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In vitro shoot production, morphological alterations and genetic instability of *Melocactus glaucescens* (Cactaceae), an endangered species endemic to eastern Brazil

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ABSTRACT

Melocactus glaucescens is a cactus endemic to Brazil. *In vitro* shoot production represents a viable alternative to conventional propagation, since *M. glaucescens* grows slowly and requires about ten years to reach its reproductive phase. *M. glaucescens* can only reproduce by seed and does not ramify or emit lateral shoots. The present work evaluated the morphogenic potential of *M. glaucescens* by (i) using variations in explant fragmentation and orientation; (ii) the use of plant growth regulators (PGRs); (iii) and the occurrence of somaclonal variation. The results suggest that number of shoots decreased with greater explant fragmentation. Shoot induction can be achieved in culture media with or without PGRs. The evaluation of the morphogenic responses of *M. glaucescens* explants to PGRs revealed that, when combining naphthalene acetic acid (NAA) with benzylaminopurine (BAP) or kinetin (KIN), BAP yielded a greater number of shoots than KIN. However, with BAP concentrations of 4.44, 8.88, and 17.76 μM, shoots were morphologically different from the control plants. The ISSRs analysis showed polymorphisms in shoots produced with or without PGRs and suggests that other factors might affect the genetic stability of *M. glaucescens*. Our results indicate that *M. glaucescens* can be propagated using *in vitro* techniques with or without PGRs. Apical cladode segments can grow in PGR-free medium and can be used as a source of explants. The technique established in this work reduces the removal of seeds from natural populations, and therefore, does not interfere in the reproduction dynamics of this species in its natural habitat.

1. Introduction

The family Cactaceae comprises a large number of species showing wide diversity, many of which have high commercial value as ornamental plants and are illegally collected from their natural habitats (Goettsch et al., 2015; Pérez-Molphe-Balch et al., 2015). The genus *Melocactus* comprises 37 species that are largely concentrated in eastern Brazil, the primary center of diversity for this genus (Taylor, 2000). In nature, most members of the genus reproduce sexually. In addition, these species do not ramify or produce lateral shoots, unless the plant suffers some injury (Machado, 2009). There are 23 *Melocactus* species and 11 subspecies occurring in Brazil, of which 21 and 11, respectively, are endemic to this country, including *Melocactus glaucescens* Buining & Brederoo (Zappi et al., 2017). The high degree of endemism of these plants associated with factors, such as habitat degradation and harvesting for commercial and ornamental purposes have resulted in numerous Cactaceae species being threatened with extinction (Goettsch et al., 2015). This is the case of *M. glaucescens*, which has been listed as endangered in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017) and in the IUCN Red List of Threatened Species (IUCN, 2017).

Tissue culture techniques represent a viable alternative to the conventional propagation of this species, which grows slowly and requires about ten years to reach its reproductive phase (Lema-Rumińska and Kulus, 2014; Pérez-Molphe-Balch et al., 2015).

The number of shoots produced by tissue culture techniques can vary according to plant genotype, explant type, as well as concentration and nature of plant growth regulators (PGRs) used (Rocha et al., 2014; Kulus, 2015; Krishna et al., 2016). In order to increase *in vitro* shoot production and reduce the number of original plants utilized, additional strategies, including fragmentation and various explant orientation in the culture media, can be also considered. The use of PGRs, such as auxins and cytokinins, usually increases the quantity of shoots (Rocha et al., 2014). In general, absence or low levels of auxin, together with

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moderate to high levels of cytokinins increase shoot production in cacti (Pérez-Molphe-Balch et al., 2015). However, other combinations are also possible (Lema-Rumińska and Kulus, 2014).

Although it is possible to produce genetically uniform plants as a product of clonal multiplication, somaclonal variation may occur (Larkin and Scowcroft, 1981; Krishna et al., 2016). Therefore, it is important to select a multiplication method that minimizes risks of genetic changes and provides a high number of shoots produced *in vitro* (Grover and Sharma, 2016). Molecular markers can be efficiently used to analyze genetic variation of *in vitro* shoots. Among DNA molecular markers, Inter Simple Sequence Repeats (ISSRs) is notable due to its repeatability, relatively low-cost and easy analysis, added to its capacity to reveal polymorphisms (Grover and Sharma, 2016). This marker has been commonly used to evaluate plants produced with *in vitro* tissue culture techniques, because it allows for the differentiation between closely related genotypes, regardless of the developmental stage (Bhattacharya et al., 2010; Grover and Sharma, 2016).

In spite of the high commercial appeal of many cactus species, relatively few attempts have been made to use tissue culture for reproduction, in contrast to the extensive efforts made with other plant families (Lema-Rumińska and Kulus, 2014; Pérez-Molphe-Balch et al., 2015). In the *Melocactus* genus, few studies of *in vitro* shoot production are known (Retes-Pruneda et al., 2007; Monostori et al., 2012; Pérez-Molphe-Balch et al., 2015). Currently, there are no studies related to *in vitro* shoot production of *Melocactus glaucescens*. Therefore, basic studies are needed so that future studies can explore the ornamental and economic potential of the species.

