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ARTICLE

Optimizing an *In Vitro* multiplication protocol using cytokinins for *Schizanthus hookeri* Gillies *ex* Graham, a native chilean ornamental

Otimização de um protocolo de multiplicação in vitro utilizando citocininas para *Schizanthus hookeri* Gillies ex Graham, uma planta ornamental nativa do Chile

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Abstract
Schizanthus Ruiz & Pav. (Solanaceae) is a genus endemic to South America, comprising 12 native species distributed mainly in Chile. *Schizanthus hookeri* stands out for the colour of its flowers, generating high value from an ornamental point of view. Since there are few studies related to the propagation and establishment of this species, *in vitro* micropropagation arise as an efficient alternative. Plant Growth regulators (PGR), particularly cytokinins, are determinants for the *in vitro* multiplication. Thus, the present study was aimed at evaluating different concentrations (0.0, 0.5, 1.0, and 2.0 mg L^{-1}) of 6-benzylaminopurine (BAP) and kinetin (KIN) on the micropropagation of *S. hookeri*. Every 3 weeks, shoot length (cm) and shoot number were evaluated, in addition a photographic record of the plantlets was taken. The statistical analysis performed confirmed an interaction between the factors for the number of shoots in weeks 6 and 9, showing the highest number of shoots for the plantlets cultured with the media supplemented with 1.0 mg L^{-1} BAP (11.6) and 2.0 mg L^{-1} KIN (9.9). Regarding the type of cytokinin, KIN obtained the highest means for shoot length, while BAP obtained the highest means for the number of shoots. The present research provides preliminary results for a massive micropropagation protocol for *S. hookeri*, contributing to the conservation and domestication of this native species with ornamental value.
Keywords: 6-benzylaminopurine (BAP), *In vitro* culture, Kinetin (KIN), Micropropagation, Ornamental plant.

Resumo
Schizanthus Ruiz & Pav. (Solanaceae) é um gênero endêmico da América do Sul, compreendendo 12 espécies nativas distribuídas principalmente no Chile. *Schizanthus hookeri* se destaca pela coloração de suas flores, gerando alto valor ornamental. Como existem poucos estudos relacionados à propagação e estabelecimento desta espécie, a micropropagação *in vitro* surge como uma alternativa eficiente. Reguladores de crescimento (PGR), particularmente citocininas, são determinantes para a multiplicação *in vitro*. Assim, o presente estudo teve como objetivo avaliar diferentes concentrações (0.0; 0,5; 1,0 e 2,0 mg L^{-1}) de 6-benzilaminopurina (BAP) e cinetina (KIN) na micropropagação de *S. hookeri*. A cada 3 semanas, foram avaliados o comprimento dos brotos (cm) e o número de brotos, além de um registro fotográfico dos mudas. A análise estatística realizada confirmou uma interação entre os fatores para o número de brotos nas semanas 6 e 9, mostrando o maior número de brotos para os mudas cultivados com os meios suplementados com 1,0 mg L^{-1} de BAP (11,6) e 2,0 mg L^{-1} de KIN (9,9). Em relação ao tipo de citocinina, KIN obteve as maiores médias para comprimento do broto, enquanto BAP obteve as maiores médias para o número de brotos. A presente pesquisa fornece resultados preliminares para um protocolo de micropropagação massiva para *S. hookeri*, contribuindo para a conservação e domesticação desta espécie nativa com valor ornamental.
Palavras-chave: 6-benzilaminopurina (BAP), Cinetina (KIN), Cultura *in vitro*, Micropropagação, Planta ornamental.

