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In vitro conservation and propagation of the endemic species of "floating hearts" (*Nymphoides krishnakesara* Joseph and Sivar. - Menyanthaceae) as a conservation strategy

Swetha Thilak T[1], Anusree P[1], Saranya Babu Jayaprakash CM[1], Smitha RB[1], Minoo Divakaran[2], Madhusoodanan PV[1], Prakashkmar R[1]*

[1]Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode, Kerala, [2]Department of Botany, Providence Women's College, Kozhikode, Kerala, India.

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ABSTRACT

Nymphoides krishnakesara Joseph and Sivar. (Menyanthaceae), an endangered aquatic angiosperm, endemic to Kerala, was multiplied and propagated through *in vitro* culture. It is a rare species with very restricted distribution occurring in a single location. It is an annual herb, grows in shallow temporary pools on laterite. The nodal explants when cultured in Murashige and Skoog (MS) medium attained bud break in 20 days. Maximum multiple shoot proliferation observed in 1.0 mg/L benzyl amino purine (80 shoots/explant). Shoots developed *in vitro* were rooted in MS medium with both indole 3-butyric acid (IBA) (1.0 mg/L) and naphthalene acetic acid (mg/L). Roots developed in IBA are found to be more favorable based on the histological studies. The rooted plantlets were then transferred to the field after hardening and they flowered after 2 months. Total time taken from explants to flowering is 10 months. This work standardizes an easy protocol for mass production of plantlets, and thus enhances conservation of this endemic and rare aquatic plant. The hardened plants were successfully reintroduced and recorded 100% survival.

1. INTRODUCTION

India is rich in its aquatic flora, of which majority comprises from South India. Among the aquatic plant diversity *Nymphoides* Seg. is an interesting genus of about 20 spp. [1], widely distributed in the tropical and temperate regions of both the Old World and the New World. *Nymphoides krishnakesara,* an endemic emergent herb found in shallow waters of seasonal ponds of lateritic hills, is an interesting dioecious plant [2]. *N. krishnakesara* was originally reported from Madayipara, a midland lateritic hill in Kannur District, Kerala, South India [2]. Habitat of the species is threatened due to environmental modifications and urbanization. Its occurrence in a single location made this plant endemic to Northern Kerala and also included in the IUCN Red list of threatened species version 2011 [3]. The plant being dioecious and unavailability of male and female plants in the same location restricts the natural propagation through seeds. In traditional folklore medicine, the flowers and roots of this plant are used as a febrifuge [4].

*Corresponding Author:
Prakashkmar R,
Malabar Botanical Garden and Institute for Plant Sciences,
Kozhikode, Kerala, India.
Phone: 9446556113
E-mail: rprak62@gmail.com

The genus can be easily distinguished from the similar looking water lilies (*Nymphaea* spp.) by its petiole-like uniphyllous sympodial branches bearing a cluster of flowers at the nodal region. Only few works were undertaken on the micropropagation of *Nymphoides* spp. The genus *Nymphoides* is a less exploited one. A protocol for rapid shoot organogenesis from petiole explants of the ornamental aquatic plant *Nymphoides indica* L. [5] and indirect regeneration of *Nymphoides cristatum* floral buds [6,7] are some works on the genus. The application of tissue culture as a tool for the conservation of rare and endangered plants has gained huge trust in the recent decades. The present study was undertaken to formulate a standard protocol for micropropagation of the endangered and endemic species *N. krishnakesara* Joseph and Sivar. and its introduction to the field, thereby helping habitat restoration.

