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In vitro propagation of *Byblis liniflora* Salisb

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Abstract

This study was conducted to develop an *in vitro* propagation procedure for *Byblis liniflora* Salisb. The process of seed sterilization and *in vitro* sowing achieved 95% efficiency. From the material aseptic stem segments, the shoot proliferation program was determined with the rate of 33.67 shoots/explants after 4 weeks of culture on nutrient medium (1/3 strength MS basal salts plus full Morel and Wetmore's vitamins) with addition of a mixture of cytokinin (0.5 mg/L Kin and 2 mg/L BA) and then transferred to culture for another 4 weeks on nutrient medium. The rooting program from aseptic stem segments was to culture in nutrient medium supplemented with 0.5 mg/L NAA for 8 weeks. To produce a complete plant, single shoot separated from the proliferative clusters was transferred to a culture flask containing nutrient medium and cultured for 4 weeks. The *in vitro* shoot developed more branches and self-rooted to form a complete plant. The suitable growing substrate in the acclimatization and outdoor stages was a mixture of peat moss and perlite (1 : 2 w/w). The climatic conditions of Ho Chi Minh City and the Southeast of Vietnam were suitable for growing *Byblis liniflora* trees outdoors all year round.

Keywords: *Byblis liniflora* Salisb; Ho Chi Minh City; *in vitro* propagation; MS medium; Shoot proliferation

1. Introduction

Byblis Salisb was the only genus in the family Byblidaceae that included eight recognized species. This genus was also commonly known as the "rainbow plant" because the stems and leaves were covered with a layer of glittery trichomes in the sunlight. *Byblis liniflora* was one of four species of the *Byblis liniflora* complex along with *B. rorida*, *B. filifolia* and *B. aquatica*. The genus *Byblis* was naturally distributed from Australia to New Guinea [1-5]. With a beautiful appearance and the ability to attract and digest insects of a carnivorous plant, *Byblis liniflora* and other plants of the same genus were popular as ornamental plants and had been introduced to Vietnam.

In addition, many compounds presented in carnivorous plants and *Byblis liniflora* in particular had been discovered and proposed to apply, especially the role of substances in bio-glue [6-8]. Secondary compounds with medicinal uses such as plumbagin, anthraquinone, and quinone had been identified and obtained from micropropagation tissue of *Drosera burmanni* in Vietnam [9, 10]. Four types of phenylethanoid glycosides including acteoside, isoacteoside, desrhamnosylacteoside and desrhamnosylisoacteoside had also been isolated from *Byblis liniflora* plants grown *in vitro* [4]. The medicinal value of many carnivorous plants (of two genera *Utricularia* and *Drosera*) had also been documented [11].

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Micropropagation or *in vitro* cell and tissue culture of plants had been widely applied. This method allowed rapid propagation of rare species or species that were difficult to propagate by traditional methods. The individuals created from micropropagation had high uniformity and were pathogen-free. Furthermore, it also facilitated the extraction of secondary compounds [12]. For the genus *Byblis*, several *in vitro* tissue culture studies had been reported (*B. liniflora*, *B. filifolia* and *B. gigantea*) [4, 8, 13]. This was the basis for carrying out *in vitro* propagation research of *Byblis liniflora* to serve the needs of ornamental plants in Vietnam, and at the same time providing tissue materials for studying the extraction of secondary compounds later.

2. Material and methods

2.1. Samples collection and preparation

The original source of *B. liniflora* was the seed that was commercialized in Vietnam (https://www.caybatmoi.net/). Seeds were sown on a 1:2 (w/w) peat moss (dried *Sphagnum* moss) mixed perlite substrate, and moistened by water tray method. The pots were left outdoors in the climate of Ho Chi Minh City, Vietnam. The average sunshine hour per month was 160 – 270, the average temperature was 27.6°C, the average air humidity was 79.5% [14]. The re-identification of this generation of *B. liniflora* was done by Dr. Pham Van Ngot, Faculty of Biology, Ho Chi Minh City University of Education. Seeds collected from this generation were input materials for *in vitro* research.