Introduction

The *Schizanthus* Ruiz & Pav. Genus (Solanaceae), endemic to the Andean region of southern South America, comprises 12 species distributed mainly in Chile (Grau and Gronbach, 1984; Pérez et al., 2006; Christen et al., 2020). These species inhabit from Tarapacá (22°S) to Los Lagos (40°S) regions in Southern Chile, growing in a great diversity of vegetation formations and habitats (Rodriguez et al., 2018). *Schizanthus* species are herbaceous, annual or biennial plants with wide morphologic diversity among species, exhibiting zygomorphic and bilabiate flowers of diverse and varied sizes, colours and shapes (Chinga et al., 2021). These characteristics render the genus high ornamental value for cut flowers and/or garden plants (Cuesta et al., 2021; Rivas et al., 2021). Furthermore, chemical studies have shown that species belonging to this genus accumulate a wide variety of tropane and pyrrolidine alkaloids (Cretton et al., 2021), compounds of great importance in the pharmacological market (Christen et al., 2020). Some of these species were domesticated in the Northern hemisphere, and the hybrid *S. x wisetonensis* Mast., coming from the cross between *S. pinnatus* Ruiz & Pav. × *S. retusus* Hook. cv Grahamii, has been cultivated as an ornamental plant (Andersen, 2019). However, currently there are no varieties registered as *Schizanthus* is not on the list of taxa protected by the UPOV (2024). Within the *Schizanthus* genus, *S. hookeri* Gillies ex Graham is distributed from the Coquimbo (29°20' S) to the La Araucanía region (39°37' S) in Chile, on mountainous areas ranging from 1,300 to 3,000 m above sea level. This species stands out for its characteristic violet, pink or purple flowers with a large yellow spot on the central lobe, very attractive from an ornamental point of view (Muñoz-Schick and Moreira-Muñoz, 2008; Rivas et al., 2021). Considering its potential in the ornamental plant market and the necessity of conservation efforts, developing an efficient propagation system is required. There are few studies related to the propagation of *S. hookeri* including *ex vitro* (Cuesta et al., 2021), *in vitro* seed culture (Rivas et al., 2021) and *in vitro* organogenesis from callus tissue, where a combination of NAA and BA induced more than 10 shoots/callus (Jordan et al., 2006).

In vitro tissue culture is an efficient option for native species propagation and domestication due to the advantages over conventional propagation (Mehbub et al., 2022), allowing a wide range of species to be propagated in considerably shorter periods of time, with a high multiplication rate and under strict aseptic conditions (Anis and Ahmad, 2016). One of the most important factors for the micropropagation process is the culture medium, made up of inorganic salts and organic compounds, providing sources of carbon, nutrients and growth regulators (Mehbub et al., 2022), allowing the establishment and development of plant material. Plant Growth regulators (GR) are essential as they directly influence several physiological processes, allowing the manipulation of cell growth and tissue differentiation. Within PGR's, cytokinins are widely applied in micropropagation as they promote cell division and proliferation, the formation and multiplication of shoots and stimulate the growth of lateral buds by inhibiting apical dominance (Alcantara-Cortes et al., 2019). Among cytokinins, 6-benzylaminopurine (BAP) and kinetin (KIN) are two of the most used for the *in vitro* multiplication of plants with the

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use of different concentrations of these cytokinins directly affecting the micropropagation efficiency of several species. In *Alstroemeria pallida* Graham, proliferation rate increased with higher concentrations of BAP (2.0 mg L^{-1}), while low concentrations of BAP promoted longer shoots (0.5 and 1.0 mg L^{-1}) (Aros et al., 2017). Moreover, formation of new protocorms of *Trichopilia suavis* Lindl. & Paxton was promoted by the supplementation of 0.5 mg L^{-1} of BAP (Hussien et al., 2023). Similarly, a previous study reported that by increasing the concentration of KIN (2.0 mg L^{-1}) in Cavendish Banana (*Musa acuminata* Colla), the highest shoot proliferation (14.67 shoots per plantlet) was observed with media supplemented with 2.5 mg L^{-1} of BAP, while higher concentrations of this PGR (5.5 mg L^{-1}) generated a decrease in the shoot number (6.67) (Chhon et al., 2025). To date, available studies on *S. hookeri* have not thoroughly addressed its in vitro propagation; therefore, our hypothesis was that BAP will promote higher shoot multiplication rates compared to KIN due to its greater biological activity in solanaceous species, and the main objective was to evaluate the effect of different types and concentrations of cytokinins (BAP and KIN) in the micropropagation of *S. hookeri*.

Material and Methods

Plant Material

Schizanthus hookeri embryos were extracted from immature fruits collected in January 2023 from Farellones, Metropolitan Region, Chile (33°20'46.6"S 70°18'12.2"W), at an altitude of 2300 m (Fig. 1). The harvested fruits were immersed in 95% (v/v) ethanol, and disinfected via direct flaming for 5 s. The embryos were extracted from the disinfected fruits and cultured in Petri dishes using ½ strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} of sucrose and 6.5 g L^{-1} of agar (Phytotechnology Laboratories®) with the pH adjusted to 5.7. Petri dishes with the embryos were kept under complete darkness at 23 °C. After 30 days the germinated embryos were transferred to 200 mL flasks containing 30 mL of MS medium supplemented with 30 g L^{-1} of sucrose, 7.0 g L^{-1} of agar, and 0.5 mg L^{-1} of BAP, with the pH adjusted to 5.7. *In vitro* cultures were kept under controlled conditions at 23 °C and a photoperiod of 16/8 hours light/dark (light provided by fluorescent tubes with density photosynthetic photon flux of 45 µmol photons m^{-2} s^{-1}) for eight weeks, subculturing every four weeks to obtain the number of individuals needed for the experiment.



