inflammatory and antioxidant properties of the ethanol extract of *Clerodendrum cyrtophyllum* Turz in copper sulfate-induced inflammation in zebrafish, *Antioxidants (Basel)*, 9(3), 192. **5**. Nguyen T. H., Nachtergael A., Cornet V., Kestemont P., (2020), Anti-inflammatory properties of the ethanol extract from *Clerodendrum cyrtophyllum* Turz based on *invitro* and *invivo* studies, *Journal Ethnopharmacology*, 254, 112739. **6**. Nair AB., Jacob S. (2016), A simple practice guide for dose conversion between animals and human, *Journal Basic and Clinical Pharmacy*, 7(2), 27-31. **7.** Zhang L., Hu J. J., Lin J. W., Fang W. S., Du G. H. (2009), Anti-inflammatory and analgesic effects of ethanol and aqueous extracts of *Pterocephalus hookeri* (C.B. Clarke) Höeck, *Journal Ethnopharmacology*, 123(3), 510-514. **8.** Ishola I. O., Agbaje O. E., Narender T., Adeyemi O. O., Shukla R., (2012), Bioactivity guided isolation of analgesic and anti-inflammatory constituents of *Cnestis ferruginea* Vahl ex DC. (Connaraceae) root, *Journal Ethnopharmacology*, 142(2), 383-389. **9**. Winter C. A., Risley E. A., Nuss G. W., (1962), Carrageenin induced edema in hind paw, *Experimental Biology Medicine*, 3(111), 544-547. **10**. Das S., Haldar P. K., Pramanik G., (2010), Evaluation of anti-inflammatory activity of *Clerodendron infortunatum* Linn. extract in rats, *Global Journal of Pharmacology*, 4(1), 4850.

Journal of Medicinal Materials, 2024, Vol. 29, No. 5 (pp. 316 - 320)

MICROPROPAGATION OF *STEPHANIA BRACHYANDRA* DIELS, A RARE MEDICINAL PLANT OF THE NORTH-WESTERN VIETNAM

Le Thi Quynh Nga[1], Nguyen Thi Xuyen[1], Hoang Thi Nhu Nu[1], Nguyen Thi Lam Hai[2], Nguyen Minh Khoi[1], Vu Hoai Sam[1,*]

[1]*National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam;*

[2]*Vietnam National University of Agriculture, Hanoi, Vietnam*

*Corresponding author: samthavn@yahoo.com

(Received June 14th, 2024)

Summary

Micropropagation of *Stephania brachyandra* Diels, a Rare Medicinal Plant of the North-Western Vietnam

An effective protocol for micropropagation of *S. brachyandra*, a rare medicinal plant of the North-Western Vietnam has been established. 2-3 cm long *in vivo* nodal segments were efficiently disinfected with 0.1% $HgCl_2$ solution for 10 minutes and regenerated on basic MS solid medium augmented with 0.5 mg/L kinetin. The highest *in vitro* shoot multiplication coefficient of 8.2 was obtained on the medium containing full-strength MS combined with 1.0 mg/L kinetin. The best medium for *in vitro* rooting was the half-strength MS solid medium fortified with 0.25 mg/L IBA, giving a rooting percentage of 85.1%, and an average of 3.3 roots per shoot after 4 weeks of culture. The rooted plantlets were successfully acclimatized in TRIBAT substrate with 96.4% vigor rate and then transferred to the field in Tam Dao Research Center for Medicinal Materials, Vinh Phuc province, where they thrived well.

Keywords: *Stephania brachyandra, Micropropagation, Medicinal plant, North-Western Vietnam.*

1. Introduction

Stephania brachyandra is a medicinal plant belonging to the genus *Stephania*, having common characteristics of the genus, such as perennial plant with deciduous twining and large tuberous rootstock. It was distributed mainly on ravine sides in forests, at altitudes of about 1000 m [1]. Its tuber contains the main active ingredient L-tetrahydropamatin, which has been used in Vietnam for several clinical indications under the drug Rotundine. This is a kind of sedative to treat effectively neuropathic diseases today due to its few side effects. *S. brachyandra* is being overexploited, leading to the exhaustion of its natural resources, therefore, it was listed in the Viet Nam Red Data Book at Endangered level (EN), and the list of Group II in Decree No. 84/2021/ND-CP dated September 22nd, 2021 of the Vietnam Government on the management of endangered, rare and precious species of forest fauna and flora and observation of convention on international trade in endangered species of wild fauna and flora.

