. sellowiana* after 50 and 80 days in culture in response to different GSH levels.

Treatments	Induction rate (%)	
	Day 50	Day 80
GSH 0 mM	36.67[b]	56,67[a]
GSH 0.1 mM	70.00[a]	70.00[a]
GSH 0.5 mM	66.67[a]	73.33[a]
GSH 1 mM	76.67[a]	80.00[a]

Different letters for each evaluation time (50 and 80 days in culture) in column indicate statistical difference between different treatments, compared by chi-square (χ^2) contingency test (95%; n = 30).

Although the total number of somatic embryos did not differ between the treatments evaluated after 80 days in culture, significant differences were observed in relation to the embryonic stages obtained in the different treatments (Table 2 and Figure 1). At day 80 in culture, 0.5 and 1 mM GSH-supplemented treatments indicated the largest portion of somatic embryos obtained in torpedo stage (Figure 2). Differently, treatments supplemented with 0 and 0.1 mM GSH showed equal amounts of somatic embryos at all embryonic stages.

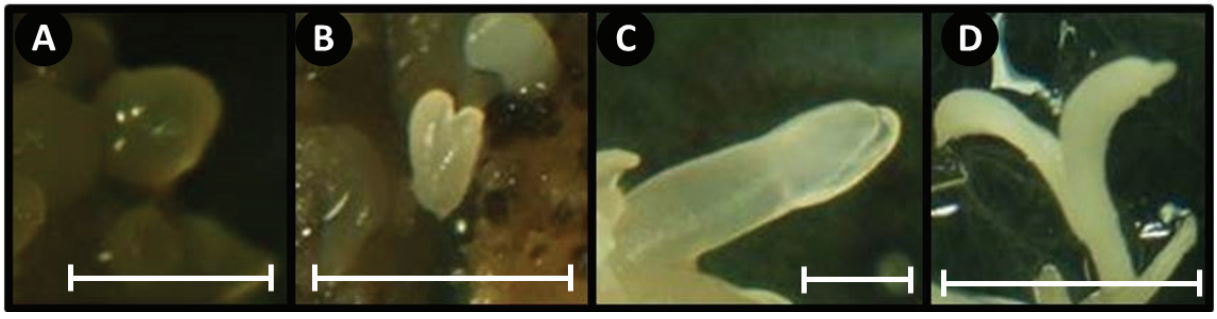

Figure 1. Morphological features of *A. sellowiana* somatic embryos in different embryonic developmental stages. A: Globular-staged somatic embryo, bar: 1.0 mm. B: Heart-staged somatic embryo, bar: 1.0 μm. C: Torpedo-staged somatic embryo, bar: 1.0 μm. D: Cotyledonary-staged somatic embryo, bar: 5 mm.