The objective of this work was to establish an efficient protocol for *in vitro* shoot production of *M. glaucescens* that can be used for commercial ornamental purposes, in cactus collections, and *in vitro* conservation. For this purpose, we evaluated 1) the morphogenic potential of *M. glaucescens* explants by using different fragmentations and orientations, 2) the effect of PGRs, 3) morphological alterations and 4) the occurrence of somaclonal variation. Currently, there are no known studies investigating the possible causes of morphological alterations in cacti. This is the first molecular work to study the relationship between morphological and genetic alterations in cacti.

2. Material and methods

2.1. Plant material

The explants were obtained from *in vitro* germination of *M. glaucescens* seeds collected from natural populations located in Morro do Chapéu from Bahia State, eastern Brazil (11°29′38.4″S; 41°20′22.5″ W). The seeds were disinfected with ethanol 96% for 1 min, NaClO 2% for 10 min, and subsequently washed tree times in sterile water. Afterwards, seeds were germinated in 250 mL flasks containing 50 mL of MS culture media (Murashige and Skoog, 1962) at half-salt concentration (½MS) supplemented with 15 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The pH of the media was adjusted to 5.7 before chemical sterilization (Teixeira et al., 2006).

2.2. Tissue culture conditions

Cultures were maintained at 25 ± 3 °C under fluorescent light with photosynthetically active radiation levels of approximately 60 μmol m^{-2} s^{-1} and a 16/8-h light/dark photoperiod during the entire experiment.

2.3. In vitro shoot induction

2.3.1. The effect of explant fragmentation and orientation

Plants germinated *in vitro* for 200 days had their apical cladode segments removed (Fig. 1A) and were initially sectioned transversally, generating six types of explants in disc format with 3–4 mm (Fig. 1B) or 1.5–2 mm of height (Fig. 1D), which were themselves sectioned either transversally (Fig. 1C) or longitudinally (Fig. 1H and E) and placed in vertical (Fig. 1B, D, F and I) or horizontal (Fig. 1G and J) position in the medium, originating six treatments (Fig. 1B, D, F, G, I and J). These explants were inoculated in glass tubes (25 × 150 mm) containing 15 mL of MS culture media supplemented with 30 g L^{-1} sucrose and 6.5 g L^{-1} agar (PGR-free). The pH of the media was adjusted to 5.7 before chemical sterilization (Teixeira et al., 2006).

The experiment was conducted in a completely randomized design, with six replicates composed of five tubes each (one explant per tube). Ninety days after inoculation, we evaluated the percentage of explants that produced shoots (%E) and the number of shoots per explant (NS). The total number of explants inoculated was considered 100%.

2.3.2. The effect of plant growth regulators

Plants germinated *in vitro* had their apical cladode segments removed (Fig. 1A) and were sectioned transversely generating explants in disc format with a height of 3–4 mm and 20 areola (meristem regions) (Fig. 1B). This type of explant was used in all experiments with PGRs. The explants were inoculated in vertical position in glass tubes (25 × 150 mm) containing 15 mL of MS culture media with PGRs added, supplemented with 30 g L^{-1} sucrose, and 6.5 g L^{-1} agar. The pH of the media was adjusted to 5.7 before chemical sterilization (Teixeira et al., 2006). Shoots originated from explants inoculated in PGR-free medium were considered as control.

2.3.2.1. BAP as source of cytokinin. The explants derived from plants germinated *in vitro* for 170 days were inoculated in media with five different concentrations of benzylaminopurine (BAP; 0.0, 2.22, 4.44, 8.88 and 17.76 μM) combined with three concentrations of naphthalene acetic acid (NAA; 0, 0.67 and 1.34 μM). The percentage of explants with shoots (%E) and number of shoots per explant (NS), and number of shoots morphologically different from the control were evaluated 110 days after inoculation.

2.3.2.2. KIN as source of cytokinin. In the first experiment, morphological alterations related to BAP were observed, so kinetin (KIN) was used as source of cytokinin. Explants derived from plants germinated *in vitro* for 340 days were inoculated in media containing five different concentrations of KIN (0.0, 2.22, 4.44, 8.88 and 17.76 μM) combined with three different concentrations of NAA (0.0, 0.67 and 1.34 μM). Evaluations of percentage of explants with shoots (%E), number of shoots per explant (NS) and number of shoots morphologically different from the control were scored 120 days after inoculation.

2.3.2.3. Experimental design. Samples were randomized and assigned to each treatment in a factorial scheme (cytokinin concentration × auxin concentration) with eight replicates composed of three tubes each (1 explant per tube).

2.3.2.4. Molecular analyses. In order to investigate if the morphological alterations observed in shoots produced from different concentrations of BAP and NAA had a genetic origin, plants germinated *in vitro* for 400 days had the apical cladode segments removed (Fig. 1A) and were sectioned transversely. Apical cladode segments of each donor plant were kept *in vitro* in ½MS supplemented with 15 g L^{-1} sucrose and solidified with 6.5 g L^{-1} agar without PGRs to allow growth and subsequent utilization as mother plants (See arrow of Fig. 1A). The explants (Fig. 1B) were inoculated on media with four different concentrations of BAP (0.0, 4.44, 8.88 and 17.76 μM) combined with three concentrations of NAA (0.0, 0.67 and 1.34 μM), generating 12 treatments. The treatment with 2.22 μM BAP was not considered in these analyses, due to the lack of morphological alterations observed in shoots produced in this treatment.