Figure 1 *B. liniflora* grown in pots in outdoor conditions

2.2. Seed sterilization, *in vitro* seedling production

Seeds of *B. liniflora* were subjected to several surface disinfection treatments as suggested in the literature [15], each treatment was performed with 30 seeds, repeated 3 times. The tested procedures differed fundamentally on: Whether the seeds were treated with soap or/and alcohol 70°; How long (1 or 5 or 15 minutes) the seeds were shaken in a mixture of Tween-20 (0.01% v/v) and Javen (10% v/v). After sterilization, seeds were sown in flasks containing one third of strength mineral salts of MS medium [16] that were solidified with 7 g/L agar, fully supplemented with Morel and Wetmore vitamins [17] (hereinafter referred to as “culture medium” or “nutrient medium”). The percentage of seeds successfully disinfected and germinated to produce seedlings were determinated. Seedlings continued to be cultured until reaching a height of about 5 cm (4 – 5 weeks). Culture conditions were established in the laboratory including 25±2°C, under white light of fluorescent lamps with illuminance of 1500 lux for 14 hours per day (used throughout the following experiments).

2.3. Induction of shoot formation from stem segments

In vitro plants obtained from the best treatment of experiment 2.2 were collected. Stems cut into 1 cm long segments containing lateral shoots were considered as explants. The explants were placed in culture flasks (5 explants per flask) containing culture medium (nutrient medium) with the addition of kinetin (6-furfurylaminopurine) (abbreviated as kin) at a fixed concentration of 0.5 mg/L and benzylaminopurine (abbreviated as BA) on a concentration scale of 0 to 2 mg/L, 0.5 mg/L for each step.

After 4 weeks of culture under established temperature and light conditions (see section 2.2), one batch of flasks (6 flasks per treatment) was transferred to new culture flasks with minerals and vitamins as described (nutrient medium) without the addition of phytohormones, and were cultured for the next 4 weeks (Batch b). The remaining batch of flasks (6 flasks per treatment) continued to be kept in the same condition and cultured for another 4 weeks (Batch a). The average number of shoots obtained from each explant and the average shoot length were recorded in the efficiency comparison between the two experimental batches.

2.4. Induction of root formation from stem segments

The explants (1 cm long stem segments) were placed in culture medium (see section 2.2) with the addition of naphthaleneacetic acid (abbreviated as NAA) on a concentration scale of 0.05 to 0.45 mg/L, 0.10 mg/L for each step, in comparison with control treatment without NAA (5 explants per dish, 6 dishes per treatment). After 8 weeks of culture under established light and temperature conditions (see section 2.2), the average number of roots obtained from each explant and the average root length were recorded.

2.5. Induction of root formation from *in vitro* shoots

The shoot clusters from the best treatment of experiment 2.3 were obtained, separated the shoots with a height of about 4-5 cm and transferred to the flasks containing the culture medium (see section 2.2) supplemented with the best rooting effective dose of NAA (best treatment in experiment 2.4). The control was shoots grown in culture media without NAA. The culture flasks were placed under the established temperature and light conditions (see section 2.2) until the plants were well rooted.

2.6. Creating products from *in vitro* seedlings

Seedlings obtained from experiment 2.5 were further cultured in flasks which would be decorated to form new products, *in vitro* ornamental plant that did not require care and watering.

Seedlings could be removed from the culture flasks, rinsed off the agar that clung to the roots and re-transplanted into a plastic pots containing peat moss and perlite (1:2 w/w), covered with nylon films to retain moisture, and placed in the shade net house (5000 - 10000 lux) [15]. After about 2 weeks of acclimatization, the plants were transferred to larger pots and officially planted.

2.7. Processing statistics

All quantitative data were analyzed for One-way ANOVA (Analysis of Variance) and Least Significant Difference (LSD) with α=0.05 by using IBM SPSS Statistics 20.0.

