



Micropropagation and genetic transformation of *Byblis liniflora*

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Abstract

Byblis, a small genus of carnivorous plants predominantly found in Australia, is characterized by its passive trapping mechanism and unique floral features. The chemical composition of *Byblis*, including identified phenylethanoid glycosides, particularly acteoside, highlights its pharmacological potential with various biological activities. *In vitro* culture techniques have been established for propagation, with micropropagation protocols developed for different *Byblis* species. However, information on genetic transformation, vital for trait modification and enhanced pharmacological interest, remains limited. This study focuses on optimizing micropropagation, adventitious regeneration, and genetic transformation methods for *Byblis liniflora*. Adventitious regeneration rates were highest in medium with reduced Murashige and Skoog salts (MS/10) and sucrose (3 gL⁻¹) concentrations. Zeatin supplementation (1 mgL⁻¹) further improved regeneration rates and bud development with 100% of regenerated root explants and 8.8 shoots per explant. Liquid MB3 medium supplemented with indole-3-acetic acid (IAA) 5 mgL⁻¹ facilitated efficient rooting and acclimatization. The establishment of an efficient *Rhizobium*-mediated genetic transformation method yielded transgenic plants expressing green fluorescent protein (GFP). Molecular analysis confirmed transgene integration, marking the first successful genetic transformation in the *Byblis* genus. These advancements pave the way for exploring gene function and enhancing pharmacological properties, thereby broadening our understanding and utilization of carnivorous plants like *Byblis*.

Keywords Acclimatization · Carnivorous plant · *In vitro* multiplication · Rainbow plant · Transgenic

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Introduction

Byblidaceae is a small family of carnivorous plants that belong to the order Lamiales (Albert *et al.* 1992; Conran 1996), predominantly distributed in the west and north of Australia. The Byblidaceae family consists solely of the genus *Byblis*, which includes eight recognized species. Six of these species are annual and grow in the semi-arid to tropical humid zone in the northern region of Australia (*B. aquatica*, *B. filifolia*, *B. rorida*, *B. guehoi*, *B. liniflora*, and *B. pilbarana*). The remaining two species (*B. gigantea* and *B. lamellate*) are perennial and grow in the southwest of Australia (Cross *et al.* 2018; Phuong *et al.* 2022; IUCN 2024; POWO 2024). *Byblis* species have slender, linear leaves that range from 2 to 25 cm in length. These leaves emerge radially from the stem and are adorned with glandular trichomes that are both stalked and sessile. The trapping mechanism employed by *Byblis* species is passive, involving the secretion of adhesive substances with both digestive and absorptive functions. The flowers are hermaphroditic, measuring less than 4 cm, and are mostly pale lilac in color.

They are characterized by domed petals (Cross *et al.* 2018). The number of chromosomes varies among groups or complexes within the *Byblis* genus. In the *B. liniflora* complex which comprises *B. aquatica*, *B. filifolia*, and *B. rorida*, the base number is $x=8$, while *B. gigantea* has a base number of $X=9$ (Hirsikorpi *et al.* 2002). Most species in this family are diploid, except for *B. guehoi* and *B. liniflora*, which are tetraploid (Fukushima *et al.* 2011). *B. liniflora* is the only species with distribution extending to the southern part of New Guinea, growing upright up to 15–20 cm. It exhibits rapid growth, and flowering can occur in the early weeks of germination. The chromosomal count for this species is $2n=32$ (Hirsikorpi *et al.* 2002; Schlauer *et al.* 2004; D'Amato 2013; Cross *et al.* 2018).

The Byblidaceae family is the subject of research aimed at understanding evolutionary adaptations, although it is not as widely recognized as other carnivorous plant families. Genetic and molecular studies on this family can offer valuable insights into evolution. The genus *Byblis* includes species that are appreciated for their exotic nature and are gaining recognition as decorative plants in various environments (Caldeira *et al.* 2021). The genus *Byblis* and its chemical composition are not well-known but four phenylethanoid glycosides have been identified, marking the first instance of these compounds in the Byblidaceae family (Schlauer *et al.* 2004). Among these compounds, acteoside, also known as verbascoside, has been identified in various species, including numerous medicinal plants, and is recognized for its diverse biological activities. Acteoside is often considered a significant chemotaxonomic marker (Jiménez and Riguera 1994; Schlauer *et al.* 2004). Furthermore, it has pharmacological benefits such as antioxidant, anti-inflammatory, neuroprotective, and anticancer activities (Xiao *et al.* 2022).

In vitro culture is widely used for propagation in the phytopharmaceutical and horticultural industries. Carnivorous species, such as *Drosera* and *Dionaea*, have extensively utilized plant tissue culture. Micropropagation protocols have also been developed for other species, including *Aldrovanda vesiculosa* (Adamec and Kondo 2002), *Drosophyllum lusitanicum* (Gonçalves and Romano 2005), and *Nepenthes khasiana* (Devi *et al.* 2013). Seeds are considered the optimal starting material as they can be easily sterilized to eliminate contaminants. However, different explants, such as leaves, stems, or apices, can also be used (Legendre and Darnowski 2018). Micropropagation protocols have been developed for various species of the genus *Byblis* such as *B. liniflora* (Schlauer *et al.* 2004) and *B. filifolia* (Pelto and Lindstrom 2003). For *B. liniflora*, propagating seedlings in an MS basal medium (Murashige and Skoog 1962) without growth regulators has been observed to be efficient (Schlauer *et al.* 2004). Several studies have demonstrated that media containing between one-tenth and one-half of MS salts concentration have shown superior effectiveness in plant growth

(Banasiuk *et al.* 2012; Northcutt *et al.* 2012; Devi *et al.* 2013; Tuleja *et al.* 2014; Miclea and Zăhan 2017). Moreover, the addition of plant regulators such as auxins enhances the development of adventitious roots, subsequently facilitating the acclimatization process to *in vivo* conditions (Phuong *et al.* 2022).