Because of the increasing demand for seedlings and medicinal materials of *S. brachyandra*, propagation of this endangered medicinal plant species for the conservation of genetic resources and to develop its production areas of medicinal materials is an urgent duty. Sexual propagation and asexual propagation using rootstocks could not provide enough seedlings for production in large-scale, because breeding materials are rare. Furthermore, it is difficult for its seeds to germinate in natural conditions. To achieve the highest rate of 90%, they require the optimal treatments such as soaking in tap water for 6 hours, 1% NPK containing substrate and 50% shading mode [2]. Micropropagation, which uses small pieces of the plant as explants under aseptic and controlled environmental conditions to produce a large number of consistently uniform and true-to-type plants, overcomes the downsides of above traditional propagation methods. Therefore, it is the most optimal method to propagate *S.*

brachyandra. There are not, however, many studies on this species. Earlier attempts have been made for the propagation of *S. cepharantha*, which belongs to the same genus *Stephania* [3]. In this study, the effects of PGRs on all stages of *in vitro* propagation of *S. brachyandra*, including establishment, proliferation, and rooting were investigated to develop an efficient protocol for this rare medicinal plant.

2. Materials and methods

Explant preparation and sterilization

The juvenile nodal segments (2 - 3 cm) with at least an axillary bud of the donor plants were collected from the Sapa Research Center for Medicinal Materials in Lao Cai province. The explants were washed thoroughly under running tap water for 5 mins and then shaken in detergent powder 0.01% for 10 mins and rinsed many times with the tap water. After that, they were disinfected with $HgCl_2$ 0.1% for 10 mins and were finally rinsed 5 times with sterile distilled water in a laminar airflow cabinet.

Culture medium and growth conditions

The basal MS (1962) medium [4] supplemented with 30 g/L sucrose and 7 g/L agar was used in this study. Different plant growth regulators (PGRs), including BAP, kinetin (KIN), IBA, and α-NAA, were used singly or in combination at different concentrations (mg/L). The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 mins. The culture room was kept under a light intensity of 2000 lux approximately for 14 h photoperiod per day at 24 ± 2°C and 70% relative humidity.

Shoots proliferation

The effect of different concentrations and combinations of PGRs in the full-strength modified MS medium on shoot bud initiation and multiple shoot induction were evaluated. Firstly, the PGRs were tested for initiation shoot establishment such as KIN at 0, 0.1, 0.3, and 0.5 mg/L concentrations, then investigated for multiple shoot induction with single BAP or KIN at 0, 1.0, 1.5, 2.0 mg/L, or in combination between the tested best single cytokinin concentration with α-NAA at 0, 0.1, 0.2, and 0.3 mg/L. The percentage of explant establishment (established explants/explants x 100%), the number of induced shoots/explants were recorded after four weeks of culture, the percentage of shoot clumps, shoot proliferation (shoots/explants), the number of nodes (stem nodes/shoot), and proliferation coefficient (= the formed shoot cluster percentage X the average number of shoots per explant X the average number of nodes per shoot) were evaluated after six weeks of culture.

Rooting induction

Individual propagated shoots (1.5-2 cm with at least one leaf) were cultured on a medium containing different levels of MS salts without PGRs to find the best appropriate strength of MS. Then, it was supplemented with different concentrations of IBA (0, 0.25, 0.5, 0.75, and 1.0 mg/L) for rooting induction. The percentage of rooting and the number of roots per shoot were observed and recorded after eight weeks of culture.

