Induction of somatic embryogenesis and evaluation of genetic stability in regenerated plants of *Magnolia dealbata.*

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Abstract

The utility of plant tissue culture for the mass propagation of trees is well known, but continuous *in vitro* multiplication of plant material may increase the possibility of somaclonal variation; therefore, it is essential to evaluate the genetic integrity of regenerants from species-specific *in vitro* protocols prior to mass production and implementation. The objectives of this study were: *1*) to determine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) concentration over two cycles of secondary somatic embryogenesis in *Magnolia dealbata*; and *2*) to verify the genetic stability of the regenerants obtained. The embryogenic response was not significantly affected by the concentration of 2,4-D but did vary across cycles of induction. The addition of 4.52 μM 2,4-D induced the highest total number of embryos (100.5), the mean number of somatic embryos (25.1) and somatic embryos per explant (80.6). In both 2,4-D concentration (2.26 or 4.52 μM), genetic integrity between the donor and the propagated clones was 0.90, and the low genetic instability (≤ 0.10 in both PGR treatments) might be due to effect of cyclic somatic embryogenesis or the different response of the explants at stress in *in vitro* culture conditions. However, it is necessary to examine more cell lines and somatic embryogenesis cycles.

Additional key words: 2,4-dichlorophenoxyacetic acid, *in vitro* culture, simple sequence repeats, somaclonal variation.

Introduction

Plant tissue culture is widely used for conservation and mass multiplication of several plant species (Kumari *et al*. 2017). *In vitro* regeneration involves two main morphogenetic routes: organogenesis and somatic embryogenesis (SE). For the propagation of some species, especially trees, regeneration *via* SE is preferred to organogenesis because *1*) large numbers of plants can be easily produced; *2*) chimerism is low; *3*) somaclonal variation is believed to be low; and *4*) fewer steps are required, which leads to lower cost (Gaj 2001).

Somatic embryogenesis can be generated from different explants. In trees (angiosperms), zygotic embryos have been preferentially used as the starting explant. Somatic embryos can be formed directly from the explant or through the formation of a callus, and it is often necessary to add plant growth regulators (PGR), principally auxins such as

2,4-dichlorophenoxyacetic acid (2,4-D). However, a close relationship has been found between 2,4-D concentration, length of time in culture, and the somaclonal variation (Clarindo *et al*. 2008). Thus, it is important to incorporate verification of the genetic integrity of propagated material into the micropropagation process (Shahzad *et al*. 2017), particularly when *in vitro* culture is used as an alternative for *ex situ* conservation of genetic diversity.

Molecular techniques are most commonly used to detect somaclonal variation, since they are efficient, unaffected by environmental factors, and the results are reproducible and trustworthy. Various conventional molecular markers, including random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and simple sequence repeats (SSR) have been used extensively in the assessment of clonal fidelity (Bhattacharyya *et al*. 2017). These have proven to be a polymorphic, rapid, and simple way to assess somaclonal variation.

Many studies on *in vitro* propagation have focused on optimal conditions for morphogenesis; however, conditions that are optimal for plant regeneration may not be optimal for maintenance of genetic integrity (Nehra *et al*. 1992). Some studies conducted with SE induction have reported ≥ 90 % genetic integrity, with little influence of type and concentration of PGR or length of time in culture (Harvengt *et al*. 2001, Lopes *et al*. 2006, 2009, Yang *et al*. 2008, Marum *et al*. 2009, Carloni *et al*. 2014, Li *et al*. 2014, Carra *et al*. 2016, Naz *et al*. 2016, Niazian *et al*. 2017, Raji *et al*. 2018). In contrast, there are also reports of high genetic variation: in *Picea mariana*, a phenotypic variation of 11 - 57 % was reported and was associated with genotype and length in culture (Tremblay *et al*. 1999). In *Quercus robur*, the duration of *in vitro* culture was correlated with changes in ploidy level (Endemann *et al*. 2001). In *Carica papaya*, different heteroploidy have been reported in 14 % of the plants analyzed (Clarindo *et al*. 2008). In *Coffea arabica*, instability in the ploidy was found after four months in a liquid medium (Clarindo *et al*. 2012).