One of the prerequisites for executing some propagation protocols, increasing genetic variability through somaclonal variation, or obtaining transgenic plants is to know how to regenerate plants from tissue culture explants. Information on carnivorous plants unfolds at a notably slow pace concerning *in vitro* regeneration methods (Caldeira *et al.* 2021). The most favorable conditions for regeneration have been investigated in various carnivorous plant species. In *Pinguicula gigantea*, optimal results were achieved with the combination of growth regulators 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) (Saetiew *et al.* 2011). *Nepenthes khasiana* exhibited superior outcomes with the application of kinetin (KIN) and 6-BA (Devi *et al.* 2013). *Dionaea muscipula* responded well to 6-BA (Anchalee 2014), while *Drosera burmanni* demonstrated successful regeneration with 6-BA and NAA (Yanthan *et al.* 2017). For *Nepenthes mirabilis*, the crucial combination involved thidiazuron (TDZ), 6-BA, and indole-3-butyric acid (IBA) (Miguel *et al.* 2020), whereas *Utricularia gibba* showed optimal results with 6-BA (Oropeza-Aburto *et al.* 2020). In other species like *Drosera rotundifolia* and *Cephalotus follicularis*, the most effective medium did not require the exogenous application of auxins and cytokinins (Ko *et al.* 2010; Jadczyk *et al.* 2017).

Genetic transformation is a crucial method for investigating gene structure and function in plants and, in this regard, traits associated with carnivory. Therefore, genetic modification could help to understand the intricate mechanisms of prey capture and digestion that these plants have developed, offering valuable insights into their evolutionary and physiological adaptations. Moreover, the insecticidal and antimicrobial properties of the digestive enzymes in carnivorous plants can be harnessed to develop novel biotechnological strategies (Wójciak *et al.* 2023). Despite its scientific and biotechnological significance, the number of publications on genetic transformation in carnivorous plants remains limited (Miguel *et al.* 2020). Although the transformation with *Rhizobium radiobacter* is efficient in many species, its application in carnivorous plants has been constrained (Oropeza-Aburto *et al.* 2020). Genetic transformation protocols have been reported in *Drosera rotundifolia* (Hirsikorpi *et al.* 2002), *N. mirabilis* (Miguel *et al.* 2020), and *Utricularia gibba* (Oropeza-Aburto *et al.* 2020). However, protocols for genetic transformation in species of the genus *Byblis* are currently unknown.

In this work, we have studied the best conditions for plant propagation, explant adventitious regeneration, shoot

rooting, and plant acclimatization for *B. liniflora*. Moreover, we established for the first time an efficient *Rhizobium*-mediated genetic transformation method. Thanks to these advances, it will be possible to modify the characteristics of this species and to improve its pharmacological interest.

Materials and methods

Plant material, disinfection, germination, clonal propagation, and acclimatization Seeds were obtained from a local carnivorous plant nursery. Axenic culture of *B. liniflora* was started by the surface disinfection of seeds with a solution of sodium hypochlorite containing 0.37% active chlorine and five drops of Tween-20 for 10 min. Disinfected seeds were rinsed three times in sterile water and sown in MB3/3 (Table 1). This medium was composed by one-third strength macro- and microelement formulation proposed by Murashige and Skoog (1962), sucrose (10 gL⁻¹), myo-inositol (100 mgL⁻¹), and thiamine hydrochloride (1 mgL⁻¹). The culture conditions were photoperiod of 16-h light/8-h dark, light intensity of 70 μEm⁻² s⁻¹, and a temperature of 25 ± 2 °C. Clonal propagation of *B. liniflora* was evaluated with the MS and/or sucrose concentration at full or one-tenth strength (Table 1).

To evaluate and improve the regeneration of adventitious roots for suitable acclimatization, meristematic apices and axillary buds were grown in MB3 liquid medium with and without the supplementation with 5 and 10 mgL⁻¹ of two different auxins, IBA and IAA. Glass marbles were used as inert support for the explants.

Once rooted, the plants were acclimatized by washing the roots with deionised water and transplanted into moist sphagnum moss. They were then covered with cling film to avoid desiccation for 1 wk in the greenhouse. This film was progressively removed to adapt the plants to the glasshouse conditions: long-day photoperiod (16-h of natural light supplemented with Osram lamps Powerstar HQI-BT, 400 W), temperature fixed at 24 °C during the day and 18 °C at night, and always keeping half a centimeter of deionized water in

the tray. Survival rate was evaluated, as well as their development 40 d after acclimatization.

Adventitious regeneration Leaf and root sections of 1.5–2 cm and transverse sections of internode explants 3–5 mm free of axillary buds were grown on different basal media (Table 1). In addition, the organogenic potential of these explants was evaluated in MB3/10 supplemented with zeatin 1 mgL⁻¹ (Duchefa, Haarlem, Netherlands) after 30 d. For the selection of transgenic *Byblis* plants, explants were grown on MB3/10 regeneration medium supplemented with zeatin 1 mgL⁻¹ with different concentrations of kanamycin (Duchefa)—0, 25, 50, 75, and 100 mgL⁻¹—to infer the minimum concentration that inhibits the regeneration of *Byblis* explants.

Ploidy determination The ploidy level of regenerated and transgenic plants was evaluated by DNA quantification in a Partec CyFlow cytometer as described in Atarés *et al.* (2011). Young leaves from these plants were sliced with a razor blade into 0.4 mL of nuclei isolation buffer (High Resolution DNA Kit, Solution A: Nuclei Isolation; Partec, Franklin Park, IL) and mixed with 1 mL of staining buffer (High Resolution DNA Kit, Solution B: DAPI Staining; Partec). The mixture was filtered through 50-mm nylon mesh (Nyblot). The filtrates (more than 5000 nuclei per extract) were analyzed.

Rhizobium-mediated genetic transformation The plasmid pK2GW7:EGFP contains the *nptII* gene and the *GFP* as a reporter gene. The plasmid construct was produced using Gateway technology (Invitrogen, Waltham, MA <http://www.invitrogen.com>) as illustrated in Fig. S1 and confirmed by sequencing. The primers used for PCR amplification of EGFP are listed in Table S1. The plasmid constructs were electroporated and propagated in *Escherichia coli* strain TOP 10. Transfer of the plasmid construct to *R. radiobacter* LBA4404 cells was carried out by electroporation and utilized to transform *B. liniflora* plants.

Root and leaf explants of 1 to 1.5 cm and stem explants of about 0.5 cm without pre-existing meristems were obtained. These explants were immersed in an inoculation medium containing a *Rhizobium* culture harboring pK2GW7:EGFP (Atarés *et al.* 2011). The resulting explants were grown on coculture solidified medium—MB3/10 (Table 1) supplemented with 3'5'-dimethoxy-4-hydroxyacetophenone 200 μM (Sigma-Aldrich, St. Louis, MO)—and maintained for 48 h in the dark at 28 °C.

