

Synthetic seeds of *Acmella oleracea* (Asteraceae) from micropropagated plants in different concentrations of 6-benzylaminopurine

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Research Article

Keywords: 6-benzylaminopurine. Encapsulation. Jambu. Nodal segment

Posted Date: September 6th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3308033/v1>

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Abstract

Acmella oleracea, a leafy vegetable native to the Amazon region, has potential for the cosmetic, pharmaceutical and gastronomic industries. Hot environments and or low relative humidity accelerate losses of vigor and quality, making unfeasible to storage seeds of this plant. This makes necessary to develop new propagation and conservation techniques for *A. oleracea*. The objective was to evaluate the biometric characteristics, describe the stomata and to verify the presence or absence of epicuticular wax on the abaxial leaf epidermis of *A. oleracea* plantlet obtained from the conversion of synthetic seeds (SS) *in vitro*. The nodal segments of micropropagated plantlets, at different concentrations of 6-benzylaminopurine (BAP) (0.000; 0.025; 0.050; 0.100 and 0.200 mg l⁻¹), were sectioned into explants of approximately 0.3 cm, encapsulated in alginate matrix to produce SS, inoculated in test tubes with Murashige & Skoog culture medium and kept in a growth room at a temperature of 25 ± 2 °C and a photoperiod of 16 hours. The percentage of SS conversion, number of leaves and shoots, height and root dry mass were higher in the treatments with BAP. Shoot dry mass was 54.5% higher on plants originated from SS obtained with 0.100 mg l⁻¹ of BAP than in the control. Anomocytic and ellipsoid-shaped stomata were well defined and epicuticular wax deposition was not observed on the abaxial epidermis of *A. oleracea* plantlets leaves. The stomata of *A. oleracea* and the absence of epicuticular wax on the leaf epidermis of this plant were similar between treatments.

Key Message

An effective synthetic seed production protocol was developed. The results of synthetic seedlings produced from explants micropropagated with 6-benzylaminopurine were better.

Introduction

Jambu or watercress from Pará state, Brazil, *Acmella oleracea* (R. K. Jansen) (Asteraceae), is cultivated for culinary, medicinal and insecticide purposes. The leaf composition of this plant includes 24.01% proteins, 1.54% lipids, 63.38% carbohydrates, besides calcium, magnesium and copper (Seal et al. 2013; Neves et al. 2019; Benelli et al. 2019).

Acmella oleracea is preferably cultivated in hot and humid conditions, typical of the Amazon region, with temperatures above 25°C, annual rainfall greater than 2,500 mm and relative humidity of 80% (Spinozzi et al. 2022). Water availability is one of the factors that most influence the germination of *A. oleracea* seeds and the rehydration intensifies respiration and the degradation of reserve tissues, producing energy and nutrients necessary for the growth of the embryonic axis (Sarmiento et al. 2019). On the other hand, very hot environments with low relative humidity reduce the water potential and, consequently, the speed, percentage and vigor of germination of *A. oleracea* seeds (Botelho and Perez 2001; Ávila et al. 2007).

In vitro cultivation, with plant tissue culture, includes different techniques and vegetative materials such as the production of synthetic seeds (SS). This technique facilitates clonal propagation for commercial

purposes, short-term conservation, *in vitro* germplasm exchange and transfer, staggered *in vitro* and *ex vitro* plantlet production, reducing time and increasing homogeneity of production with good phytosanitary condition and high productive potential (Behera et al. 2020; Singh and Singh 2020; Bellumori et al. 2022).

Protocols have been established to produce SS, using nodal segments as explants, from plants of the Asteraceae family, such as *Artemisia vulgaris*, *Spilanthes mauritiana* and *Stevia rebaudiana* (Sudarshana et al. 2013; Sharma and Shahzad 2014; Kundu et al. 2018) with higher percentages of SS conversion using the growth regulator 6-benzylaminopurine (BAP) (Lalthafamkimi et al. 2022).

The objective was to evaluate the biometric characteristics (percentage of conversion, number of leaves and shoots, stem diameter, plant height, shoot and root dry mass), to describe the stomata and to verify the presence or absence of epicuticular wax on leaves of *A. oleracea* seedlings, obtained from the conversion of SS *in vitro*, prepared with explants from micropropagation in different BAP concentrations.

