# ARTICLE

## Micropropagation and callogenesis in *Mandevilla guanabarica* (Apocynaceae), an endemic plant from Brazil

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**Abstract** – Mandevilla guanabarica *is an endemic plant from Brazil, with pharmacological and ornamental potential, both unexplored. This study established the best culture medium for in vitro plant maintenance, efficient protocol for its regeneration, and callogenesis from different explants excised from in vitro-grown plants. Woody plant medium with double boron concentration (WPM$_B$) plus 2.27 μM thidiazuron or 0.49 μM 2-isopentenyladenine provided multiplication rates higher than 1:6. Shoots were 100% rooted on WPM$_B$. After acclimatization, plants showed 83% survival. For callogenesis, the use of MS media supplemented with high concentrations of picloram or 2,4-dichlorophenoxyacetic acid produced, respectively, friable or compact non-morphogenic calluses from different types of explants. This micropropagation protocol allows the production of seedlings of* M. guanabarica *for ornamental or commercial uses, and for conservation purposes; calluses can be used to establish suspension cultures in prospecting for bioactive compounds.*

**Key words:** *Morphogenesis, tissue culture, plant regeneration, callus induction.*

## INTRODUCTION

*Mandevilla* Lindley is the largest Neotropical genus of Apocynoideae (Apocynaceae) subfamily, and at least 70 species have been reported for Brazil (Sales et al. 2006). The landscape and ornamental potential of this genus is widely recognized and exploited in Europe and the United States, through culture and development of new varieties (EPO 2013). Members of *Mandevilla* are also highly interesting for their ethnobotanical uses: several species are used as medicinal and spiritual aids by indigenous people throughout the Neotropical region (Schultes 1979, Adams et al. 2007, Bussmann et al. 2011). The pharmacological potential of *Mandevilla* is confirmed by results obtained in many studies on *M. velutina* (Mart.) Woodson and *M. illustris* (Vell.) Woodson. These species have an underground system that is widely used in folk medicine in the form of infusions or alcoholic extracts, against inflammation and snakebite (Calixto et al. 1985). For optimizing the production of secondary metabolites with pharmacological properties from the underground system of *Mandevilla* species, micropropagation protocols have been established, using *in*

*vitro* culture techniques (Handro et al. 1988, Biondo et al. 2004, Biondo et al. 2007).

According to Pilatti et al. (2011), the prioritization of endemic, threatened species, and the habitats where they live is a crucial point of conservation actions, particularly in areas with rich endemic floras. *In vitro* culture techniques are a way of *ex situ* conservation, and have been used to secure and protect the native plant species, where new species with virtually unknown ornamental and pharmacological potential are continually being discovered.

*Mandevilla guanabarica* Casaretto ex. M.F. Salles, Kinoshita-Gouvêa and A. Simões (Sales et al. 2006) is an endemic species of the Atlantic Forest. It is a liana, with slender, latescent, twining, and densely leafy lateral branches. Its distribution is restricted to the edges of shrub thickets in restingas of the states of Rio de Janeiro and Espírito Santo. Although it is not officially at risk of extinction, *M. guanabarica* is restricted to restingas, many of which have undergone intense human impact due to land speculation and extractive predatory practices. Micropropagation and *in vitro* conservation of *M. guanabarica* are strategies that

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allow its sustainable use and conserve its genetic diversity, provide protection against predatory exploitation in its natural habitats, and still allow investigations about its phytochemical characterization, through callogenesis.

The objectives of this study were i) to establish an efficient protocol for regeneration of *M. guanabarica*, either for production of seedlings for ornamental purposes, or for assays for substances of pharmacological interest, and ii) callus induction from different explants excised from *in vitro*-grown plants to use in prospecting for bioactive compounds.

## MATERIAL AND METHODS

### Plant material

*Mandevilla guanabarica* fruits and branches were collected in the Maricá Environmental Protection Area (22º 52' to 22º 54' S and 42º 48' to 42º54' W) in the municipality of Maricá, RJ. Authorization to collect was granted by INEA (Instituto Estadual do Ambiente) and IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis). Vouchers are deposited at the Herbarium Bradeanum (HB), under accession numbers HB 93039 and 93041.

