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BIOLOGY

In vitro establishment and morphogenesis of *Operculina macrocarpa* L. Urb (Convolvulaceae)

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ABSTRACT: *Operculina macrocarpa* L. Urb is a species popularly known as purge potato, a climbing plant from the Convolvulaceae family that has medicinal properties. *In vitro* cultivation could contribute to the production of seedlings on a large scale, favoring its commercialization process. This this study adjusted existing protocols for *in vitro* establishment and evaluated the effect of the interaction of growth regulators on the *in vitro* morphogenesis of *O. macrocarpa*. In the *in vitro* establishment process, the following were tested: 1x MS medium without activated charcoal, 1x MS medium with 1g.L^{-1} of charcoal, 1/2x MS medium (with 50% of the nutrient concentration) without charcoal and 1/2x MS medium with 1g.L^{-1} of charcoal. In addition to activated charcoal, the effect of gibberellic acid (GA_3) was tested under the following conditions: 1x MS medium without GA_3, 1x MS medium with 20µM of GA_3 and 1x MS medium with 40 µM of GA_3. After establishment *in vitro*, trials were carried out with different growth regulators. Initially, different concentrations of BAP were evaluated with 3 different segments of the plant (basal, median and apical). Different combinations of ANA x BAP and ANA x KIN were then tested. The experimental design was entirely randomized. The results obtained showed that it is possible to establish and multiply *O. macrocarpa in vitro* using the seed and the base of the stem, respectively, as a source of explant, in MS medium in the presence and absence of BAP.
Key words: medicinal plants, *in vitro* propagation, growth regulators.

Estabelecimento e morfogênese *in vitro* de *Operculina macrocarpa* L. Urb (Convolvulaceae)

RESUMO: *Operculina macrocarpa* L. Urb é uma espécie conhecida popularmente como batata de purga, uma trepadeira da família Convolvulaceae que possui propriedades medicinais. O cultivo *in vitro* poderá contribuir para a produção de mudas em larga escala, favorecendo seu processo de comercialização. Assim, este trabalho teve como objetivo ajustar protocolos existentes para o estabelecimento *in vitro* e avaliar o efeito da interação de reguladores de crescimento sobre a morfogênese *in vitro* de *O. macrocarpa.* No processo de estabelecimento *in vitro*, foram testados: meio de cultura 1x MS sem carvão ativado, meio 1x MS com 1g.L^{-1} de carvão, meio 1/2x MS (com 50% da concentração de nutrientes) sem carvão e meio 1/2x MS com 1g.L^{-1} de carvão. Além do carvão ativado, foi testado o efeito do Ácido giberélico (GA_3) nas seguintes condições: meio de cultura 1x MS sem GA_3, meio 1x MS com 20µM de GA_3 e meio 1x MS com 40 µM de GA_3. Após o estabelecimento *in vitro*, foram realizados ensaios com diferentes reguladores de crescimento. Inicialmente foram avaliadas diferentes concentrações de BAP com três diferentes segmentos da planta (basal, mediana e apical). Em seguida, foram testadas diferentes combinações com ANA x BAP e ANA x KIN. O delineamento experimental foi inteiramente casualizado. Os resultados obtidos permitem afirmar que é possível o estabelecimento e a multiplicação *in vitro* de *O. macrocarpa* utilizando a semente e a base do caule, respectivamente, como fonte de explante, em meio MS na presença e ausência de BAP.
Palavras-chave: plantas medicinais, propagação *in vitro*, reguladores de crescimento.

INTRODUCTION

As Brazil has the greatest plant biodiversity on the planet, it has great potential for supplying medicinal resources to the pharmaceutical industry (BRUNO et al., 2021). This diversity of medicinal species is distributed across all biomes, with special emphasis on the areas where the Amazon Forest, Atlantic Forest, Mato Grosso's Pantanal, Cerrado and Caatinga take place (PACHECO et al., 2022).

From a pharmacological point of view, most of the plant resources reported in these biomes can be used to produce medicines, due to the high pharmacotherapeutic potential of bioactive compounds (GOMIDE et al., 2016). However, due to the incipient focus on research with these species, phytochemical, pharmacological and toxicological studies still need to be carried out for most species (SOUZA et al., 2014). According to MELO et al. (2021), in Brazil, the medicinal use of most plant resources is still restricted to empirical knowledge, based on extractivism, held by traditional peoples who use teas and infusions as the main means of curing diseases and illnesses.

