In vitro micropropagation and physiological assessment of *Senecio bonariensis*

Micropropagación *in vitro* y evaluación del estado fisiológico de plantas de *Senecio bonariensis*

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Abstract

Senecio bonariensis is a plant native to South American wetlands. This plant has ecological importance, is used in traditional medicine, and is also popular for ornamental purposes. This study aimed to develop the first *in vitro* propagation protocol for *S. bonariensis*. Leaf explants were disinfected and placed on Murashige and Skoog (MS) agar medium supplemented with different combinations of growth regulators. We tested the effect of two different cytokinins: Kinetin (KIN) and 6-benzylaminopurine (BAP), in the presence of the auxin α-naphthalene acetic acid (NAA). All treatments with KIN resulted in root production, but only treatments with BAP induced shoot formation. As results, we determined the optimal concentration for maximum shoot production, achieving a 100% success in rustication while finding similar physiological traits among micro-propagated and wild-type plants. In conclusion, we developed a protocol for the large-scale production of *S. bonariensis* plants, providing an alternative source of bioactive compounds for medical and pharmaceutical purposes while preserving the natural habitat of this native plant.

Keywords

Margarita de bañado • *Senecio bonariensis* • plant growth regulator • *in vitro* tissue culture • conservation • OJIP-test

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Resumen

Senecio bonariensis Hook. y Arn. es una planta nativa que se encuentra principalmente en zonas de humedales de América del Sur. Esta planta tiene importancia ecológica y también se utiliza en la medicina tradicional. Además, es una opción popular con fines ornamentales. El objetivo de este estudio fue desarrollar el primer protocolo de propagación *in vitro* de *S. bonariensis*. Los explantos de hojas se desinfectaron y luego se colocaron en medio agar Murashige y Skoog (MS) suplementado con diferentes combinaciones de reguladores de crecimiento. Se probó el efecto de diferentes citoquininas: kinetina (KIN) y 6-bencilamino-purina (BAP) en presencia de la auxina ácido α-naftalenoacético (NAA). Encontramos que todos los tratamientos, incluido KIN, dieron como resultado la producción de raíces, pero solo los tratamientos que incluyeron BAP mostraron inducción de brotes. Como resultado, determinamos la concentración óptima para la mayor producción de brotes y la tasa de éxito del proceso de rusticación fue del 100%. También evaluamos el estado fisiológico de las plantas micropropagadas y observamos que los parámetros probados eran similares a los de las plantas silvestres. En conclusión, hemos desarrollado un protocolo para la producción a gran escala de plantas de *S. bonariensis*. Esto proporcionará una fuente alternativa de compuestos bioactivos para fines médicos y farmacéuticos y al mismo tiempo preservará el hábitat natural de esta planta nativa.

Palabras clave
Margarita de bañado • *Senecio bonariensis* • regulador de crecimiento vegetal • cultivo de tejidos *in vitro* • conservación • prueba OJIP

Introduction

The Asteraceae family is one of the largest families of dicotyledonous plants (5, 10, 14). *Senecio bonariensis* is a native plant species primarily found in wet zones of central and northern Argentina, Bolivia, Uruguay, and southern Brazil (10). This shrub is also known as Margarita de bañado, Pillahuincó, Bálsamo, Lampacillo, Lampaso, Lampazo, Lengua de ciervo, Margarita del agua, Margaritón de bañado, or Sanguinaria in Spanish; Margarida do banhado in Portuguese; and butterweed, groundsel, or ragwort in English (10). Considering *S. bonariensis* is traditionally used to treat skin, respiratory and osteoarticular diseases (5, 10, 14), there is growing interest in its cultivation to obtain bio-compounds for medicinal use and basic or applied research. Plant tissue culture is widely accepted for propagating native species. This technology has been adopted for conservation purposes by organizations such as The Botanic Gardens Conservation International (BGCI), which represents botanical gardens in 120 countries (4, 6, 11). However, only a few micropropagation and *in vitro* propagation protocols for other *Senecio* species have been previously reported (7, 16, 18) with protocols presenting bottlenecks at various stages of the propagation process, highlighting the need for specific protocols tailored to this genus. Considering that precise regulation of cytokinins and auxin levels strongly affects growth of stems, roots, and leaves, determining type and concentrations of particular plant growth regulators (PGRs) is essential. Poorly established protocols typically result in low shoot multiplication, low rooting frequency, morphological abnormalities, and high production costs (1). This study aimed to develop a protocol, particularly considering *S. bonariensis* for successful plant micropropagation and rustication. We assessed the effects of two cytokinins, Kinetin (KIN) and 6-benzyl aminopurine (BAP), in conjunction with the α-naphthalene acetic acid (NAA) auxin, on shoot production from leaf explants, aiming to identify the optimal combination for maximizing yield. Additionally, we evaluated physiological parameters of the micro-propagated plants utilizing the non-destructive and cost-effective OJIP test. This test analyzes the OJIP curve providing insights into thermal and photochemical phases of electron transport chain (17).