Fig. 1. Details of the flower (a) and the immature fruits (b) of *Schizanthus hookeri*.

Experimental design

A completely randomized design (CRD) with a factorial structure of treatments was used, where the cytokinin type factor presented 2 levels (KIN and BAP) and the concentration factor 3 levels (0.5, 1.0, and 2.0 mg L^{-1}). Moreover, a control treatment without PGR was evaluated (Table 1). For each treatment a total of 10 replicates were performed, and the experimental unit was a shoot of *S. hookeri* (2-3 cm long) cultured in a 200 mL glass jar.

Table 1. Detail of the treatments of different cytokines (BAP and KIN) and concentrations (0.0, 0.5, 1.0, and 2.0 mg L^{-1}) to evaluate the multiplication of *Schizanthus hookeri*.

Treatment	Cytokinin	Concentration (mg L^{-1})
Control	Control	0.0
BAP 0.5	BAP	0.5
BAP 1.0	BAP	1.0
BAP 2.0	BAP	2.0
KIN 0.5	KIN	0.5
KIN 1.0	KIN	1.0
KIN 2.0	KIN	2.0

***In vitro* multiplication**

Each plantlet was cultured in a 200 mL glass jar with 30 mL of MS medium culture containing 30 g L^{-1} of sucrose, 7.0 g L^{-1} of agar and the cytokinin concentrations previously described (Table 1). The pH of the medium was adjusted to 5.7 and the plantlets were cultured at 23 °C with a photoperiod of 16/8 hours light/dark. The plantlets were subcultured three times every 3 weeks, the moment at which the evaluations were performed.

Evaluations

Evaluations were performed every 3 weeks until 9 weeks were completed and considered shoot length (cm), determined as the distance from the axillary bud to the apical end of the shoot, and the number of shoots, considering only vigorous, non-necrotic shoots. A photographic record of the development of the plantlet was performed using a Nikon D5500 10 Mpx Black (Nikon Co., Tokyo, Japan). All the evaluations were performed in a sterile environment (on autoclaved cardboard trays) under a laminar flow chamber.

Statistical analysis

Normality of data and homogeneity of variances were verified according to the Shapiro-Wilk and the Brown-Forsythe tests, respectively. Since the Analysis of Variance (ANOVA) assumptions were not met, variability was analysed based on the Kruskal-Wallis test on ranks, and differences among means were evaluated using the Student-Newman-Keuls test. All statistical analyses were performed at $\alpha = 0.05$ using SigmaPlot 15 software (Impixon, USA).

Results and Discussion

Effect of cytokinins on shoot length

Plantlets that received the same treatment did not present statistically significant differences regarding their shoot length, except in the case of those treated with 1.0 mg L^{-1} BAP (Table 2). Moreover, no statistically significant differences were observed among treatments in the third week. By the sixth week, the control showed the highest mean value, similar to those of 0.5 and 1.0 mg L^{-1} KIN. On the contrary, all BAP treatments exhibited markedly lower values. At week 9, the control presented the highest value, without differing significantly to all three KIN treatments.

Overall, the plantlet's shoot length remained stable within the same treatment group, whereas BAP treatments were associated with generally lower values after the third week. In contrast, KIN treatments maintained responses closer to the control, particularly at 0.50 and 1.0 mg L^{-1}.

Table 2. Shoot length (cm; mean ± std dev) of *S. hookeri in vitro* plantlets after 9 weeks cultured under different concentrations of BAP (0.5, 1.0, and 2.0 mg L^{-1}) and KIN (0.5, 1.0, and 2.0 mg L^{-1}).