2. MATERIALS AND METHODS

2.1. Bud Break and Proliferation

A healthy growing plant in the Aquagene (Aquatic Plant Conservatory of Malabar Botanical Garden and Institute for Plant Sciences) introduced from the original locality is used as the explant source. Nodal cuttings from fresh sprouts (3rd and 4th leaf) were used as explants [Figure 1a]. The leaves were collected, washed in running water for 25 min and the nodal region was separated and treated with Tween 20 (2–3 drops in 1 L distilled water) for 15 min followed by washing with double distilled

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water 3–5 times. Surface sterilization of explants was done with 0.1% (w/v) $HgCl_2$ for 2–3 min and washed thrice with sterile double distilled water before inoculation inside the laminar air flow chamber. The nodal segments were trimmed into two equal vertical halves of about 1 cm long aseptically and used directly as the explant. The surface sterilized explants were inoculated in Murashige and Skoog (MS) basal medium [8] and MS medium with combinations of auxins (indole 3-butyric acid [IBA]*, naphthalene acetic acid [NAA]**) and cytokinins (benzyl aminopurine [BAP]***) were tried for shoot or root induction. Once shoot formation was noticed, they were subcultured in the rooting medium with auxin after the formation of roots and they were transferred to basal MS medium containing low concentration (4.5 mg/L) of agar. After 2–3 weeks, the fully developed plants were transferred to pots with sterile clay and kept in the greenhouse for hardening.

The media used were fortified with 3% sucrose and the pH adjusted to 5.8, before the addition of agar and followed by autoclaving at 121°C for 20 min. The cultures were maintained at 25 ± 1°C with 70% relative humidity and a photoperiod of 12/12 h 35–40 µ/mol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

2.2. Anatomical Studies

The roots developed *in vitro* were investigated anatomically. Hand sections of root formed in medium supplemented with 0.5 mg/L IBA and 0.5 mg/L NAA were taken, stained with 1% safranine and observed under microscope and photographed.

3. RESULTS AND DISCUSSION

3.1. Establishment of Explants and Bud Initiation

The nodal segments inoculated on basal MS medium showed bud break after 20 days. After the bud break, direct regeneration of a single shoot was noticed on the 30th day of inoculation in MS medium without any PGR [Figure 1b]. Once the regeneration is noticed, they were transferred to the multiplication medium. Different concentrations (0.25–1.0 mg/L) [Figures 2 and 3] of cytokinins (BAP) were tried for shoot proliferation. Highest shooting percentage (90%) with maximum shoot proliferation (80 shoots/explant) is attained in medium supplemented with 1.0 mg/L BAP [Figure 1c]. 0.25 and 0.5 mg/L BAP gives 20 and 50 shoots/explant and 60% and 80% of shooting response, respectively. Initially, the concentration of agar used was 6.5 g/L in which the *in vitro* shoot development showed stunted growth with brittle leaves [Figure 1d]. A combination of BAP and IBA both 0.5 mg/L showed rhizogenesis after 10 days along with multiple shoots where the shooting percentage noticed was the same as that of 0.5 mg/L fortified medium.

3.2. Effect of Auxin in the Development of Roots

The *in vitro* regenerated shoots were excised and transferred to rooting medium with different concentrations (0.5 and 1.0 mg/L) [Table 1] of auxins (IBA and NAA). Both IBA and NAA were found to be effective in rhizogenesis. *In vitro* developed shoots when subcultured in a medium with 0.5 mg/L NAA showed rhizogenesis after 5 days of inoculation [Figure 1e] with an average of 14 roots while that of media fortified with 0.5 mg/L of IBA produced roots after 1 week [Figure 1f] with an average of five roots. The average number of roots were high in NAA and the number showed a considerable increase when the concentration of NAA was raised to 1 mg/L. There was no callus formation at the base, and hence, the rhizogenesis is direct. Highest rooting percentage was noticed in medium fortified with 1.0 mg/L NAA with an average of 24 roots.