In conclusion, our study developed a protocol for large-scale production of *S. bonariensis* plants as an alternative source of bioactive compounds for medical and pharmaceutical purposes, while contributing to the preservation of the species´ natural habitat.

Material and methods

Plant material and surface sterilization of explants

Leaves of *Senecio bonariensis* were collected during springtime (2018-2022) from Laguna de Chascomús (35°35'22.92'' S 58°1'20.14'' W, Buenos Aires, Argentina). A voucher specimen was deposited in the Herbarium of the Museo de Ciencias Naturales de La Plata (Buenos Aires, Argentina), under the collection number M. G. Corigliano 1, LP 082432. Healthy young leaves were meticulously washed under running tap water to prevent damage. Subsequently, explants underwent surface sterilization using a 20% v/v solution of commercial bleach for 30 minutes, followed by four rinses with sterile distilled water in a biosafety cabinet.

Callus induction

Surface-sterilized explants were dissected into small pieces (1 cm^2) without disrupting serrated margins and subsequently placed onto sterile Murashige and Skoog (MS) basal medium (13) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. To induce callus formation, the auxin α-naphthalene acetic acid (NAA) was tested at three different concentrations (0.1, 0.5, and 1 μg ml^{-1}) in combination with two different cytokinins, BAP or KIN, each at three different concentrations (0.5, 1, and 2 μg ml^{-1}), totalling eighteen combinations. Eight leaf fragments from distinct plants were incubated in culture flasks in a growth chamber set to a 16-hour day/8-hour night photoperiod, with a photosynthetic photon flux density (PPFD) of *350 μmol quanta m^{-2} s^{-1} provided by cool-white fluorescent lamps, at a constant temperature of 24/21 ± 2°C. The percentage of callus induction (PCI) was assessed 30 days after the initial culture (d.a.i.c.), and calculated by dividing the number of explants with calli by the total cultured explants, x 100. Three independent experiments were performed.

Shoot growth study

Calli were sub-cultured onto fresh MS medium with the same hormone combination 30 d.a.i.c. The number of shoots produced per explant was evaluated at 90 d.a.i.c. We also measured shoot length and registered the tallest shoot for each treatment.

Acclimatization

Shoots obtained at 90 d.a.i.c. were transplanted into plastic pots filled with a sterile mixture of sand, soil, and perlite (1:1:1 ratio) and watered with Hoagland nutrient solution (8) every 2 days. The pots were placed in a growth chamber with a 16-hour day/8-hour night photoperiod, provided by cool-white fluorescent lamps, and maintained at a temperature of 24/21 ± 2°C. Plant survival and phenotypic variation were recorded. After 12 weeks, the plants were transferred to field conditions and flowering ability was assessed.