After the co-culture period, they were washed in liquid MB3/10 medium with cefotaxime 500 mgL⁻¹ (Duchefa) and cultured in solid MB3/10 medium supplemented with kanamycin 200 mgL⁻¹ for selection of transformed cells; zeatin 1 mgL⁻¹ for regeneration and timentin 300 mgL⁻¹ (Duchefa) prevent *R. radiobacter* growth. They were maintained under photoperiod conditions (16-h light/8-h dark,

Table 1. Medium composition used for clonal multiplication of *Byblis*. All the media were adjusted to pH=5.7 and gelified with bacteriological agar (6.8 gL⁻¹)

	Mineral salts	Sucrose	THCl	Myo-inositol
MB3	MS	30 gL ⁻¹	1 mgL ⁻¹	100 mgL ⁻¹
MB3/10	MS 1/10	3 gL ⁻¹		
MB0.3	MS	3 gL ⁻¹		
MB3 MS/10	MS 1/10	30 gL ⁻¹		
MB3/3	MS 1/3	10 gL ⁻¹		

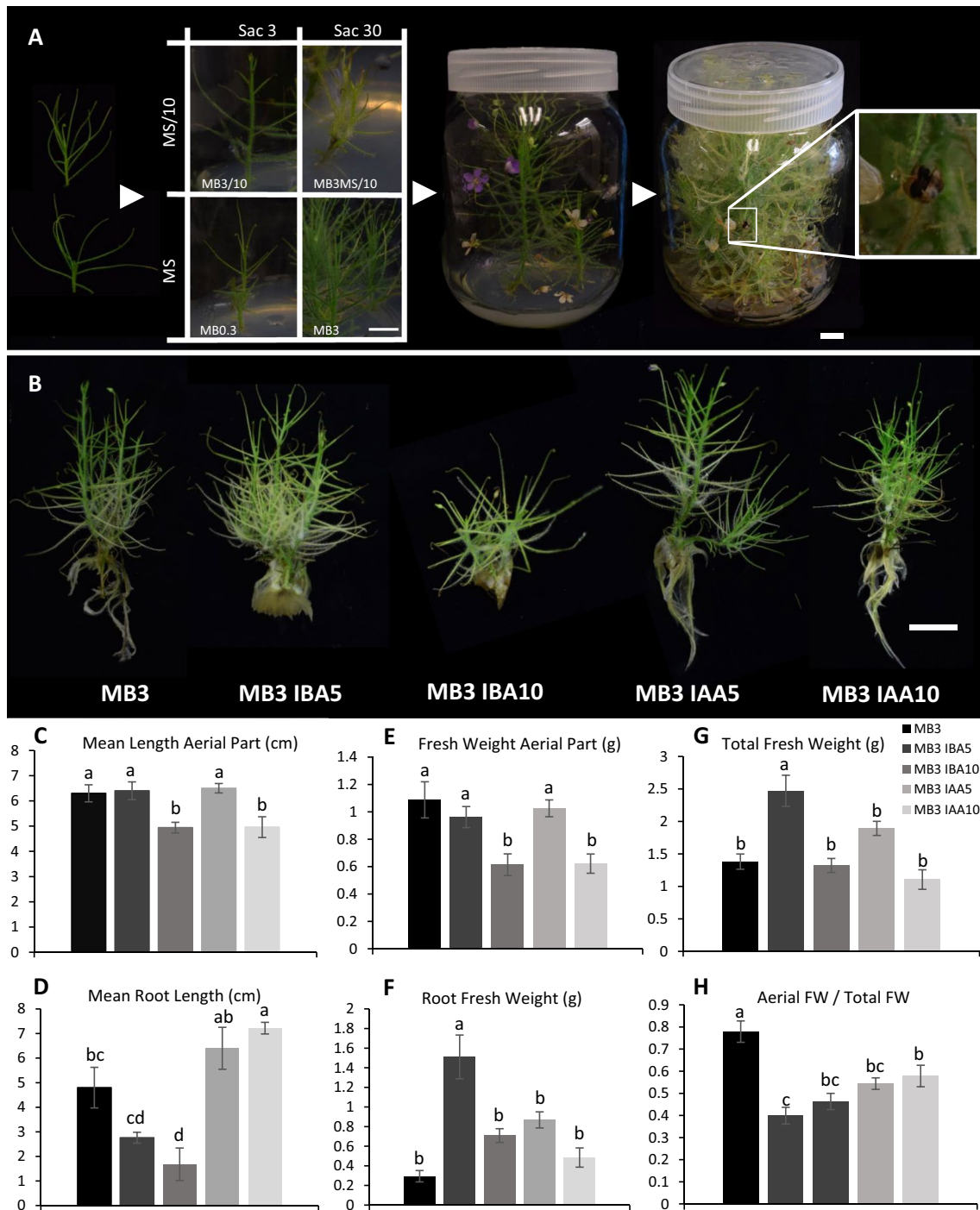


Figure 1. Clonal propagation. (A) Developing a method for clonal propagation based on the combination of different MS salt and sucrose concentrations, and plant development on the selected MB3 medium where it blooms and fructifies. Scale bar: 1 cm. (B) Clonal propagation on liquid basal MB3 and supplemented with 5 and 10 mgL⁻¹ of IBA or IAA for the rooting of explants. Scale bar: 1 cm.

(C–H) Quantification of the length and weight of the aerial part and root system of plants cultivated in liquid media. Error bars represent the relative error of each mean, and the significant difference—*p*-value < 0.05—among the different groups is represented by Tukey’s HSD compact letter display.

light intensity of $70 \mu\text{Em}^{-2} \text{s}^{-1}$) by subculturing every 2 wk to the same medium until the formation of organogenic structures. Transformation efficiency was calculated as the rate between number of explants with one or more transgenic events and number of transformed explants.

GFP expression and molecular analysis of transgenic plants Fluorescence in regenerated transgenic plants was detected using different microscope techniques including a MZ16F LEICA stereomicroscope and a confocal Zeiss Axio Observer 780 equipped with a light source or laser capable of exciting this protein at 488 nm and allowing its detection between 500 and 520 nm. A lambda scan was performed to confirm the spectral pattern of our signal that corresponded to GFP 509 nm.

Genomic DNA was extracted from different transgenic lines of *B. liniflora* leaves and nodes using The REDExtract-N-Amp Plant PCR Kits from Sigma-Aldrich, following the instructions described in the protocol. PCR analysis of genomic DNA from WT plants and plants expressing *35S:EGFP* was performed using the oligonucleotide primers P3 for EGFP 5' end and P4 for EGFP 3' end (Table S1) can amplify 480-bp PCR fragment in transgenic plants, but not in WT plants. PCR products were separated by electrophoresis on 1% (w/v) agarose gels containing Midori Green Advance (nucleic acid stain) and visualized by ultraviolet light (Fig. 5).