Among the species with known medicinal potential, one can highlight plants belonging to

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the genus *Operculina*, which are distributed in regions between the Antilles and Brazil, in addition to occurring in temperate regions of the Mexican Andes (COELHO et al., 2011). In Brazil, species of the genus *Operculina* have a wide geographical distribution, occurring in the North, Northeast, Mid-West and Southeast regions, and are present in the Amazon, Caatinga, Cerrado and Atlantic Forest biomes (SIMÕES & PETRONGARI, 2022).

Currently, 430 species have been classified as belonging to the genus *Operculina*, of which 193 are endemic and predominantly found in environments with a higher incidence of light (BELO, 2020). In this genus, the species *O. macrocarpa* L. Urb stands out, characterized by having white flowers and fruits with four hard and black seeds (BRASILEIRO, 2009), tuberous roots and resinous cells, which give it a purgative and laxative action, which is why it is popularly known as "purge potato" (FONTELES et al., 2008).

Despite being widely used in folk medicine, *O. macrocarpa* has difficulty germinating due to the hardness of its seeds (BRASILEIRO, 2009). As a result of this integumentary dormancy, the seed is impermeable, which reduces the entry of water and gases necessary to start the germination process (BORGES et al., 2022). According to GOMES et al. (2021); although, dormancy is an evolutionary strategy for the survival and temporal distribution of germination, it is an undesirable characteristic agronomically, as it delays the production of seedlings.

As a way of overcoming the difficulties that can arise in producing seedlings and obtaining healthy plants, techniques such as micropropagation have been widely used for various species of agronomic and/or medicinal interest. For example, for *Ipomoea sepiaria Roxb.*, a medicinal species belonging to the family *Convolvulaceae*, *in vitro* propagation has been tested with a view to producing seedlings on a large scale (CHERUVATHUR et al., 2015). For *Ipomoea turpethum* (synonymous with *O. turpethum*), the applicability of this technique has been tested with a view to preserving the plant genetic resource, given that this is an endangered species (GURAV et al., 2019). Nonetheless, despite its potential for use in seedling production, the success of *in vitro* micropropagation depends on biotic and abiotic factors.

The factors that affect *in vitro* propagation include the type of explant used, the species, the culture medium used to grow the explants, temperature and photoperiod, as well as the concentration of growth regulators added to the medium. Regulators such as auxins and cytokinins are decisive in *in vitro* cultivation, as they are responsible for various functions in the multiplication and rooting stages (CID & TEIXEIRA, 2015). They also modulate various biochemical processes and play a role in plant growth and development (BEKIRCAN et al., 2018).

In vitro cultivation has great potential for large-scale multiplication and plant conservation (SILVA et al., 2020), because, in addition to being a propagation method that allows control of the determining factors in seedling production, it also allows the propagation of species for which seed reproduction is difficult (OSENI et al., 2018). Despite the advantages of this type of propagation, studies on *in vitro* cultivation are still incipient for many species. In the case of *O. macrocarpa*, in addition to the efficiency of producing seedlings, *in vitro* cultivation could contribute to the *ex situ* conservation of the species, considering its intense use in folk medicine and the fact that it is not yet a cultivated species.

In this context, this this research adjusted existing protocols for *in vitro* establishment and evaluated the effect of the interaction of growth regulators on the *in vitro* morphogenesis of *O. macrocarpa*.

MATERIALS AND METHODS

Obtaining the material

The seeds were collected from mother plants kept in the medicinal and aromatic plant collection of the Forest Garden Experimental Unit, belonging to the Universidade Estadual de Feira de Santana (UEFS), and the experiments were carried out in the Plant Tissue Culture Laboratory (LCTV, as per its Portuguese acronym), located in the same unit (12º16'9"S / 38º56'19"W).

In vitro establishment

The collected seeds were previously washed in running water and neutral detergent and; subsequently, disinfected in a laminar flow chamber by immersion in 70% alcohol for 1 minute, followed by washing with sodium hypochlorite (2.5% active chlorine) for 15 minutes. Three washes with autoclaved distilled water were carried out to remove possible hypochlorite residues.

