Notas Científicas

Salt concentrations in culture media for the development of *Dipteryx alata* in vitro

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Abstract – The objective of this work was to evaluate the effect of salt concentrations in culture media on the early development of "barueiro" (*Dipteryx alata*) seedlings, obtained from the shoot apex, in vitro. Two different in vitro culture media – Murashige & Skoog (MS) and wood plant medium (WPM) – in different salt concentrations (25, 50, 75, and 100%) were used. At 120 days, the *D. alata* seedlings in the MS medium, in its original concentration (100%), developed better than in other concentrations or in the WPM. Therefore, the MS medium was considered the most suitable for ensuring in vitro establishment because it provided greater root length (27.65 cm) and number of leaves per plant (26 leaves) than the other medium.

Index terms: Cerrado biome, culture media, osmoregulation, plant growth, tissue culture.

Concentrações de sais em meios de cultivo para o desenvolvimento de barueiro in vitro

Resumo – O objetivo deste trabalho foi avaliar o efeito de diferentes concentrações de sais em meios de cultivo no desenvolvimento inicial de mudas de barueiro (*Dipteryx alata*), provenientes de ápice caulinar, in vitro. Foram utilizados dois meios de cultivo in vitro – Murashige & Skoog (MS) e "wood plant medium" (WPM) – em diferentes concentrações de sais (25, 50, 75 e 100%). Aos 120 dias, as plântulas de *D. alata* no meio MS, em sua concentração original (100%), apresentaram melhor desenvolvimento que as em outras concentrações ou em meio WPM. Portanto, o meio MS mostrou ser o mais indicado para garantir o estabelecimento in vitro, uma vez que propiciou maior comprimento da raiz (27,65 cm) e número de folhas por planta (26 folhas) que o outro meio.

Termos para indexação: Cerrado, meio de cultura, osmorregulação, crescimento vegetal, cultura de tecidos.

"Barueiro" (*Dipteryx alata* Vog.), a plant species belonging to the family Fabaceae, found in the Cerrado biome, has multiple uses (Sano et al., 2004) and a growing market demand (Magalhães, 2014). However, effective off-site production of this species is challenging (Magalhães, 2014), owing to the limited knowledge of propagation techniques for the species.

Among various plant propagation techniques, in vitro cultivation has allowed for the large-scale production of seedlings with superior phytosanitary and genetic qualities (Shahzad et al., 2017). The few known reports of micropropagation of *D. alata* relate to seed germination. However, these reports do not specify which culture medium is the most suitable for the in vitro development of the species (Silva et al., 2016b). Other authors have proposed alternatives for providing nourishment in barley explants, such as supplementation of the Murashige & Skoog (MS) medium with banana pulp and coconut water (Silva et al., 2016a); this study however, could not infer on the most promising protocol for the in vitro development of *D. alata*. This gap in knowledge also exists for other species of the genus *Dypteryx*, such as *D. odorata* (Aubl.) Willd. and *D. oleifera* Benth.; for these species, protocols for cells in suspension culture have been defined, but with a focus on pharmacochemical aspects (Gómez & Atehortúa, 2013). In fact, the determination of the ideal nutrient medium (including nutrient

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concentration) for in vitro development is a necessary first step for any micropropagation protocol.

Culture media are the basis of in vitro development protocols because they provide explants with substances essential for growth and development, and the growth response can be differentiated between media. This response is based on the interaction of explants with different mineral components and concentrations of plant hormones and/or salts and/or other elements present in the medium. The most common culture media in studies used to determine in vitro multiplication protocols are MS and woody plant medium (WPM) (Yeung et al., 2015). The MS medium (Murashige & Skoog, 1962) formulated for in vitro culture of tobacco (*Nicotiana* sp.) cells is useful for most herbaceous plant species; however, woody species are often not responsive to the original composition of this medium. For the cultivation of woody species, modifications, such as a reduction in the macronutrient content, result in a better growth/development rate (Yeung et al., 2015; Shahzad et al., 2017). This is because concentration of salts or other osmotically active compounds that constitute the nutrient medium may trigger the activation/inactivation of metabolic pathways that culminate in embryo and/or seedling development.

However, substituting the MS medium with WPM (Lloyd & Mccown, 1980), which was originally formulated for woody species and has diluted nutrient concentrations (Melo et al., 1998), may also be a viable option. Notably, positive results have been obtained with the WPM, unlike the MS medium, when used for micropropagation of some woody species of the savanna, such as *Eugenia involucrata* O. Berge (Anis et al., 2010; Golle et al., 2012). These differences constitute the main reason for the constant search for alternatives and/or adaptations of protocols based on both media, to determine the best in vitro development conditions for a plant species that has not yet been studied.