### Culture media and conditions

Basal media consisted of LS (Linsmaier and Skoog 1965), WPM (Lloyd and McCown 1980) or MS (Murashige and Skoog 1962) salts, supplemented respectively with LS, MS or WPM vitamins and 2%, 2% or 3% sucrose (w/v), and solidified with 0.75% agar (w/v). Other variants of MS and WPM media were supplemented with twice the original boron concentration, termed $MS_B$ and $WPM_B$, respectively. To shoot production, different concentrations of the plant growth regulators were added to one of the basal media: 0, 4.44 or 8.87 µM benzyladenine (BA); 0, 4.65 or 9.29 µM kinetin (KIN); 0, 4.56 or 9.12 µM zeatin (Zea); 0, 0.49, 2.46, 4.92, 14.76, 24.61 µM 2-isopentenyladenine (2-iP); or 0, 0.45, 2.27, 4.54, 13.62 or 22.71 µM thidiazuron (TDZ). For callus induction, 20 or 40 µM of picloram (PIC), or 2,4-dichlorophenoxyacetic acid (2,4-D) were added to MS medium. The pH of all media was adjusted to 5.8, and the liquid medium was dispensed in glass tubes (2.0 x 15.0 cm) for seed germination, or in glass bottles (7.5 x 13.5 cm) for the establishment of culture, shoot production, rooting, or callus induction, and autoclaved for 15 min at 121 °C/1.0 atm. All cultures were maintained in a growth room at 25±1 °C, under a 16h photoperiod with an irradiance of 23 µmol m$^{-2}$ s$^{-1}$ provided by daylight fluorescent lamps.

### Culture establishment

Seeds were obtained after the dehiscence of fruits. Tuft of trichomes, which assist in seed dispersal, was removed by hand, and seeds were surface-sterilized, under agitation, with 10% (v/v) commercial detergent for 10 min, 70% (v/v) ethanol for 10 min, and 30% (v/v) sodium hypochlorite for 5 min, and then washed three times with sterile distilled water. In laminar air flow, disinfested seeds were inoculated into glass tubes containing 10 ml of MS medium, and were maintained in a growth room for 60 days. After this period, plantlets obtained were used as a source of explants for the experiments.

### Establishment of culture medium

Isolated nodal segments (1.0 cm long) were inoculated into glass bottles with 50 mL of LS, MS, $MS_B$, WPM or $WPM_B$ medium and maintained in a growth room for two months. The experimental design was fully randomized, with 6 replicates per treatment; each replicate consisted of a bottle of 5 nodal segments (n = 30). After this period, plant development was evaluated regarding multiplication rate, and root or callus formation.

### Shoot production

The culture medium which presented the best results for multiplication rate ($WPM_B$) was used in the experiment on shoot production. Isolated nodal segments (1.0 cm long) were inoculated into glass bottles with 50 ml of $WPM_B$ medium, supplemented with the concentrations mentioned above for BA, KIN, Zea, 2-iP and TDZ. The experimental design was fully randomized, consisting of 10 replicates per treatment; each replicate consisted of a bottle of 3 nodal segments (n = 30 / treatment). All cultures were maintained in a growth room for two months, and after this period, shoot development was evaluated with respect to the number of nodal segments per explant (multiplication rate), and root or callus formation.

### Rooting

Shoots (3-4 cm) were excised from plants grown in the culture medium which were most efficient in inducing buds, and placed in glass bottles with 50 ml of $WPM_B$, according to the results described in the section *Establishment of culture medium*. Cultures were maintained in a growth room for three months.

### Acclimatization

*In vitro*-grown plants with roots and/or callus, shoot development, and more than 4.0 cm high were removed

from the glass bottles, washed in tap water to remove excess medium, and carefully transferred to plastic pots containing autoclaved vermiculite. Pots were placed in a plastic box covered with plastic film, for acclimatization in a greenhouse at a temperature of $28 \pm 2$ °C. During the course of one month, the plastic film cover was removed gradually; by the end of the month, pots were uncovered. Survival rate was evaluated after two months.

### Callus induction

Several types of explants were excised from plantlets grown *in vitro*: apical meristems (5 mm), leaves (1 cm$^2$), nodal segments, internodal segments, and roots (1 cm), and epicotyl and hypocotyl (0.5 cm). These explants were inoculated into glass bottles with 50 ml of MS medium supplemented with two different concentrations of 2,4-D or PIC (20, 40 µM), and maintained in a growth room under light (16h photoperiod) or in darkness. The experimental design was fully randomized, with 5 replicates per treatment and type of explant; each replicate consisted of a bottle of 2 explants (n = 10/treatment). Cultures were maintained in a growth room for two months, and after this period, they were evaluated regarding the type of callus developed (friable or mucilaginous, non-organogenic or embryogenic), and biomass accumulation, based on fresh and dry weights. Dry weights were determined after drying at $70 \pm 2$ °C for 24 h to constant weight.