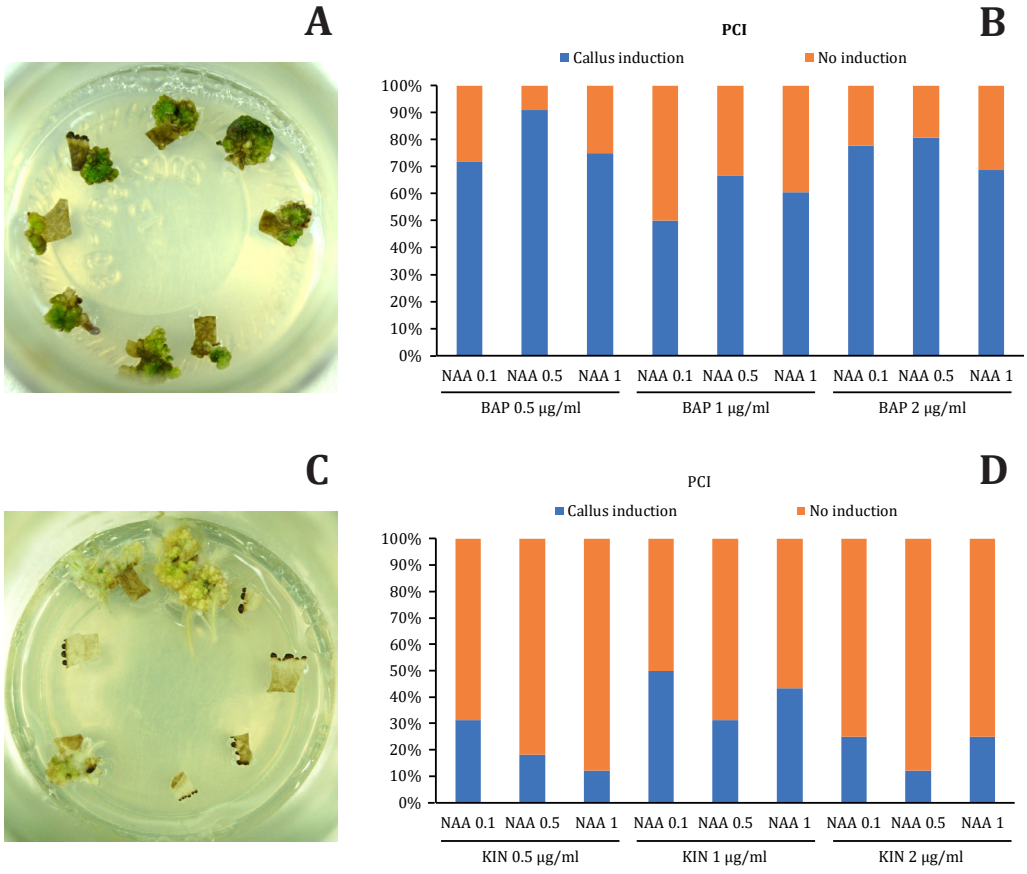
Chlorophyll fluorescence fast-transient analysis

The non-invasive OJIP test (16) was conducted on 6 to 7-month-old plants using a portable chlorophyll fluorometer (Pocket PEA v.1.1, Hansatech Instruments Ltd.), as described by Corigliano *et al.* (2019). Briefly, the youngest fully developed leaf was dark-adapted for 20 minutes before analysis. Subsequently, leaf samples were exposed to a 3-second pulse of light at an intensity of 3500 μmol photons m^{-2} s^{-1} (peak wavelength: 637 nm). Data were analyzed using PEA Plus software (Hansatech Instruments Ltd.). Maximum quantum yield of primary PSII photochemistry (Fv/Fm) and dissipation energy flux per active reaction center of PSII (DIo/RC) were determined. Additionally, we analyzed the contribution to photosynthesis regulation of two functional steps, namely ABS (absorption of light energy) and TRo (trapping of excitation energy) by RC (reaction center), and CSo (cross-section).

Results

Callus induction

Effective surface sterilization was achieved for *S. bonariensis* leaf explants. The effect of two cytokinins, BAP and KIN, in combination with the NAA auxin, was tested. Figure 1 shows calli induction 30 d.a.i.c. Although MS medium without PGR did not lead to callus generation (data not shown), callus induction was observed for all hormone combinations, either BAP-NAA (figure 1A) or KIN-NAA (figure 1C). However, the BAP-NAA combination resulted in a higher calli percentage compared to KIN-NAA in any combination. The PCI was determined for various BAP-NAA combinations. The highest PCI obtained was about 90% when calli were cultured in MS medium supplemented with NAA 0.5 µg ml^{-1} and BAP 0.5 µg ml^{-1} (figure 1B). On the other hand, the highest PCI was only 50% when calli were cultured with NAA 1 µg ml^{-1} and KIN 0.5 µg ml^{-1} (figure 1D). Initial callus induction was observed at the serrated edge of leaves (figure 1A, 1C).