Given the risk of somaclonal variation in any of the morphogenetic routes of propagation and in the subsequent manipulation of the cultures in repeated cycles, the evaluation of the genetic integrity of regenerated plants is necessary to establish limits for the application of the species-specific protocols of propagation. This is of the utmost importance when the objectives include the uniform production of genotypes, such as in conservation studies and integrated management plans. Based on this, the potential use of repetitive somatic embryogenesis was evaluated as an alternative propagation method for the conservation of *Magnolia dealbata*, a species endemic to Mexico and cataloged as endangered (SEMARNAT 2010) and near threatened (IUCN) (Rivers *et al*. 2016). *M. dealbata* has ornamental, medicinal, and pharmacological importance (Alonso-Castro *et al*. 2014). Studies conducted on this species have mainly focused on demographic, ecological (Gutierrez and Vovides 1997, Corral-Aguirre and Sánchez-Velásquez 2006, Valladares *et al*. 2006, Velazco-Macías *et al*. 2008, Sánchez-Velásquez and Pineda-López 2010) and pharmacological (Martínez *et al*. 2006, Domínguez *et al*. 2009, 2010, Flores-Estévez *et al*. 2013, Alonso-Castro *et al*. 2014) aspects, as well as on *in vitro* propagation (Mata-Rosas *et al*. 2006). These latter authors established a micropropagation protocol *via* somatic embryogenesis and suggested its use for propagation, conservation, and sustainable use. However, they did not study the genetic stability of individuals generated by this method, and there is little information available in the literature. The objective of this study was, therefore, to determine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) concentration on two repeated cycles of secondary somatic embryogenesis and to characterize the genetic stability of the regenerants obtained; this will help to establish the utility and application limits of propagation *via* somatic embryogenesis in *M. dealbata*.

Materials and methods

Somatic embryo induction: Mature fruits were collected of *Magnolia dealbata* Zucc. in Chilijapa, Hidalgo, Mexico. The criterion for the harvest was that the fruits showed the exposure of the seeds. Cultures were established according to Mata-Rosas *et al*. (2006) as follows: the sarcotesta was removed from the seeds and then the seeds were washed with liquid commercial detergent for 20 min and rinsed for 10 min with sterile distilled water. Surface disinfection was then performed with 70 % (v/v) ethanol for 2 min, followed by a commercial solution of 30 % (v/v) *Clorox* [5.25 % (m/v) sodium hypochlorite, Oakland, CA, USA] containing 0.1 % (v/v) *Tween 80* (*Sigma-Aldrich*, Saint Louis, MO, USA) for 15 min, and finally the seeds were rinsed three times with sterile distilled water, 5 min each. All steps were conducted with continuous stirring.

Embryos were excised aseptically from surrounding tissue (endosperm) under a stereomicroscope using fine forceps and scalpel and cultured on Woody Plant medium (WP; Lloyd and McCown 1980), with 0, 2.26, or 4.52 μM 2,4-D and 3 % (m/v) sucrose for induction. The pH was adjusted to 5.5 before the addition of 6.5 g dm^{-3} of agar (*Caisson Laboratories*, Smithfield, UT, USA). The culture media were sterilized at 120 ºC for 15 min. The cultures were incubated at 25 ± 1 ºC in darkness for 60 d. For each treatment, 60 embryos were cultured (2 embryos per culture flask and 30 repetitions per treatment). After 60 d, the resulting calluses were transferred to WP medium supplemented with 0.05 % activated carbon for 30 d for development and maturation of the somatic embryos.

Repetitive somatic embryogenesis: For induction of two cycles of secondary somatic embryogenesis, nine explants with somatic embryos were randomly chosen from each treatment (2.26 or 4.52 μM 2,4-D) from previous cycle induction, and ten primary torpedo to cotyledonary somatic embryos were individually selected from each explant and cultured on the induction medium as described above (5 embryos per culture flask and 2 repetitions per callus line).

Conversion of somatic embryos into plantlets: After one month in a WP medium supplemented with activated carbon, 30 individual torpedo to cotyledonary somatic embryos formed in each embryogenic induction cycle, and from each treatment (2.26 and 4.52 μM 2,4-D) were germinated on the WP medium (one embryo per tube culture). The cultures were incubated at 25 ± 1 ºC with a 16-h photoperiod provided by *T8 24W LED* tube lights (an irradiance of 55 μmol·m^{-2}·s^{-1}). Plantlets completely formed with a height of at least 5 cm were selected, and the agar was carefully removed from the roots with tap water, transferred to forest soil (loam / compost / vermicompost / pumice, 1:1:1:1), and cultured in a greenhouse at 28 °C and a relative humidity of ~80 %.