### Statistics

Data were subjected to analysis of variance (ANOVA), and means were compared with the Tukey-Kramer test at 0.05% significance level, using the software *GraphPad InStat* v. 3.01 and *Sigma Plot*™ for Windows, version 11.0.

## RESULTS AND DISCUSSION

The disinfestation method applied to seeds of *M. guanabarica* provided 100% uncontaminated seeds. After two months of culture, plantlets showed fully development of roots and shoots. The establishment of a protocol for micropropagation from seeds allows the maintenance of genetic variability, which is essential for germplasm conservation, particularly in the case of endangered species. Other advantages of seeds are the ability to obtain a large number of explants from plantlets germinated *in vitro*, young tissues that are highly responsive to growth regulators, and lack of need for the difficult process of disinfestation of explants from woody plants from the field, or for phenolic oxidation, which can negatively affect the development of the explant *in vitro* (Bonga 1982, Grattapaglia and Machado 1998).

Multiplication rate and rooting of plants cultivated on LS, MS, MS$_B$, WPM and WPM$_B$ media are shown in **Table 1**. All media promoted the development of pre-existing meristems. LS, MS, MS$_B$ and WPM media induced multiplication rates of 1:1.8 to 1:2.2 and rooting of 51 to 70%, which did not differ significantly among each other. In plants cultivated on MS and MS$_B$, leaf blade was smaller, and shoot apex died in 83% of the plants. WPM$_B$ (Figure 1a) was the most effective for shoots, with a multiplication rate of 1:3.5 and 100% rooting. MS$_B$ and WPM$_B$ were used since apical death occurred in approximately 70% of plantlets grown for two months on MS medium. Although the symptoms of boron deficiency are related to species and plant age, the characteristic symptom is observed mainly in shoots since boron deficiency limits leaf expansion, resulting in small leaves, necrosis and death of young leaves and terminal buds, and consequent loss of apical dominance, forming a highly branched plant (Dell and Huang 1997).

Supplementation of WPM medium with boron was effective for *in vitro* development of *M. guanabarica*. This medium, indicated for woody plants, has lower concentration of nutrients than the salt composition of MS, with 25% of the nitrate and ammonia contained in LS and MS, but with high concentrations of potassium and sulfate (Linsmaier and Skoog 1965). Studies on the micropropagation of other *Mandevilla* species obtained positive results with LS medium

**Table 1.** Multiplication rate and rooting of nodal segments of *Mandevilla guanabarica* cultured on different media, after two months of culture

| Culture medium | Multiplication rate[1] | | Rooting[1] (%) | |
| --- | --- | --- | --- | --- |
| LS | 1.92 ± 0.86 | b | 58 | b |
| MS | 1.88 ± 0.83 | b | 51 | b |
| MS$_B$ | 2.04 ± 0.88 | b | 70 | b |
| WPM | 2.23 ± 1.52 | b | 64 | b |
| WPM$_B$ | 3.52 ± 1.75 | a | 100 | a |

Different letters, in each column, indicate statistical differences by the Tukey-Kramer test (p< 0.05).



**Figure 1.** Plants of *Mandevilla guanabarica in vitro* and acclimatized: **a** - plants developed from nodal segments cultivated on WPM$_B$ medium (after two months); **b** - plants developed from nodal segments cultivated on WPM$_B$ medium + 0.49 µM 2-iP (after two months); **c** - acclimatized plants in plastic pots containing vermiculite, after two months of acclimatization.

(Handro et al. 1988) and MS medium with the concentration of salts reduced to one-third (Biondo et al. 2004, Biondo et al. 2007). Similarly superior results with diluted media were obtained here for *M. guanabarica*.