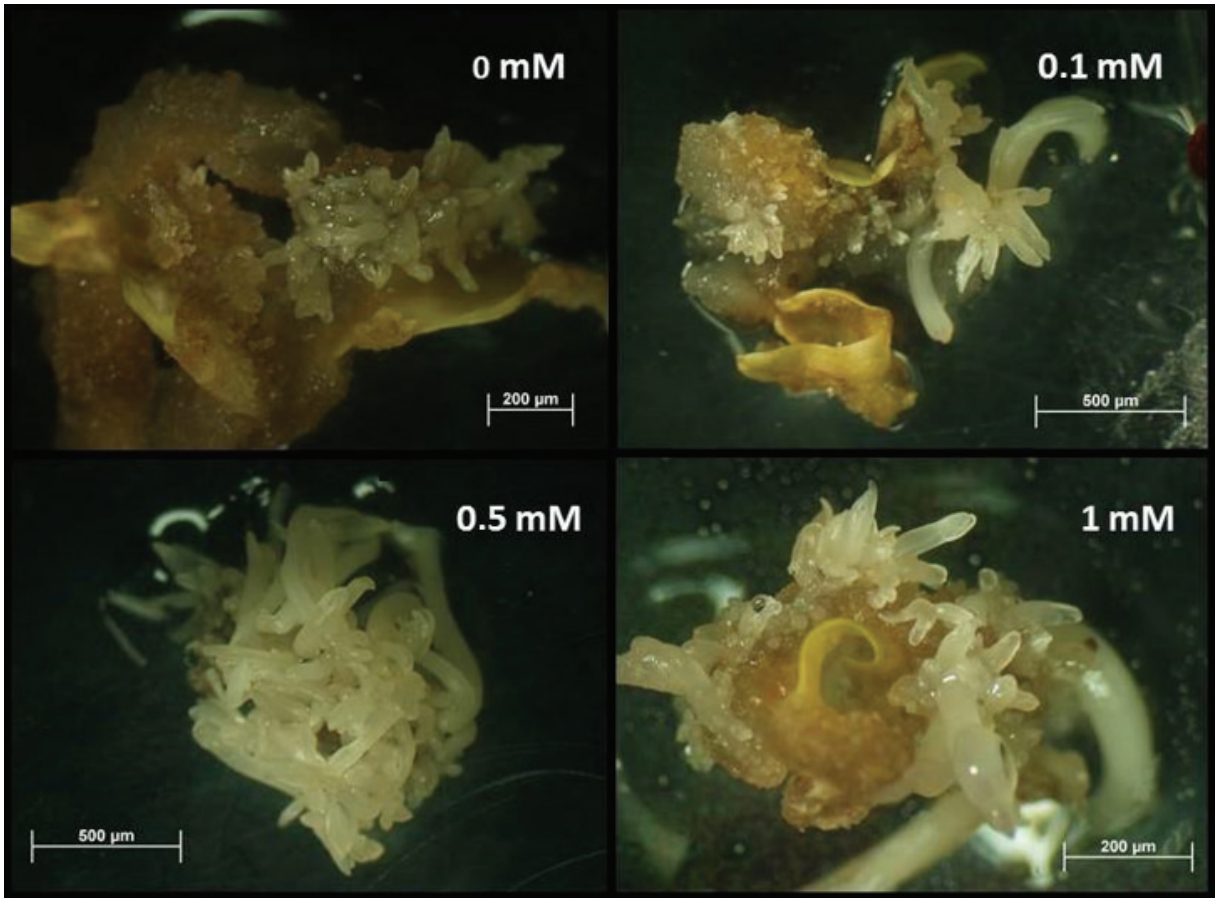

Figure 2. Somatic embryos of *Acca sellowiana* obtained from the different induction treatments evaluated (0, 0.1, 0.5 and 1 mM GSH) indicating expressive differences in the degree of maturity and homogeneity after 80 days in culture.

Table 2. Mean number of *A. sellowiana* somatic embryos in different embryonic developmental stages obtained in different GSH concentrations evaluated.

Treatments	Embryonic stage			
	Globular	Heart	Torpedo	Cotyledonary
GSH 0 mM	38.83 ± 14.72[a]	21.83 ±8.92[a]	20.83 ± 10.40[a]	9.17 ± 4.96[a]
GSH 0.1 mM	56.17 ± 20.18[a]	34.83 ± 8.96[a]	57.17 ± 12.09[a]	9.17 ± 1.85[a]
GSH 0.5 mM	14.67 ± 3.78[b]	16.00 ±4.98[b]	39.83 ± 8.98[a]	9.67 ± 3.28[b]
GSH 1 mM	6.00 ± 1.37[c]	28.67 ± 6.20[b]	50.33 ± 8.62[a]	13.00 ± 4.13[bc]

Mean values ± standard error followed by different letters in line represent differences between embryonic developmental stages for each evaluated treatment, according to SNK test ($p < 0.05$; $n = 6$).

Effects of different carbon sources on somatic embryos conversion

During the somatic embryos conversion step, different plantlets phenotypes were observed: normal chlorophyllous, normal achlorophyllous and abnormal somatic plantlets (Figure 3). The percentage of conversion to normal somatic plantlets of both treatments evaluated, supplemented with sucrose or maltose, did not indicate significant differences, with both treatments showing about 20% conversion rate (Table 3). However, a higher percentage of normal achlorophyllous plantlets were observed in maltose-supplemented treatment. Among the rate of abnormal somatic plantlets obtained, no statistical differences were observed among the evaluated treatments (Table 3).

Table 3. Somatic embryos conversion rate of *A. sellowiana* in sucrose- or maltose-supplemented treatments after 4 weeks in culture. Different categories based on somatic plantlet phenotype is indicated (normal chlorophyllous, normal achlorophyllous, abnormal and abnormal achlorophyllous).