Genetic stability: Fresh leaves of 90 plants from each treatment (2.26 and 4.52 μM 2,4-D) were collected from SE regenerated plants. Total genomic DNA was isolated
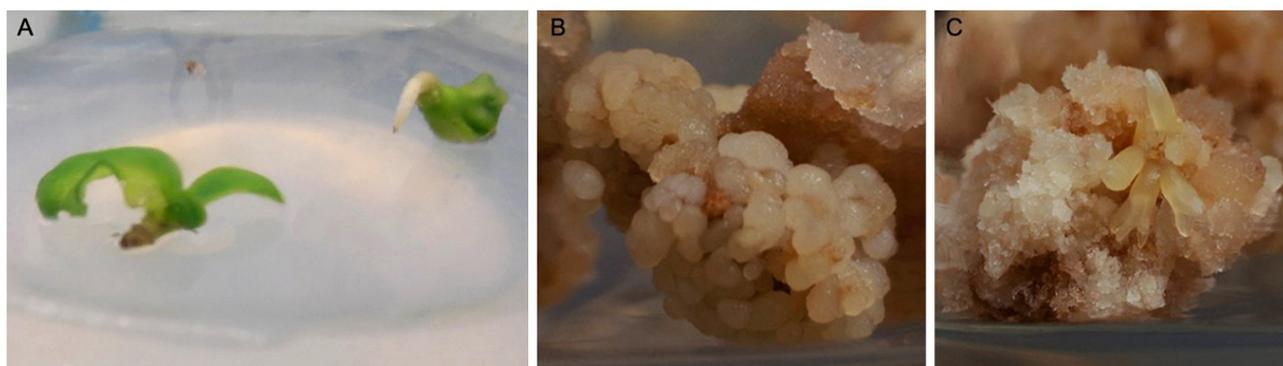
Fig. 1. *A* - Germination of the zygotic embryo of *Magnolia dealbata* cultured in Woody Plant (WP) medium without plant growth regulators. *B,C* - Induction of somatic embryos in WP medium with 2.26 and 4.52 µM 2,4-D, respectively.

following the CTAB protocol (Doyle and Doyle 1987). In order to evaluate genetic stability, six microsatellite loci specific for *Magnolia dealbata* were used (Veltjen *et al.* 2019; Table 1 Suppl.). One of the primers of each pair was marked with 1 of 4 fluorescent dyes (ABI: 6-FAM, VIC, NED or PET). The reaction was conducted in a volume of 5 mm³ containing 5 ng DNA, 0.2 µM of each primer and 2.5 mm3 of 2× *QIAGEN Multiplex* (3 mM MgCl$_2$). The amplification conditions were one cycle at 95 ºC for 15 min, 30 cycles at 94 ºC for 30 s, 58 ºC for 30 s, and at 72 ºC for 1 min, followed by a final extension step at 72 ºC for 30 min. The PCR amplification was repeated twice. The microsatellite fragment lengths were determined using capillary electrophoresis with an *ABI Prism 3100* genetic analyzer (*Applied Biosystems*, Foster City, CA, USA), using *LIZ 600* as a marker and the program *Peak Scanner 2.0* (*Applied Biosystems*). A positive control was used for the correct assignment of the alleles, while genotyping was conducted according to Selkoe and Toonen (2006).

Statistical analysis: Every three months of culture, the response of somatic embryogenesis was considered as the percentage of explants that presented somatic embryos, the mean and total number of somatic embryos, as well as the number of somatic embryos per explant, and the number of somatic embryos at each developmental stage (globular, heart, torpedo, and cotyledonary). All data were analyzed using a non-parametric Kruskal-Wallis test for *n* independent groups and a Mann-Whitney test for analysis of two independent groups. Differences were considered significant at the level of 5 %. The program *SPSS 24* was used. Graphs were produced with the package *Excel 2011*.

With the online program *GenePop* (Raymond and Rousset 1995), deviation from the Hardy-Weinberg equilibrium (HWE) was recorded at each locus, assuming as an alternative hypothesis the equilibrium deficiency of heterozygotes and the linkage disequilibrium (LD) between each pair of loci with 1 000 dememorization steps, 100 batches and 1 000 iterations. Besides, the frequency of null alleles was estimated for each locus using *FreeNA* (Chapuis and Estoup 2007) with 0.2 as a threshold for retaining the markers for use. The program *Micro-Checker 2.2.3* (Van Oosterhout *et al*. 2004) was used to review possible errors of genotypification. The genotype of each

explant donor of the first cycle was used as reference to evaluate the genetic similarity between donor and their clones (Table 2 Suppl.). A genetic distance tree was constructed to assess genetic relatedness between isolates using Provesti's distance with 1 000 bootstrap replicates.

Results and discussion

Development of somatic embryos: The formation of callus and embryogenic masses was recorded from the two treatments with 2,4-D. In the absence of 2,4-D, the zygotic embryo germinated, and no additional morphogenetic response was observed (Fig. 1). At 30 d, callus formation was evident along the length of the explant. At 45 d, the formation of embryogenic masses and some somatic embryos in the globular stage were observed. After 30 d on WP medium without 2,4-D and with activated carbon, embryos from the globular to cotyledonary stages were observed. The formation of somatic embryos from the two 2,4-D treatments was asynchronous, without favouring any stage of development (Fig. 2). Concerning the repetitive cycles of SE, at 4.52 µM 2,4-D, a high number of embryos was observed in both first and second cycle, although their number decreased in the third cycle. The same pattern was presented only in the heart and torpedo stages using 2.26 µM 2,4-D. When we compared embryo formation per stage between the two concentrations of 2,4-D, a significant difference was established only for the torpedo stage at the second cycle, where a higher number of embryos was induced per explant at 4.52 µM 2,4-D (288 embryos in total and an average of 36 embryos per explant). In the induction cycles at 2.26 µM 2,4-D, a statistically significant difference for embryo number was only observed at the torpedo stage of the first cycle, where the lowest average was recorded (6.3 embryos per explant). In the treatment with 4.52 µM 2,4-D, the cycle two was statistically different for the torpedo stage, and in the heart stage, the third cycle differs from the second (Fig. 3).