After two months of culture, all plants grown on WPM$_B$ medium supplemented with cytokinin showed shoot production by direct organogenesis, via the development of preexisting meristems, with basal and friable calluses and without roots. Plants cultivated on WPM$_B$ without growth regulators showed 100% rooting, and calluses in only 30% of them. All media supplemented with cytokinin allowed the development of friable basal calluses (Table 2), and induced changes in the morphology of leaves, making them small and tapered. Plants cultured on WPM$_B$ supplemented with 2-iP at low concentrations (0.49 and 2.46 µM) also showed high multiplication rate, above 1:6 (Figure 2a); plants cultured on WPM$_B$ supplemented with 2.27 or 4.54 µM TDZ showed the highest multiplication rates, on the order of 1:7.5 (Figure 2b). Plants cultured on media supplemented with 2-iP (Figure 1b), although they showed a lower multiplication rate than plants cultured on media supplemented with TDZ, presented less alteration in leaf morphology; thus, 2-iP was considered the most effective plant growth regulator for regeneration of *M. guanabarica*.

Shoots were rooted from cultures on WPM$_B$ medium, the best for this purpose as previously evaluated in the section *Establishment of culture medium*. After three months, all plants showed rooting by indirect organogenesis, with roots arising from the basal callus or from a large thickening at the base of the stem. Rooting was not affected by the absence of growth regulators in the medium, which allowed these plants to acclimatize. Two months after transfer to soil, the *ex vitro* survival rate was 83%, and the aerial parts were fully developed (Figure 1c).
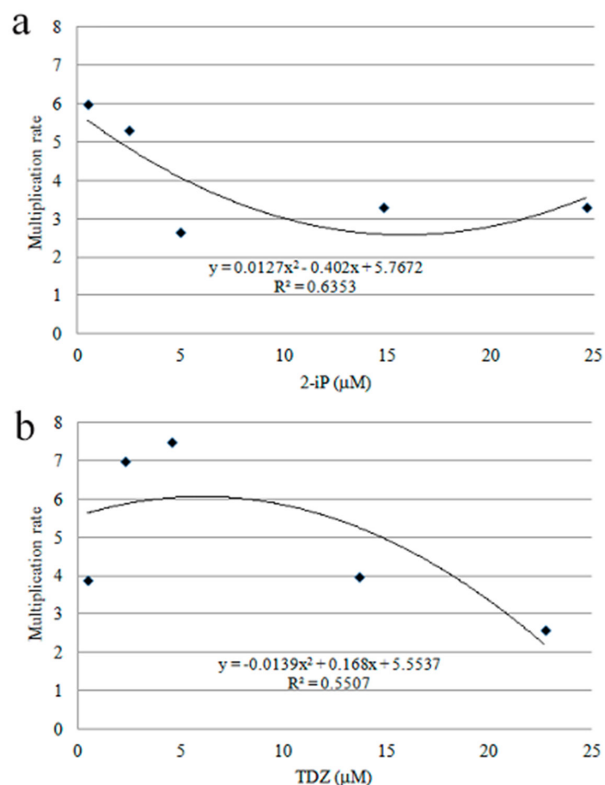
Shoot production by direct organogenesis via the development of preexisting meristems was observed in all treatments with cytokinins. TDZ cytokinins, which are a potent regulator and more active than BA and Zea, and 2-iP have been recommended for micropropagation in order to induce axillary bud development and shoot elongation (Pereira-Netto 1996, Biondo et al. 2007). High rates of shoot multiplication were obtained, by direct organogenesis, with the same concentrations used here, 0.49 and 2.46 µM 2-iP for *M. illustris* (Biondo et al. 2004), and for other species of Apocynaceae, such as *Holarrhena antidysenterica*, with 15 µM 2-iP (Raha and Roy 2001). Although BA has not been effective in shoot production for *M. guanabarica*, this regulator in the range of 4.44 to 8.87 µM promoted multiplication rates of 1:21 for *M. moricandiana* (Cordeiro et al. 2012).

**Table 2**. Effect of growth regulators on *in vitro* nodal segments of *Mandevilla guanabarica*, after two months of culture