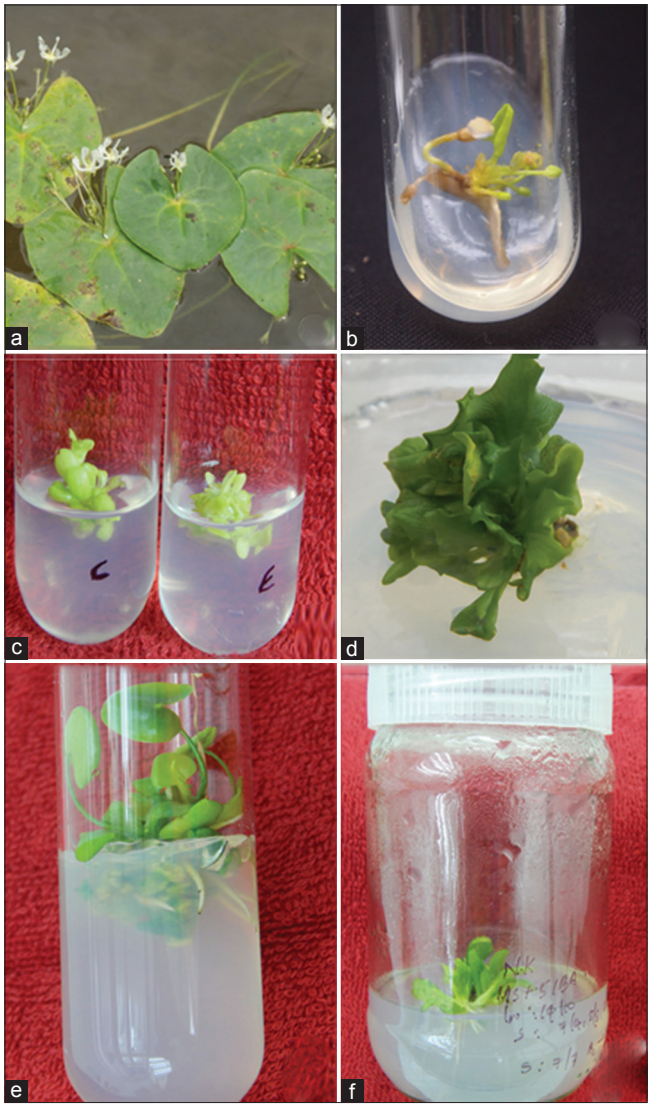

Figure 1: (a) Habit, (b) direct organogenesis, (c) multiple shoot formation in 1mg/L benzyl amino purine, (d) short and brittle leaves in 6.5 mg/L Agar, (e) induction of roots in 0.5 mg/L Naphthalene acetic acid. (f) induction of roots in 0.5 mg/L Indole 3- butyric acid.

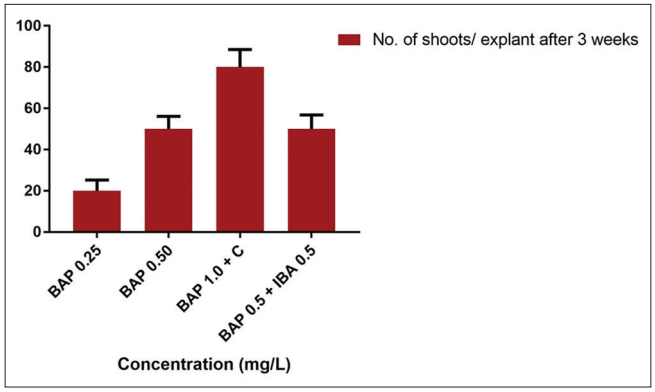

Figure 2: The number of shoots in different concentration of benzyl aminopurine.

Table 1: Effect of different concentration of auxins promoted rooting of the plant.

Hormone	Concentration	Root induction (%)	Rooting in number of days	Number of roots
IBA	0.5	80	7	5
	1.0	80	7	10
NAA	0.5	70	5	14
	1.0	80	7	24

IBA: Indole 3-butyric acid, NAA: Naphthalene acetic acid.

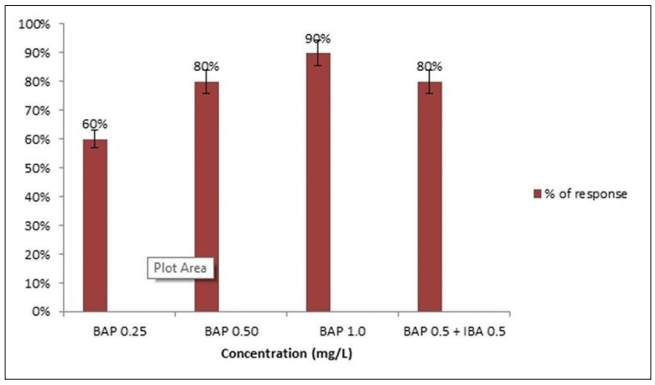

Figure 3: Percentage response of shoot formation in different concentration of benzyl aminopurine.