Material and Methods

In vitro establishment of *A. oleracea*

The experiment was conducted at the Plant Tissue Culture Laboratory of the Department of Plant Science in the Federal University of Ceará (UFC) in Fortaleza, Ceará state, Brazil. *Acmella oleracea* (R.K. Jansen) seedlings were established *in vitro* from seeds inoculated in Murashige and Skoog medium (MS) culture medium (Murashige and Skoog 1962), added with 30 g l⁻¹ of sucrose and solidified with 5.8 g l⁻¹ of agar (HIMEDIA®).

Production of nodal segments

SS of *A. oleracea* were produced with explants of approximately 0.3 cm from nodal segments of micropropagated seedlings in the concentrations of 0.000; 0.025; 0.050; 0.100 and 0.200 mg l⁻¹ 6- BAP.

Production of synthetic seed

The explants of *A. oleracea* were mixed with the sodium alginate matrix (2% w/v) (NEON®) dissolved in MS medium and, individually, rescued with the aid of a 3 ml Pasteur pipette, sterilized in 99% alcohol, and dripped into solution of CaCl₂·2H₂O (75 mM) (NEON®) where they remained for 20 minutes for complexation and SS formation. The SS were washed three times in distilled and autoclaved water, to remove excess CaCl₂·2H₂O, and immersed in a KNO₃ solution (200 mM) (EXODO CIENTÍFICA®) for 15 minutes for decomplexation.

Synthetic seed inoculation and growth conditions

The SS (Fig. 1) were inoculated in test tubes with 10 ml of MS medium and kept in a growth room at a temperature of 25 ± 2 °C, photoperiod of 16 hours and light intensity of 35.1 W m⁻²s⁻¹.

At 30 days after inoculation, biometric and ultrastructural characteristics of the leaves and plantlets of *A. oleracea*, obtained from the SS conversion (Fig. 2), were evaluated.

Biometric characteristics evaluated

The percentage of conversion, number of leaves and shoots of the plants were counted. The stem diameter and plant height were measured using a digital caliper. The dry mass of shoots and roots were obtained using a precision scale, after drying the plantlets in a stove with forced air circulation at a constant temperature of 65°C for 72 hours.

Leaf preparation for analysis of the leaf epidermis

Leaf samples of *A. oleracea* were prepared per treatment (Dias et al. 2017), dried in a critical point apparatus (CDP 030) using liquid CO₂ and mounted in 'stubs' for silver metallization and subsequent descriptive analysis of the epidermis in scanning electron microscopy (Quanta 450-FEG – FEI).

Statistical Analysis

The experimental design was completely randomized, with the treatments being the concentrations of BAP (0.000; 0.025; 0.050; 0.100 and 0.200 mg l⁻¹) in the micropropagation of nodal segments of *A. oleracea*, with 11 replications, each with a SS. The data of the biometric characteristics were submitted to the Shapiro-Wilk test (1965) via the *Shapiro-test* function using the base package *stats* in the R Software, to verify if the data followed normal distribution, before proceeding with the analyzes (R Core Team 2021).

The characteristic numbers of shoots and the percentage of data converted did not follow a normal distribution and, therefore, were transformed via Box-Cox with the aid of the *bestNormalize* package (Peterson 2021). All data, transformed or not, were submitted to analysis of variance and the means compared by Tukey's test at $p < 0.05$ via the *HSD.test* function of the *agricolae* package (Mendiburu 2021).

Results and Discussion

The conversion percentage, number of leaves and shoots, stem diameter, plant height, shoot and root dry mass of SS of *A. oleracea* differed between treatments (Table 1). The higher percentage of SS conversion with all BAP doses than in the control, is due to the stimulation of cell division and early vascularization of tissues by this growth regulator, facilitating the translocation of nutrients from the culture medium to the plant (Sosnowski et al. 2023). The technique with growth regulators and non-embryogenic explant (vegetative segments) has also been reported for *Althaea officinalis*, *Plumbago rosea* and *Tylophora indica* (Gantait et al. 2017; Naz et al. 2018; Prakash et al. 2018). The high efficiency of this method in producing SS is also due to the high cellular competence at early stages of organ development and tissue differentiation (Bustillo-Avendaño et al. 2018; Priyadharshini et al. 2020; Bertsouklis and Tsopela 2023).