Plantlets hardening

The well-development regenerated plantlets (after eight weeks of rooting induction) were removed from the culture flasks and washed carefully to get rid of the agar medium. Then, the plantlets were transplanted to sand lanes and covered with transparent plastic for acclimatization. After 7 days, transparent plastic bags were removed, and the seedlings were transferred to plastic pots containing different substrates (sand, clay loam, TriBAT nutrient soil (TriBAT company in Vietnam) to evaluate the effect of the substrates on the growth of the seedlings. The experiment was conducted in February 2024 under a nursery in Thanh Tri district, Ha Noi. The vigor plant rate, plant height, and the number of leaves were recorded after four weeks.

Statistical analysis

The experiment followed a completely randomized design (CRD) with three replicates. Each replicate of the disinfection experiment included 37 explants, and the others included 27 explants. Data were analyzed statistically by SPSS software (version 20.) followed by Duncan's test at $\alpha = 0.05$. The results were represented as Mean ± Standard deviation of the three replicates.

3. Results and discussion

Explant establishment

The study results showed that the percentage of regenerated shoots was 100% in all experiments, including the control (the hormone-free treatment) (Table 1). Besides, there was an increase in the average number of shoots per explant and the average length of shoots as the concentration of KIN increased in culture media. The statistical analysis results displayed that the number of shoots per explant was similar on the media supplemented with 0,

0.1, and 0.3 mg/L KIN at the P-value < 0.01 level after six weeks of culture. The highest number of shoots/explant (2.07 shoots) was observed on the medium supplemented with 0.5 mg/L KIN, and this value was statistically significant (P-value < 0.01) in comparison with the other concentrations in the experiment. Hence, KIN (0.3 mg/L) was chosen for *in vitro* shoot induction from the nodal segments of *S. brachyandra*.

Table 1. Effect of KIN on *in vitro* shoot regeneration of *S. brachyandra* after 6 weeks of culture

KIN (mg/L)	Regeneration (%)	Shoots/explant	Shoot length (cm)
0	100	$1.31^{b} \pm 0.10$	$2.17^{c} \pm 0.16$
0.1	100	$1.31^{b} \pm 0.12$	$2.20^{c} \pm 0.10$
0.3	100	$1.48^{b} \pm 0.07$	$2.77^{b} \pm 0.15$
0.5	100	$2.07^{a} \pm 0.15$	$3.30^{a} \pm 0.20$
P-Value		< 0.01	< 0.01

Notes: Different lower-case letter in each column indicate that these values are statistically different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

Shoot proliferation

Cytokinin (BAP, KIN) plays a crucial role in *in vitro* shoot regeneration and proliferation as they can metabolize immediately in plant tissue. BAP and KIN were, therefore, used as major factors for the induction of multiple-shoot clusters in this study. In the above experiment, the number of shoots per explant was still increased on the medium supplemented with 0.5 mg/L KIN, so this treatment served as the control to the following experiments of the shoot proliferation stage (Table 2).

Table 2. Effect of BAP and KIN on shoot multiplication of *S. brachyandra* after 8 weeks of culture

Cytokinins (mg/L)		Cluster Rate (%)	Shoots/explant	Nodes/Shoot	Proliferation coefficient
BAP	KIN				
	0.5	100^{a}	$2.1^{c} \pm 0.1$	$3.0^{b} \pm 0.2$	$6.5^{b} \pm 0.7$
1.0		100^{a}	$2.7^{a} \pm 0.2$	$1.5^{d} \pm 0.3$	$4.0^{c} \pm 0.7$
1.5		$86.4^{ab} \pm 13.0$	$2.8^{a} \pm 0.2$	$1.2^{de} \pm 0.2$	$3.4^{cd} \pm 0.9$
2.0		$77.8^{ab} \pm 22.2$	$2.5^{ab} \pm 0.3$	$1.0^{e} \pm 0.1$	$2.7^{d} \pm 0.2$
	1.0	100^{a}	$2.3^{bc} \pm 0.2$	$3.5^{a} \pm 0.1$	$8.2^{a} \pm 0.9$
	1.5	$70.4^{b} \pm 18.5$	$2.0^{c} \pm 0.2$	$2.7^{b} \pm 0.1$	$5.6^{b} \pm 0.3$
	2.0	$27.2^{c} \pm 14.0$	$1.4^{d} \pm 0.2$	$2.1^{c} \pm 0.3$	$3.1^{cd} \pm 0.6$
P-Value		< 0.01	< 0.01	< 0.01	< 0.01