The roots formed in NAA were soft, spongy, and numerous while the roots formed in IBA were strong with lateral roots. Once the roots were formed, they were transferred to medium with low concentration of agar (4.5 g/L) for the better absorption and root development [Figure 4a and b]. In this concentration of agar, the normal leaf with long petiole, which helps the lamina to float on the water surface, was developed. Among the plants developed *in vitro,* those developed roots in medium containing IBA showed better establishment under field conditions.

3.3. Acclimatization of Plantlets Developed *In Vitro*

For the hardening process, the individual plantlets were separated from the medium and cleaned with sterile water to get rid of the excess agar [Figure 4d]. The plants were first transferred to sterilized tap water and kept in culture conditions [Figure 4c]. After 2 weeks, they were planted in cups with sterile clay with 1 cm deep water and kept in the greenhouse [Figure 4e].

The plants developed *in vitro* flowered within 2 months of introduction to the field [Figure 4f]. Of these, the plants rooted in medium with IBA showed better development in the field conditions, and initially, these were smaller in size than the normal ones.

3.4. Histological Studies

In histology, the root developed in NAA has more number of aerenchyma (air spaces) in the cortical region than that of the roots developed in the medium with IBA [Figure 4h and i]. The C.S. of the control root also showed a lesser number of aerenchyma [Figure 4g] indicating that lesser number of aerenchyma favored better establishment of roots in field conditions.

4. DISCUSSION

The aquatic systems in Kerala currently face a serious threat of extinction due to rapid urbanization and industrialization. Developmental initiatives by filling wetlands have seriously affected the rich aquatic biodiversity of Kerala which include several endemic and endangered species, many of which are reported from transient pools developed on the laterite hills of midland region during the rainy seasons but become dry during the summer season. *N. krishnakesara* is reported from such a unique habitat which survives the summer drought through dormant shoots and regenerates during next monsoon with vigor. Since the plant is a dioecious one, seeds develop only when both the male and female plant exists in the same region. This taxon is highly endemic and reported from only one locality in the Northern Kerala.

Only few works were undertaken on the micropropagation of aquatic plants. Of these, major works were on the micropropagation of medicinal plants such as *Bacopa monnieri* [9,10] and *Acorus calamus* [11,12]. For *in vitro* clonal propagation, the common explants used are the nodal segments. In the present study also, the explant selected was the nodal region, from where direct organogenesis is achieved, similar result was obtained in *N. indica* [5] also, while floral buds of *N. cristatum* produced friable callus from which organogenesis was achieved [6,7]. In this study, even though growth regulator-free MS medium was able to induce bud break and shoot formation, the number of shoots formed from single explant was found to be less in number. BAP was the single cytokinin used for the multiple shoot formation. Several reports point out the capacity of BAP for bud proliferation and multiple shoot formation in many plants such as *B. monnieri* [12] and *Avicennia marina* [13]. Averages of 80 shoots were obtained from 2-week-old cultures in medium with 1.0 mg/L BAP while 60 shoots were obtained from per piece in 50 days of culture in *Passiflora caerulae* L. [14].

Two auxins (IBA and NAA) were tried for the root induction, both showed favorable results. It is reported earlier that the auxins at lower concentration facilitate better root formation [15]. Even though the time taken for rhizogenesis in MS medium with NAA was less and produced more roots, the roots produced in IBA were strong both in morphological and anatomical studies. Similarly, the increase in the rooting percentage and the better rooting in the medium containing IBA were reported in *Alnus glutinosa* [16]. Earlier reports indicate that NAA also induces callus tissue; hence, establishment of plants in the field is hindered by the interfering callus tissue [17,18].