Bars represent percentage of one experiment. Blue represents callus induction, while orange indicates no callus induction. The assay was performed in triplicate. Significant differences were observed between NAA 1 µg ml^{-1} - KIN 0.5 µg ml^{-1} and NAA 0.1 µg ml^{-1} - KIN 1 µg ml^{-1} (P= 0.04, X2= 16.1,8).

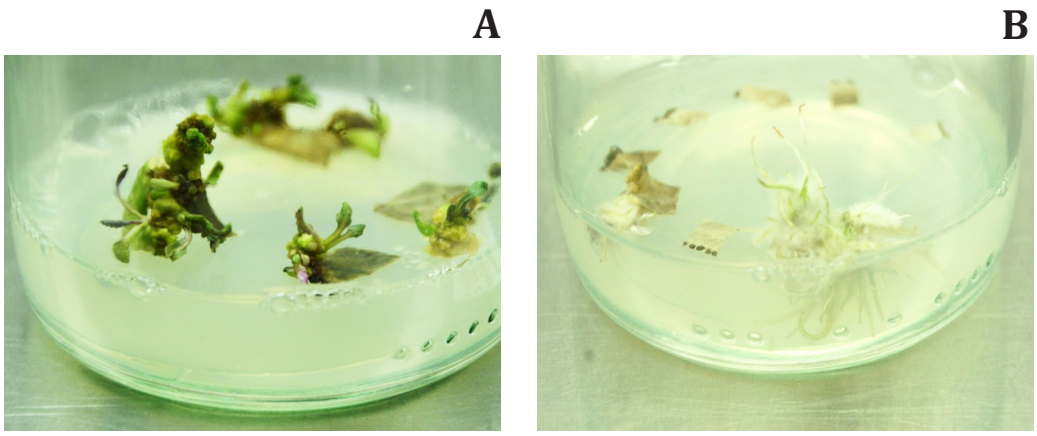
Las barras representan los promedios de un experimento y se graficaron como porcentajes. El color azul representa la inducción de callos, mientras que el anaranjado indica que no hay inducción de callos. Este ensayo se realizó por triplicado. Se observaron diferencias significativas entre NAA 1 µg ml^{-1} - KIN 0,5 µg ml^{-1} y NAA 0,1 µg ml^{-1} - KIN 1 µg ml^{-1} (P= 0,04, X2= 16,1,8).

Figure 1. Effect of NAA and BAP on callus induction. A Callus induction from leaf explants on MS medium supplemented with either 0.5 µg ml^{-1} NAA and 0.5 µg ml^{-1} BAP (C), or with 0.1 µg ml^{-1} NAA and 1 µg ml^{-1} KIN, 30 days after culture initiation. The PCI from leaves cultured on MS and supplemented with NAA at three different concentrations (0.1, 0.5, and 1 µg ml^{-1}) and combined with either BAP (B) or KIN (D) at three different concentrations (0.5, 1, and 2 µg ml^{-1}) was evaluated 30 d.a.c.i.

Figura 1. Efecto de NAA y BAP en la inducción de callos. (A) Inducción de callos a partir de explantos de hojas en medio MS suplementado con 0,5 µg ml^{-1} de NAA y 0,5 µg ml^{-1} de BAP (C) o con 0,1 µg ml^{-1} de NAA y 1 µg ml^{-1} de KIN, 30 días luego de la inducción del callo (d.l.i.c). Evaluación del porcentaje de inducción de callos en hojas cultivadas con MS y suplementadas con NAA en tres concentraciones diferentes (0,1, 0,5 y 1 µg ml^{-1}) y combinadas con BAP (B) o KIN (D) en tres concentraciones diferentes: 0,5, 1, y 2 µg ml^{-1}, a los 30 d.l.i.c.