Notes: Different lowercase letters in each column indicate that these values are statistically different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

The study results showed that the percentage of shoot cluster induction reached its maximum (100%) on the control medium and media amended with 1.0 mg/L IBA or KIN (Table 2). Higher concentrations of them beyond this 1.0 mg/L level inhibited the overall shoot sprouting frequency, leading to the shoot cluster rate parameter decline. It is observed that there is slight variation in the number of regenerated shoots per explant among the treatments. They ranged from 1.4 to 2.8 after eight weeks of culture. On the media containing three different concentrations of BAP (1,0; 1,5; 2.0 mg/l), the shoots/explant achieved the highest value of 2.5-2.8 shoots found to be statistically insignificant among them. The number of shoots per explant on medium fortified with 2.0 mg/L KIN was the lowest value and differed statistically in comparison to the other treatments. Each *in vitro* nodal segment could be used as an explant in the subculture of *S. brachyandra* thus, it was counted to build up the proliferation coefficient. Although the cluster rate and the number of shoots per explant were the highest values on the second treatment (1.0 mg/L BAP), the highest proliferation coefficient (8.2 times) was recorded on the fifth one (1.0 mg/L KIN) in this experiment. Therefore, it was the best treatment for the shoot proliferation stage in *S. brachyandra*.

Rooting

Reducing MS salt strength triggered

adventitious root formation in various plants in previous studies [5],[6]. Besides, IBA stimulated the *in vitro* rooting regeneration of *S. japonia* and *S. dentifolia* [5],[6]. In the present study, we, therefore, investigated the effects of different concentrations of IBA (0-1.0 mg/L) in combination with 1/2 MS on the rooting induction of *S. brachyandra* shoots. The response of individual shoots (1.5-2 cm) after eight weeks of culture was displayed in Table 3.

Table 3. Effect of IBA on rooting induction of *S. brachyandra* after 8 weeks of culture

IBA (mg/L)	Rooted Rate (%)	Roots/shoot	Root length (cm)	Plant height (cm)
0.00	$55.5^{bc} \pm 16.9$	$2.9^{ab} \pm 0.2$	$5.8^{a} \pm 0.5$	$5.0^{a} \pm 0.1$
0.25	$85.1^{a} \pm 12.8$	$3.3^{a} \pm 0.2$	$3.6^{b} \pm 0.3$	$4.7^{ab} \pm 0.3$
0.50	$76.5^{ab} \pm 13.0$	$3.1^{a} \pm 0.2$	$3.2^{b} \pm 0.2$	$4.2^{b} \pm 0.3$
0.75	$58.0^{abc} \pm 14.0$	$2.5^{b} \pm 0.1$	$2.5^{c} \pm 0.2$	$3.4^{c} \pm 0.2$
1.00	$35.8^{c} \pm 7.7$	$1.3^{c} \pm 0.3$	$1.9^{d} \pm 0.1$	$2.8^{d} \pm 0.2$
P-value	< 0.01	< 0.01	< 0.01	< 0.01

Notes: Different uppercase letters in each column indicate that these values are statistically different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