The institute has undertaken several studies on different aspects of the aquatic vegetation of South India with a view to develop a comprehensive conservation protocol for aquatic and wetland plants of the country. The study also involves micropropagation of aquatic plants of rare, endangered, and threatened category through *in vitro* cloning. The present study facilitates an easy protocol for the production of rooted multiple shoots up to 80 from a single explant in basal MS medium supplemented with 1.0 mg/L BAP. It is therefore established that the most reliable way for rapid clonal propagation of *N. krishnakesara* is through direct organogenesis.

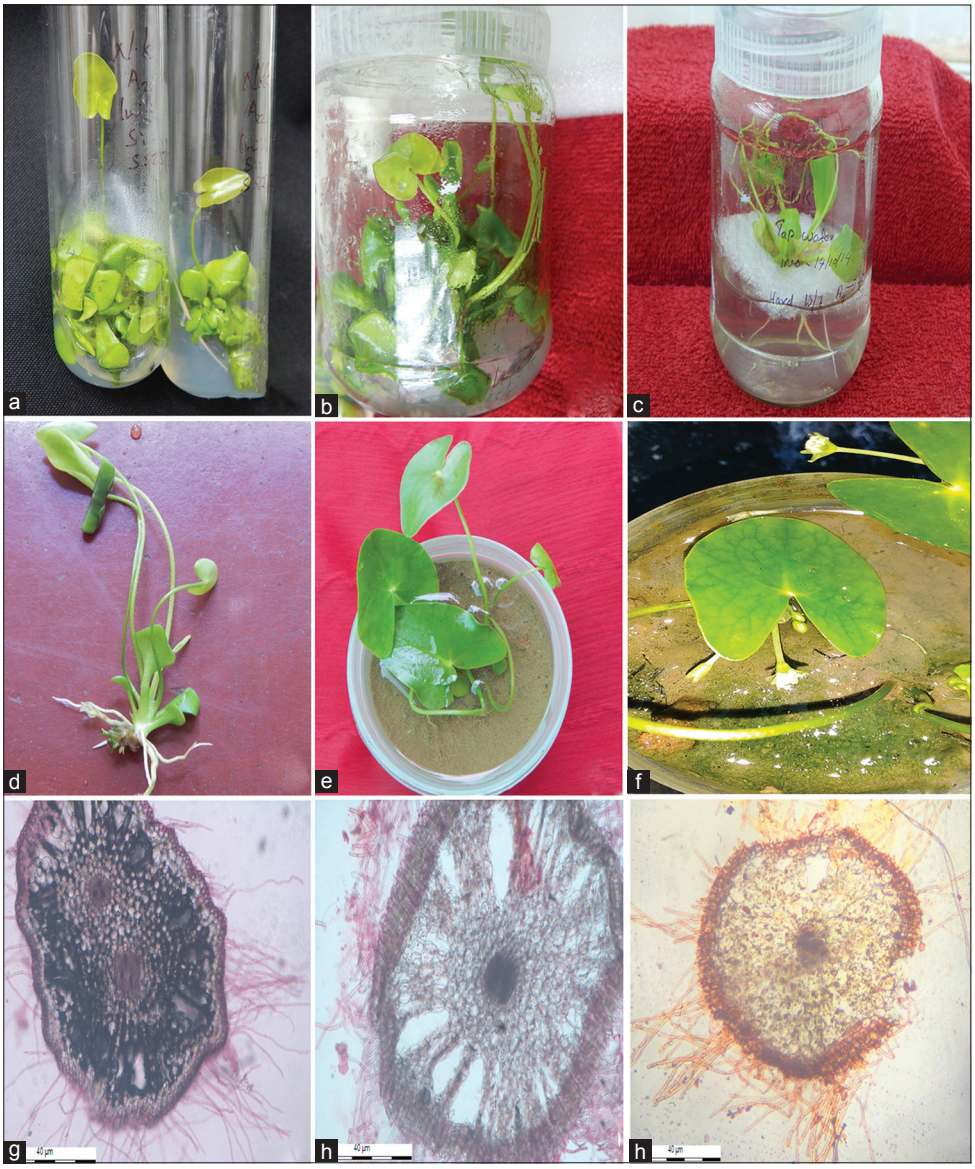

Figure 4: (a and b) Normal leaves in 4.5 mg/L agar, (c) hardening in tap water, (d) plantlet before planting, (e) plant in greenhouse for hardening, (f) flowering in field, (g) C. S. of control root, (h) C. S. of root in Naphthalene acetic acid and (i) C. S. of root in Indole 3- butyric acid showing lesser number of air chambers.