Effects on BAP, KIN, and NAA on shoot multiplication

Treatments including BAP led to shoot induction (figure 2A). Conversely, even though different concentrations of KIN induced callus formation, they did not result in shoot production, but in roots (figure 2B). Shoots per cultured explant were counted at 90 d.p.i.c (figure 3, page 142). Even without statistical differences among groups with different BAP-NAA combinations, the highest number of shoots per explant was observed with BAP 0.5 µg ml^{-1} and NAA 0.5 µg ml^{-1} (figure 3A, page 142). Interestingly, the longest shoots were both obtained with the BAP 0.5 µg ml^{-1} and NAA 0.5 µg ml^{-1} combination (figure 3B, page 142).



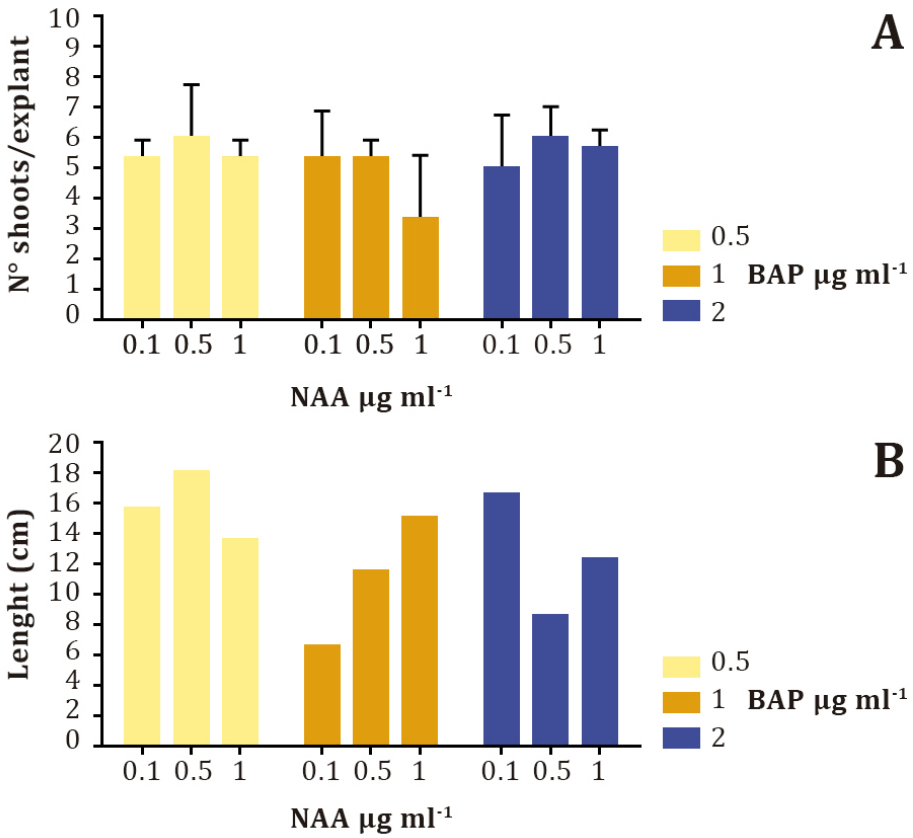
(A) Shoot induction from NAA-BAP treated calli 45 d.a.i.c. (B) Root induction from NAA-KIN treated calli 45 d.a.i.c.

(A) Inducción de brotes de callos tratados con NAA-BAP luego de 45 días. (B) Inducción de raíces tratadas con NAA-KIN luego de 45 días.

Figure 2. Effects of NAA and BAP on shoot multiplication of *S. bonariensis*.
Figura 2. Efecto de NAA y BAP en la multiplicación de brotes de *S. bonariensis*.

