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## *In vitro* culture of jambu with different growth regulators

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### ABSTRACT

The aim of this study was to establish the best concentrations of growth regulators for *in vitro* cultivation of jambu for a subsequent elaboration of an efficient micropropagation protocol. After sterilized, the seeds were inoculated on different media (MS, ½MS and water-agar) for *in vitro* germination. Nodal segments of *in vitro* germinated jambu seedlings were used as explants in the micropropagation with different concentrations of 6-benzylaminopurine (BAP) (0.0; 0.125; 0.25; 0.50 and 0.75 mg L<sup>-1</sup>) and callus induction with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.0; 0.25; 0.50; 0.75 and 1.0 mg L<sup>-1</sup>) on Murashige & Skoog's (MS) medium. The highest germination rates were obtained on MS medium with better seedling development and greater height (3.7cm). In micropropagation, the best treatment was obtained on 0.125 BAP (T2), with an average of 2.2 sprouts/explant, average height of 2.4 cm and vigorous sprouts. In callus induction, all treatments with 2,4-D had developed friable calluses in 30 days and using doses of 0.25 and 0.50 mg L<sup>-1</sup> provided greater fresh matter. The induction of friable callus and the root production occur without supplementation of exogenous growth regulator.

**Keywords:** *Acmella oleracea*, micropropagation, callus induction, BAP, 2,4-D, nodal segments.

### RESUMO

#### Cultivo *in vitro* de jambu com diferentes reguladores de crescimento

Objetivou-se estabelecer as melhores concentrações de reguladores de crescimento para o cultivo *in vitro* de jambu, visando a posterior elaboração de um protocolo eficiente de micropropagação. Após assepsia, sementes foram inoculadas em meios diversos (MS, ½MS e água-ágar) para germinação *in vitro*. Segmentos nodais de plântulas germinadas *in vitro* foram utilizados como explante na micropropagação com diferentes concentrações de 6-benzilaminopurina (BAP) (0,0; 0,125; 0,25; 0,50 e 0,75 mg L<sup>-1</sup>) e na indução de calos com ácido 2,4-diclorofenoxiacético (2,4-D) (0,0; 0,25; 0,50; 0,75 e 1,0 mg L<sup>-1</sup>) em meio MS. Maiores taxas de germinação foram obtidas em meio MS, com melhor desenvolvimento das plântulas e maior altura (3,7cm). Na micropropagação o melhor tratamento foi em 0,125 mg L<sup>-1</sup> BAP (T2), com média de 2,2 brotações/explante e altura média de 2,4 cm e brotações vigorosas. Na indução de calos todos os tratamentos com 2,4-D formaram calos friáveis em 30 dias e as doses de 0,25 e 0,50 mg L<sup>-1</sup> de 2,4-D proporcionaram maior massa da matéria fresca. A indução de calos friáveis e a produção de raízes ocorrem sem a suplementação de regulador de crescimento exógeno.

**Palavras-chave:** *Acmella oleracea*, micropropagação, indução de calos, BAP, 2,4-D, segmento nodal.

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*Acmella oleracea* (*Spilanthes acmella*), popularly known in Brazil as “jambu” and in India as “toothache plant”, is an important medicinal herb belonging to Asteraceae family being a non-conventional vegetable found in tropical and subtropical regions. It is originated from the Brazilian tropics, widely grown as ornamental and medicinal plant in various parts of the world (Nabi & Shrivastava, 2015) and used as spice in the Northern part of the country (Favoreto & Gilbert, 2010; Gusmão & Gusmão, 2013; Prushothaman *et al.*, 2018).

Jambu has attracted the interest among researchers and of national and multinational companies, due to the properties of its extract which presents antioxidants, analgesics, antibacterial, diuretics and anti-inflammatory functions; it also has ovicidal and larvicidal activities against *Aedes aegypti* and *Anopheles culicifacies* mosquitoes. Several of these properties are due to the presence of spilantol alkaloid, with numerous applications in pharmaceutical industries (Cheng *et al.*, 2015; Barbosa *et al.*, 2016; Dallazen *et al.*, 2019). This species is

also promising in cosmetics area, since some studies have been proving that spilantol has shown potential anti-skin effect, relaxing the microtensions, acting as “anti-wrinkles” (Demarne & Passaro, 2009).