While it has been suggested that PGR can have multiple effects depending on their concentration or the developmental stage of the embryo (Giridhar *et al*. 2004), in this study, the percentages of explants with formation
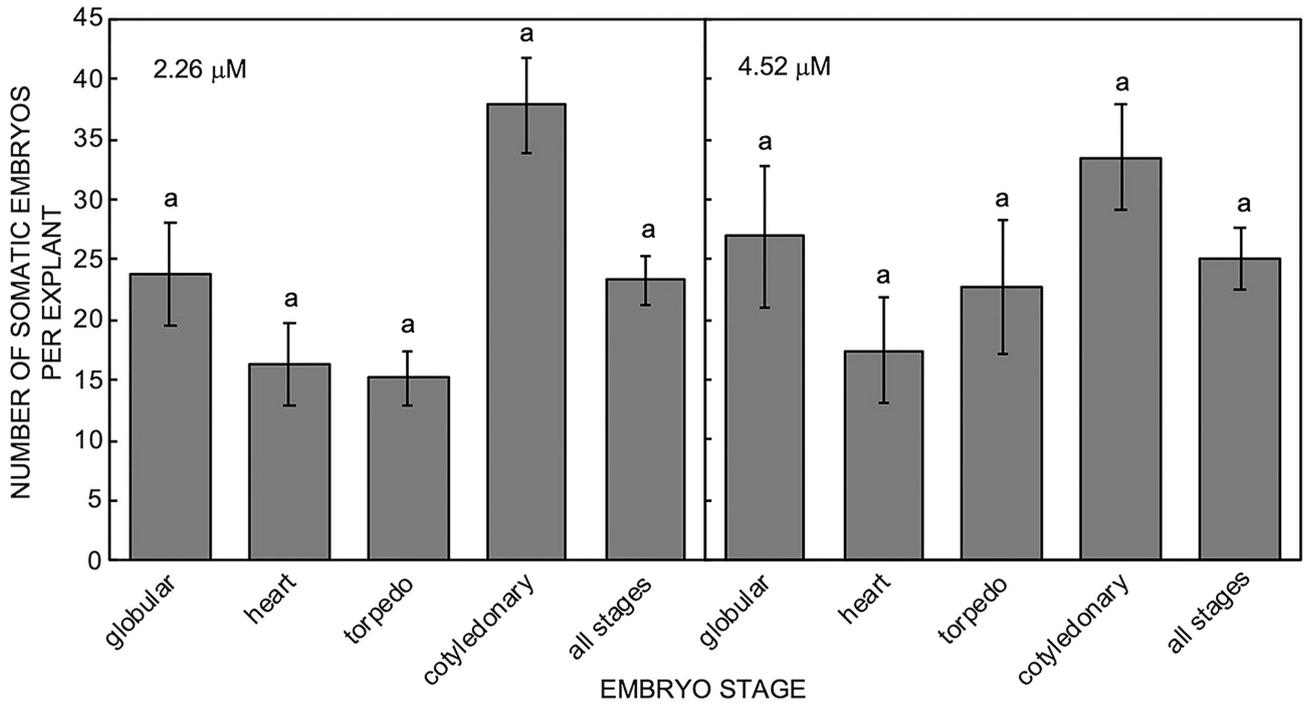
Fig. 2. Effect of 2,4-D concentration on the development of somatic embryos of *Magnolia dealbata* across all cycles. Means ± SEs, $n = 60$. Values marked with the same letter do not differ significantly at $P > 0.05$ (the Mann-Whitney test).
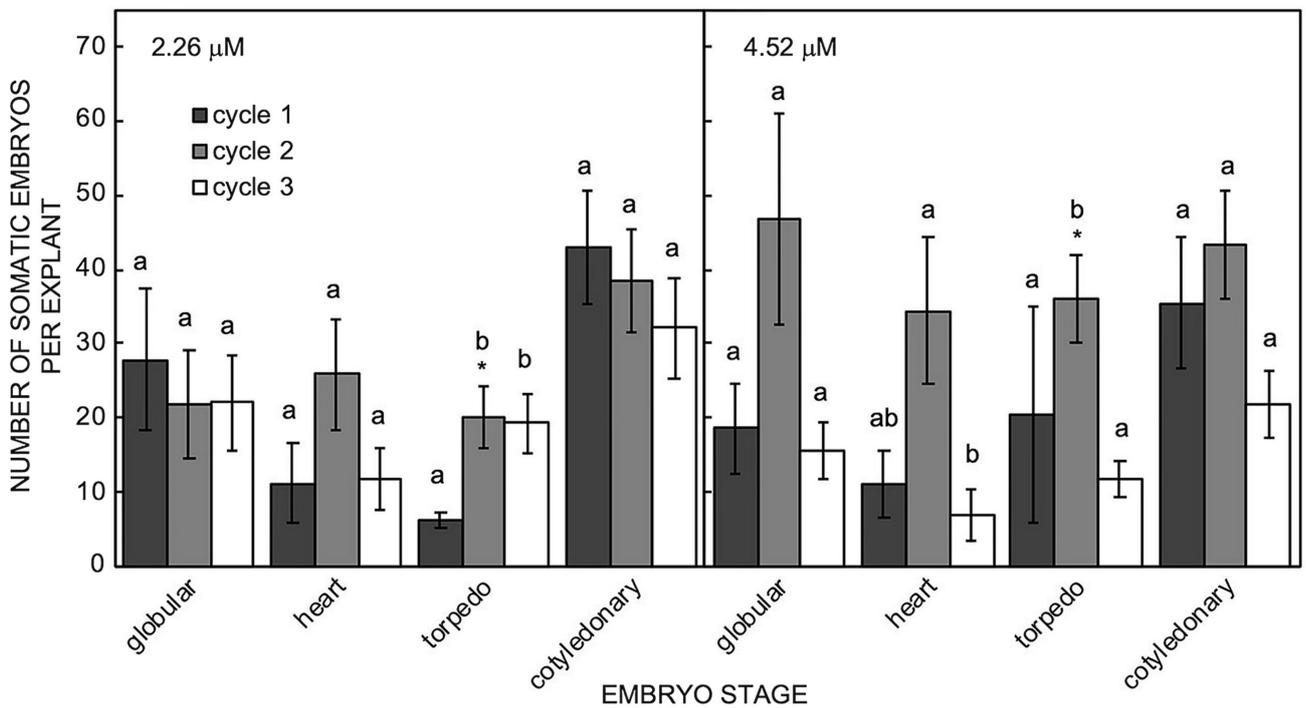