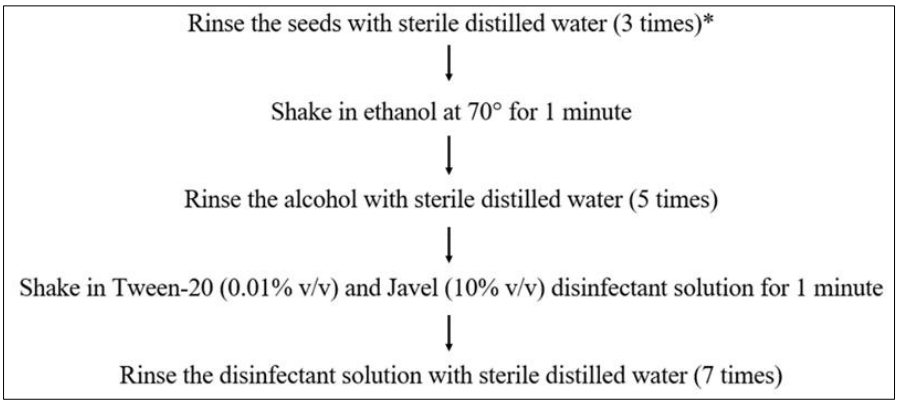
3. Results and discussion

3.1. Efficacy of *in vitro* seed sterilization

The seeds of the *Byblis liniflora* plants were obtained by self-pollination. Shaking the seeds with soap for 2 minutes before treating the seeds with 70° alcohol and increasing the seed treatment time with a mixture of Tween-20 and Javen from 5 to 15 minutes was not suitable for the seeds of *B. liniflora* in this experiment. The efficiency of the seed treatment group following this procedure only resulted in a seed germination rate of 55 to 87%. The seed sterilization procedure that resulted in 95% germination and seedling formation in vitro at 28 days after sowing did not include the washing of seeds with soap and had one minute left for the seed shaking time in the Tween-20 and Javen (Figure 1).

Reducing the time and concentration of disinfectants and still obtaining a clean sample was most desirable in tissue culture because of the reduced potential for damage to the sample [15]. Therefore, for the seed source of plants with capsules such as *Byblis* or *Drosera*, it was necessary to take advantage of the sterilization of the capsule surface and then remove the seeds under aseptic conditions to increase the sterilization effect. Quach Ngo Diem Phuong and Bui Van Le took advantage of disinfecting the fruit surface of *Drosera burmanni* with a mixture of 10% Javen (v/v) and Tween-20 (0.01%) in 10 minutes, produced 94.09% sterile and germinated seeds after 3 weeks of sowing on half strength MS medium [9]. In the present experiment, the seeds were separated from the newly picked fruit, so the sterilization time with the mixture of Tween-20 and Javen as mentioned above (Figure 1) gave the good result.

Some literature had also mentioned that seeds of carnivorous plants such as *Byblis*, *Drosera*, *Dionaea* need to be treated with smoke or GA_3 (gibberellic acid) to break dormancy and stimulate seed germination [8]. However, in practice with the *Byblis liniflora* species in the present experiment that seed treatment step was unnecessary.



(*): If there was a soap washing phase, it should be done first, before rinsing the sample with water for 3 times

Figure 2 Procedure for seed sterilization of B. *liniflora*

3.2. Efficiency of *in vitro* shoot cluster formation from stem segments

In the same experimental group ('Batch a' or 'Batch b'), increasing the concentration of BA from 0 to 2.0 mg/L on a 0.5 mg/L kinetin background resulted in an increase in the number of shoots. Comparing between two experimental batches, keeping the explants for 8 weeks (Batch a) or after 4 weeks culturing in hormone medium and transferring the samples to nutrient medium for 4 weeks (Batch b), it could be seen that the number of shoots in Batch b was significantly higher than in Batch a. The best treatment (NA5b) was 1/3 mineral of MS medium and full of Morel and Wetmore vitamins, supplemented with 0.5 mg/L kinetin and 2.0 mg/L BA, cultured for 4 weeks then transferred the sample to new nutrient medium without hormone, cultured for an additional 4 weeks. The highest multiplication rate was 33.67 shoots/explant (Table 1).