Table 1
Summary of the analysis of variance of the data of biometric characteristics evaluated in the development of synthetic seeds of *Amella oleracea* in the different treatments

Mean Square								
FV	gl	CO	NL	S	SD	H	SDM	RDM
Trat.	4	0.056*	163.50**	1.39**	8.90**	50.50**	5.03**	9.7×10^{-5} **
Res.	50	0.105	18.41	0.1726	0.326	3.56	3.44×10^{-5}	8.73×10^{-6}
Mean	-	0.84	13.91	1.50	1.96	7.22	0.028	0.0063
CV (%)	-	37.36	30.85	27.66	29.10	26.12	20.68	46.96

** and * significant effects at 0.1% ($p < 0.001$) and 1% ($p < 0.01$) probability levels, respectively, by the F test. *CO* Conversion percentage, *NL* number of leaves and *S* shoots, *SD* stem diameter, *H* plant height, *SDM* shoot and *RDM* root dry, *Treat* treatments, *Res* residues.

The percentage of SS conversion (CO) in *A. oleracea* plantlets was higher in the treatments with the BAP growth regulator, 81.8–100.00%, than in the control (Table 2).

Table 2
Biometric characteristics of *Acmella oleracea* synthetic seeds produced with various concentration of 6-benzylaminopurine

BAP (mg l ⁻¹)	0.000	0.025	0.050	0.100	0.200	DMS
CO (%)	45.4 ^{52.22} _b	81.8 ^{40.45} _a	100.0 ^{0.01} _a	90.9 ^{30.15} _a	100.0 ^{0.01} _a	39.18
NL	7.55 ^{6.72} _b	14.18 ^{2.14} _a	14.64 ^{1.86} _a	18.00 ^{3.10} _a	15.18 ^{5.42} _a	5.1774
SN	0.93 ^{0.67} _b	1.55 ^{0.17} _a	1.64 ^{0.24} _a	1.90 ^{0.26} _a	1.49 ^{0.51} _a	0.5012
SD (mm)	2.86 ^{1.44} _a	1.71 ^{0.20} _{bc}	1.43 ^{0.09} _c	1.67 ^{0.11} _{bc}	2.14 ^{0.51} _b	0.6889
H (cm)	3.41 ^{2.83} _b	8.13 ^{1.12} _a	8.26 ^{1.16} _a	8.45 ^{0.73} _a	7.87 ^{2.58} _a	2.2763
SDM (g)	0.022 ^{0.006} _c	0.027 ^{0.004} _{bc}	0.027 ^{0.005} _{abc}	0.034 ^{0.007} _a	0.032 ^{0.007} _{ab}	0.0071
RDM (g)	0.001 ^{0.001} _b	0.007 ^{0.002} _a	0.006 ^{0.002} _a	0.008 ^{0.003} _a	0.008 ^{0.005} _a	0.0036

Means, per line, followed by the same letter do not differ by Tukey's test. Superscript values represent the standard error. *CO*- Conversion percentage, *NL*- number of leaves and *S* shoots, *SD*- stem diameter, *H*- plant height, *SDM*- shoot and *RDM*- root dry, *Treat*- treatments, *Res*- residues.

The number of leaves and shoots, plant height and root dry mass were higher for plantlets originated from SS in the BAP treatments than in the control (Table 2). Shoot dry mass was 54.5% higher for plantlets originated from SS in the treatment with 0.100 mg l⁻¹ of BAP than in the control. The highest number of leaves and shoots, plant height and shoots and roots dry mass of *A. oleracea* plantlets using BAP agrees with that observed *in vitro* culture of *Artemisia laciniata* (Asteraceae) with 0.1 mg l⁻¹ of this growth regulator and is due to the greater organogenesis in *A. oleracea* plantlets (Kodym et al. 2018). BAP acts in the regulation of plant growth, cell division, regeneration, organ formation and induction of multiplication and development of shoots in SS, as reported for the *in vitro* cultivation of *Achillea fragrantissima* and *Ansellia africana* with concentrations of 0.1 and 0.225 mg l⁻¹ of BAP, respectively (Bhattacharyya et al. 2018; Goda et al. 2023; Asghar et al. 2022). Synthetic growth regulators of the cytokinin class, including BAP, increase the translocation of nutrients from the culture medium, photosynthesis and the production and transport of photoassimilates increasing shoot and root development (Sosnowski et al. 2023).