In order to test the effect of activated charcoal and gibberellic acid (GA_3) on the germination process, immediately after disinfestation, the seeds were inoculated into test tubes (25 x 150 mm) containing 10 mL of MS culture medium (Murashige and Skoog, 1962) added with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar under the following conditions: 1x MS culture medium without activated

charcoal, 1x MS medium with 1g L^{-1} charcoal, 1/2x MS medium (with 50% of the nutrient concentration) without charcoal and 1/2x MS medium with 1g L^{-1} charcoal. In addition to activated charcoal, the GA_3 effect was tested as follows: 1x MS medium without GA_3, 1x MS medium with 20µM GA_3 and 1x MS medium with 40µM GA_3. In order to prevent the growth of pathogens, 1 mL/L of the biocide PPM (Plant Preservative Mixture) was added during the preparation of the different culture medium. The pH of the culture medium in all treatments was adjusted to 5.7 ± 0.1, which were then autoclaved at 121 ºC and 1 atm for 15 min.

After inoculation, the test tubes were kept in a growth room under a 16-hour photoperiod at 25 ºC ± 2. After 45 days, the percentage of germinated seeds, contamination and normal plants was evaluated. The plants obtained were used as sources of explants for the next stage.

Multiplication

The influence of different types of stem explants and concentrations of 6-benzylaminopurine (BAP) on the induction of sprouts in nodal explants of *O. macrocarpa* was evaluated.

From the seeds *in vitro* germinated, three types of stem segments (basal, median and apical) were obtained from the plants *in vitro* germinated. After obtaining the explants, they were cultivated in MS medium combined with different concentrations of BAP (0, 0.5, 1.0, 2.0 and 4.0 µM). The experimental design was completely randomized (DIC) in a 3x5 factorial arrangement (three types of stem segment x five concentrations of BAP) with five replicates, each replicate consisting of four seeds per plot. After 30 days of inoculation, the shoots were evaluated, where the following variables were quantified: percentage of explants that formed shoots, number of shoots per explant, number of leaves per explant, length of the greatest shoot, percentage of shoots with hyperhydricity, callus formation at the base of the explant and number of roots per explant.

The concentrations of 6-benzylaminopurine (BAP) and/or kinetin (KIN) combined with different concentrations of naphthaleneacetic acid (ANA) were also evaluated in the induction of sprouts. The most responsive type of explant, obtained from the previous experiment, was inoculated into test tubes containing 10 mL of MS culture medium plus different concentrations of the cytokinins 6-benzylaminopurine (BAP) or kinetin (KIN) (0; 0.5; 1.0; 2.0 and 4.0 µM), combined with different concentrations of the auxin naphthaleneacetic acid (ANA) (0; 0.5 and 1.0 µM). In this case, the experimental design was completely randomized in a 5x3 factorial arrangement (five cytokinin concentrations x three ANA concentrations) with five repetitions, consisting of four seeds per plot.

After 45 days of inoculation of this material, the following variables were analyzed: frequency of responsive explants, i.e., the percentage of explants that formed shoots, number of shoots per explant, number of leaves per explant, length of the greatest shoot, percentage of shoots with hyperhydricity, callus formation at the base of the explant and number of roots per explant.

Statistical analysis

The data was tested for normality through the Shapiro-Wilk test, submitted to analysis of variance (ANOVA) and Tukey's test or regression at 5% probability, using SISVAR 5.6 software. Data in percentages was evaluated using analysis of variance and then transformed into the square root of Y + 1.0 - SQRT (Y + 1.0).

RESULTS AND DISCUSSION

In vitro establishment

O. macrocarpa seeds had a germination rate of over 60%, without statistical differences between the different types of medium tested (Table 1).

When evaluating the *in vitro* germination of O. *odorifera* and *Ocotea catharinensis Mez*, MORITZ et al. (2009) did not observe a positive effect on the induction of new shoots when BAP was used in the presence of activated charcoal, suggesting that the charcoal may adsorb cytokinin and neutralize its effect on the induction of new sprouts. When evaluating the germination rate of *Muntingia calabura L.*, PIERINE et al. (2019) observed that the 1/2x MS medium (with half the concentrations of macro and micronutrients) plus activated charcoal provided a germination rate of approximately 100% in *in vitro* cultivation, which demonstrates how variable the response is, depending on the species and the medium used.

For the percentage of abnormal plants, shoots and root length, there were also no statistical differences depending on the absence or presence of activated charcoal in the medium. Overall, the lack of increase in the percentage of shoots and root length shows that the addition of activated charcoal does not offer any advantages for germinating *O. macrocarpa* seeds that would justify its use when the aim is to improve the root system and the percentage of aerial parts of the seedlings produced. However, when the aim is to accelerate seedling growth and obtain

Table 1 - Percentages of germination, contamination, abnormalities, explants with shoots and root length of *Operculina macrocarpa* L. Urb. cultivated in complete MS (Murashige and Skoog) medium with half the nutrient concentration (1/2x MS) with and without the addition of activated charcoal.