Table 1 Effect of kinetin and BA on shoot formation

Hormone concentration (mg/L)	Treatments	Transferred to nutrient medium*	Average number of shoots**	Average height of shoots (cm) **	Other characteristics
0.5 kin + 0 BA	NA1a	No	3.67$_{\gamma}$ ± 0.57	4.70 ± 1.25	Having some long roots (>5 cm), no callus.
	NA1b	Yes	6.33 ± 0.57	6.23$_{\Omega}$ ± 1.01	Having some short roots (<3 cm), no callus.
0.5 kin + 0.5 BA	NA2a	No	3.33$_{\gamma}$ ± 1.16	3.93$_{И}$ ± 0.55	Having some long roots (>5 cm), creating callus.
	NA2b	Yes	9.33* ± 0.57	5.77$_{\Omega}$ ± 0.51	Having some short roots (<3 cm), no callus.
0.5 kin + 1.0 BA	NA3a	No	4.67$_{\gamma}$ ± 0.57	3.53 $_{И}$ ± 0.21	Having some long roots (>5 cm), creating callus.
	NA3b	Yes	10.67* ± 0.57	3.97$_{N}$ ± 0.06	Having some short roots (<3 cm), no callus.
0.5 kin + 1.5 BA	NA4a	No	9.00 ± 0.57	3.10 ± 0.57	Having some short roots (<3 cm), creating callus.
	NA4b	Yes	24.67 ± 0.57	4.03 $_{N}$ ± 0.15	Having some short roots (<3 cm), no callus.
0.5 kin + 2.0 BA	NA5a	No	15.67 ± 3.79	2.17 ± 0.55	Having some short roots (<3 cm), creating callus.
	NA5b	Yes	33.67 ± 1.53	4.93 ± 0.59	Having some short roots (<3 cm), no callus.

(*) Nutrient medium containing one third of strength mineral salts of MS medium that were solidified with 7 g/L agar, fully supplemented with Morel and Wetmore vitamins. (**) Values followed by the same symbol were not statistically different at the alpha level of 0.05 by Duncan's test.

Pelto and Lindstrom found that in *Byblis filifolia* tissue culture, an increase in BA concentration from 1 to 2 µM reduced shoot number but increased shoot size. The presence of hormones also promoted better growth of shoot clusters compared with a germination medium consisting of one-fifth the mineral strength of MS and full of vitamins [8]. However, the combination of two commonly used cytokinins, BA and kinetin, in the present study had produced some new morphogenesis. Although both of those hormones were thought to reduce apical dominance and stimulate lateral shoot formation, kinetin also had callus-inducing effects and stimulated shoot formation from callus (in combination with auxin had lower concentration) while BA had the added ability to form roots [12, 18, 19]. *Byblis liniflora* explants in the present study reproduced rooting under the effect of kinetin in the absence of BA (NA1a and NA1b) and the longer it was left (8 weeks), the longer the roots grew without any more shoots (NA1a in contrast to NA1b). Only in combination with BA at equal or greater concentrations (within 8 weeks) did the explant induce callus. Transfer of explants to new nutrient medium for 4 weeks after hormone exposure for 4 weeks (Plot b) increased the number and quality of shoots, no further root development and no callus formation.