	Somatic embryos converted into plantlets (%)			
Treatment	Normal chlorophyllous	Normal achlorophyllous	Abnormal	Abnormal achlorophyllous
Sucrose	23.02[a]	10.94[b]	34.55[a]	45.32[a]
Maltose	18.58[a]	19.47[a]	29.60[a]	48.93[a]

Mean values followed by different letters in column represent differences between different phenotype categories for each evaluated treatment, according to SNK test ($p < 0.05$; $n = 8$).

Effects of different GSH concentrations on SE induction

Our results indicated an improved induction rate in GSH-supplemented treatments at day 50 of induction. Fraga et al. (2016) performed experiments with different GSH concentrations (0, 0.1 and 0.5 mM) during the initial maturation stage of *Podocarpus lambertii* embryogenic cultures and obtained a higher rate of somatic embryos

formation at 0.5 mM GSH treatment after 10 days of maturation. The results of these authors are in agreement with those found in the present study, suggesting that GSH has an effect on somatic embryos formation of *A. sellowiana*, by accelerating this process.

Vieira et al. (2012) observed an increased initial somatic embryo formation of *A. angustifolia* in culture medium supplemented with low GSH concentrations (0.01 and 0.1 mM). However, when embryogenic cultures were maintained in the same culture medium for more than seven days of culture, there was a decrease in polarization and reduction of normal somatic embryos.

Results found in the present study also indicated that GSH supplementation improves the synchrony in the somatic embryo formation of *A. sellowiana*, increasing the number of somatic embryos in more advanced developmental stages (Table 2 and Figure 2). Similarly, GSH supplementation in the somatic embryo maturation stage increased the number and quality of *Podocarpus lambertii* somatic embryos (Fraga et al., 2016). In *Picea glauca*, GSH addition to the maturation culture medium increased the subsequent somatic embryo conversion rate, and the authors related this result to the expression of genes associated to embryonic development and morphological changes during the maturation stage (Stasolla et al., 2004).

The manipulation of GSH/GSSG (glutathione disulfide) ratio during the final maturation process may be an interesting strategy to improve the rate of normal embryos at maturation stage (Stasolla, 2010; Vieira et al., 2012; Fraga et al., 2016). According to Stasolla (2010), the somatic embryos transfer during the maturation stage to a more oxidized culture medium supplemented with GSSG favors the tissue differentiation and further somatic embryos maturation.

In experiments with *Picea glauca*, an increased formation of somatic embryos in culture medium supplemented with 0.1 mM GSH was observed, and when somatic embryos were subsequently transferred to culture medium supplemented with GSSG, the number of cotyledonary-staged somatic embryos was improved compared to GSSG-free treatment (Belmonte & Yeung, 2004; Belmonte et al., 2005). Similarly, Pullman et al. (2015) also obtained increased mature somatic embryos of *Pinus taeda* in culture medium supplemented with GSSG. Thus, the manipulation of GSH/GSSG ratio during maturation stage may be an appropriate strategy to improve SE in *A. sellowiana*.

Effects of different carbon sources on somatic embryos conversion

In the present work, the conversion percentage in normal chlorophyllous plantlets was about 20% in both treatments evaluated (Table 3), which is equivalent to previous reports for this species, varying between 20 and 35% (Cangahuala-Inocente, Dal Vesco, Steinmacher, Torres, & Guerra, 2007; Fraga et al., 2012). Furthermore, no differences could be observed between conversion treatments regarding the number of normal chlorophyllous plantlets obtained.

Conflicting results can be found in the literature regarding the use of sucrose or maltose in the somatic embryos conversion. Different carbon sources, including maltose and sucrose, were evaluated in the conversion culture medium of *Catharanthus roseus* somatic embryos into plantlets, and the best results were obtained with 3-6% sucrose or 3% fructose (Junaid, Mujib, Bhat, & Sharma, 2006). In contrast, the highest number of mature somatic embryos from *Ahirs nordmanniana* and the highest conversion percentage into plantlets were obtained in maltose-supplemented culture medium, as compared to sucrose (Norgaard, 1997). Similarly, an improved number of mature somatic embryos and conversion rate of *Castanea sativa* were obtained in culture medium supplemented with maltose (Corredoira, Ballester, & Vieitez, 2003).