| Media | Multiplication rate | Root and callus formation (%) | |
| --- | --- | --- | --- |
| | | Roots | Callus |
| WPM$_B$ | 3.83 ± 1.80 cdef | 100 | 30 |
| WPM$_B$+ 4.44 µM BA | 4.73 ± 2.15 bcde | 0 | 100 |
| WPM$_B$+ 8.87 µM BA | 2.86 ± 1.48 ef | 0 | 100 |
| WPM$_B$+ 4.65 µM KIN | 4.33 ± 2.40 bcdef | 0 | 100 |
| WPM$_B$+ 9.29 µM KIN | 3.46 ± 2.40 def | 0 | 100 |
| WPM$_B$+ 4.56 µM Zea | 3.06 ± 1.94 ef | 0 | 100 |
| WPM$_B$+ 9.12 µM Zea | 3.80 ± 1.79 cdef | 0 | 50 |
| WPM$_B$+ 0.49 µM 2-iP | 6.00 ± 2.27 abc | 0 | 100 |
| WPM$_B$+ 2.46 µM 2-iP | 5.33 ± 0.95 abcd | 0 | 100 |
| WPM$_B$+ 4.92 µM 2-iP | 2.66 ± 1.27 ef | 0 | 100 |
| WPM$_B$+ 14.76 µM 2-iP | 3.33 ± 1.12 ef | 0 | 100 |
| WPM$_B$ + 24.61 µM 2-iP | 3.66 ± 2.25 def | 0 | 100 |
| WPM$_B$+ 0.45 µM TDZ | 3.90 ± 2.05 cdef | 0 | 100 |
| WPM$_B$+ 2.27 µM TDZ | 7.50 ± 1.99 a | 0 | 100 |
| WPM$_B$+ 4.54 µM TDZ | 7.00 ± 2.36 a | 0 | 100 |
| WPM$_B$+ 13.62 µM TDZ | 4.00 ± 2.69 cdef | 0 | 100 |
| WPM$_B$+ 22.71 µM TDZ | 2.60 ± 3.09 f | 0 | 100 |

Different letters in multiplication rate column, indicate statistical differences by the Tukey-Kramer test (p<0.05).

a

$$y = 0.0127x^2 - 0.402x + 5.7672$$
$$R^2 = 0.6353$$

Multiplication rate / 2-iP (µM)

b

$$y = -0.0139x^2 + 0.168x + 5.5537$$
$$R^2 = 0.5507$$

Multiplication rate / TDZ (µM)

**Figure 2.** Multiplication rate of nodal segments of *Mandevilla guanabarica* after two months of culture on $WPM_B$ medium supplemented with different citokinins: **a** – $WPM_B$ supplemented with different concentrations of 2-iP; **b** - $WPM_B$ supplemented with different concentrations of TDZ.

Other studies have been successful in producing shoots of *Mandevilla*, although via indirect organogenesis from friable and basal calluses. With *M. velutina,* multiple shoots were obtained using combinations of BA-NAA (Handro et al. 1988) or BA in the range of 0.44 to 4.44 µM, with a maximum multiplication rate of 1:6.7; Zea, TDZ and 2-iP were not effective in producing shoots of this species (Biondo et al. 2007).

In these studies with *Mandevilla*, rooting was improved by the addition of auxins to the culture medium; the best results were obtained with NAA and IBA for *M. illustris* and *M. velutina* (Handro et al. 1988, Biondo et al. 2007), and IAA and IBA for *M. moricandiana* (Cordeiro et al. 2012). For *M. guanabarica*, $WPM_B$ without plant growth regulators allowed 100% rooting, which was fundamental for successful acclimatization. According to Handro et al. (1988), Biondo et al. (2004) and Biondo et al. (2007), plants with an underground system composed by a xylopodium or tuberous roots need more studies of *in vitro* rooting, which is fundamental for successful micropropagation.

Regarding the callogenesis, all explants grown under light developed non-morphogenic calluses, which differed in type and color according to the growth regulator, regardless of the concentration or type of explant. The calluses developed in 2,4-D were compact and colored green to brown (Figures 3a and 3b), while those developed in PIC were friable and yellowish-white (Figures 3c and 3d). All explants maintained in the dark, except the root explants cultured with 2,4-D, showed senescence after two to three weeks of culture. After two months in a growth room, regardless of the concentration of the growth regulator, calluses developed from roots and maintained in the dark were friable and brown, and showed intense development of roots with negative geotropism. Regardless of the light conditions, type of explant or growth regulator, none of the calluses developed somatic embryos.