	Germination (%)	Contamination (%)	Shoots (%)	Explants with shoots (%)	Root length (cm)
MS medium without charcoal	90.59[a]	10.0[a]	18.28[a]	26.00[a]	24.28[a]
MS medium with 1g charcoal	61.63[a]	11.65[b]	14.48[a]	13.50[ab]	28.4[a]
MS medium ½ without charcoal	79.73[a]	10.62[ab]	13.00[a]	13.00[ab]	39.2[a]
MS medium ½ with 1g charcoal	58.48[a]	11.44[b]	12.50[a]	12.50[ab]	18.7[a]
CV	55.06	14.99	15.00	75.49	48.2

Means followed by the same letter in the column do not differ by Tukey's test at 5% probability.

greater vigor, activated charcoal can bring good results. Contrasting with the results obtained for this study, for *in vitro* strawberry culture, CAMARGO et al. (2020) observed that the addition of activated charcoal favored an increase in shoot length, average root length and number of roots.

Despite the lack of significance, the results obtained still showed a high contamination rate (18.19 to 46.19%). The occurrence of contamination may have resulted from the existence of pre-existing pathogens inside the seeds, as already demonstrated by ESPOSITO-POLESI (2020). In this case, given that *O. macrocarpa* seeds have a hard tegument, washing with sodium hypochlorite solution may not have been enough to eliminate the pathogen before inoculation into culture medium. More efficient disinfestation protocols can be established by increasing the time the seeds are immersed in the disinfesting solution (PIERINE et al., 2019; SOARES et al., 2020), by increasing the concentration of the disinfesting solution or by testing the addition of different fungicides to the culture medium (CID & TEIXEIRA, 2015). For example, in order to increase the efficiency of decontamination and break the dormancy of *Myrciaria floribunda* (H. West Ex Willd. O. Berg.) seeds before washing with 70% alcohol and sodium hypochlorite, the seed coat was broken with a penetrometer (SANTOS et al., 2022).

Contamination of the culture medium is an occurrence that can lead to unsuccessful *in vitro* establishment (ESPOSITO-POLESI, 2020). As a way of avoiding contamination, disinfestation of the material to be cultivated is essential and must be carried out rigorously. However, even when washing with sodium hypochlorite, contamination has been reported in many studies in the pertinent literature (ANSILIERO et al., 2020; BOTELHO et al., 2009; MORITZ et al., 2019).

In addition to the occurrence of contamination during material handling, another factor that influences its occurrence is the type of explant used for *in vitro* cultivation. For example, explants from leaf segments are easier to disinfect due to the greater permeability of the tissues; while, in the case of seeds, the tegument or external coating of the seeds can hinder the penetration of the disinfectant solution, thus resulting in greater chances of contamination of the culture medium, given the presence of pre-existing pathogens inside the seeds (CID & TEIXEIRA, 2015; ANSILIERO et al., 2020). The efficiency of the decontamination solution also depends on factors inherent to the species. For example, for *in vitro* germination of *Lychnophora pohlii Sch. Bip.*, sulphuric acid resulted in lower contamination rates than sodium hypochlorite (GONZAGA et al., 2021).

For *O. macrocarpa*, seed germination, both in basic MS medium and in MS medium with activated charcoal, resulted in plants with morphologically normal aerial parts and without hyperhydric or necrotic shoots. Although, growth parameters were not quantified, the plants cultivated on MS medium with activated charcoal showed better appearance, were more robust and had improved development. The apparent lack of effect of activated charcoal on the germination of *O. macrocarpa* seeds and its apparent effect on plant growth after germination

suggested that charcoal may be more involved in the seedling growth process than germination for this species. When evaluating the effects of the interaction between activated charcoal and different concentrations of BAP on the *in vitro* multiplication of banana trees, COSTA et al. (2006) reported that the addition of charcoal to the MS culture medium significantly reduced the multiplication rate of banana trees, but that taller and more vigorous shoots with a greater number of roots were obtained in MS medium with activated charcoal.

According to GALDIANO-JÚNIOR et al. (2010), activated carbon can adsorb phenolic compounds, thus preventing the oxidative process in the *in vitro* environment. In addition, FAGUNDES et al. (2017) reported that activated charcoal is also capable of adsorbing hormones (auxins and cytokinins), plant exudates and toxic substances released by the explants or produced during autoclaving. However, SANTOS et al. (2016) state that activated carbon has a non-selective effect, which can alter the pH, remove organic nutrients and inhibit growth and morphogenesis. Therefore, the use of this additive in the culture medium must be adjusted with the aim of providing maximum efficiency, without allowing the negative effects to considerably influence the development of the species.