Week	Treatment						
	Control	BAP 0.5	BAP 1.0	BAP 2.0	KIN 0.5	KIN 1.0	KIN 2.0
3	3.28 ± 0.50 Aa	2.82 ± 0.19 Aa	2.74 ± 0.19 Aa	2.61 ± 0.18 Aa	3.19 ± 0.21 Aa	3.16 ± 0.25 Aa	3.01 ± 0.26 Aa
6	4.52 ± 1.48 Aa	1.70 ± 0.25 Bc	1.65 ± 0.31 Ac	1.69 ± 0.13 Ac	3.20 ± 0.63 Aab	2.84 ± 0.68 Aab	2.65 ± 1.40 Abc
9	4.55 ± 1.04 Aa	2.16 ± 0.27 ABbc	1.87 ± 0.32 Ac	2.15 ± 0.44 Abc	3.33 ± 0.54 Aa	2.97 ± 0.43 Aab	2.94 ± 0.59 Aab

Upper- and lowercase letters indicate statistically significant differences ($p \leq 0.05$) among means in the same column and line, respectively, according to the Student-Newman-Keuls test.

These results are consistent with those previously obtained in *Staurogyne repens (Nees)* Kuntze, where KIN obtained higher average shoot lengths compared to BAP (Kose et al., 2021). Likewise, other authors observed that *Glinus lotoides* L. shoots developed in media containing KIN had a higher average length than shoots obtained in media supplemented with BAP, with the latter cytokinin reducing apical dominance to a greater extent compared to kinetin (Teshome and Feyissa, 2015). The results of the present study are partially in accordance with those reported for *Physalis angulata* L. (Solanaceae), where plantlets without PGRs showed the highest value of shoot length (13.05 cm) compared to treatments supplemented with different concentrations of KIN and BAP (0.5, 1.0, and 1.5 mg L^{-1}) (de Jesús Romo-Paz et al., 2021). This response can be explained because one of the effects of cytokinins is to generate a loss or reduction of apical dominance in plants, promoting the formation of lateral shoots through the induction of cell division, generating a reduction in shoot length (Asghar et al., 2023).

Effect of cytokinins on number of shoots

Plantlets that received the same treatment presented statistically significant differences regarding the number of shoots only in the cases of those treated with 1.0 and 2.0 mg L^{-1} BAP and 0.5 and 1.0 mg L^{-1} KIN (Table 3).

Comparison among treatments showed that at week 3 no statistically significant differences were observed, indicating that neither BAP nor KIN produced an early effect on the number of plantlets´ shoots. By the sixth week, the mean values of the control remained significantly lower than those of 0.5 mg L^{-1} BAP and 2 mg L^{-1} KIN.

At week 9, the highest mean value was recorded for 1.0 mg L^{-1} BAP, differing significantly from the control and the PGRs treatments with 2.0 mg L^{-1} BAP and 0.5 and 1.0 mg L^{-1} KIN.

These results indicate that cytokinin supplementation promoted the number of shoots over time, with BAP treatments, particularly 0.5 and 1.0 mg L^{-1} BAP and 2 mg L^{-1} KIN.

Similar effects have been earlier reported, where concentrations greater than or equal to 0.5 mg L^{-1} BAP significantly promoted the development of lateral shoots in *Vestia foetida* (Ruiz & Pav.) Hoffmanns, a Chilean native species (Uribe et al., 2012). This can be attributed to the effect of cytokinins on plants, as they promote cell proliferation and division, enhancing the formation of axillary shoots, and ultimately suppressing or eliminating apical dominance (Asghar et al., 2023). Specifically, the superior shoot proliferation observed with BAP compared to KIN (yielding up to 11.6 shoots per plantlet) can be explained by fundamental physiological differences in their chemical structure, receptor affinity, and morphogenic potency. Although both BAP and KIN belong to the aromatic class of cytokinins -which generally exhibit lower susceptibility to enzymatic degradation by cytokinin oxidase/dehydrogenase (CKX) (Galuszka et al., 2007). BAP frequently demonstrates higher efficiency in shoot organogenesis and axillary bud release compared to weaker cytokinins like KIN (Spíchal et al., 2004; Aremu et al., 2012). This enables BAP to effectively overcome auxin-mediated apical dominance and promote active cell division in lateral buds, yielding a higher number of secondary shoots. In contrast, KIN acts more moderately, favouring cellular differentiation and shoot extension over rapid bud proliferation.

Table 3. Number of shoots of *S. hookeri in vitro* plantlets after 9 weeks cultured under different concentrations of BAP (0.5, 1.0 and 2.0 mg L^{-1}) and KIN (0.5, 1.0 and 2.0 mg L^{-1}).