The stem diameter of *A. oleracea* was greater in the control than in the treatments with different BAP concentrations (Table 2). The larger stem diameter of the *A. oleracea* seedlings in the control than in the treatments with BAP is due to the smaller number of shoots, reducing the mobilization of nutrients from the culture medium and photoassimilates for different parts of the plant, compared to the treatments with BAP. The greater number of shoots increases the competition for photoassimilates between expanding young leaves, decreasing mass accumulation per shoot and reducing plantlets diameter, as reported with nodal segments of *Manihot esculenta* (Alves et al. 2018; Feyisa 2023).

The stomata of *A. oleracea* plantlets are anomocytic (subsidiary cells absent), ellipsoid with an average length of 40 µm and were open and well defined (Fig. 3A, B, C, D and E). Epicuticular wax was not observed in the abaxial epidermis of *A. oleracea* leaves (Fig. 3F, G, H, I and J). The presence of anomocytic-type stomata, with an ellipsoid shape, open and well defined, is associated with their normal functionality in *in vitro* culture (Khan et al. 2002; Afreen 2005). Environmental conditions inside the test tube did not affect stomatal size, and high humidity kept them open. The size and shape of the stomata in the control and treatments with BAP indicate that this growth regulator did not alter them in *A. oleracea* plantlets produced *in vitro*, as already reported in its *ex vitro* cultivation (Matyushin and Evdokimova 2017). The absence of epicuticular wax on the abaxial surface of *A. oleracea* leaves is due to morphological adaptations, associated with the *in vitro* culture condition, reducing transpiration control mechanisms and facilitating the continuous loss of water from the leaf (Lando et al. 2016; Vieira et al. 2020; Garvita and Wawanngrum 2020).

Conclusion

The mean value of the biometric characteristics of *A. oleracea* plants produced by SS, with the exception of the larger stem diameter higher in the control and shoot dry mass 54.7% higher in the treatment with 0.100 mg l⁻¹ of BAP compared to the control, were higher with the use of BAP in micropropagation. The

stomata of *A. oleracea* plants and the absence of epicuticular wax in the leaf epidermis were similar between treatments.

Statements & Declarations

Abbreviations

SS	Synthetic seeds
BAP	6-benzylaminopurine
MS	Murashige and Skoog medium

Declarations

Funding The “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)” and the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)” for funding.

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jorge Braga Ribeiro Junior, Gabrielen de Maria Gomes Dias and Lailla Sabrina Queiroz Nazareno. The first draft of the manuscript was written by Jorge Braga Ribeiro Junior and all authors commented and wrote previous versions of the manuscript. All authors read and approved the final manuscript.”