Micro-propagated plants displayed purple phenotype with no physiological disturbances

Shoots obtained 90 d.a.i.c. were transferred to plastic pots and placed in a plant room with a 16 h day/ 8 h night photoperiod and 24/21 ± 2°C. Plant survival and rustication were 100% successful (data not shown). A variety of physiological parameters validated the micropropagation protocol. Some plants grown *in vitro* showed purple colorations on leaf abaxial face, prompting a comparative analysis of physiological traits of green and purplish leaves from micro-propagated plants, and green leaves from non-propagated (wild-type, WT) control plants (figure 4A, page 143). We assessed maximum quantum yield of primary photochemistry (Fv/Fm) and examined photosynthesis regulation by the two functional steps, namely ABS (absorption of light energy) and TRo (trapping of excitation energy) by RC (reaction center) and CSo (cross-section). The Fv/Fm values for green, purple, and WT plants were almost 0.8, considered normal (figure 4B, page 143). Other energetic parameters (ABS/RC, ABS/CSo, TRo/RC, and TRo/CSo) showed no statistical differences among plants (figure 4B, page 143). However, the dissipation energy per reaction center (DIo/RC) was statistically lower in green and purple plants than in WT plants ($p<0.01$) (figure 4C, page 143).



(A) Assessment of the number of shoots obtained per explant using NAA at three different concentrations (0.1, 0.5, and 1 µg ml^{-1}) and combined with BAP at 0.5 µg ml^{-1} (yellow), 1 µg ml^{-1} (orange), and 2 µg ml^{-1} (blue) obtained 60 d.a.i.c. This experiment was conducted in triplicate. Statistical analysis was performed by one-way ANOVA and no statistical differences were observed among groups. (B) Shoot length at different hormone combinations achieved 60 d.a.i.c. The longest shoot per group in three independent experiments is plotted in the figure.

(A) Evaluación del número de brotes obtenidos por explanto utilizando NAA en tres concentraciones diferentes (0,1, 0,5 y 1 µg ml^{-1}) y combinado con BAP a 0,5 µg ml^{-1} (amarillo), 1 µg ml^{-1} (anaranjado), o 2 µg ml^{-1} (azul) luego de 60 días. Este experimento se realizó por triplicado. El análisis estadístico se realizó mediante análisis de varianza unidireccional (ANOVA) y no hubo diferencias estadísticas entre los grupos. (B) Gráfico de la longitud máxima de los brotes con diferentes combinaciones de hormonas alcanzada a 60 d.l.i.c. La longitud del brote más largo de cada grupo en tres experimentos independientes se midió y se representó en la figura.

Figure 3. Effects on BAP and NAA on shoot multiplication.
Figura 3. Efectos de BAP y NAA en la multiplicación de brotes.

Acclimatization of regenerated shoots

After 12 weeks, the plants were transferred to field conditions, and phenotypic variation was visually assessed. Notably, purple colorations on leaf abaxial sides disappeared after one or two weeks in the field.