Statistical analysis results and data trends showed that various concentrations of IBA created different rooting induction responses. The control treatment resulted in a rooting rate of 55%. The highest number of rooting rates (85.1%) was observed on 1/2 MS with IBA (0.25 mg/L). In the treatments containing higher concentrations of IBA (0.5-1.0 mg/L), this one tends to decrease. The lowest percentage of rooting regeneration was recorded on the medium containing the highest concentration of IBA (1.0 mg/L). The maximum numbers of roots per shoot were observed on the treatment containing 0.25 or 0.5 mg/L IBA, however, there was an insignificant difference in comparison with that on the control treatment. The highest values of root length (5.8 cm) and plant height (5.0 cm) were obtained on the medium without IBA. The gradual decrease of these parameters with increasing IBA concentration might be due to the competition of nutrients or the endogenous content of auxin. In the rooting induction, the number of rooted shoots and the number of roots per shoot are the major parameters for evaluation of rooting effectiveness, hence, the half-strength MS medium fortified with 0.25 mg/L IBA was the best appropriate medium for adventitious rooting of *S. brachyandra* shoots. In the current study, the higher concentration of IBA (> 0.25 mg/L) halted the growth of roots, while the higher concentrations of IBA (0.5 - 1.0 mg/L) were also applied in *in vitro* rooting induction for *S. japonica* and *S. dentifolia* [3],[5], and even for *S. brachyandra* in recently another study [7]. The different results of these studies indicated that the effects of different IBA concentrations may vary according to the plant species, the rooting culture time, and the size of explants.

Hardening and transferring plantlets to soil

One of the usual problems of plant propagation using the tissue culture method is the acclimatization to external conditions, which adds to high mortality of plantlets caused by fungal contamination to which *in vitro* raised plants are very vulnerable. Planting substrate is one of the affective factors playing an important role in the acclimatization of plantlets. A study on the effect of sand, garden soil, and TriBAT on the vigor of *S. brachyandra* plantlets was conducted.

Table 4. Effect of substrates on the acclimatization of *S. brachyandra* plantlets after 4 weeks

Plating substrates	Survival rate (%)	Plant height (cm)	Leaves/plantlet
Sand	$93.69^{a} \pm 1.56$	$8.58^{b} \pm 0.66$	$7.10^{a} \pm 0.24$
Garden soil	$76.58^{b} \pm 10.81$	$9.97^{a} \pm 0.68$	$4.06^{c} \pm 0.21$
TriBAT	$96.40^{a} \pm 4.13$	$10.17^{a} \pm 0.56$	$6.08^{b} \pm 0.26$
P-Value	< 0.05	< 0.05	< 0.01

Notes: Different uppercase letters in each column indicate that these values are statistically different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

The results showed there were significant differences in the survival rate, plant height, and the number of leaves of *S. brachyandra* plantlets among the applied substrates (Table 4). The vigor percentage of the plantlets on the sand and TriBAT substrates reached a quite high level, 93.69% and 96.40%, respectively. The plant heights after four weeks on garden soil and on the TriBAT were equivalent because the difference was not statistically significant. The plant height increasing on these two substrates was much higher than it was on the sand, while the average number of leaves per plantlet on the sand was the most, following up the TriBAT, and then garden soil. In short, TriBAT was the best substrate for the acclimatization of *S. brachyandra* plantlets derived from *in vitro* propagation to *ex vitro* conditions (Fig. 1). Then, these plantlets were transferred to the field in the Tam Dao Research Center for Medicinal Materials, Vinh Phuc province with a 100% survival rate and thrived well.