Identification of *Rhizobium* contamination in the genomic DNA of the *B. liniflora* transformed cells was analyzed by PCR using specific primers (P5 and P6) for spectinomycin-resistant gene (Table S1), which lies outside of the right and left borders of pK2CW7:EGFP. pK2GW7:EGFP plasmid was used as a positive control for the PCR reaction. PCR products were separated by electrophoresis on 1% (w/v) agarose gels containing Midori Green Advance (nucleic acid stain) and visualized by ultraviolet light (Fig. S6A). Additional screening was performed by carrying out a 48-h LB supplemented with rifampicin 40 mgL^{-1} and streptomycin 50 mgL^{-1} culture. We used *Rhizobium* pK2GW7:EGFP as a positive control, tissue from wild type as a negative control, and transgenic plant tissue (Fig. S6B–C).

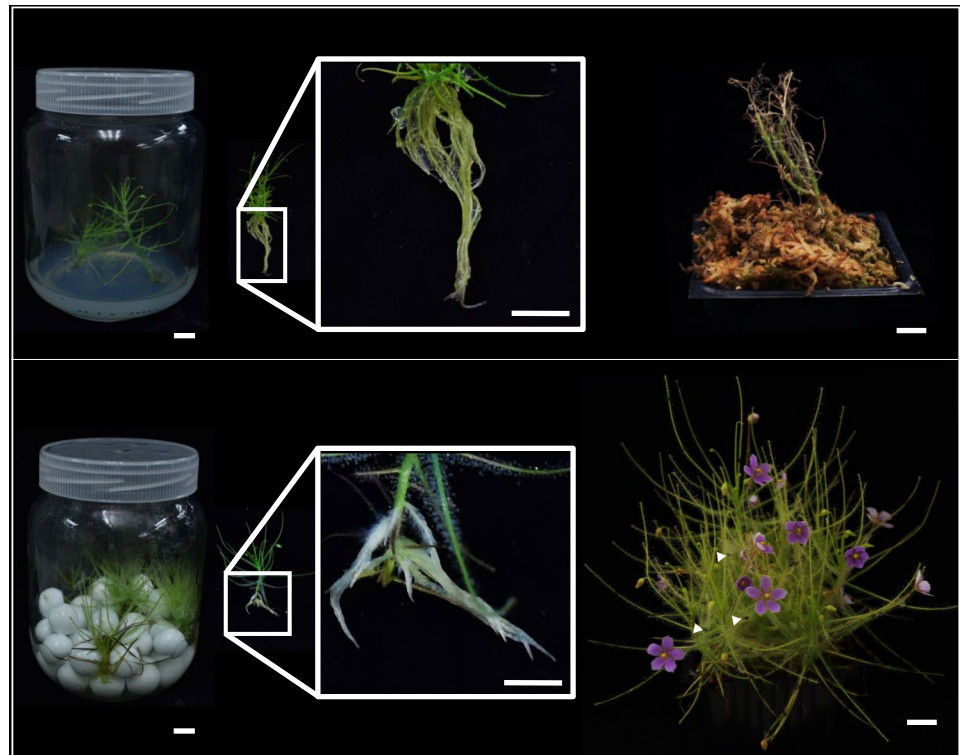
Statistical analysis Statistical analysis was performed when required. A Tukey HSD test was used to compare the means and determine the significant differences among the different media tested for the liquid medium clonal propagation assay. Five individuals for each condition were evaluated. Unpaired Student *t*-test was used to determine significant differences in the adventitious shoot regeneration rate (ASRR) and the mean shoot number (MSN) in the zeatin regeneration assay, by comparing the zeatin-supplemented medium with the non-supplemented one. For each condition, 30 individual explants—10 explants/plate—were evaluated.

Results and discussion

Clonal propagation Effective surface disinfection of *B. liniflora* was achieved in all tested seeds. Moreover, the disinfectant partially degraded the outer seed coat enabling the visualization of the endosperm within (Fig. S2A). Seeds germinated, rooted, and developed in MB3/3 medium (Fig. S2B). Viable axenic seeds were obtained from MB3 *in vitro* flowering plants. Flowers, fruits, and seeds in MB3 axenic plants exhibited similar number, shape, and color to those in *in vivo* conditions (Fig. S2C). Shoot tip explants and axillary buds were subcultured on MB3/3 medium for clonal propagation. After two or three subcultures, the tips of the youngest leaves began to undergo necrosis, eventually affecting the apical meristem (Fig. S2D). Rooting was unaffected, and this clonal propagation method facilitated the establishment of a clone collection. Nevertheless, the severity of apical necrosis increased with prolonged exposure to MB3/3. To mitigate leaf necrosis, various modifications in the concentration of MS salts (full and 1/10) and sucrose (full and 1/10) were investigated (Fig. 1A). The full concentration of MS salts (MB0.3 and MB3) showed the best results. In these two media, plants exhibited a significant reduction in meristem and leaf necrosis, along with an intense green coloration. Conversely, plants grown on media with MS salts reduced to one-tenth showed chlorotic coloring (Fig. 1A). Notably, MB3 cultured plants exhibited a close development to that in *in vivo* conditions. In this medium, plants demonstrated the ability to flower and produce fruits containing viable seeds (Fig. 1A). Consequently, this medium was established for the maintenance of the clonal collection.

Many researchers have observed that carnivorous plants show better development in tissue culture by reducing salt concentrations in the medium (Banasiuk *et al.* 2012; Northcutt *et al.* 2012; Devi *et al.* 2013; Tuleja *et al.* 2014; Miclea and Zăhan 2017). Thus, clonal multiplication of *Byblis filifolia* is based on a medium with MS/5 salts (Pelto and Lindstrom 2003). This response could be related to the typical environmental conditions where this type of plants thrive with a low concentration of mineral salts and absorbable nutrients by the roots. However, our results with *B. liniflora*, contrary to what happens with the rest of carnivorous plants we work with in our laboratory, have shown better development in media with undiluted MS salts. This unexpected response could help to elucidate the mechanisms that have been modified in carnivorous plants to survive in environments deprived of salts in the soil. In addition, this is the first report of the production of viable axenic seeds in plants of this genus. *In vitro* flowering is sometimes associated with senescence processes in other species despite *Byblis* showing normal development. *In vitro* flowering, fruiting, and seed production opens new alternative ways for pollination

Figure 2. Acclimatization of *B. liniflora* from solid and liquid media. (A) Acclimatization of plants rooted in solid MB3 medium and plant appearance 40 d after transplanting to substrate. Central image shows the remains of culture medium on the roots. Bar: 1 cm. (B) Acclimatization of plants rooted in liquid MB3 and plant growth 40 d after acclimatization. The plant is flowering vigorously and is able to catch small flies. In this case, no traces of agar are observed in the root system. Scale bar: 1 cm.



between parents or obtaining offspring under axenic conditions. After checking the low germination rate also of seeds produced *in vitro*, we have ruled out that sterilizing treatments are the reason for this. In fact, in nature, it is common for the seeds of these species to need stimuli such as fires or the action of compounds present in smoke to favor the germination. In this context, D'Amato (2013) proposes the use of cigarette smoke, matches, liquid smoke, and other commercial derivatives to favor germination of *Byblis gigantea*.