Although jambu is conventionally propagated by seeds and cuttings (Gusmão & Gusmão, 2013), micropropagation is a technique of great importance for large-scale multiplication of the species and an alternative which can prevent genetic erosion caused by indiscriminate collection and frequent deforestation in areas of natural

occurrence of the species. Moreover, *in vitro* plant cell and tissue culture can standardize and even increase synthesis of bioactive substances in plants (Wang *et al.*, 2017).

Thus, considering the economic and medicinal importance of this plant (*Acmella oleracea*), the aim of this study was to establish the best concentrations of phytohormones for *in vitro* cultivation of the species, aiming a subsequent elaboration of an efficient micropropagation protocol.

## MATERIAL AND METHODS

The experiment was carried out in Laboratório de Biotecnologia Vegetal do LABISIBIO (Laboratories of Systematic Researches in Biotechnology and Molecular Biodiversity) at Universidade Federal do Pará (UFPA).

### *In vitro* germination

Jambu seeds obtained from hydroponic cultivation in a laminar flow cabinet were immersed in 70% alcohol (v/v) for 1 minute, then in sodium hypochlorite (NaClO) (v/v) containing 2.0% active chlorine, for 20 minutes. Afterwards, the seeds were washed three times in sterile distilled water. After asepsis, the seeds were inoculated in bottles containing 30 mL of different media in order to evaluate germination [T1= MS medium (Murashige & Skoog, 1962), T2= medium containing half concentration of macro- and micronutrient salts of MS (½ MS) and T3= water-agar medium]. All the media used in the experiments were supplemented with 30 g L<sup>-1</sup> sucrose and solidified with 8 g L<sup>-1</sup> agar. The medium pH was adjusted to 5.8 and the bottles were autoclaved for 15 minutes at 121°C (1.0 atm pressure). The inoculated seeds were kept in a BOD incubator at 25 ± 2°C, 16 h light photoperiod and 25 µmol m<sup>-2</sup> s<sup>-1</sup> luminous intensity for 30 days. A completely randomized design, with three treatments, was used. Each treatment consisted of 25 seeds, five replicates. Observations were made every seven days for 30 days. Germination percentage variables, height (cm) and quality of seedlings obtained were evaluated by Scott-Knott

test, at 5% probability using RStudio software.

### Micropropagation

Nodal segments of *in vitro* germinated jambu seedlings were inoculated on basic MS medium supplemented with 30 g L<sup>-1</sup> sucrose and solidified with 8 g L<sup>-1</sup> agar and supplemented with different concentrations of BAP (T1= 0.0 mg L<sup>-1</sup>; T2= 0.125 mg L<sup>-1</sup>; T3= 0.25 mg L<sup>-1</sup>; T4= 0.5 mg L<sup>-1</sup>; T5= 0.75 mg L<sup>-1</sup>). Explants were inoculated on the media and kept in a BOD incubator at 25 ± 2°C, 16 h light photoperiod and 25 µmol m<sup>-2</sup> s<sup>-1</sup> luminous intensity for 25 days. A completely randomized design with five treatments was used, which consisted of six explants for each treatment, three replicates. Each replicate consisted of one bottle with two nodal segments. Observations were made every seven days for 25 days and the number of shoots and height (cm) were evaluated by Scott-Knott test, at 5% probability using RStudio software.

### Callus induction

Nodal segments were inoculated on basic MS medium supplemented with 30 g L<sup>-1</sup> sucrose and solidified with 8 g L<sup>-1</sup> agar and supplemented with different concentrations of auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (T1= 0.0 mg L<sup>-1</sup>; T2= 0.25 mg L<sup>-1</sup>; T3= 0.5 mg L<sup>-1</sup>; T4= 0.75 mg L<sup>-1</sup>; T5= 1.0 mg L<sup>-1</sup>). After inoculation, the explants were kept in an incubator at 25 ± 2°C, 16 h light photoperiod for 25 days. A completely randomized design with five treatments and three replicates was used, which consisted of six explants for each treatment, two explants per replicate. We compared average fresh mass (mg) by Scott-Knott test, at 5% probability using RStudio software. Friability and callus induction percentage were also evaluated.