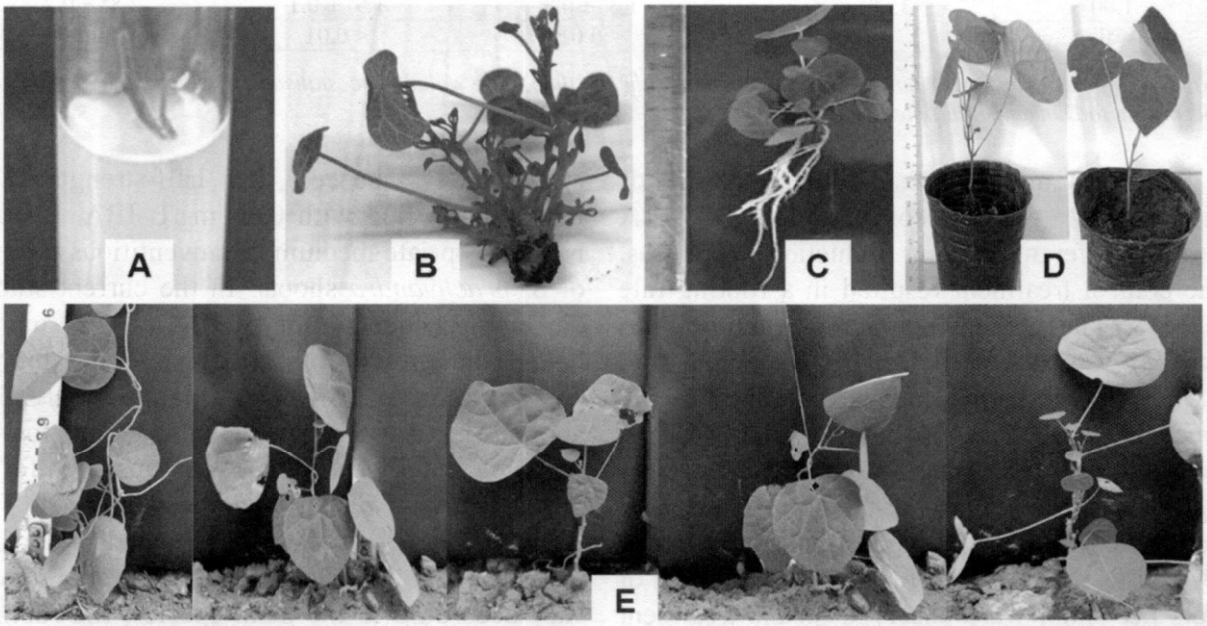

Fig. 1. Different stages of micropropagation of *S. brachyandra*; (A) initial shoot establishment; (B) shoot proliferation; (C) rooting induction; (D) plantlets acclimatization in plastic pots; (E) Cultured seedlings on the field

4. Conclusion

In the present study, we improved the efficiency of micropropagation of *S. brachyandra*. The maximum proliferation (100%) and highest shoot multiplication coefficient (8.2 times) were achieved on the full-strength MS medium amended with 1.0 mg/L KIN. Rooting induction rate of 85.1% on half-strength MS solid medium fortified with 0.25 mg/L IBA. The TriBAT was the suitable substrate for transferring plantlets to *ex vitro* conditions net house. These plantlets were planted and well-expanded on the field of Tam Dao Research Center for Medicinal Materials, Vinh Phuc province.

Acknowledgments: *This research was supported by the Ministry of Science and Technology, Vietnam (grant No. NVQG-2023/ĐT.08).*

References

1. Flora of China (2008), *Stephania brachyandra* Diels in Engler, Pflanzenr 46(IV.94)275.1910, *FOC*, 7, 17. **2.** Duong Van Thao (2023), Propagation of *S. brachyandra* in Thai Nguyen province in Vietnam, *Vietnam Journal of Forest Science*, 1, 25-31. **3.** Pham Thi Thanh Nhan, Tran Thi Hong, Hoang Phu Hiep, Cao Thi Phuong Thao, Tu Quang Tan, Chu Hoang Mau (2020), Study on sterilizing plant materials and the *in vitro* culture medium of *S. cepharantha* Hayata, *Proceeding of National Conference on Biotechnology*, 914-919. **4.** Murashige T., Skoog F. (1962), A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiologia Plantarum*, 15, 473-497. **5.** Handique P. J., Sharma D. (2010), Micropropagation of *S. japonica*, a rare medicinal plant of north-east India, *Journal of Hill Agriculture*, 1(2),102-106. **6.** Yu L., Duan Y., Zhan H. (2023), An improved micropropagation of a medicinal plant *S. dentifolia*, *Plant Cell, Tissue and Organ Culture* (PCTOC), 153(1), 219-224. **7.** Chu Thi Thuong (2023), *Study on propagation of Stephania brachyandra* Diel *by tissue culture*, Master's thesis of Thai Nguyen University of Agriculture and Forestry - Thai Nguyen University.