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Conservation of an endangered ornamental and medicinal aquatic plant, *Nymphoides macrosperma* Vasudevan through *in vitro* propagation

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Abstract

Nymphoides macrosperma is a valuable aquatic plant from Kerala, South India which is at the risk of extinction in the nearby future. A protocol for rapid multiple shoot proliferation from the basal buds of this plant was developed successfully. Treating the explants with 15% concentrated solution of commercial bleach (Robin liquid bleach, *Reckitt Benckiser, India*), followed by a quick dip in absolute alcohol was proved to be the best sterilization procedure to obtain clean cultures. After proper *in vitro* stabilization on plant growth regulator-free (PGR-free) Murashige and Skoog (MS) basal medium, earliest shoot bud regeneration was obtained on a medium containing full strength MS inorganic salts, 100 mg/l myo-inositol, 0.1 mg/l thiamine-HCl, 3% sucrose, 0.8% agar as gelling agent and supplemented with a combination of 0.5 mg/l 6- Benzyl aminopurine (BAP) and 1.0 mg/l 6-furfurylaminopurine (kinetin). Maximum multiplication of the *in vitro* shoots was obtained on MS medium supplemented with 1.0 mg/l BAP. Lowering the concentration of BAP from 1.0 mg/l favored shoot elongation and maximum elongation of the *in vitro* developed shoots was obtained on MS medium supplemented with 0.5 mg/l BAP as well as on PGR-free MS basal medium. MS medium supplemented with 0.15mg/l indole-3-butyric acid (IBA) was the best medium for *in vitro* root formation. The *in vitro* developed plantlets were successfully hardened and planted out in the aquarium. *N. macrosperma* can be mass propagated through the procedure standardized here so that the plant can be saved from the danger of extinction while being exploited commercially.

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Introduction

Nymphoides sp., belonging to the family Menyanthaceae, are valuable aquatic plants having bright potential to become a multimillion-dollar commodity in the aquarium trade. *Nymphoides macrosperma* Vasudevan, is an endangered species reported as endemic to Ernakulam in Kerala, South India (Vasudevan, 1968). It is a dioecious rhizomatous herb with petiole-like branches bearing single floating leaves at the apex and flower clusters at the nodes. The white fringed floral petals and the light green floating leaves (Vasudevan, 1968) make the plant attractive in water gardens. The plant is also used in aquaria as fillers (Ansari and Jeeja, 2006). Besides being ornamental, *N. macrosperma* has medicinal importance too. In South India, a drug by name 'Granthika Tagara' belonging to *N. macrosperma* (Yoganarasimhan *et al*., 1979) is used in several therapeutic preparations for the treatment of various diseases such as anaemia, epilepsy, fever, cough, jaundice and mental disorders (Murali *et al*., 2007; Veesam *et al.,* 2012).

N. macrosperma has growing demands in the aquarium (Personal communication; Unnikrishnan S. K., Oriental Aquatics, Singapore) and pharmaceutical industries (Murali *et al*., 2007; Veesam *et al.,* 2012) due to its ornamental and medicinal properties. But as the plant has been facing the challenges of habitat destruction and water pollution for the past few decades, it has become rare in its natural habitat and has been enlisted as 'endangered' (Ansari and Jeeja, 2006). Therefore immediate measures should be adopted for large scale propagation of this plant to meet the market demands while conserving it in its natural habitat. It has been reported that, most of the aquatic plants are difficult to be propagated through conventional methods as they show infrequent seed production and slow rate of propagation through rhizome (Kane *et al.,* 1991; Dissanayake *et al*., 2007). It is in this scenario, mass propagation of *N. macrosperma* by using the technique of tissue culture has become a necessity.

The technique of micropropagation offers large scale production of plantlets within a very short span of time irrespective of the climatic conditions. Moreover, the plantlets produced through tissue culture will be free from pests and diseases. In microprogation, a plant part (explant) is cultured on a medium containing optimum concentration of plant growth regulators so as to induce direct or indirect (through callus formation) shoot and root organogenesis. But the exogenous hormone requirement for shoot and root organogenesis varies with the species and with the explant that is used for culture initiation (Chawla, 2002). Moreover, a standardized surface sterilization is also needed for getting axenic cultures from the explant. Therefore, standardization of an appropriate protocol is necessary for the micropropagation a particular plant. So far no reports are available on the micropropagation of *N. macrosperma* nevertheless Jenks *et al*., (2000) and Niranjan and Sudarshana (2000) have published papers on the *in vitro* micro propagation of two other species, *Nymphoides indica* and *Nymphoides cristatum* respectively. The present study was aimed at standardizing an efficient protocol for the micropropagation of *N. macrosperma*.