## RESULTS AND DISCUSSION

### *In vitro* germination

*In vitro* germination of jambu began from the second day of inoculation and on the fourth day, 64% of the seeds of T1 (MS medium) were already germinated, showing that *in vitro* germination process is pretty fast. According to Gusmão & Gusmão (2013), the average

germination of jambu in nurseries is 90% and occurs only from the fourth day after sowing in the field at temperatures from 25°C to 33°C. Environmental factors such as temperature, water and light availability considerably influence germination process, considering that the quantity of these factors ranges according to the species. Moreover, factors like medium chemical composition and hormonal balance also strongly influence germination process (Carvalho & Nakagawa, 2012). Thus, controlled conditions in *in vitro* germination favored the acceleration of germination.

We noticed a difference in *in vitro* germination of jambu using different culture media (Table 1). MS medium stood out among the others; this treatment showed the highest germination (88%), with the highest average of germinated seeds (4.4). Whereas the medium containing half concentrations of macro and micronutrient salts of MS (½ MS) showed the lowest germination (64%) and average of germinated seeds (3.2%). This result did not differ statically from treatment T3, using water-agar medium, showing 48% *in vitro* germination and 2.4 average of germinated seeds.

The highest percentage of *in vitro* germination of jambu, using MS medium can be explained by the fact that this medium is quite rich in macronutrients, micronutrients and vitamins (Dezan *et al.*, 2012); however, nutritional demands are very specific, widely varying among different species.

In relation to *in vitro* seedling development, the least efficient treatment was the one using the water-agar medium, showing undeveloped seedlings measuring 0.28 cm-high, since the germinated seedlings did not find nutrients for its development in this medium. MS and ½MS media showed the best result with seedlings presenting good development and vigor, 3.7 cm and 3.96 cm respectively, showing that a medium with nutrients is more conducive to the development of jambu seedlings.

In germination, initially, seeds need good aeration and availability of water to germinate, since they use nutrients from their own resources. However,

when their resources are used up, the germinated seedlings will need nutrients to develop properly (Vidal & Vidal, 2000).

Cultivation conditions, mainly the culture medium used in the experiment, are essential in this first moment of *in vitro* establishment, since the appropriate combination of the components is decisive for the success of germination and obtaining a greater number of seedlings. So, the MS medium showed higher germination percentage, probably due to the presence of higher quantity of macro- and micronutrients and vitamins, appropriate substances which make the seed develop efficiently after germination under *in vitro* conditions. Silva & Ferreira (2016) assert that the nutrients used for cultures provide the essential substances for tissue development and control the growth and pattern of *in vitro* development by the presence of hormones or growth regulators.

### Micropropagation

**Table 1.** Percentage of germination and average of *in vitro* germination, seedling height and contamination (%) of *Acmella oleracea* after 30 days treatment in different culture media. UFRA, Belém, 2019.

Treatments	Germination (%)	Average germination	Seedling height (cm)	Contamination (%)
MS medium	88a	4.4a	3.70a	0
½MS medium	64b	3.2b	3.96a	0
Water-agar medium	48b	2.4b	0.28b	20

\*Average values followed by same lowercase letters in a column do not differ, Scott-Knott test.

**Table 2.** Number of shoots, shoot height and seedling morphology of *Acmella oleracea* seedlings on MS micropropagation medium with different BAP concentrations after 30 days. UFRA, Belém, 2019.

BAP concentrations (mg L <sup>-1</sup> )	Nº of shoots	Height (cm)	Morphology
0.0	1.4a	7.0a	Many roots
0.125	2.2a	2.4b	Shoots showing good development and vigor
0.25	3.0a	1.8b	callus at the base and glazed appearance
0.5	2.5a	0.66c	callus at the base and glazed appearance
0.75	2.0a	2.5b	callus at the base and glazed appearance

\*Average values followed by same lowercase letters in a column do not differ, Scott-Knott test.

Nodal segments of jambu used as explants in this experiment showed good response in relation to *in vitro* micropropagation. Micropropagation of jambu using nodal segments, leaves and meristematic portions of the branches are viable and easy to form plants (Gusmão & Gusmão, 2013).

The authors noticed differences in shoot height among the treatments used (Table 2). When BAP was not used, we observed greater shoot growth (7.0 cm) and the development of many adventitious roots, which allows to infer that jambu plants present high contents of endogenous hormones, mainly auxins which are used for root induction. This was not observed with *Spilanthus acmella*, as Nabi *et al.* (2018) induced organogenesis from calluses obtained from leaves on MS medium supplemented with BAP and 2,4-D. Algabri & Pandhure (2019) also micropropagated *S. acmella* using shoots as an explant in liquid MS medium supplemented with BAP.