5. CONCLUSION

In the present situation, there is a great need for the conservation of aquatic plants since the rapid urbanization has led to the loss of our valuable aquatic and wetland habitats. In this context, the present study suggesting a simple protocol for micropropagation of rare and endemic aquatic plants owes its importance. This study is the first attempt on the micropropagation and the successful field establishment of the endemic plant *N. krishnakesara* Joseph and Sivar.

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7. REFERENCES

1. Cook CD, Gut BJ, Rix EM, Schneller J, Seitz M. Water Plants of the World. Dr. The Hague: Junk Publisher; 1974.
2. Joseph KT, Sivarajan VV. A new species of Nymphoides from India. Nordic J Bot 1990;10:281-4.
3. Karuppasamy S, Rao MLV. Nymphoides Krishna kesara. The IUCN Red List of Threatened Species 2011: E.T194157A8884723. DOI: 10.2305/IUCN.UK.2011-1.RLTS.T194157A8884723.en.
4. Divakar MC, John J, Devi V, Poornima, Anisha, Subhash A, Govindan V. Herbal remedies of Madayipara hillock tribals in Kannur district, Kerala, India. J Med Plant Stud 2013;1960:34-42.
5. Jenks MA, Kane ME, McConnell DB. Shoot organogenesis from petiole explants in the aquatic plant *Nymphoides indica*. Plant Cell Tiss Organ Cult 2000;63:1-8.
6. Niranjan MH, Sudarashana MS. *In vitro* plant regeneration in *Nymphoides cristatum* (Roxb.) O. Kumtze. Phytomorphology 2000;50:343-4.

7. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
8. Behera S, Nayak N, Shasmita Barik DP, Naik SK. An efficient micropropagation protocol of *Bacopa monnieri* (L.) Pennel through two stage culture of nodal segments and *ex vitro* acclimatisation. J Appl Biol Biotechnol 2015;3:16-21.
9. Kaur J, Nautiyal K, Pant M. *In vitro* propagation of *Bacopa monnieri* (L.) Wettst- A medicinally priced herb. Int J Curr Microbiol Appl Sci 2013;2:131-8.
10. Anu AK, Babu N, John CZ, Peter KV. *In vitro* clonal multiplication of *Acorus calamus*. J Plant Biotechnol Biochem 2001;10:53-5.
11. Verma S, Singh N. *In vitro* mass multiplication of *Acorus calamus* L. - An endangered medicinal plant. Am Eur J Agric Environ Sci 2012;12:1514-21.
12. Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, *et al*. *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst. Afr J Biotechnol 2010;9:8318-22.
13. Al-Bahrany AM, Al-Khayri JM. Micro propagation of grey mangrove *Avicennia marina*. Plant Cell Tiss Organ Cult 2003;72:87-93.
14. Gattuso S, Severin C, Salinas A. Micro propagation *Passiflora caerula* L. And histological studies of tissue regeneration. J Trop Med Plant 2003;4:249-56.
15. Pandey P, Mehta R, Upadhyay R. *In vitro* propagation of an endangered medicinal plant *Psoralea coryfolia* Linn. Asian J Pharm Clin Res 2013;6:115-8.
16. Jose SC, Romero L, Janeiro LV. Effect of Indole-3-Butyric Acid on root formation in *Alnus glutinosa* microcuttings. Silva Fennica 2012;46:643-54.
17. Biondi S, Thorpe TA. Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus rudiatus*. Bot Gaz 1982;143:20-5.
18. Kapai VY, Kapoor P, Rao IU. *In vitro* propagation for conservation of rare and threatened plants of India. Int J Biol Technol 2010;1:1-14.

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