Byblis explants spontaneously rooted in a gelified basal medium. However, the survival rate after acclimatization was very low. The use of liquid media for rooting propagules has been previously described in *Darlingtonia californica* (Uhnak 2003). In these media, the cleaning of the roots is easier because the culture medium is completely removed, and the acclimatization is more efficient. After testing various liquid media, MB3 with IBA or IAA at 0, 5, and 10 mgL⁻¹, most explants developed roots with different characteristics (Fig. 1B). Plants grown in MB3 or supplemented with either auxin 5 mgL⁻¹ exhibited similar aerial part lengths. The aerial part length and fresh weight decreased significantly with the highest auxin concentration (Fig. 1C, E). However, these two auxins produced different root developments. Plants supplemented with IBA showed a very compact root development while larger roots were observed when using IAA (Fig. 1D). Plants cultivated in IBA 5 mgL⁻¹ had many hairy and extremely compacted roots (Fig. 1B) and their root fresh weight doubled compared to the other

conditions (Fig. 1F). In contrast, at IBA 10 mgL⁻¹, the formation of a semi-organised rhizogenic callus was observed at the base of the explant (Fig. 1B). On the other hand, plants grown on IAA medium showed longer roots with higher fresh weight than those in MB3 (Fig. 1F). Plants supplemented with 5 mgL⁻¹ IBA had the highest total fresh weight (Fig. 1G). In addition, the ratio of the fresh weight of the aerial part to the total weight showed that the use of auxins decreased this parameter, probably by diverting the plant's resources toward root formation (Fig. 1H). In conclusion, the results suggest that the best medium for rooting propagated plants to be acclimatized is liquid MB3 supplemented with IAA 5 mgL⁻¹. *In vivo* propagation rooting has been previously reported in *B. filifolia* (Pelto and Lindstrom 2003). They successfully acclimatized 28 out of 30 propagules that were immersed in a commercial IBA solution before cultivation in the substrate. Other authors have reported high survival rates in other carnivorous plants, such as *Drosophyllum lusitanicum*, by immersing the basal end of the propagule in IBA for 2 min prior to acclimatization (Gonçalves and Romano 2005).

Plant acclimatization The initial attempts to acclimate *Byblis* involved plants grown on solid MB3 medium. The roots were rinsed with distilled water just before being planted into *Sphagnum* moss. Out of the 50 plants that were rooted, only three survived the first month. However, these three seemingly acclimatized plants failed to grow and eventually

succumbed after 2 mo (Fig. 2A). The roots exhibited clear signs of rotting, with the growth of various fungi likely attributed to the remnants of medium and agar adhering to the roots surface. After using plants that had been rooted in a liquid medium, 70% were successfully acclimatized 40 d after transplantation (Fig. 2B). This approach aimed to leverage the benefits of an already established root system while avoiding the fungal issues associated with agar residues. The acclimatized plants quickly flowered upon transitioning from *in vitro* conditions (Fig. 2B) and soon attracted insects from our greenhouse. These flowers ultimately produced seeds, which were utilized to sustain and replenish the collection of *in vitro* material.

Other species of carnivorous plants have been successfully acclimatized with other substrates. In *B. filifolia*, inert substrates such as sponges suitable for plant rooting have been used (Pelto and Lindstrom 2003). In *Dionaea muscipula* and *Drosera peltata*, acclimatization was achieved with 1:1 mixtures of blond peat and sand (Jang *et al.* 2003; Kim and Jang 2004). Finally, different species of sarracenias were successfully acclimatized with complex mixtures of sand, pumice, blond peat, perlite, and *Sphagnum* moss (Northcutt *et al.* 2012).

Adventitious regeneration The organogenic potential of leaf, internode lacking pre-existing meristems, and root explants was evaluated using the same tested media during clonal propagation: MB3, MB0.3, MB3 MS/10, and MB3/10 (Fig. 3A). Callus formation and adventitious shoot regeneration rates were measured after 30 d of culture (Fig. 3B). The regeneration rates concerning explant type were 53.1% in roots, followed by 31.0% in internodes. No regeneration was observed in leaf explants. Regarding regeneration capacity, MB3/10 was the most effective medium, with a regeneration rate of 55.0% for the cultured explants, followed by 43.2% in MB3 MS/10, 12.2% in MB0.3, and 3.6% in MB3. Notably, this regeneration occurs directly and predominantly in wounded areas, with minimal unorganized callus formation (Fig. 3B). These results suggest that media with full concentrations of MS salts and sucrose while promoting plant growth during clonal propagation were not the most suitable for inducing adventitious regeneration. Mineral salt concentration appears

to be the critical factor influencing organogenic potential. When sucrose is reduced to one-tenth, root regeneration rates reach 35%, whereas reducing MS salts to one-tenth increases this percentage to 88%. Additionally, there appears to be a synergistic effect between the reduction of MB3 of MS salts and sucrose concentration, as evidenced by MB3/10, the medium with both reduced salts and sucrose, being the most effective for inducing regeneration in stem (73%) and root (93%) and forming disorganized callus in leaf (29%).