Only calluses cultured under light were evaluated about biomass accumulation since those kept in the dark had senesced. The accumulation of fresh and dry biomass of calluses was evaluated statistically and the results did not allow determination of a culture medium or explant more efficient in promoting callus, since the obtained data were not statistically significant. The explants cultured in the presence of 2,4-D or PIC developed calluses, and although each type of explant had a standard size, there was wide variation in the size of the developed calluses, which caused the high standard deviation observed in all the samples (Figure 4). However, statistical analysis just enabled to state that roots grown in medium supplemented with 40 mM PIC did not promote callus formation or accumulation of biomass as observed for other types of explants.
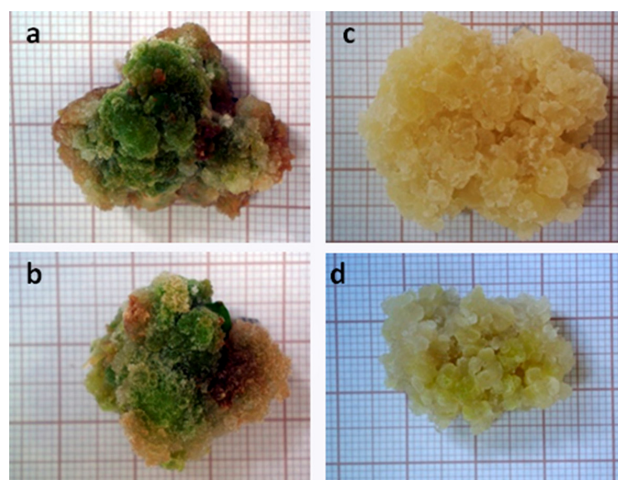


**Figure 3.** Aspect of calluses developed from explants of *Mandevilla guanabarica* cultivated on MS medium after two months of culture, under light: **a.** hypocotyl segment cultivated on MS + 20 µM 2,4-D; **b.** leaf segment cultivated on MS + 40 µM 2,4-D; **c.** epicotyl segment cultivated on MS + 20 µM PIC; **d.** internodal segment cultivated on MS + 40 µM PIC.
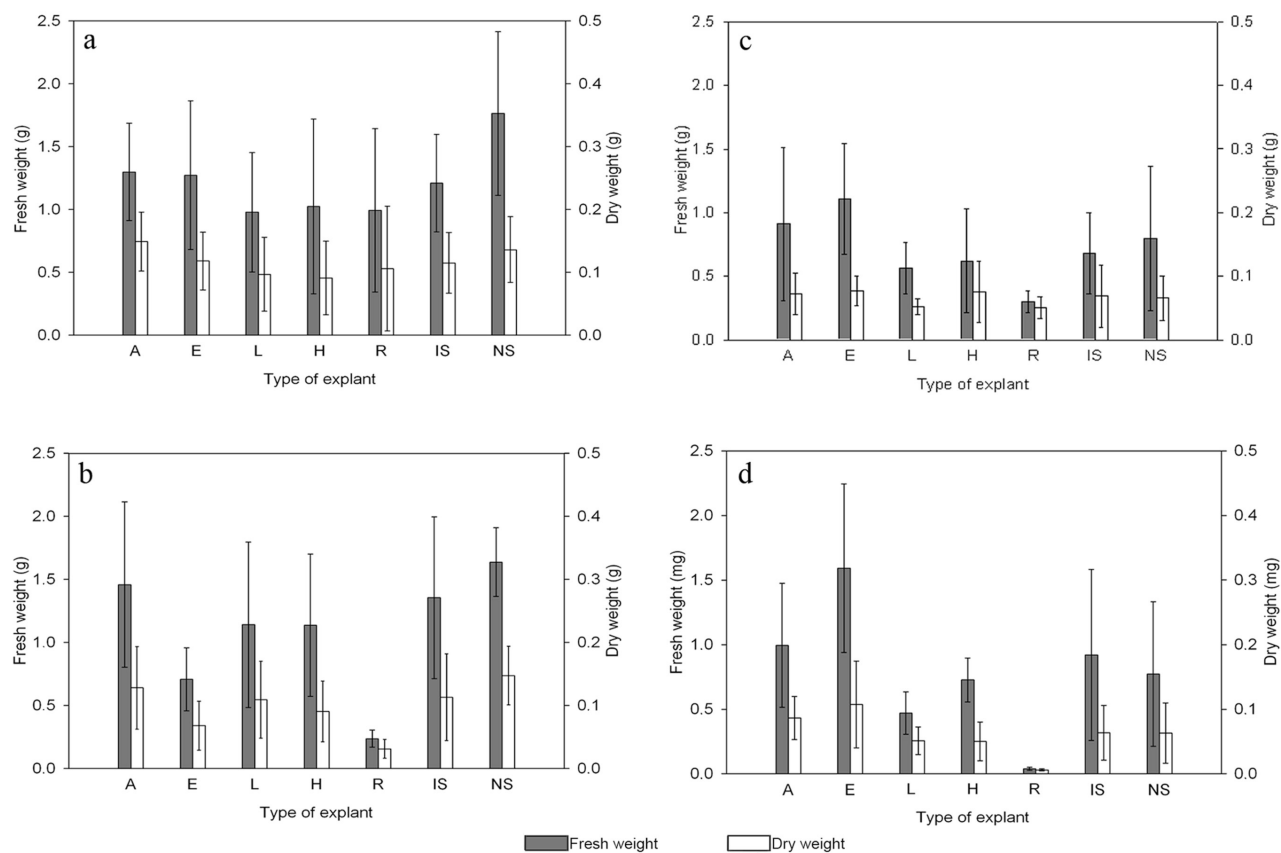
**Figure 4.** Biomass accumulation (g) of calluses derived from explants of *Mandevilla guanabarica*, inoculated on MS medium supplemented with different concentrations of 2,4-D or PIC, after two months of culture under light. **a.** 20 μM 2,4-D; **b.** 40 μM 2,4-D; **c.** 20 μM PIC; **d.** 40 μM PIC. Type of explant: A - apical meristems, E - epicotyl, L - leaf, H - hypocotyl, R - root, IS - internodal segment, and NS - nodal segment.