The objective of this work was to evaluate the effect of salt concentrations in culture media for the early development of *D. alata* seedlings in vitro, obtained from the shoot apex.

To this end, ripe *D. alata* fruits, dispersed by the mother plant, were collected across four matrices located in the municipality of Uberlândia, in the state of Minas Gerais, Brazil (19º08'46"S, 48°22'41"W). Each one of the matrix plants was between 2 and 8 m apart. After collection, the fruits were taken to the laboratory, and the seeds were extracted using mechanical propulsion (the machine is in the process of patent registration).

For the production of shoot apex donor seedlings, the seeds were subjected to two selection steps prior to inoculation in media. In the first pre-selection, seeds were discarded if they had been damaged by machine cutting, had been consumed by insects, or were obviously malformed. The seeds selected in this first stage (n=500) were left in running water for 5 min, immersed in 70% pure alcohol (v/v) for 2 min, and then immersed in 2% sodium hypochlorite solution of active chlorine (v/v) for 30 min. Finally, these seeds were rinsed thrice in distilled and autoclaved water in a laminar flow cabinet. At the end of the asepsis process, seeds that were visibly imbibed (second pre-selection), were discarded.

According to laboratory protocol (Yeung et al., 2015), inoculation to obtain seedlings of explants was performed only in the MS medium (Murashige & Skoog, 1962) supplemented with 0.4 mg L^{-1} thiamine, 1 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid, 100 mg L^{-1} myo-inositol, and 0.5 g L^{-1} hydrolysed casein, plus 30 g L^{-1} sucrose, 3 g L^{-1} activated carbon, and 8 g L^{-1} agar. The pH was adjusted to 5.7. Seed germination and plant development occurred at 25±1°C on a photoperiod of 16 hours and photosynthetic photon flux density (PPFD) of 25 µmol m^{-2} s^{-1}. Sixty days after inoculation, the plants developed were ranked in terms of development, with the more vigorous plants considered suitable donors of shoot apex for the experiment per se.

The replicate of the sterile plant material (shoot apex) was carried out under conditions similar to those used for inoculation described above, and plant establishment occurred in development conditions similar to those of the seedlings that supplied the explant (also described above). The treatments were MS media (Murashige & Skoog, 1962) and WPM (Lloyd & Mccown, 1980), supplemented with 8 g L^{-1} agar, at different salt concentrations, i.e., 25, 50, 75, and 100% of the original concentration. Therefore, a factorial arrangement of treatments with 2 (media) × 4 (concentrations) was obtained, constituting eight treatments (t=8) with five replicates each (r=5). The design was completely randomized. The replicate plots comprised ten flasks, each containing two explants (a=20). For all treatments, the pH was adjusted to 5.7.

At 120 days after explant inoculation, the following growth and development measurements were analysed: stem diameter (SD), length of aerial shoots of the primary root, number of leaves, fresh mass, and dry mass. The data collected were statistically processed using the Kolmogorov-Smirnov test for normality of residuals and Levene's test of homogeneity of variance (α=0.01). When all assumptions were met, the analysis of variance was used (Snedecor's F; α=0.05). A comparison of means was performed for all types of medium using Tukey's test (α=0.05). Estimates of the range of salt concentrations studied were determined using polynomial regression models (α=0.05). According to Kutner et al. (2004), for quantitative and qualitative factors (in this study, the salt concentrations in the culture medium and type of culture medium, respectively), the nature of the characteristic and factor should be considered for the statistical analysis, even if the interaction is significant.

Of the characteristics analysed, only the SD was affected by the interaction between salt concentration and medium type (Table 1 and Figure 1). In general, the WPM resulted in increased root growth at extreme doses (SD25%=0.87 mm and SD100%=2.16 mm). However, at intermediate doses, the SD did not differ between the culture media; it was approximately 2.0 mm for both media. The SD increased linearly with an increase of 0.0154 and 0.024 mm for every 1% of salt concentrations in MS and WPM, respectively (R^2=76.18% in MS and R^2=82.21% in WPM; Figure 1). These results confirm that salt solutions in culture media exert not only a nutritional effect but also an osmoregulatory effect, which affects cell growth and morphogenesis (Maldaner et al., 2006), by interacting with the pressure potential that governs the stability and extensibility of cell walls. High osmotic pressure limits water absorption from the medium to the cell (Beauzamy et al., 2015) and, therefore, the lower salt concentration of the WPM than that of the MS medium improves water availability to the cell and positively affects stem differentiation.