In contrast to other plants requiring exogenous growth regulators for adventitious shoot formation, *Byblis* has demonstrated an acceptable regeneration capacity in the plant regulator-free medium. Once the optimal basal medium for *Byblis* regeneration was identified, we decided to supplement it with zeatin 1 mgL^{-1} to enhance the previously obtained results. The addition of zeatin allowed for regeneration from leaf explants. Regeneration rates with this medium were 27%, 93%, and 100% for leaf, stem, and root, respectively (Table 2). Zeatin also positively influenced the development of adventitious buds, with the highest numbers observed in all three explant types with 0.3, 2.0, and 8.8 shoots per total number of explants in leaf, stem, and root, respectively (Table 2). None of the regenerated shoots showed any change in their ploidy level (Fig. S3). In conclusion, the optimal medium for inducing organogenesis in all the tested explants in *B. liniflora* is the MB3/10 medium (Table 1) supplemented with zeatin 1 mgL^{-1} . Other authors have shown differences in the morphogenetic capacity of other carnivorous plants such as *Drosera* when performing the dissolution of MS salts (Perica and Berljak 1996; Rejthar *et al.* 2014; Krongtam and Junkasiraporn 2019). The use of cytokinins such as zeatin and others such as benzyladenine or kinetin to improve organogenesis of leaf explants in *Drosera* has also been described with equally effective results (Perica and Berljak 1996; Rejthar *et al.* 2014). In other carnivorous plants that present more difficulties for *in vitro* multiplication, such as *Drosophyllum lusitanicum*, organogenesis has only been described using zeatin (Gonçalves and Romano 2007).

Genetic transformation Once we developed a method for the adventitious regeneration of *B. liniflora*, we empirically

Table 2. Differences in the adventitious shoot regeneration rate (ASRR) and the mean shoot number (MSN) between MB3/10 medium and MB3/10 supplemented with 1 mgL^{-1} zeatin, MB3/10 Z

	Leaf		Internode		Root	
	MB3/10	MB3/10 Z	MB3/10	MB3/10 Z	MB3/10	MB3/10 Z
ASRR(%)	0.0 ± 0.0	$26.7 \pm 8.8^*$	53.3 ± 8.8	$93.3 \pm 3.3^*$	96.7 ± 3.3	100 ± 0.0
MSN	0.00 ± 0.00	$0.30 \pm 0.09^{**}$	0.60 ± 0.11	$2.00 \pm 0.23^{***}$	3.20 ± 0.28	$8.80 \pm 0.49^{***}$

Asterisks represent significant differences in the unpaired Student *t*-test when comparing each type of explant in both media: **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.0001

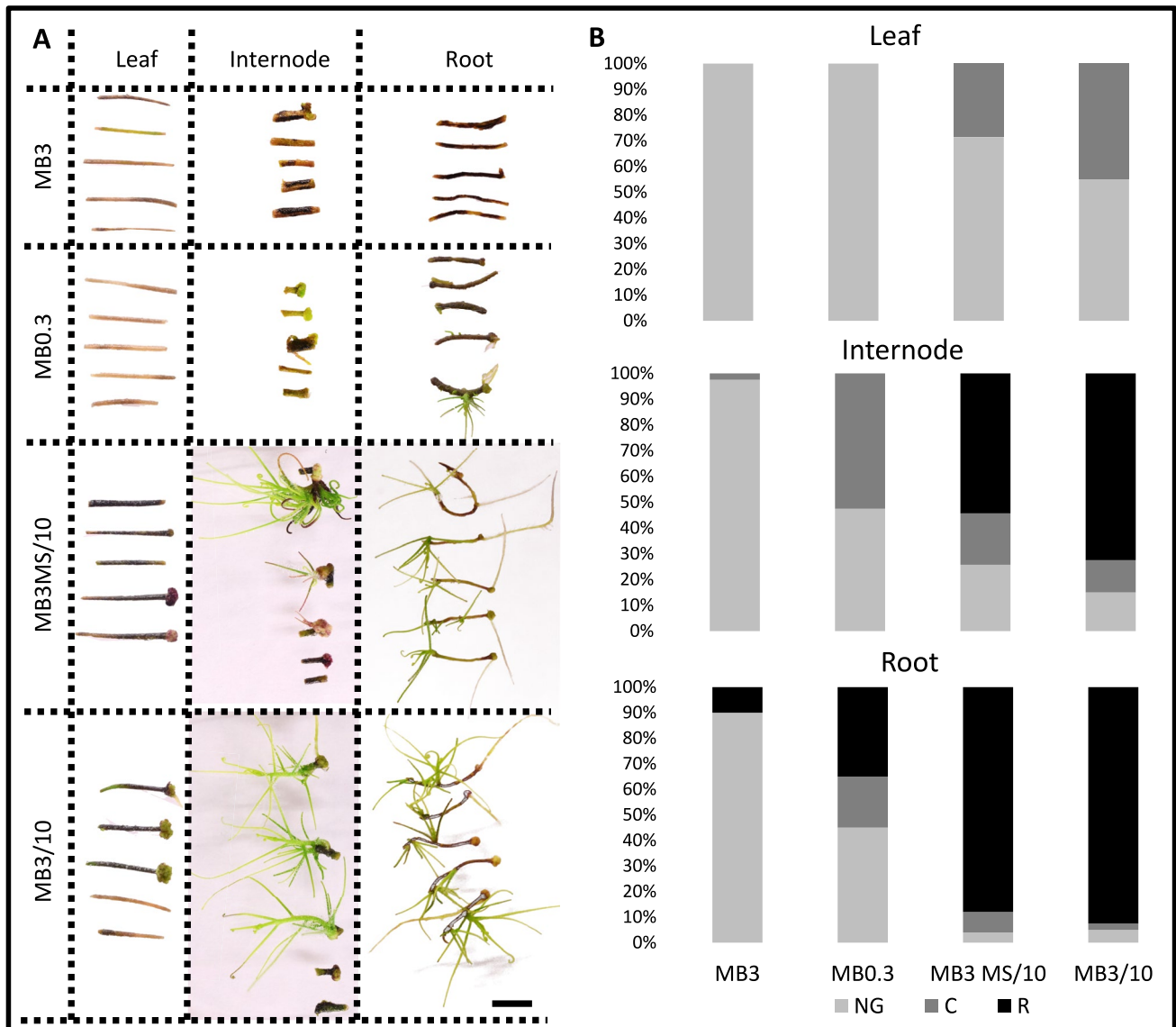


Figure 3. Adventitious regeneration assays. (A) Explants from leaves, internodes, and roots cultured in different media for 30 d. Scale bar: 1 cm. (B) Percentage of explants with no growth (NG), disorganized callus formation (C), and adventitious regeneration (R), for the four tested media.

estimated the concentration that inhibits such regeneration at kanamycin 200 mgL^{-1} (Fig. S4). Similar pre-transformation trials have been described in other carnivorous plant species. Thus, *Utricularia gibba* is resistant to kanamycin 80 mgL^{-1} (Oropeza-Aburto *et al.* 2020) and in *Drosera rotundifolia* kanamycin 400 mgL^{-1} was established as an inhibitory concentration (Hirsikorpi *et al.* 2002). Other authors have used different concentrations during different stages of transformation. For example, Miguel *et al.* (2019) used kanamycin 60 mgL^{-1} for the regeneration of *Drosera capensis* inoculated with *Rhizobium* and 300 mgL^{-1} for the cultivation of potentially transgenic shoots. In any case, it will be necessary to adjust the selection conditions according to the behavior of the controls to assess whether the

regenerated structures are escapes or putatively transformed plants.