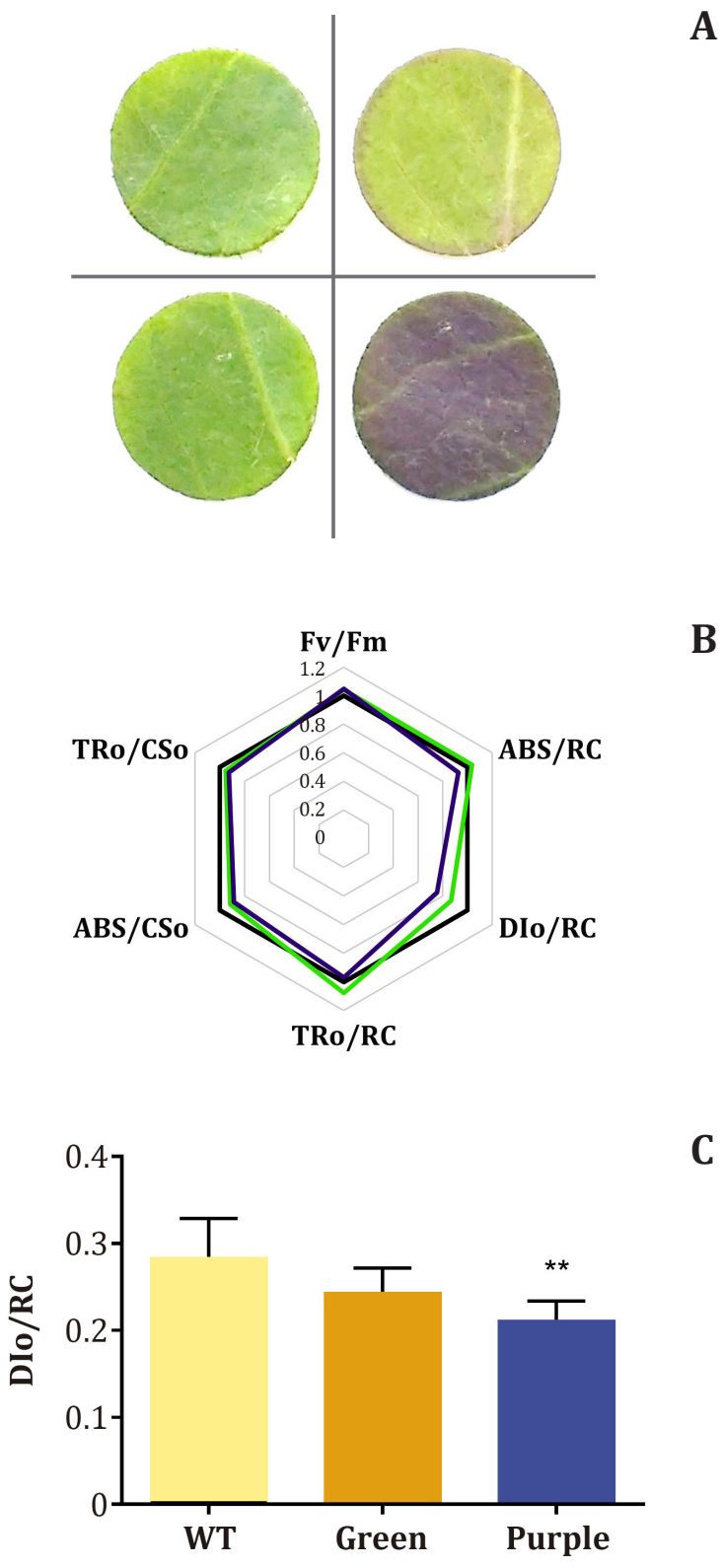


Figure 4. Physiological performance of micro-propagated *S. bonariensis* plants.
Figura 4. Desempeño fisiológico de plantas micropropagadas de *S. bonariensis*.

(A) Adaxial face (upper left and right) and abaxial face (lower left and right) of green and purple leaves from micro-propagated plants, respectively. (B) Mean values of six OJIP parameters are shown in radar charts for WT (black line), micro-propagated green leaf (light grey line), and micro-propagated purple leaf (dark grey line). Results are expressed relative to WT, assigned as 1. Each parameter is defined in the text. (C) Dissipation energy per reaction center determination in WT (yellow), micro-propagated green leaf (orange), and micro-propagated purple leaf (blue). Results are means of 7 biological replicates ± SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's Multiple Comparison Test using Prism 5 (GraphPad Software, CA, USA). ** $p < 0.01$.

(A) Cara adaxial (arriba a la zquierda y derecha) y cara abaxial (abajo a la izquierda y derecha) de hojas verdes y moradas de plantas micropropagadas, respectivamente. (B) Los valores promedios de 6 parámetros OJIP se muestran en gráficos de radar para WT (línea negra), hoja verde micropropagada (línea gris claro) y hoja púrpura micropropagada (línea gris oscuro). Los resultados se expresan en relación con WT, que se asignó a 1. La definición de cada parámetro se proporciona en el texto. (C) Energía de disipación por determinación del centro de reacción en WT (amarillo), hoja verde micropropagada (anaranjado) y hoja morada micropropagada (azul). Los resultados muestran el promedio de 7 réplicas biológicas ± DS. El análisis estadístico se realizó mediante análisis de varianza unidireccional (ANOVA) seguido de una prueba de comparación múltiple de Tukey utilizando Prism 5 (GraphPad Software, CA, EE. UU.). **p<0,01.