With tissues fully hydrated, the demand for hydraulic conductance of the xylem, required to improve the capillarity of the water from the medium to the leaves, is reduced. Thus, the number of xylem veins in the stem, which is positively correlated to the stem diameter (Tyree & Sperry, 1988; Nobel, 2017), is lower in seedlings without water restriction. This explains the differences in stem development of *D. alata* plants, which showed greater stem thickening with the WPM that had a lower salt concentration than the MS medium.

Other physiological and molecular characteristics, such as ethylene content, and stress indicators, like cytosolic calcium and reactive oxygen species (Miller et al., 2010; Baxter et al., 2014), which may help plants with thicker stems to better withstand the acclimatization phase, may affect the relationship between increased water availability (i.e., a lower osmotic pressure) of the medium and stem thickening of the WPM-grown seedlings. However, these analyses were not the focus of the present work, which is intended to be a practical study for protocol determination. In this sense, this precedent should be explored in future research on the development and in vitro establishment of *D. alata* and other native plants of the Cerrado biome.

The MS medium favoured better development of the primary root (Table 1), leading to a root system

Table 1. Stem diameter, length of aerial shoots of the primary root, number of leaves, fresh mass, and dry mass of young "barueiro" (*Dipteryx alata*) plants developed in MS medium and wood plant medium (WPM) in different salt concentrations (25, 50, 75, and 100%), evaluated at 120 days after inoculation[(1)].

Media	Stem diameter (mm)				Length (cm)		Mass (g)		Number of leaves
	25%	50%	75%	100%	Aerial shoots	Root	Dry	Fresh	
MS	0.87b	1.89a	1.88a	2.16b	5.55a	27.65a	0.92a	2.87a	26.00a
WPM	1.54a	1.88a	2.06a	3.48a	6.50a	23.30b	0.85a	2.35b	21.15b
K-S	0.216				0.128**	0.132**	0.149**	0.154**	0.121**
F'	2.821**				0.789**	3.632	4.569	3.005**	1.035**

[(1)]Means followed by equal letter, in each column, do not differ by Tukey's test, at 5% probability. **Values indicate homogeneous variance and residuals with normal distribution by the Shapiro-Wilk and Levene's tests, respectively, both at 1% probabilty.

with length 4.35 cm greater than that in the WPM. Consequently, plants growcn in the MS medium presented greater development (NL=26 leaves per plant and MF=2.87 g). Leaf growth was not affected by the type of medium used for in vitro cultivation (Table 1), but did improve linearly with increasing salt concentrations, resulting in an increase of 0.3136 cm for MS and 0.2648 cm for WPM (Figure 1 B). Thus, dry mass also increased linearly with increasing salt concentration (Figure 1 D). This can be explained by two facts: the MS medium, even at diluted concentrations, provides a higher concentration of nutrients than the WPM (Yeung et al., 2015), and for *D. alata*, additional macronutrients are significant for