Data availability The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

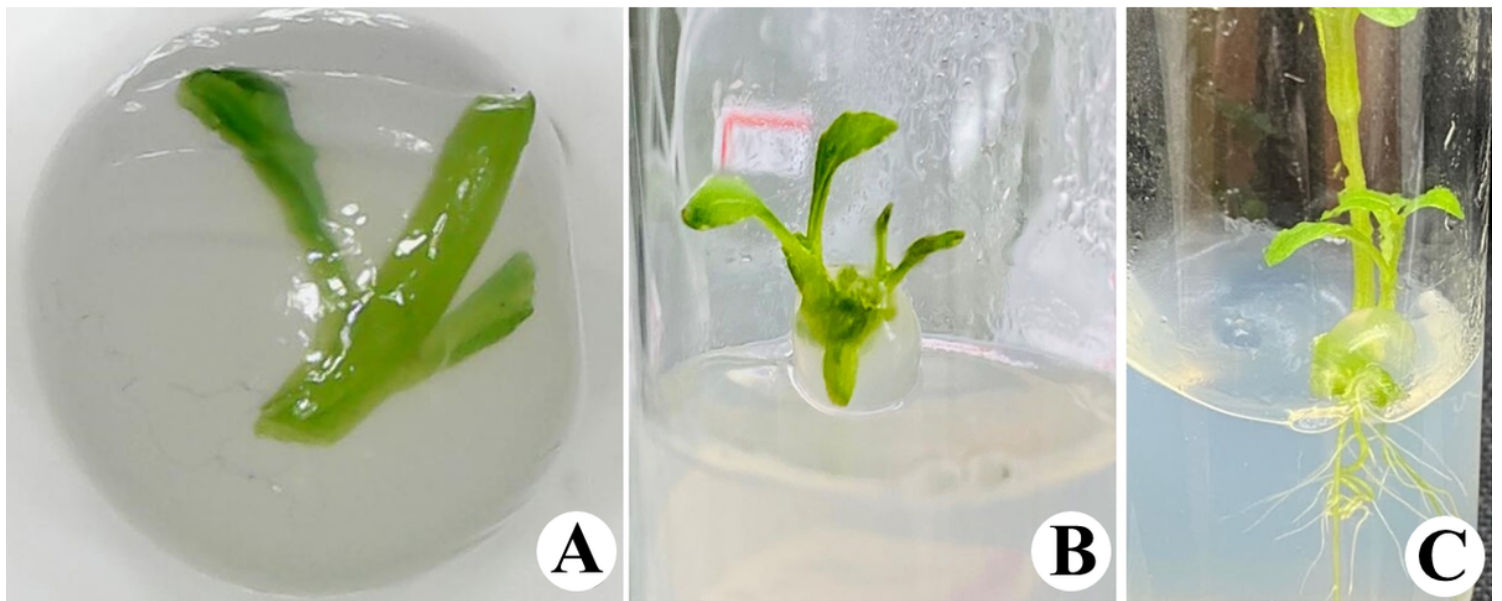


Figure 1

Synthetic seed of *Acmella oleracea* (A), converted after six days of inoculation (B) and rooted after 10 days of inoculation (C).