Fig. 3. Effect of cycles on the development of somatic embryos of *Magnolia dealbata* treated with 2,4-D. Means ± SEs, $n = 9$. Values marked with the same letter within the same concentration and embryo stage do not differ significantly at $P > 0.05$ (the Mann-Whitney test); * - difference between concentrations for the developmental stage compared within the same cycle.
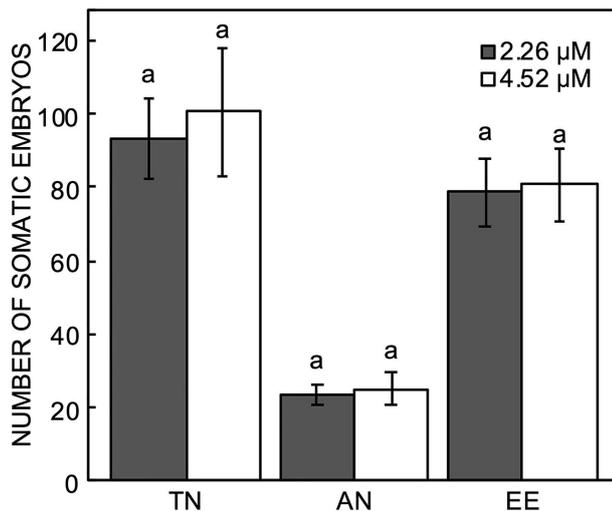
Fig. 4. Effect of 2,4-D concentration on embryogenic response: total (TN) and average (AN) numbers of somatic embryos and somatic embryos per explant (EE) in *Magnolia dealbata*. Means ± SEs, *n* = 60. Values marked with the same letter within response types do not differ significantly at *P* > 0.05 (the Mann-Whitney test).

(100.5), the mean number of somatic embryos (25.1), and number of somatic embryos per explant (80.6) (Figs. 4 and 5).

In the present study, the percentages of explants with somatic embryos were lower compared to the 85 % response reported by Mata-Rosas *et al.* (2006) for the same species with 4.5 μM 2,4-D. It is possible that the different percentages of somatic embryo formation recorded in the present study and that reported by Mata-Rosas *et al.* (2006) was related to genotype and physiological stage of the explant, since the biological material was collected from different localities (Coyopolan, Veracruz *vs.* Chilijapa, Hidalgo) and maybe from fruits with different stage of maturation. Some studies indicated that the somatic embryogenesis is determined mainly by the genotype and physiological stage of the explant (Molina *et al.* 2002, Wilhelm *et al.* 2005, Li *et al.* 2014, Henao *et al.* 2018). In some cases, induction is higher when young zygotic embryos are used, but in other species, the maximum efficiency of SE is reached in the most advanced stage of development (Gaj 2004, Priyono *et al.* 2010).