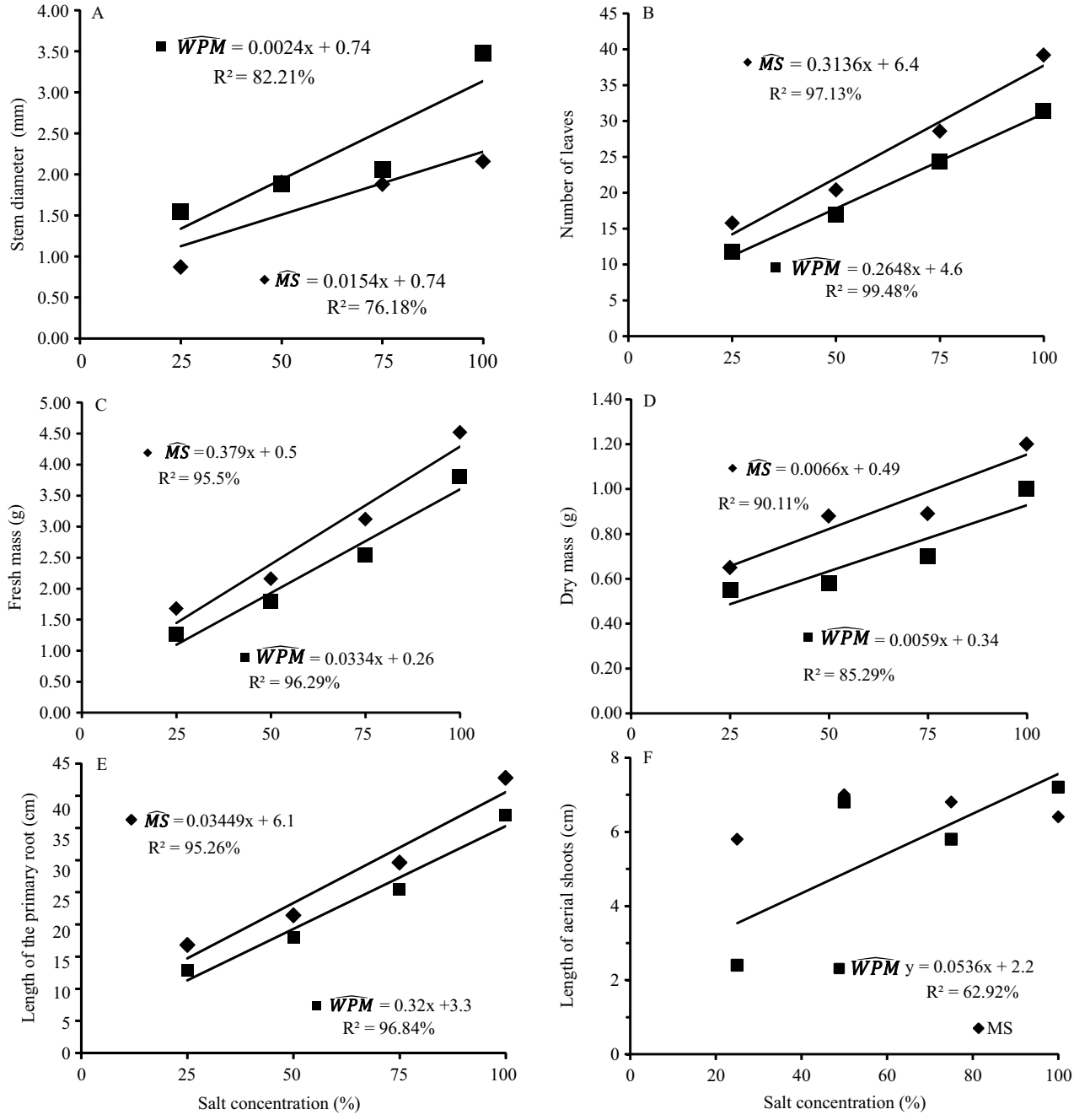

Figure 1. A, stem diameter; B, number of leaves; C, fresh mass; D, dry mass; E, length of the primary root; and F, length of aerial shoots of "barueiro" (*Dipteryx alata*) developed in MS medium and wood plant medium (WPM), and evaluated at 120 days after inoculation.

growth, increasing the stem diameter, leaf number, leaf area, and the production of dry mass of leaves, stem, and roots (Silva et al., 2016a, 2016b). This confirms the close relationship between an increase in salt concentration and the potential development of young plants in this species under in vitro culture. Thus, it is possible to affirm that the *D. alata* is a woody species responsive to the greater availability of nutrients in the medium, as long as the salinity threshold is respected. This is, to a certain extent, expected because, despite being a species from the Cerrado biome, a region of dystrophic soils and very low pH, *D. alata* is normally found in regions of soils with intermediate to high fertility (Sano et al., 2004).

The length of the longest root had linear increments of 0.344 cm when grown in MS medium and of 0.0322 cm when grown in WPM (Figure 1 E) for each unit of increase in salt concentration. This behavior was also observed for the length of the aerial part in WPM (an increase of 0.0536 cm with increasing salt concentrations), although with high dispersion (Figure 1 F). In general, a greater development of the root system results in a greater shoot development, because of the increased nutrient interception (Pérez-Harguindeguy et al., 2013). This idea was consistent with the finding of the present study, which showed linear increases in shoot length with increasing salt concentration. As for the MS medium, no regression analysed was adequate to fit the data (Figure 1).

The above results suggest that the MS medium at 25% concentration is superior to WPM at any concentration of salts, and that this relationship is maintained with increasing salt concentrations in MS medium. In optimal conditions, at 120 days, *D. alata* seedlings from the shoot apex inoculated with MS medium at its original concentration (100%) showed a root length of 27.65 cm and 26 leaves per plant, characteristics that signal successful in vitro establishment. Therefore, the better culture medium for robust protocols for the in vitro development of *D. alata* is the MS medium at the original concentration, i.e., MS as defined by Murashige & Skoog (1962).

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