When evaluating the effect of different concentrations of activated charcoal supplemented to the culture medium for different cultivars of *Rubus idaeus* L. (Rosaceae), FAGUNDES et al. (2017) reported that a dose containing 4 g L^{-1} of activated charcoal was effective in reducing *in vitro* oxidation for the cultivars Golden Bliss, Polana, Fallgold, Schönemann and Bababerry. However, for the cultivars Indian Summer, Heritage and Willamette, the 2 g L^{-1} dose of activated charcoal was the most suitable for controlling phenolic oxidation in explants. Aiming to establish an *in vitro* micropropagation protocol for *Colubrina glandulosa Perkins* (Rhamnaceae), HASS et al. (2022) showed that the addition of activated charcoal to the culture medium negatively influenced the rooting percentages of the sprouts. According to the authors, the negative effect of charcoal was due to its ability to adsorb components of the culture medium, such as, for example, growth regulators, reducing their effects on the sprouts.

The results obtained in this study showed that gibberellic acid significantly influenced the germination of *O. macrocarpa* seeds, with the highest concentration of gibberellin resulting in 100% germination (Table 2).

Different results to those obtained in this study were observed by GONZAGA et al. (2021) for Lychnophora pohlii Sch. Bip., where the addition of gibberellin did not result in higher germination rates. According to ALMEIDA & RODRIGUES (2016), gibberellic acid is a hormone involved in regulating seed germination, leaf expansion, flowering and fruit development. The main functions of gibberellins include stimulating plant cell elongation, leaf expansion and promoting the growth of a seed's embryo.

The addition of GA_3 to the culture medium significantly affected the percentages of germination, contamination and explants with shoots, without contamination and oxidation when 40 µM GA^3 was added to the MS medium, showing a highest percentage of explants with sprouts (Table 2). Based on the results obtained, it is possible to state that the use of MS medium with the addition of 40 µM gibberellin (GA_3) allows healthy plants to be obtained without the occurrence of pathogens. Moreover, the difference in the elongation of the shoots was visually evident with the use of 40 µM GA_3. A similar result was obtained by STEFANEL et al. (2022), when they evaluated different concentrations of gibberellin in

Table 2 - Percentages of germination, oxidation, contamination of the culture medium and explants with shoots of *Operculina macrocarpa* L. Urb. in MS (MURASHIGE SKOOG) medium prepared with and without the addition of gibberellin.

Treatment	Germination (%)	Contamination (%)	Explants with shoots (%)
MS medium 0 µM GA_3	50^b	40^b	$12,0^b$
MS medium 20 µM GA_3	55^b	40^b	$14,0^b$
MS medium 40 µM GA_3	100^a	0^a	19^a
CV	13.49	15.31	26.37

Means followed by the same letters do not differ at 5%.

the *in vitro* cultivation of *Eugenia involucrata D.C.*, where they observed the positive effect of gibberellin on the elongation of the species.

Among the concentrations of 6-benzylaminopurine (BAP) combined with different types of explants in *in vitro* morphogenesis, considering the different types of segments tested, only the segment was regenerative, i.e., it emitted new sprouts in all the concentrations of BAP used. Accordingly, 4, 3, 1.25, 5 and 4.66 sprouts were obtained from the segments cultivated in MS medium supplemented with 0 (control), 0.5, 1.0, 2.0 and 4.0 µM BAP, respectively. However, no significant differences were observed in relation to the concentrations of the regulator. Although, no dose-dependent effects of BAP on any morphogenetic process were detected, a high number of oxidized shoots, malformed shoots and root formation were observed in the explants.

However, the beneficial effect of 6-benzylaminopurine (BAP) on *in vitro* sprout multiplication has been reported and is related to the influence of this growth regulator on cell division and bud release inhibited by apical dominance. According to REIS (2017), the responses of cells, tissues and organs in the *in vitro* environment to the effect of growth regulators can vary depending on the culture conditions, the type of explant and the genotype of the plant used. For *Ceiba rubriflora Carv.-Sobr. & L. P.*, the addition of 2.0 mg L^{-1} BAP to the medium only resulted in increased callogenesis at the base of the shoots, but the emergence of new shoots was not favored (DOCHA et al., 2020). Contrasting with this research, the *in vitro* propagation of *Aechmea miniata* and *Aechmea blanchetiana* explants resulted in a greater number of shoots when the concentration of BAP was increased. However, this increase had a reverse effect on the number and length of roots and the height of the plants obtained (GARCIA et al., 2021). Data like this reinforces the idea that the response of explants to the growth regulator is variable and depends on the phytohormonal characteristics of each species.