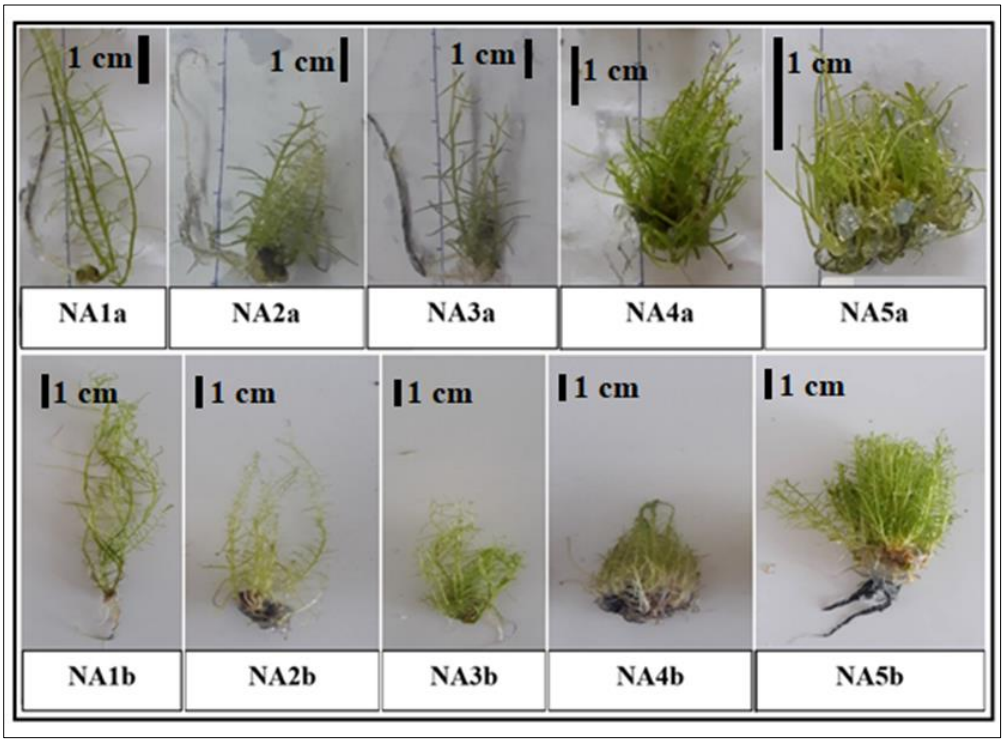


Figure 3 Efficiency of induction of shoot formation from stem segments

3.3. Efficiency of *in vitro* root formation from stem segments and shoot clusters

After 8 weeks of culture in nutrient medium supplemented with different concentrations of NAA, the results showed that from NAA concentration of 0.25 – 0.45 mg/L, the efficiency in producing the average number of roots per explant was from 5.33 – 6.67, the difference was not statistically significant while the root length was decreasing (Table 2).

The best treatment for root number and root length was NB1, with an additional NAA concentration of 0.05 mg/L which produced an average of 16.67 roots/explant and an average length of 7.73 cm. The control not supplemented with NAA did not induce roots from *in vitro* cultured stem segments. It was also important to note callus formation on explants, especially from NAA concentrations from 0.25 to 0.45 mg/L. Rooting efficiency decreased while callus formation increased with NAA concentration (Figure 3).

The combination of the best budding treatment (NA5b) with the concentration of NAA 0.05 mg/L (NB1) did not give the expected effect on *in vitro* seedlings as expected. The plant continued to grow, produced a few weaker shoots and many short roots. The stems and leaf were pale green (Figure 4 a). In contrast, with the control treatment, transferring the single shoots from the shoot cluster obtained in the NA1b treatment after 8 weeks of experimentation to the nutrient medium without NAA gave the best *in vitro* seedling efficiency. Seedlings had a characteristic green colour, continued to grow taller, generated more shoots and created roots well (Figure 4 b).

Table 2 Effect of NAA on root formation

Treatments*	NAA concentration (mg/L)	Average number of roots**	Average length of roots (cm)	Rooting properties
NB1	0.05	16.67 ± 1.53	7.73 ± 0.68	Creating the most and the longest roots, and some callus.
NB2	0.15	9.33 ± 0.58	1.70 ± 0.20	Creating fewer roots and shorter than NB1; creating more callus.
NB3	0.25	6.67ǂ ± 1.53	1.03 ± 0.15	Creating fewer and shorter roots, and more callus.
NB4	0.35	6.33ǂ ± 1.53	0.93 ± 0.21	Creating fewer and shorter roots, and more callus.
NB5	0.45	5.33ǂ ± 0.58	0.37 ± 0.21	Creating fewer roots and the shortest roots; creating the most callus.