Only at 4.52 μM 2,4-D did the cycles of induction differ significantly among all variables evaluated. The results suggested that the percentage of explants with somatic embryos decreased with each cycle of induction in both 2,4-D concentrations, although only the third cycle was significantly different at 4.52 μM 2,4-D (Fig. 6). The total and the average number of somatic embryos and the number of embryos obtained per explant all increased in

of somatic embryo was similar in the two concentration of 2,4-D (38.4 and 36.7 % at 2.26 μM and 4.52 μM, respectively). However, the addition of 4.52-μM 2,4-D induced the highest values on the total number of embryos

Fig. 5. Effect of 2,4-D concentration on the total number of embryos of *Magnolia dealbata*. Treatment with 2.26 μM (*A*) and 4.52 μM (*B*) 2,4-D in Woody Plant medium.
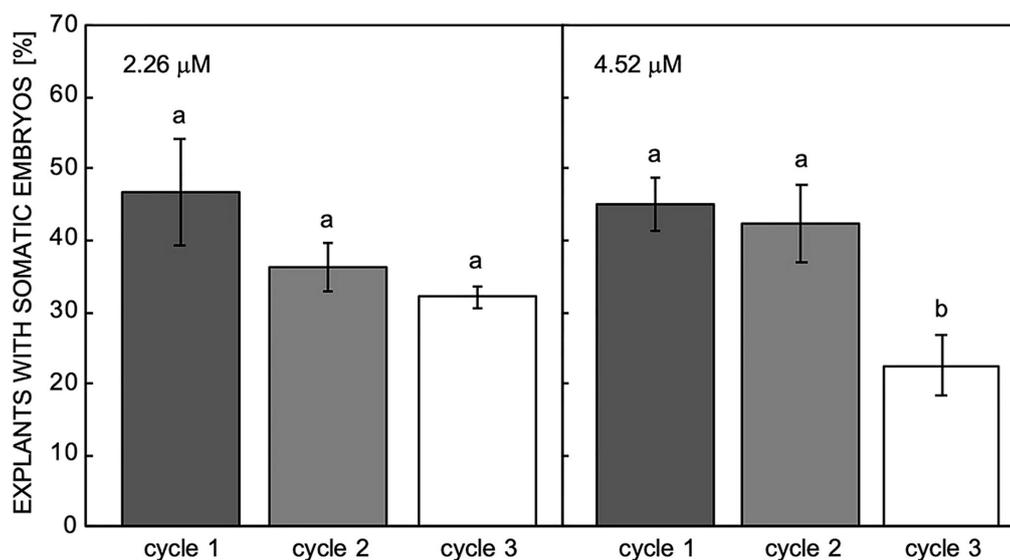
Fig. 6. Effect of cyclic embryogenesis on percentage of explants that presented somatic embryos in *Magnolia dealbata*. Means ± SEs, $n = 9$. Values within the same concentration and response types marked with the same letter do not differ significantly at $P > 0.05$ (the Mann-Whitney test).
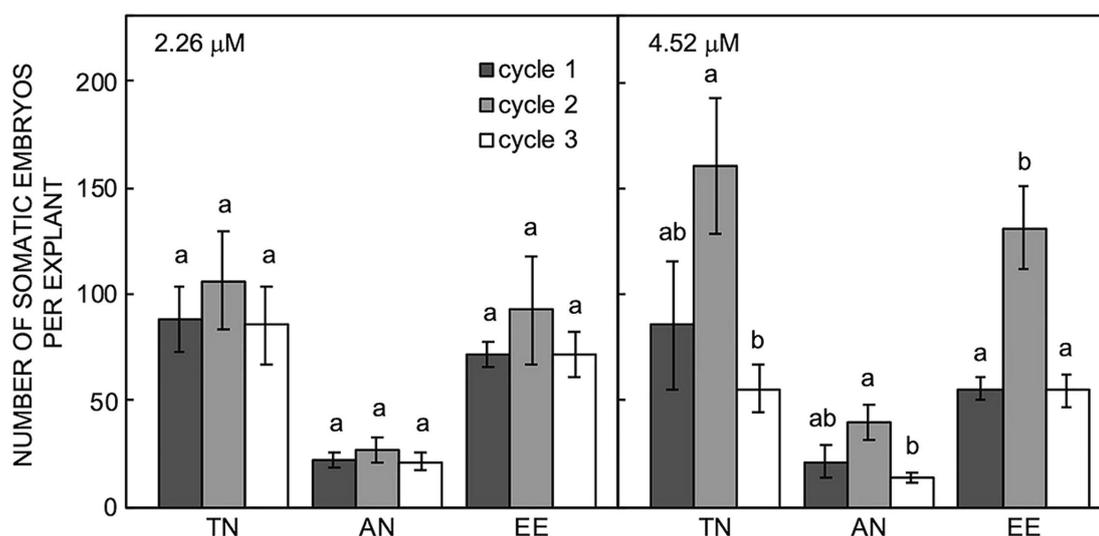