In the present study, no differences were observed between treatments for the percentage of abnormal and abnormal achlorophyllous somatic embryos, indicating that the use of maltose instead of sucrose does not interfere in abnormal somatic embryos formation. However, when the number of normal achlorophyllous somatic embryos was evaluated, maltose-supplemented treatment resulted in higher rates (19.47%, as compared to 10.94% in sucrose-supplemented treatment). This result suggests that maltose may have a detrimental effect on the chlorophyll biosynthesis during somatic embryos conversion in *A. sellowiana*.

There are no reports in the literature about inhibition of chlorophyll formation due to maltose supplementation, and according to Thorpe et al. (2008), sugars do not inhibit the chlorophyll synthesis, except for sucrose in high concentrations. In the present work, the highest percentage of normal chlorophyllous somatic plantlets in response to sucrose treatment can be explained by the fact that sucrose represents the most efficient and abundant translocated sugar in plants, being the most important carbon source for non-photosynthetic tissues and for plant respiration (Taiz & Zeiger, 2009).

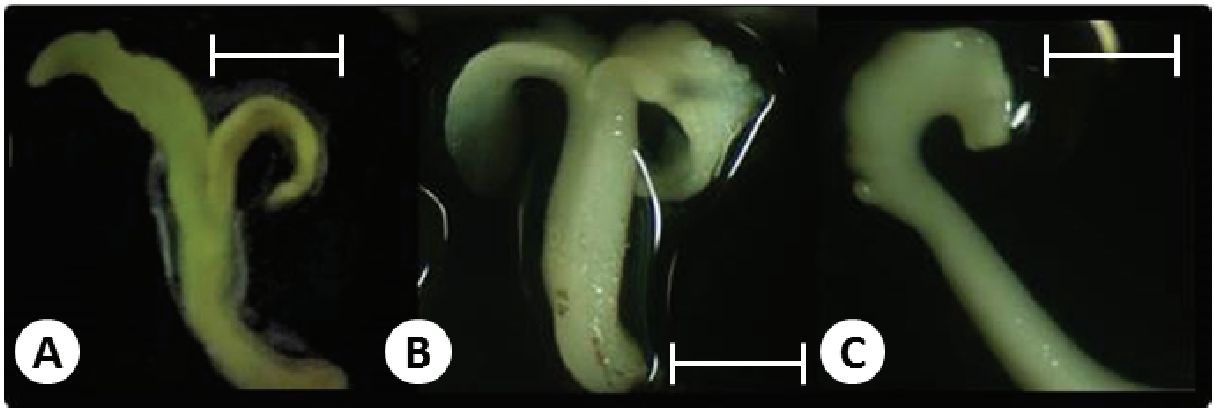

Figure 3. Morphological features of *A. sellowiana* somatic plantlets. A: Normal chlorophyllous somatic plantlet; B: Normal achlorophyllous somatic plantlet; C: Abnormal somatic plantlet. Bar: 1 mm.

In addition to being essential carbon sources, sugars also act as osmotic regulators in the culture medium, which may also have an effect on the percentage of chlorophyllous somatic plantlets observed in the present study. In an experiment to convert wheat somatic embryos, Zhou, Zheng, and Konzak (1991) observed a higher number of chlorophyllous plantlets in the sucrose-supplemented treatment compared to maltose. These authors attributed this result to the increased osmolarity of the culture medium in the sucrose-supplemented treatment due to its rapid hydrolysis and conversion to fructose and glucose (Zhou et al., 1991). Thus, the sucrose supplementation during somatic embryo conversion of *A. sellowiana* may be more suitable for chlorophyllous somatic plantlets formation.

Conclusion

The results indicated that GSH supplementation to the induction culture medium accelerates the process of somatic embryos formation and increases the embryonic synchrony in *A. sellowiana*. The use of maltose during somatic embryos conversion into plantlets showed no superior effect on the obtainment of normal chlorophyllous somatic plantlets compared to sucrose-supplemented treatment; however, this supplementation increased the formation of normal achlorophyllous somatic plantlets. Thus, the use of sucrose during somatic embryos conversion step has an apparently physiological advantage over maltose, besides its low cost and easy accessibility. As future perspectives, the manipulation of GSH/GSSG ratio during somatic embryo maturation may be an appropriate strategy to improve SE in this species. In addition, the use of other carbon sources and in different concentrations is seen as a possibility to improve the rate of somatic embryos conversion for this species.

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