In the culture medium, a high concentration of cytokinin in relation to auxin promotes the proliferation of shoots and suppresses root development, whereas a low concentration of cytokinins in relation to a higher concentration of auxins in the medium favors root development. According to Grattapaglia & Machado (1998), excessive concentrations of auxins can inhibit multiplication and favor rooting or callus formation. Therefore, it can be inferred that the treatment (in this study) with *A. oleracea* must have a low concentration of cytokinin and a high endogenous amount of auxin in plant, due to the proliferation of a large quantity of roots observed.

We noticed no differences in number of shoots in any of the treatments proposed (Table 2). The treatment with 0.25 mg L<sup>-1</sup> BAP presented callus at the base, glazed appearance and low growth, characteristics which does not favor micropropagation.

Using 0.125 mg L<sup>-1</sup> BAP, despite no differences between the other BAP concentrations in relation to the number of shoots per explant (2.2) had been noticed, this was the only treatment which presented more vigorous shoots and well developed leaves, without callus and vitrification, in addition to having good growth (2.4 cm) compared to treatment 0.25 mg L<sup>-1</sup> BAP (Table 2).

Roman *et al.* (2016) stated that shoot growth is controlled by environmental and endogenous factors and that light acts mainly as a morphogenic signal in triggering shoot proliferation via cytokinins.

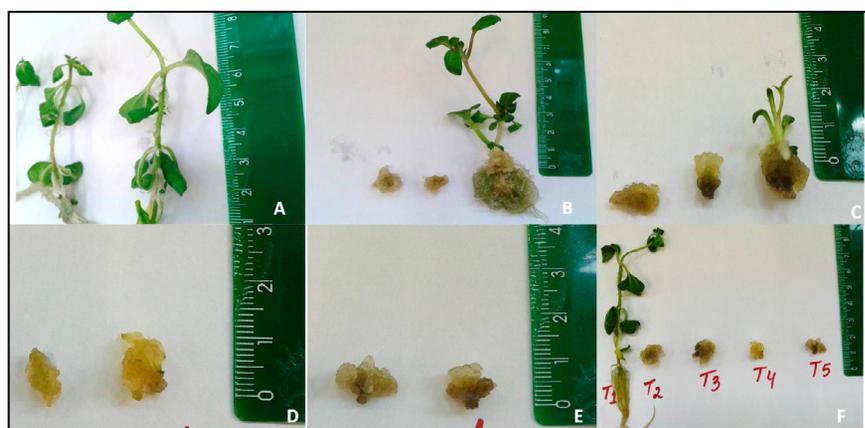
Crispim *et al.* (2014), evaluating different concentrations of BAP in sugar cane observed that the medium supplemented with BAP, at doses 0.5 or 1.0 mg L<sup>-1</sup>, provided better shoot development and greater gain of fresh mass than in the medium without addition of phytohormones.

High contents of exogenous hormonal supply can interfere with morphogenesis and form undifferentiated and disorganized cell masses, called callus, which are undesirable when we sought to develop a micropropagation procedure, which occurred using 0.25, 0.50 and 0.75 mg L<sup>-1</sup> BAP (Figure 1).

**Table 3.** Average callus induction (%), fresh mass and callus morphology of *Acmella oleracea* after 30 days on MS medium with different 2.4-D concentrations. UFRA, Belém, 2019.

Concentrations of 2.4-D (mg L <sup>-1</sup> )	Induction (%)	Fresh mass (mg)	Morphology
0.0	0	0.0b	Many adventitious roots, without callus development
0.25	100	0.4a	Friable callus, beige color, small root
0.50	100	0.3a	Friable callus, beige color, small root and leaves
0.75	100	0.1b	Friable callus, beige color, small leaves
1.0	100	0.2b	Friable callus, dark brown color

\*Average values followed by same lowercase letters in a column do not differ, Scott-Knott test.



**Figure 1.** Callus induction of *Acmeila oleracea* after 30 days at different concentrations of 2.4-D. A (T1)= 0.0 mg L<sup>-1</sup>; B (T2)= 0.25 mg L<sup>-1</sup>; C (T3)= 0.50 mg L<sup>-1</sup>; D (T4)= 0.75 mg L<sup>-1</sup>; E (T5)= 1.0 mg L<sup>-1</sup>; F= all treatments of callus induction. UFRA, Belém, 2019.