Fig. 7. Effect of cycles on embryogenic response: total (TN) and average (AN) numbers of somatic embryos and somatic embryos per explant (EE) in *Magnolia dealbata* treated with 2,4-D. Means ± SEs, $n = 9$. Values within the same concentration and response types marked with the same letter do not differ significantly at $P > 0.05$ (the Mann-Whitney test).

the second cycle of induction and subsequently diminished (Fig. 7). Similar responses have been reported for cabbage and cauliflower (Pavlović *et al*. 2013). Our results contrast with some other studies, such as in *Musa acuminata* AAA cv. Grand Naine, where embryogenic potential did not decline with increased cycles of multiplication (Remakanthan *et al*. 2014) or the efficiency of secondary SE is higher than primary SE, such as in *Arachis hypogaea*, *Beta vulgaris*, *Camellia japonica*, *Glycine max*, *Picea abies*, *Medicago sativa, Vitis* sp. and *Helianthus maximiliani* (Raemakers *et al*. 1995, Vasic *et al*. 2001).

In the somatic embryos transferred to the WP medium, the presence of the cotyledons was evident after two weeks. After one month, the embryos formed roots and in some cases, true leaves. Germination of the embryos was 90 and 80 %, from the treatments with 2.26 and 4.52 μM 2,4-D, respectively. After six months, the plantlets were transferred to trays with forest soil for acclimatization and growth.

The regenerants of *M. dealbata* presented polymorphism at all loci examined except for locus Mmc493 at 2.26 μM 2,4-D. The degree of variability differed among loci, with locus Mmc059 presenting the highest rate of mutation.

Genetic stability can be described as the fidelity or unaltered maintenance of the genetic profile following successive cycles of multiplication. However, a 15 - 10 % genetic variability among regenerants and maternal plants might be considered as low (Zoghlami *et al*. 2001, Sedov

Fig. 8. A dendrogram generated for data from microsatellite marker illustrating the Provesti's distance with 1 000 bootstrap replicates among regenerated plants from three embryogenic calli in three culture times. Treatment with 2.26 µM 2,4-D in Woody Plant medium.
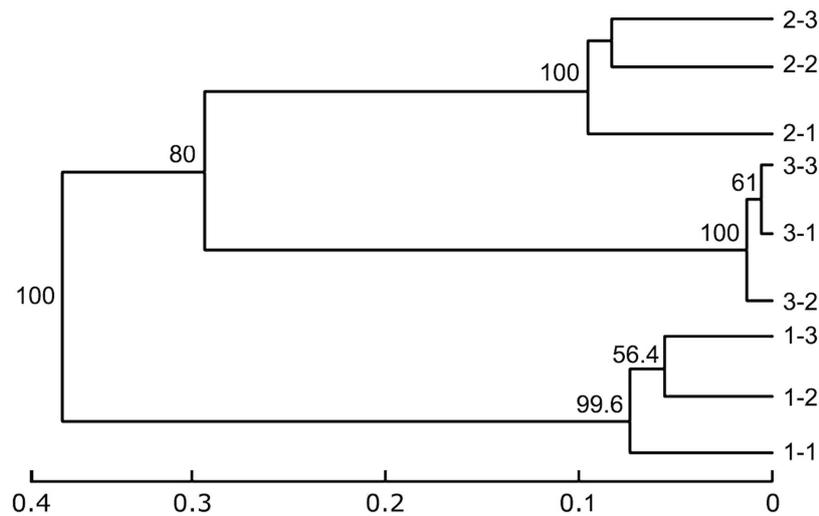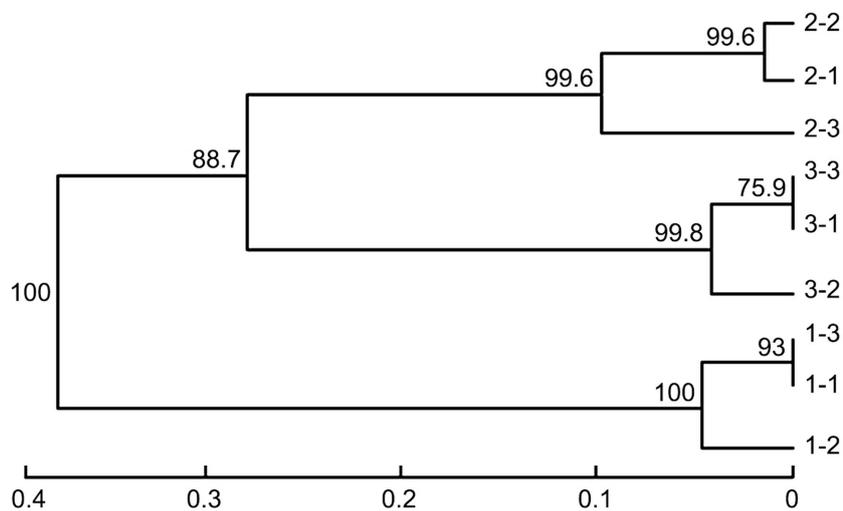