According to BASSAN et al. (2006), phenolic oxidation can happen during the initial establishment of *in vitro* culture, mainly due to the action of phenols that form quinones, which are responsible for the inhibition and death of the segments. This process was seen in the median and apical segments of *O. macrocarpa*, which prevented the emission of sprouts, resulting in the death of the explants. Still according to BASSAN et al. (2006), in *Peltophorum dubium*, a significant effect was observed for the emission of shoots from the apical segment, generating more vigorous shoots, when compared to the explant from the median segment after sprouting. However, in *O. macrocarpa*, the apical segment showed no sprouts or development of the segments (both oxidized).

Belonging to the group of synthetic cytokinins, BAP is directly related to the process of cell division and differentiation of calluses into adventitious shoots, as a result of inhibiting apical dominance in plants cultivated in the *in vitro* mode (CID & TEIXEIRA, 2015). In the *in vitro* propagation of *O. turpethum* (L.), GURAV et al. (2019) observed differences in the morphogenetic responses in relation to the concentrations of BAP, with the concentration of 3 mg L^{-1} of the regulator being indicated as the most viable for the *in vitro* multiplication of this species. They also reported that all the concentrations tested promoted induction of the aerial part and the root, but greater induction was observed for the aerial part.

When studying the processes of differentiation and redifferentiation in explants of the species *Parapiptadenia rigida* (Benth.) *Brenan (*Fabaceae), KIELSE et al. (2009) reported that, in addition to choosing the most suitable explant, i.e., the explant that responds best in terms of shoot emission, it also requires the use of phytoregulators capable of stimulating plant regeneration. In this study; although, there was no significant difference between the treatments, the induction of sprouts in the basal segment took place from the third week onwards. It was possible to observe that there were no statistical differences between the treatments, which is why it was not necessary to use the regulator BAP for *O. macrocarpa*. However, the effectiveness of BAP in *in vitro* multiplication has been demonstrated for several other species.

When evaluating the influence of BAP on the induction of *Anadenanthera macrocarpa* (Benth) shoots, MIRANDA et al. (2020) reported a greater number of shoots per explant in the treatment using the cotyledon segment in medium supplemented with 2 mg L^{-1} BAP. Similar results were found by SOARES et al. (2011), who obtained a greater number and length of shoots with the addition of BAP (2 mg L^{-1}) to the nutrient medium, as well as a reduced number of roots, inducing the best responses in the culture of segments of *Hancornia speciosa Gomes*.

Growth regulators are very important for cell development, and their balance can directly influence the behavior of plant cells, as they interfere directly with plant physiology. According to CORDEIRO et al. (2004), phytoregulators that may be present in the culture medium are directly related to growth and most of the development patterns of *in vitro* cultures.

Among the concentrations of 6-benzylaminopurine (BAP) combined with different

concentrations of naphthaleneacetic acid (ANA) in *in vitro* morphogenesis, the analysis of variance (ANOVA) for the combined effect of different concentrations of the regulators BAP and ANA on sprout induction and growth showed a high interaction and significant effect at 5%. A similar result was obtained by DIAS et al. (2020) for the *in vitro* culture of bromeliads.

The data obtained for the interaction between BAP and ANA does not show a response pattern for the variables analyzed. However, it is possible to see that the mediums with 2 and 4 mg L^{-1} BAP and 0 mg L^{-1} ANA were the ones that gave the best results for the number of shoots. Following the same trend, for the number of leaves, the medium with 0 mg L^{-1} ANA and 4 mg L^{-1} BAP resulted in the highest value. For this same variable and for shoot length, good results were obtained for the medium containing 1 mg L^{-1} ANA and 0 mg L^{-1} BAP.

The fact that the best results were obtained in mediums without one of the regulators shows that the interaction between BAP and ANA does not favor the development of the aerial part for *O. macrocorpa*. Accordingly, for this species, when the aim is to induce an increase in the number of shoots, only BAP should be added to the medium. However, when the expected response is an increase in shoot length, the most suitable medium should be prepared with ANA alone, which suggested the need for a sprout induction phase, using a cytokinin, followed by transfer to a medium with an auxin source.