According to Guerra et al. (1999), media with a high concentration of salts, such as MS, in combination with high concentrations of auxins, have shown positive effects on the growth and development of calluses and somatic embryos. Light is also an important factor in somatic embryogenesis, and somatic embryos are generally induced in darkness. For *M. guanabarica*, auxins promoted calluses without regeneration of somatic embryos.

In the only reference to the use of 2,4-D in *Mandevilla*, Handro et al. (1988) found that this growth regulator was more efficient for growth of subcultured calluses than α-naphthalene-acetic acid and indole-3-acetic acid. Somatic embryogenesis in *Mandevilla* has not been studied. Most studies of somatic embryogenesis in members of Apocynaceae have treated aspects of the production of secondary metabolites by embryogenic calluses of medicinal plants, where BA-NAA combinations promoted somatic embryogenesis from root segments of *Catharanthus roseus* (Aslam et al. 2010), *Rauvolfia micrantha* (Sudha and Seeni 2006)

and *Tylophora indica* (Sahai et al. 2010).

The micropropagation protocol established here is efficient for the production of *M. guanabarica* plants since it utilizes low concentrations of 2-iP in WPM supplemented with boron for plant regeneration and no growth regulator for rooting, and 83% of the plants successfully acclimatized. Produced plants can be used for ornamental purposes and for assays for substances of pharmacological interest, without affecting the natural populations of this endangered species. Since the protocol was developed from seeds, the maintenance of the genetic variability of the explants further allows them to be used for *in vitro* conservation of germplasm. Further optimization is needed for effectively promote somatic embryogenesis in *M. guanabarica*. Although embryogenic calluses were not produced, different growth regulators led to the formation of calluses with different characteristics, with intense cell proliferation and biomass accumulation, which makes them suitable for the establishment of cell-suspension cultures, for use in studies of the

pharmacological potential of *Mandevilla guanabarica*.

## Micropropagação e calogênesis em *Mandevilla guanabarica* (Apocynaceae), uma planta endêmica do Brasil

**Resumo -** Mandevilla guanabarica *é uma planta endêmica do Brasil, com potencial farmacológico e ornamental inexplorados. Este estudo estabeleceu um eficiente meio de cultura para manutenção da planta in vitro e eficazes protocolos de regeneração e calogênese a partir de diferentes explantes. O meio para plantas lenhosas suplementado com o dobro da concentração de boro (WPM$_B$) e com 2,27 µM de thidiazuron ou 0,49 µM 2-isopenteniladenina proporcionou taxas de multiplicação maiores que 1:6. Os brotos foram 100% enraizados em WPM$_B$, com 83% de sobrevivência após aclimatização. Na calogênese, o meio MS suplementado com altas concentrações de picloram ou ácido 2,4-diclorofenoxiacético produziu, respectivamente, calos não morfogênicos friáveis ou compactos. Este protocolo de micropropagação permite a produção de mudas de* M. guanabarica *para uso ornamental, comercial e com finalidades de conservação; os calos podem ser utilizados para o estabelecimento de culturas em suspensão na prospecção de compostos bioativos.*

**Palavras-chave:** *Morfogênese, cultura de tecidos, regeneração vegetal, indução de calos.*

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