Besides formation of calluses, these treatments also showed seedlings with abnormal development, reduction in size and vitrification.

According to Vasconcelos *et al.* (2012), hyperhydricity, formerly called vitrification, is considered a physiological, biochemical and morphological disorder resulting from the abnormal accumulation of water inside cells and tissues. This disorder can be triggered by physical factors, related to the environment of the culture vessels and consistency of the culture medium or by chemical factors such as the components of the culture medium, especially growth regulators in high concentrations.

Gao *et al.* (2018) analyzing physiological and structural alterations in hyperhydric blueberry seedlings

suggested that the excess of ethylene and EROS (reactive oxygen species) produced in response to the stress resulting from *in vitro* cultivation could lead to abnormal stomatal closure, causing the accumulation of water in tissues, oxidative stress due to hypoxia and symptoms of hyperhydricity.

The increase in BAP concentration is considered to be responsible for the increase in the percentage of vitrification. Grattapaglia & Machado (1998) reported that the use of cytokinins stimulates greater shoot production; the excess is toxic and is mainly characterized by shortened internodes, excessive stem thickening, reduction in size and generalized vitrification, though.

**Callus induction**

Callus induction occurred in all treatments with auxin phytohormone.

In the absence of 2.4-D, containing MS medium without regulator, it was the only one which did not induce calluses, due to its non-supplementation with the hormone auxin 2.4-D. However, at the end of 30 days, it showed a large amount of adventitious roots in its plant material, confirming that the explants used in jambu had shown endogenous hormones in their constitution.

Auxins act in growth and control of cell differentiation, regulating the activity of protein kinases dependent on cyclin type A (CDK/a) together with the cytokinins which activate D3 cyclins (CYC/D3). When active complex CDK/a-CYC/D<sub>3</sub> is formed, cells acquire the ability to enter the cell cycle (Mercier, 2012).

Using 0.25 mg L<sup>-1</sup> of 2.4-D, the authors observed that even in low concentrations, 2.4-D favors the development of friable calluses, beige in color, with small roots (Table 3). This reinforces Grattapaglia & Machado's (1998) statement that 2.4-dichlorophenoxyacetic acid (2.4-D) tends to stimulate callus formation, even at low concentrations.

Treatments 0.25 and 0.5 mg L<sup>-1</sup> 2.4-D presented the best results, in addition to reaching 100% callus induction, they had a greater amount of fresh mass (0.4 mg and 0.3 mg, respectively) without differing among each other. These treatments also demonstrated calluses with a tendency for organogenesis, as they exhibited the formation of roots and leaves in their structures.

Treatments 0.75 and 1 mg L<sup>-1</sup> 2.4-D induced 100% callus formation, with 0.1 and 0.2 mg fresh mass, respectively, neither differing from each other, nor among the treatments without regulator.

Callus induction requires explant selection, proper supply of nutrients in the environment and appropriate environmental conditions. Phua *et al.* (2016) obtained callus from the medicinal plant *Clinacanthus nutans* with a higher average of callus fresh mass using explants of young leaves from MS medium supplemented with 0.5 mg/L 2.4-D. Soorni & Kahrizi (2015) showed that using MS medium supplemented with 2.4-D, the genotype and type of explant interfere with both

callus induction and growth in *Cuminum cyminum* accessions.

Thus, MS medium was the most efficient for jambu seed germination. Even without any supplementation of phytohormone BAP or 2,4-D, the species *A. oleracea* induced the formation of shoots and roots, showing the presence of high levels of endogenous hormones. Nodal segments of jambu in MS medium supplemented with 0.125 mg L<sup>-1</sup> BAP showed higher shoot quality. BAP contents higher than 0.25 mg L<sup>-1</sup> formed calluses and leaves with abnormal development and vitrification. MS medium supplemented with concentrations ranging from 0.25 to 1.0 mg L<sup>-1</sup> 2,4-D favored the development of friable calluses in nodal segments of jambu and doses of 0.25 and 0.50 mg L<sup>-1</sup> 2,4-D provided greater fresh mass. Some studies have been carried out in order to verify the influence of other growth regulators and other concentrations to obtain an efficient protocol for an *in vitro* cultivation of this species.

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