In this study, the addition of 0.5 or 1.0 ANA in association with all the BAP dosages tested favored *in vitro* callogenesis. For root formation, the most responsive medium was prepared with 1.0 µM ANA and 4.0 µM BAP.

The effect of ANA on micropropagation is directly related to both the process of callus formation and the process of callus differentiation and root formation. The correct balance between auxins and cytokines is crucial for the formation of normal plants, i.e., with good crown and root architecture. From a physiological point of view, phytohormones are naturally present in plant tissues, but their quantities vary depending on the species and type of explant used (CID & TEIXEIRA, 2015; GOELZER et al., 2019). Despite this occurrence, in many cases, the quantities of phytohormones are not enough to cause the process of cell dedifferentiation and redifferentiation, which is why it is necessary to add regulators to the medium. In the process of *in vitro* morphogenesis, in addition to acting in isolation, the regulators act synergistically, and it is based on this action that the number of regulators added will or will not result in successful micropropagation (MONFORT et al., 2012; CID & TEIXEIRA, 2015).

In addition to seedling quality, the correct balance between cytokinins and auxins can increase the number of shoots and reduce the number of subcultures, thus resulting in a greater number of plants obtained through micropropagation. In the case of the species *Angelonia integerrima*, a greater number of sprouts were induced when 0.5 mg L^{-1} ANA and 1.0 mg L^{-1} BAP were added to the medium. However, the mediums with 0.1 mg L^{-1} ANA and 0.5 mg L^{-1} BAP and the medium with 0.1 mg L^{-1} ANA and 1.0 mg L^{-1} BAP also showed good results and no statistical differences when compared to the first medium mentioned (WINHELMAN et al., 2019).

If the tests carried out between different types of culture medium indicate positive results without statistically significant difference between them, it is wise to choose the medium with the lowest concentration of regulators. This is because increasing the number of regulators results in higher costs in seedling production. Unlike the results obtained by WINHELMAN et al. (2019), the analysis of the interactions between the concentrations of 0.0, 0.5, 1.0, 1.5 and 2.0 for BAP and 0.0, 0.1 and 0.25 for ANA did not result in an increase in the number of shoots for two varieties of *Opuntia ficus-indica* (L.) *Mill*. In addition to the number of shoots, in this study, the growth in shoot height and root number also did not show a positive response as a result of the interaction between BAP and ANA (DUTRA et al., 2020).

The effect of the association between BAP and ANA is not the same for all species and, depending on the dosages associated between these two regulators, can result in different morphogenic responses in the explants. Depending on the type of *in vitro* response expected, the dosage of the same regulator must be adjusted. Different responses to the dosage of ANA and BAP can be seen in the research carried out by GATO et al. (2019) on *Ananas erectifolius L.B.Sm*. The authors report that higher percentages of shoots are obtained in response to the addition of 0.19 mg L^{-1} ANA and 1.5 mg L^{-1} BAP for this species. However, when the aim is to achieve a greater number of leaves and greater root length, both objectives can be achieved by adding 0.06 mg/L^{-1} ANA and 0.5 mg/L^{-1} BAP to the medium. In order to increase shoot height, the recommended dosages are 0.12 mg/L^{-1} ANA and 1.0 mg/L^{-1} BAP. According to GOELZER et al. (2019), in addition to the concentration, the effect of ANA depends on the type of cytokinin added to the medium.

In the *in vitro* cultivation of nodal segments of *Melanoxylon brauna Schott*, the best responses for number of sprouts and number of leaves were observed at the highest concentration tested (0.27 µM ANA and 3.33 µM BAP). Despite the greater number of leaves observed at this dosage, this increase did not result in a statistical difference when compared to the treatment with 0.27 µM ANA and 2.22 µM BAP and the treatment with 0.27 µM ANA and 1.11 µM BAP (ALMEIDA et al., 2020). Insufficient doses and the interaction between the regulators may not result in the formation of parts or roots, in which case often only callus multiplication is favored.

Among the concentrations of kinetin (KIN) combined with different concentrations of naphthaleneacetic acid (ANA) in *in vitro* morphogenesis, the combined effect of different concentrations of KIN and ANA on the induction and growth of sprouts showed a significant effect for the interaction involving number of shoots, number of roots and presence of callus. Among these three characteristics, the highest coefficient of variation (CV) was obtained for explants with callus (81%), followed by number of roots (approximately 60%) and number of shoots (16.47%).