Week	Treatment						
	Control	BAP 0.5	BAP 1.0	BAP 2.0	KIN 0.5	KIN 1.0	KIN 2.0
3	2.0 ± 0.8 Aa	3.5 ± 1.0 Aa	3.4 ± 1.1 Ba	3.1 ± 0.8 Ba	3.1 ± 0.6 Ba	2.7 ± 0.8 Ba	3.9 ± 1.1 Aa
6	3.3 ± 1.0 Ab	7.0 ± 2.0 Aa	4.6 ± 2.0 Bab	5.0 ± 1.4 ABab	5.3 ± 2.0 ABab	5.2 ± 1.6 ABab	8.2 ± 2.7 Aa
9	5.0 ± 1.0 Ab	10.6 ± 3.6 Aab	11.6 ± 4.5 Aa	9.0 ± 3.1 Ab	8.0 ± 3.0 Ab	7.0 ± 2.7 Ab	9.9 ± 2.9 Aab

Upper- and lowercase letters indicate statistically significant differences ($p \leq 0.05$) among means in the same column and line, respectively, according to the Student-Newman-Keuls test.

Furthermore, the temporal accumulation of these growth regulators over subcultures plays a crucial role in shoot response. In this regard, a previous study observed no effects of different concentrations of KIN after 4 weeks, but a significant and positive effect after 8 weeks over the number and length of *Petunia* Juss. (Solanaceae) *in vitro* shoots (Bashi et al., 2023). Moreover, previous studies in *Pisum sativum* L. have confirmed that long-term *in vitro* shoot cultures grown on cytokinin-supplemented media show a gradual rise in endogenous cytokinin levels, which eventually stabilize at a high concentration (Plačková et al., 2015).

Visual assessment of plantlets

Representative plantlets from each treatment were photographed at each evaluation time point. Substantial differences are observed among the different treatments applied. Thus, at week 3 some morphological differences were already appreciated between the plantlets treated with BAP and KIN (Fig. 2). By week 6 these differences became even more evident. Although the treatments supplemented with BAP presented more shoots, these were smaller, thickened and slightly chlorotic in appearance. Previous studies have observed an adverse effect on the growth and development of the plantlets at higher concentrations of BAP (Sosnowski et al., 2023). This response occurs because high concentrations of BAP in the culture medium can induce ethylene biosynthesis by being involved in the four catalytic steps of the synthesis pathway of this molecule (Žd›árská et al., 2013), which causes an inhibitory effect on proliferation, regeneration and shoot length. In this sense, although BAP is a widely used cytokinin due to its efficacy and affordability, it is important to consider that its use tends to stimulate greater ethylene production compared to other growth regulators such as KIN, considered a less potent cytokinin (Polivanova and Bedarev, 2020).