Discussion

The growing interest in medicinal bio-compounds of *S. bonariensis* underscores the necessity for a large-scale production protocol. While micropropagation and *in vitro* propagation protocols have been documented for other Senecio species (7, 16, 18), a particular protocol for *S. bonariensis* is currently lacking. We successfully identified the optimal conditions for producing *S. bonariensis* plants by investigating eighteen combinations of two cytokinins and one auxin on shoot production. All tested combinations resulted in callus formation, while the percentage of callus induction (PCI) varied according to hormone group. Remarkably, we found that MS medium lacking plant growth regulators did not induce shoot formation. However, combinations of NAA-BAP proved more effective in inducing callus compared to NAA-KIN. In a study by Hariprasath *et al.* (2015), *S. candicans* exposed to NAA 0.5 µg ml^{-1} and either 1 or 2 µg ml^{-1} BAP resulted in 37% and 47% average PCI, respectively. In contrast, we achieved a 1.7-fold higher callus induction (66% and 80%, as shown in figure 1B, page 140). Notably, MS supplemented with NAA 0.5 µg ml^{-1} and BAP 0.5 µg ml^{-1} achieved 90% PCI. Other authors observed no differences in PCI for *S. candicans* between NAA-BAP and NAA-KIN combinations. However, for S*. bonariensis*, PCI was lower when MS was supplemented with NAA-KIN (figure 1D, page 140). We observed a twofold lower PCI with NAA-KIN combinations (0.5 µg ml^{-1} NAA - 2 µg ml^{-1} KIN and 1 µg ml^{-1} NAA - 2 µg ml^{-1} KIN), although similar PCI was observed when KIN was tested at 1 µg ml^{-1}. Notably, these calli did not produce shoots. After obtaining calluses, we evaluated shoot induction. NAA-BAP combinations resulted in 100% shoot induction for *S. bonariensis* (figure 2A, page 141), as previously found on *S. macrophyllus* M. Bieb, with 100% shoot induction (18), and *S. cruentus* cv. Tokyo Daruma, with 86.4% to 98.4% shoot induction (16). In contrast, shoot formation in *S. candicans* ranged from 48% to 76% (7). All NAA-BAP combinations were highly effective for inducing *S. bonariensis* shoots. In contrast with other Senecio species, *S. bonariensis* did not produce shoots from calli treated with NAA-KIN (figure 2B, page 141), (7, 16, 18). This may be attributed to KIN being a weaker cytokinin than BAP (1, 2). Further studies should consider the effect of different concentrations of KIN to determine optimal conditions for significant shoot production.

Shoot number per explant was assessed after 60 days of incubation. Shoot induction occurred in the presence of NAA-BAP. Mean shoot number per explant ranged from 3.3 to 6, similar to Trejgell *et al.* (2010). Shoot length was comparable to other Senecio species (7, 16, 18).

Leaf purplish coloration during rustication could constitute a form of plant photoprotection, of the immature photosynthetic apparatus, dissipating high irradiance and mitigating potential damage from solar radiation (15). We evaluated *in vitro*-propagated plants using a non-invasive OJIP test, ideal for researching valuable plant material that should not be destroyed. Considering our results regarding physiological and energetic parameters, *i.e.* Fv/Fm, ABS, TRo, RC and Cso in green and purple leaves under high-light stress, the maximum quantum yield of photosystem II (PSII) (Fv/Fm) decreases due to photo-oxidative damage, as previously reported (9, 12). However, the non-significantly different physiological state among leaves, followed by a significant decrease in DIo/RC in purple leaves of micro-propagated plants compared to unpropagated (WT) and green leaves, indicated that purple coloration did not provide photoprotection. This is because dissipation prevents photodamage. Interestingly, some leaves of micro-propagated plants displaying purple phenotype showed no physiological disturbances. This phenotype disappears one or two weeks after being transplanted to the field. Further studies are required to understand these findings. Notably, micro-propagated plants successfully flowered and attracted pollinators.

Conclusion

We created a straightforward and efficient procedure for the large-scale propagation of *S. bonariensis*. This protocol holds promise for diverse applications, ranging from medicinal research and ecological studies to commercial landscaping. Furthermore, it offers valuable insights into other Senecio species.

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Declarations

All the authors declare that they have no conflicts of interest.