Leaf, internode, and root explants were inoculated with the *R. radiobacter* strain LBA4404 that was transformed with the Pk2GW7-eGFP plasmid. We used MB3/10Z medium (MB3/10 with zeatin 1 mgL^{-1}) with a selective pressure of kanamycin 200 mgL^{-1} . Non-inoculated explants were cultured with and without kanamycin as a negative (NSP) and positive (WSP) control for regeneration, respectively (Fig. 4A–C). Thanks to the use of the GFP, the presence of transformation events was analyzed shortly after inoculation. No GFP was detected in either of the NSP and WSP explants. The first signs of transgenic cells were observed in inoculated explants after 2 wk.

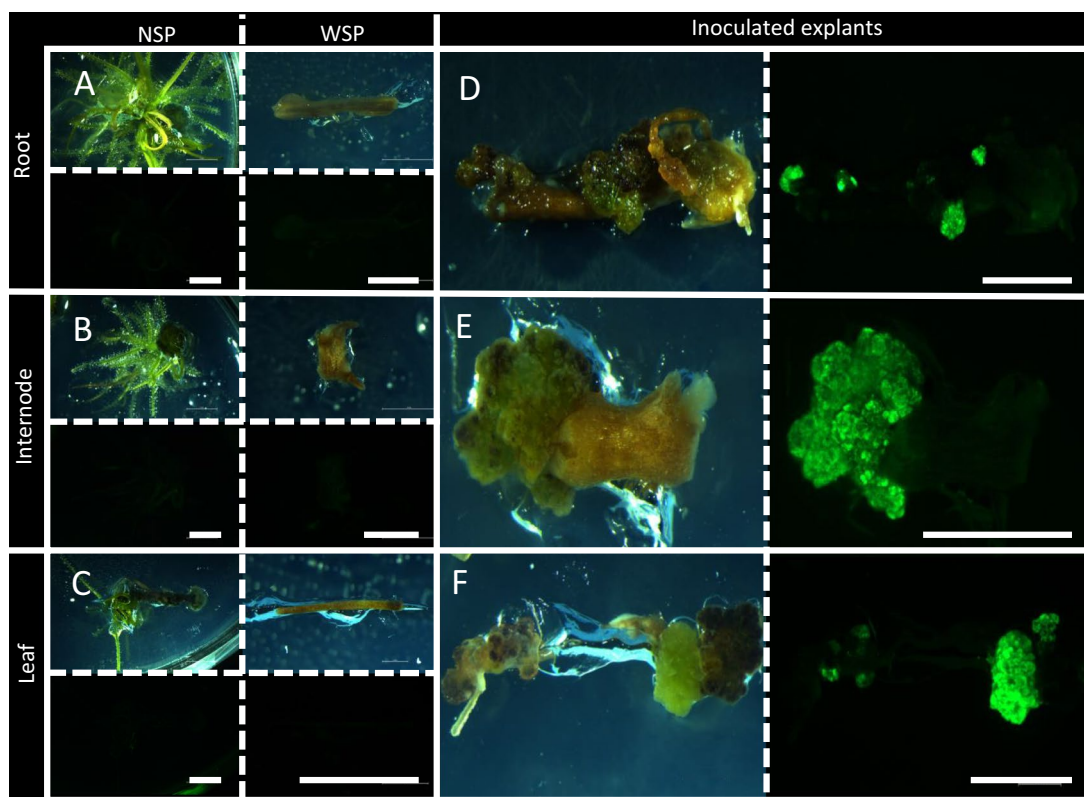


Figure 4. Obtention of transgenic disorganized callus from root, internode, and leaf explants. (A–C) Control with no selective pressure, NSP (left), and control with selective pressure, WSP (right), in brightfield (up) and under GFP filter (down) of root, internode, and

leaf explants, respectively. (D–F) Inoculated root, internode, and leaf explants in brightfield (left) and under GFP filter (right). In inoculated explants, fluorescent disorganized callus formation is observed 2 mo after transformation. Scale bar: 5 mm.

These events corresponded to transformed cells in which fluorescence could be detected. Prior to the regeneration of transgenic shoots, we could identify unorganized transgenic callus formations in the root, internode, and leaf explants (Fig. 4D–F).

Regeneration of the first transgenic shoots was observed after 3 mo of cultivation under selective conditions. Transformation efficiencies were 5.8% in root explants, 7.7% in leaf explants, and 25.5% in internode explants. None of the regenerated shoots showed any change in their ploidy level (Fig. S3). Fluorescence in adult plants was ubiquitously detected in different plant tissues using different microscope techniques (Fig. 5A–F). We discarded endogenous fluorescence by analyzing WT plants in a confocal microscope (Fig. S5). Additionally, a lambda scan was performed to confirm the spectral pattern of our signal that corresponded to GFP 509 nm (Fig. S6).

Additionally, we confirmed the transgenic nature of 16 independent transgenic plants by the amplification of a fragment of the *GFP* gene (Fig. 5G). Moreover, the *B. liniflora* transgenic lines had been additionally assessed for the presence of bacterial contamination. Preliminary screening by culturing transgenic plant material in LB medium has proven

the absence of any *Rhizobium* contamination (Fig. S7A–B). Additional screening was carried out by performing PCR using specific primers for the spectinomycin resistance gene which lies external to T-DNA. No amplified band was noticed in the samples of the *B. liniflora* transformants (L1–L10). These data indicate the absence of *Rhizobium* strain (LBA4404) within the transformants (Fig. S7C).

All these verifications allowed us to affirm that we have obtained the first transgenic plants in the *Byblis* genus. In this way, this genus joins other previously transformed such as *Drosera* for engineering its biochemical pathways (Hirsikorpi *et al.* 2002), *Nepenthes* for studying carnivory (Miguel *et al.* 2019), *Dionaea* for obtaining some bactericidal compounds (Makowski *et al.* 2021), and *Utricularia* for studying developmental processes involved in trap formation (Oropeza-Aburto *et al.* 2020).