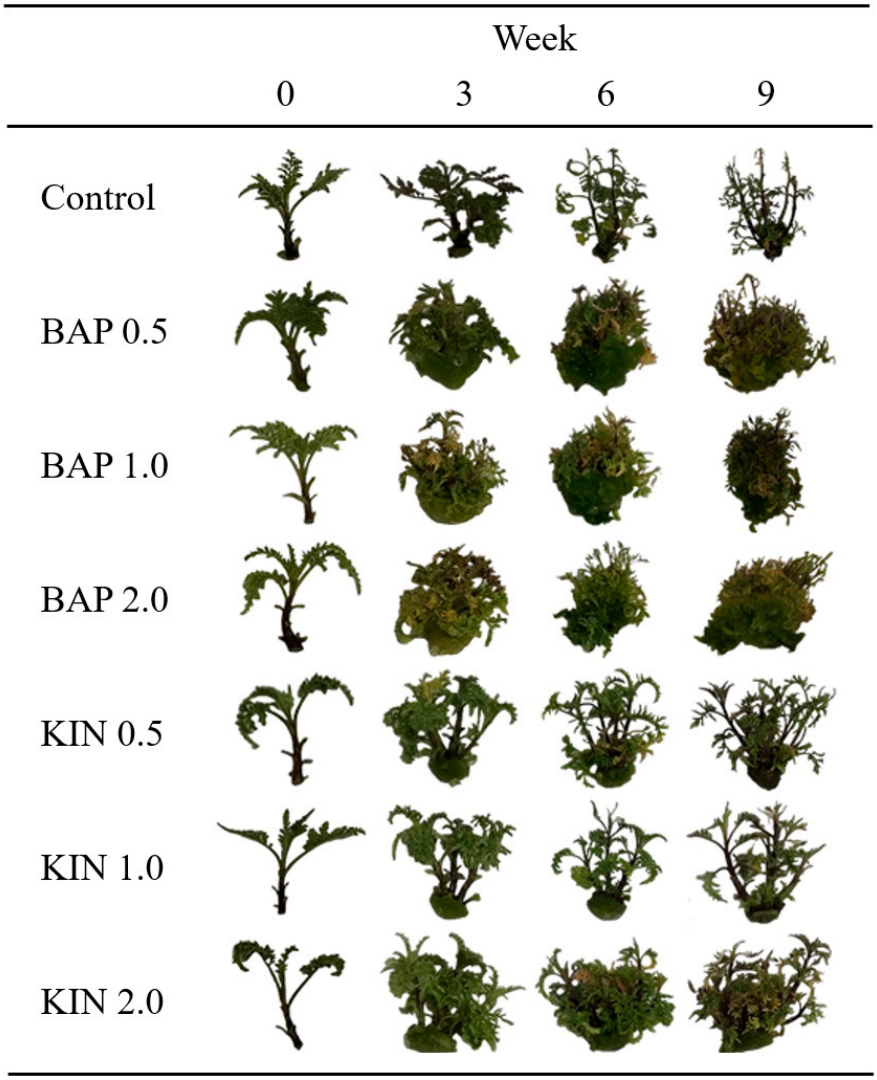


Fig. 2. Development of *in vitro* plantlets of *Schizanthus hookeri*, after nine weeks of being cultured under three concentrations of BAP (0.5 mg L^{-1}, 1.0 mg L^{-1}, and 2.0 mg L^{-1}), and three concentrations of KIN (0.5 mg L^{-1}, 1.0 mg L^{-1}, and 2.0 mg L^{-1}), plus a control where no PGRs were applied.

At week 9 all treatments supplemented with BAP showed a massive production of shorter shoots, associated with the high rate of cell multiplication promoted by this cytokinin, generating multiple points of meristematic growth. These shoots have shorter internodes and rosettes, with some level of vitrification (Fig. 3). In this sense, a similar response has been observed in *Aronia melanocarpa* (Michx.) Elliott (black chokeberry) *in vitro* plants where a high BAP concentration (5 mg L^{-1}) induced vitrification in up to 77% of the plantlets (Bayhan and Yücesan, 2024). The resulting abnormal morphogenesis is associated with hyperhydricity, a physiological disorder frequently triggered by elevated cytokinin levels (Polivanova and Bedarev, 2022).

On the other hand, treatments supplemented with KIN yielded better results in terms of shoot growth and development. However, as the concentration of this PGR increased, a decline in shoot quality was observed, expressed by reduction of vigour and leaf yellowing. These outcomes can be attributed to the cumulative effect of KIN in the culture medium, as reported in *Cucumis sativus* L., where increasing concentrations of KIN led to inhibitory physiological responses, reducing regeneration rates, shoot number, and shoot length. This phenomenon is likely related to the so-called "saturation point" of the PGR in the plantlet, which diminishes the physiological response of the in vitro plantlet (Abu-Romman et al., 2015).

Finally, considering that using a combination of two cytokinin types can sometimes produce better outcomes than using a single cytokinin alone (Nowakowska et al., 2022; Bhat et al., 2022), future studies could be focused on the supplementation of BAP and KIN together in order to obtain better results.

Conclusions

A significant effect between the interaction of the cytokinin type and concentration was observed, particularly for number of shoots in weeks 6 and 9. Thus, the treatment supplemented with 1.0 mg L^{-1} BAP produced the highest mean number of shoots after nine weeks (11.6 shoots per plantlet). Furthermore, plantlets exposed to BAP clearly showed shorter shoots in comparison with the control, whereas no such reduction was observed in the KIN treatments. In contrast, BAP application promoted a greater shoot proliferation response. This study presents a starting point on the micropropagation of *Schizanthus hookeri*, a Chilean native species with ornamental value. Lower concentrations of BAP are recommended for further studies, alongside the evaluation of other factors on the composition of the growing media.

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Author Contribution

DA: Conceptualization**,** Methodology, Formal Analysis, Visualization, Funding Acquisition, Writing-Original Draft, Writing-Review & Editing. **SL:** Methodology, Data Curation, Writing-Original Draft. **PA:** Formal Analysis, Visualization, Writing-Review & Editing. **DC:** Formal Analysis, Visualization, Writing-Review & Editing. **CM:** Methodology, Formal Analysis, Writing-Review & Editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Data will be made available upon request to the authors.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

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