(*) The control treatment without hormone did not induce rooting (not shown in Table). (**) Values followed by the same symbol were not statistically different at the alpha level of 0.05 by Duncan's test.

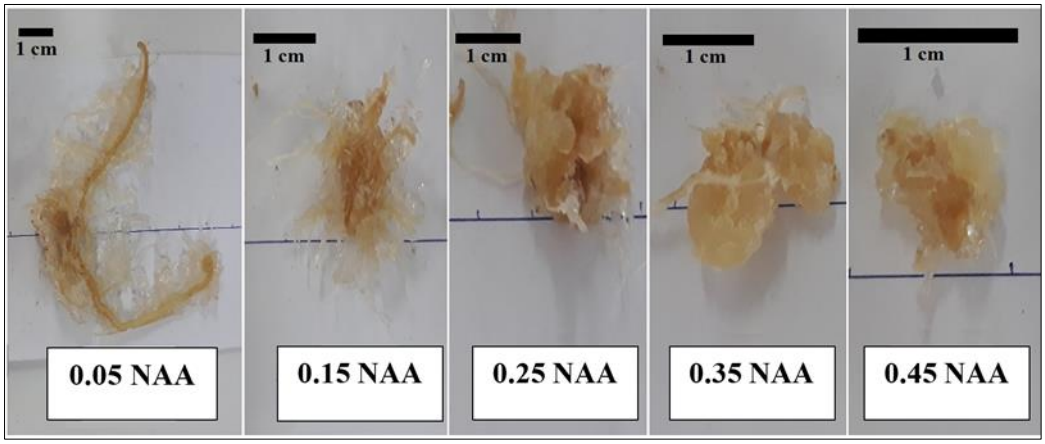


Figure 4 Efficiency of induction of root formation from stem segments

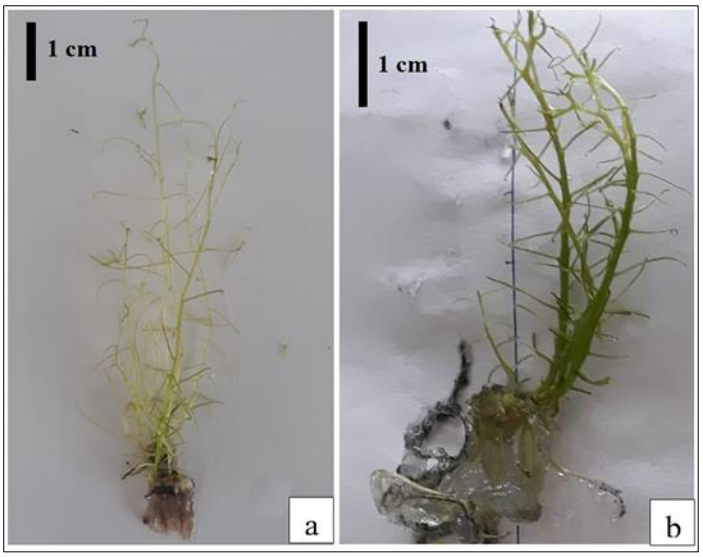


Figure 5 Efficacy of root induction from single shoots in nutrient medium supplemented with 0.05 mg/L NAA (a) and without NAA (b)

If the multiplication phase was mainly based on cytokinin addition or in combination with lower amounts of auxin, in the rooting phase of the explant, the use of cytokinin was not necessary in many cases. Auxin had the effect of inducing callus, forming lateral and adventitious roots but inhibiting the formation of adventitious and axillary shoots [12]. In which NAA was a synthesized substance but had similar efficacy to natural auxins. The recommended dose of NAA for rooting stimulation was 0.1 to 2.0 mg/L alone or in combination with another auxin such as IAA or IBA [12]. The amount of IBA present in commercial rooting medium (Clonex ©, Australia) suitable for shoot clusters of *Byblis filifolia* was 14.7 µm [8], which was a very small dose. In the present study, NAA concentration of 0.05 mg/L gave the best rooting effect for stem explants of *B. liniflora*, but for single shoots separated from the shoot cluster, this auxin supplement was not necessary. *In vitro* growth and morphogenesis were also subject to interactions and balances between artificial complement regulators and endogenous hormones in the seedling [12].