Genetic transformation has become an indispensable tool for the study of model species, allowing the overexpression of exogenous and endogenous genes, as well as their total or partial knockout. This technique is being widely applied in species such as *Arabidopsis*, *Nicotiana*, and tomato. Nonetheless, the development of these techniques in species more distant from the model systems opens the door to the knowledge, utilization,

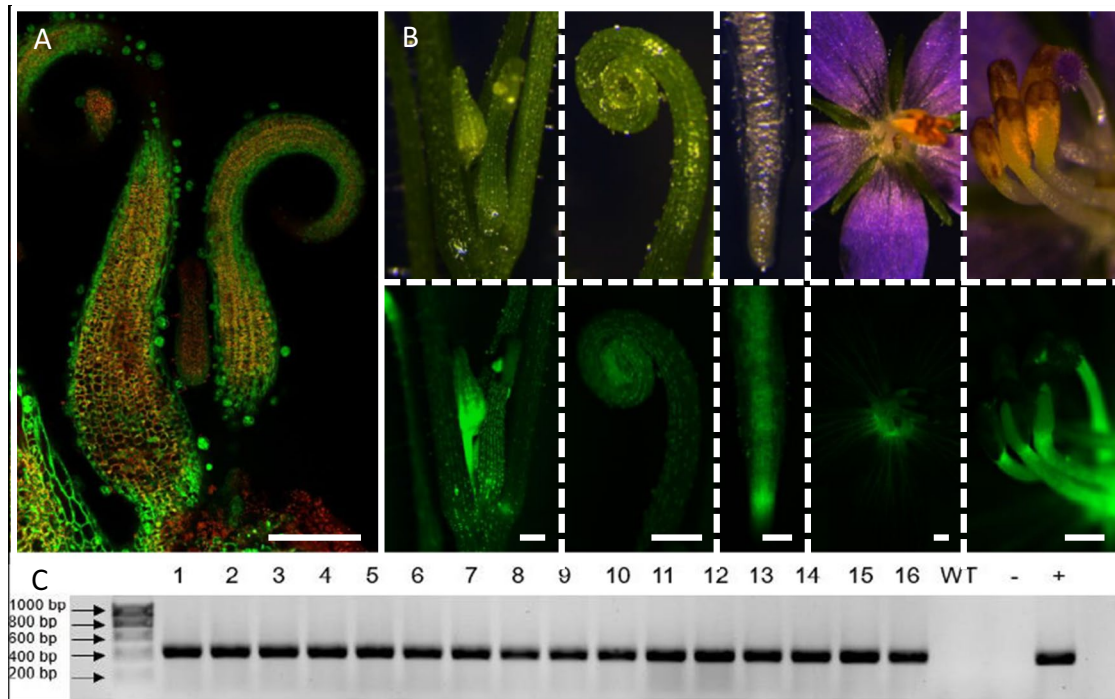


Figure 5. Phenotypic and genotypic (molecular) evaluation of the regenerated transgenic plants. (A) Detection of GFP (green) and chlorophyll (red) in the early stages of adventitious shoot regeneration by confocal microscopy. Scale bar: 0.5 mm. (B) Ubiquitous expression in different tissues of an adult plant in brightfield (top) and under GFP filter (bottom) of meristems and flower buds, leaves, roots, flowers and stamens and pistil (left to right). Scale bar: 0.5 mm. (C) The transgenic *B. liniflora* lines carrying the 35S:EGFP reporter construct were verified by PCR using primers targeting the EGFP fragment.

Amplified product after PCR in 1% agarose gel electrophoresis. Sixteen representative transgenic lines, expressing 35:EGFP, were used in this test (lane 2 to lane 17), wild-type *B. liniflora* plants (lane 18), and no-DNA sample (lane 19) were used as negative controls. An EGFP containing plasmid pK2GW7 (lane 20) was used as positive control in this test. Using specific primers (P3 and P4) within the marker EGFP produced specific bands of 480 bp present in transgenic plants, but not in WT plants. A DNA molecular size marker (MM) is shown at the leftmost lane 1.

and exploitation of plant material with unique characteristics in scientific, biotechnological, and pharmacological fields.

In this context, carnivorous plants possess some of the most unique mechanisms within the plant kingdom, such as the use of rapid movements and the secretion of enzymatic cocktails for the digestion and absorption of small animals. Genome sequencing technologies along with recent advances in bioinformatics have become accessible tools in basic research as they allow for the sequencing and assembly of reference genomes of less-studied plants at a relatively low cost. This facilitates the discovery of new genes related to the biosynthesis of enzymes, proteins, and secondary metabolites, as well as the study of the evolution of this singular group of plants.

Genomes of carnivorous plants such as *Utricularia* (Ibarra-Laclette *et al.* 2013), *Genlisea* (Leushkin *et al.* 2013), *Cephalotus* (Fukushima *et al.* 2017), *Dionaea* and *Drosera* (Palfalvi *et al.* 2020), and *Nepenthes* (Gao *et al.* 2023) have been sequenced, enabling a more precise tracing of the evolution of these plants and the development of certain strategies such as digestive enzyme secretions and absorption systems distinct from roots. The discovery

and isolation of these genes will open new avenues for the customized modification of other agronomically significant species with previously unstudied characteristics.

Additionally, the development of genetic transformation methodologies is the first step in obtaining plants with high ornamental value, enabling impossible modifications through conventional hybridization and breeding techniques. Recently, the first bioluminescent plants (Mitiouchkina *et al.* 2020), “The Firefly Petunia,” have appeared on the market, demonstrating a significant interest in developing plants with novel ornamental characteristics based on genetic transformation.

Conclusion

In view of the above, we conclude that we have achieved the development of a protocol for both plant micropropagation and genetic transformation of *B. liniflora* for the first time. The presented cloning and regeneration protocols set the

basis for the rapid and efficient multiplication of this plant for both conservation and ornamental production. The liquid-medium-based acclimatization protocol has proved to be the most effective way for obtaining greenhouse growing and flowering plants from those micropropagated ones. On the other hand, the genetic transformation of *B. liniflora* along with bioinformatics and genome sequencing will enhance the study of these plants in both basic research and practical applications, opening new frontiers in plant knowledge and biotechnology. These advancements not only lay the groundwork for investigating gene function but also for the study of the secondary metabolism and exploitation of their pharmacological characteristics. In summary, these results will address new breeding objectives in *Byblis* and expand our comprehension and utilization of carnivorous plants.

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Data Availability Not applicable.

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