Fig. 9. A dendrogram generated for data from microsatellite marker illustrating the Provesti's distance with 1 000 bootstrap replicates among regenerated plants from three embryogenic calli in three culture times. Treatment with 4.52 µM 2,4-D in Woody Plant medium.

et al. 2014). Based on this, the genetic profile obtained in this study indicates that there are small changes between the donor and the propagated clones at both concentrations of 2,4-D used (90 % of genetic stability, Figs. 8 and 9), but this can be considered acceptable polymorphism. Although PGR level may affect somaclonal variation indirectly by stimulating a rapid and disordered cell proliferation (Soares *et al*. 2016), the two auxin concentration used did not have a differential effect on genetic stability in *Magnolia dealbata*.

Within the concentrations of 2,4-D, genetic stability varied between explants (embryogenic callus line); although these variations are acceptable (< 20 %) (Figs. 8 and 9), genetic distances among explants varied from 0.02 to 0.18 with 2.26 µM 2,4-D while from 0.05 to 0.13 at 4.52 µM 2,4-D. The differential response of explants might

be attributable to differences in sensitivity to the stresses caused by *in vitro* culture (Bradaï *et al*. 2016). Other studies have indicated that the genotype/species plays an important role in the frequency of genetic alterations during somatic embryogenesis (Burg *et al*. 2007). The genotype, conditions and culture of the *in vitro* culture are considered to be parameters that influence genetic stability; however, some studies do not show a significant effect from these factors (Burg *et al*. 2007, Carloni *et al*. 2014, Mamedes-Rodrigues *et al*. 2018). In the present study, although the explants showed different responses to the time of propagation, in general, we observed a reduction in genetic fidelity with increased cycles of induction at 2.26 µM 2,4-D (≈3 %). With 4.52 µM 2,4-D, the genetic stability decreased only at the first cycle of secondary somatic embryogenesis (≈1 %). The indirect

SE in *M. dealbata* may reduce the genetic uniformity of the regenerants through the callus phase presented both during primary and secondary SE (Sharma *et al.* 2007, Rai *et al.* 2012). The culture environment might also present stresses leading to a loss of programmed cellular control (Mehta *et al.* 2011). In *Pinus pinaster* and *Quercus robur*, prolonged propagation reduced genetic stability (Endemann *et al.* 2001, Marum *et al.* 2009).

The values of genetic instability in this study (≤ 10 %) correspond to absent to low levels of variation reported for SE (0 - 10 %) as in *Cassia occidentalis*, *Ipomoea batatas*, *Picea abies*, *Distylium chinense*, *Quercus robur*, *Psidium guajava*, *Olea maderensis*, *O. europea*, etc. (Fourré *et al.* 1997, Lopes *et al.* 2006, 2009, Valladares *et al.* 2006, Triqui *et al.* 2008, Yang *et al.* 2008, Mehta *et al.* 2011, Rai *et al.* 2012, Bobadilla *et al.* 2013, Li *et al.* 2014, Carra *et al.* 2016, Morais-Lino *et al.* 2016, Naz *et al.* 2016, Vinoth and Ravindhran 2016). It is possible that the explants selected may be less susceptible to genetic instability (DeVerno *et al.* 1999) and, as a consequence, the results differ from those reported by others using the same morphogenetic route with intermediate levels of variation (17 - 31 %) (Müller *et al.* 1990, López *et al.* 2004, Wilhelm *et al.* 2005, Burg *et al.* 2007, Prado *et al.* 2010, Dey *et al.* 2015).

This report provides important and valuable information on secondary somatic embryogenesis and somaclonal variation in the species *Magnolia dealbata*. Only induction cycles had a significant effect on secondary somatic embryogenesis. Molecular analysis on 180 regenerants from two concentrations of 2,4-D showed that polymorphism between donor and clones was low (≤ 10 %), with differences observed with the number of cycles of induction and explants. Based on our findings, we conclude that this method of propagation *via* somatic embryogenesis in this species is associated with low risk of genetic variability, although it might increase over successive subcultures. For this reason, we recommend evaluating this propagation method for *M. dealbata* in more than three cycles of culture. Levels of genetic stability differ among explants, and it is therefore suggested to evaluate the feasibility of micropropagation systems in more than one cellular line, to avoid overestimation of their application in studies of conservation, restoration or management of resources and thus ensure the preservation of the genetic integrity of the regenerants.

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