3.4. Efficiency in creating ornamental plants

(*) Nutrient medium containing one third of strength mineral salts of MS medium that were solidified with 7 g/L agar, fully supplemented with Morel and Wetmore vitamins.

Figure 6 *Byblis liniflora* plants grown in used infusion bottles on nutrient medium (*)

The used infusion bottle (200 – 500 mL) was to be substituted for the *Byblis liniflora* culture flask. The product bottles were decorated in an eye-catching way and were commercialized to serve the new bonsai hobby in Vietnam (Figure 5). This ornamental plant did not need to be watered and fertilized, and was suitable for display in the office for a period of 6 months or more. At the same time, this practice had also helped to reuse used infusion bottles in addition to recycling glass waste.

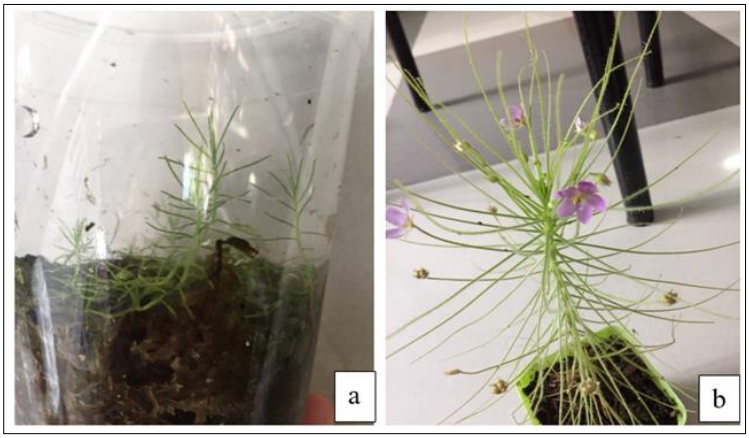


Figure 7 Seedlings obtained from tissue culture were in the process of acclimatization (a) and after growing outdoors (b)

Besides the above-mentioned ornamental method, after the adaptation period of seedlings (Figure 6 a), plants grown on moss and perlite mixed substrate (1:2 w/w) with self-watering thanks to water trays also gave an efficiency of over 95%. The natural temperature, light and humidity regime of the Southeast region, Vietnam was completely suitable for *Byblis liniflora* plants to be grown outdoors (Figure 6 b). If fed with insects, plants could live up to 2 years because they were not restricted by the dry season as in the wild in the native area.

4. Conclusion

The study developed in vitro successful micropropagation procedure of *Byblis liniflora*. Aseptic seedlings of *Byblis liniflora* were produced from seeds sterilized in ethanol 70% for one minute, then in mixture Tween-20 0.01% and Javen 10% for one minute and sown on one third of strength mineral salts of MS medium complemented Morel and Wetmore vitamins and 7g/L agar (the nutrient medium). Stem segments with length of 1.0 cm from the seedlings were cultured on the nutrient medium added Kin 0.5mg/L and BA 2mg/L for four weeks to produce shoot clusters. The clusters sub-cultured on the nutrient medium for the next four weeks. Single shoots separated from the proliferative clusters were sub-cultured on the nutrient medium for four weeks to form complete seedlings. The mixture of peat moss and perlite (1 : 2 w/w) was suitable growing substrate in the acclimatization and outdoor stages to the seedlings of *Byblis liniflora*. Culture conditions were established in the laboratory at 25±2°C, with illuminance of 1500 lux for 14 hours per day. The protocol could be used for rapid propagation of *Byblis liniflora* for the needs of ornamental plants in Vietnam, and providing aseptic tissue material for further studies of extracting useful secondary compounds from it.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

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