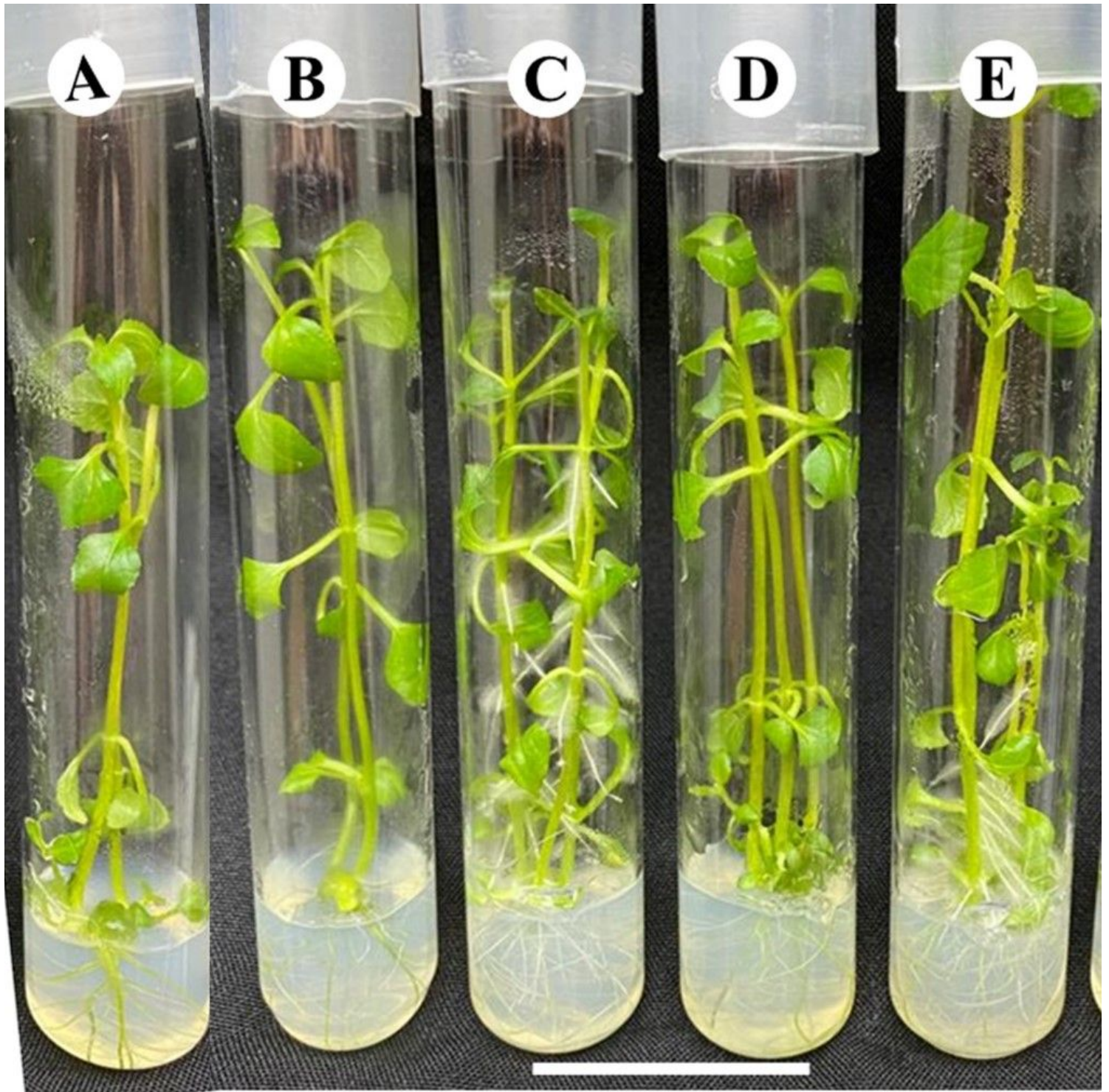


Figure 2

Plantlets of *Acmella oleracea* 30 days after inoculation of synthetic seeds produced with micropropagated explants at the concentrations of 0.000 (A), 0.025 (B), 0.050 (C), 0.100 (D) and 0.200 (E) mg l⁻¹ of 6-benzylaminopurine. Bar = 3 cm.

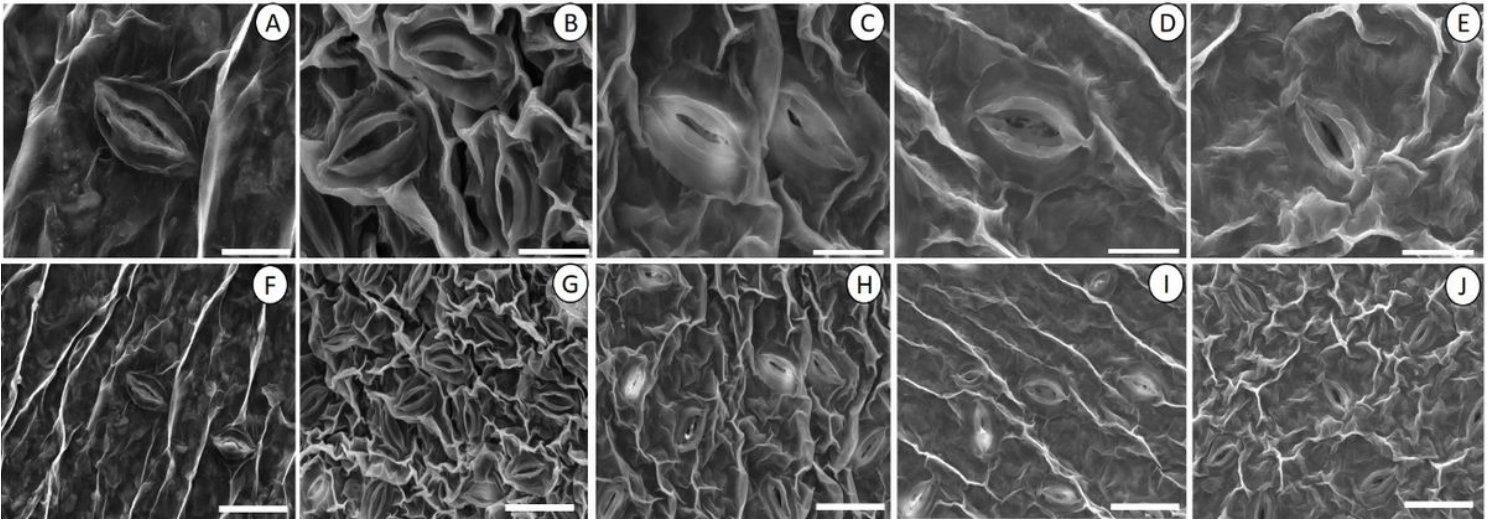


Figure 3

Scanning electron micrograph of the epidermis of the abaxial surface of *Acmeilla oleracea* plantlets leaves 30 days after inoculation of the synthetic seeds, showing anomicytic-type stomata (A, B, C, D, E) and adaxial epidermis without wax (F, G, H, I, J) in the treatments with 0.000 (A) and (F), 0.025 (B) and (G); 0.050 (C) and (H); 0.100 (D) and (I); and 0.200 (E) and (J) mg l⁻¹ BAP in MS. Bar $\approx$ 20 μ m (A, B, C, D, E) and 50 μ m (F, G, H, I, J).