All the variables analyzed were responsive to the ANA X KIN interaction. The surface-response graph shows that the dosages of 0.5 mg L^{-1} ANA and 4 mg L^{-1} KIN gave the best results for most of the variables tested, except for the number of shoots and the number of roots. Regarding to shoot number, the best results were observed for the dosages of 0 and 0.5 mg L^{-1} ANA, without the addition of KIN. In the case of root number, the best results were observed for the medium prepared with 1 mg L^{-1} ANA and 4 mg L^{-1} KIN and for the medium prepared without the addition of both regulators. The dosages of 0.5 mg L^{-1} ANA and 2 mg L^{-1} KIN also proved to favor callogenesis.

Although, they act synergistically and, in many cases, it is necessary to add both cytokinins and auxins to the culture medium, the endogenous balance and the high concentration of some phytoregulators in the explant tissue can favor *in vitro* propagation, even in the absence of one of the regulators. When testing the interdependence of ANA and KIN in the *in vitro* propagation of *Eugenia involucrata*, STEFANEL et al. (2022) concluded that the growth of the aerial part can be enhanced by using culture medium with 2 µM ANA and without the addition of KIN. *For Leptochloa crinita* (Lag.) *P.M. Peterson* and *N. Snow*, the combination of ANA and KIN resulted in a low percentage of *in vitro* callogenesis for the dosages tested (1 mg L^{-1} ANA and 1 mg L^{-1} KIN; and 2 mg L^{-1} ANA and 1 mg L^{-1} KIN) by CARBONELL et al. (2022). However, for the medium prepared with 1 mg L^{-1} ANA and without the addition of KIN, callus formation on the explants was statistically higher. Among all the mediums tested; although, the percentage of callogenesis in the medium with 2 mg L^{-1} ANA and 1 mg L^{-1} KIN was the highest, the authors observed that this concentration was also sufficient to induce *in vitro* morphogenesis of *L. crinita.*

The results obtained in this research reinforce the idea formulated by CANDIDO (2013), who pointed out that the significant effect of the interaction also depends on the regulators used. Differentiated responses due to the association between different cytokinins, auxins and cultivation conditions can be observed in the work carried out by PAULA (2019) with the species *Libidibia ferrea* (Mart. ex Tul.) *L.P. Queiroz*, where the medium prepared with ANA and BAP resulted in higher percentages for the regeneration of shoots than the medium prepared with ANA and KIN, both kept under white LED light. Even under a blue-red LED light condition, the medium prepared with ANA (0.05) and BAP (0.1) showed better percentages of sprout regeneration when compared to the medium with ANA and KIN (MATHEUS, 2019).

Bearing in mind that the success of *in vitro* morphogenesis depends on the condition of the phytohormonal balance, the lack of a positive response from the simultaneous addition of different regulators may suggest that the combined addition of cytokinins and auxins has the opposite effect to that expected for some species, thus resulting in a greater phytohormonal imbalance (STEFANEL et al., 2022). However, despite the lack of a positive effect when testing a few combined doses, as seen in several studies in the pertinent literature, this may not mean that the combined addition of auxins and cytokinins to the medium cannot have a positive effect, in which case testing smaller dosage ranges could provide better and more accurate results. When testing dosages that are generally not widely reported in the pertinent literature, SCHMIDT et al. (2007) observed that the best results for multiplication rate were obtained by adding 0.093 mg L^{-1} ANA and 5.380 mg L^{-1} KIN to the medium for the *in vitro* propagation of 'Tainung 01' papaya trees. Conversely, with a view to holding the indirect induction of organogenesis and embryogenesis, the most responsive combination of ANA and KIN was 1.862 mg L^{-1} and 5.38 mg L^{-1} respectively.

There are several contrasting results in the scientific literature regarding the best dosage, where the most effective regulators vary for each species

and type of explant, as well as for the best cultivation conditions, thus demonstrating the need to test the ideal combination of growth regulators for *in vitro* cultivation for each species. Conducting more research like this, which is the first for *O. macrocarpa*, can encourage large-scale seedling production and help to reduce the pressure to explore areas where the species naturally takes place, as well as its *ex situ* conservation.

CONCLUSION

The *in vitro* establishment of *O. macrocarpa* is possible using seeds in MS medium. The addition of activated charcoal and gibberellin (GA_3) to the MS culture medium allows us to obtain more vigorous and elongated plants. The basal segment of the stem allows the regeneration of plants in the *in vitro* environment and should be used during the multiplication phase. At the concentrations used, the cytokinin x auxin interaction does not increase the proliferation of sprouts in the basal explant of the species.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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