Materials and methods

Explant sterilization and culture initiation

Mother plants (Figure 1) were collected from River Periyar in Aluva, Kerala, India. Leaf clusters and roots were removed from the stolon and the stolons were washed under running tap water for 10 minutes, soaked in 5% detergent solution (Cleansol, India) for 10 minutes and again washed thoroughly in running tap water to remove the superficial dirt. Surface sterilants tried to obtain axenic cultures included *0.1% mercuric chloride ($HgCl_2$) and commercial bleach containing 5.25% sodium hypochlorite as active ingredient (Robin Liquid Bleach, Reckitt Benckiser, India). To standardize the most suitable surface sterilization procedure, basal bud explants were treated with 0.1% $HgCl_2$ for 5 different durations (1 min, 2 min, 3 min, 4 min, 5 min) or with* six different dilutions of commercial bleach solution

(5%, 10%, 15%, 20%, 25% and 30 %) for six different durations (1 min, 5 min, 10 min, 15 min, 20 min and 25 min). Each treatment was done with 10 replications and number of survival and formation of healthy cultures with each treatment was recorded after 4 weeks of explant inoculation. After discarding the surface sterilant, explants were washed thrice with sterile water, subjected to a quick dip in 70% ethanol, and again washed thrice with sterile water. Inside the laminar air chamber, the basal buds were isolated and inoculated on medium containing full strength Murashige and Skoog (MS) inorganic salts (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 0.1 mg/l thiamine-HCl, 3% sucrose, 0.8% agar as gelling agent and devoid of any plant growth regulators (PGRs). The pH of the media was adjusted to 5.8 using 0.1 N HCl, dispensed in 150×25mm culture tubes (15 ml medium per tube), and autoclaved at 121° C for 15 minute. Cultures were maintained in a culture room at 25 ± 2°C under a 16 hour photoperiod with light intensity of 35 µmol photons $m^{-2}s^{-1}$ from Philips cool white fluorescent tubes. The cultures were subcultured twice at an interval of 4 weeks on fresh media with the same media composition for attaining *in vitro* stabilization and were then subcultured onto media containing various PGRs at varying concentrations.

Media used

The media tried to get earliest culture initiation and shoot regeneration included PGR-free MS basal medium and MS media supplemented with 0.5, 1.0, 1.5 and 2.0 mg/l BAP or Kinetin or nine different combinations of BAP and kinetin (Table 1). All the media used for the *in vitro* propagation of *N. macrosperma* contained MS mineral salts (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 0.1 mg/l thiamine-HCl, 3% sucrose and 0.8% agar. Number of days taken for bud release with each media was recorded; and the *in vitro* shoots from each media were subjected to repeated subculture on the same media to analyze the consistency in shoot multiplication rate. Observations on shoot multiplication, shoot elongation and root formation on each medium were recorded after 4 weeks of third subculture on the media. In addition to the above said cytokinin-containing media, *in vitro* root formation on MS media supplemented with 0.1, 0.15, 0.2, 0.25 or 0.3 mg/l Indole-3-acetic acid (IAA), IBA or Naphthalene acetic acid (NAA) was also studied.

Data analysis

To analyze the effect of various concentrations of BAP (0.5, 1.0, 1.5, 2.0 mg/l) and various concentrations of kinetin (0.5, 1.0, 1.5, 2.0 mg/l) on culture initiation, two experiments were set up separately in a Completely Randomized Design (CRD), each with 5 treatments (including PGR-free MS basal as control) and 6 replications. The experimental set up to analyze the effect of various combinations of BAP and kinetin on culture initiation was in CRD with 10 treatments (including PGR-free MS basal as control) and 6 replications. Number of days taken for bud release on each media was taken as the observation. In a similar way, separate experiments were set up each in a CRD to analyze the effect of various concentrations of BAP (5 treatments and 10 replications), various concentrations of kinetin (5 treatments and 10 replications) and various combinations of BAP and kinetin (10 treatments and 10 replications) on various aspects like shoot multiplication, shoot elongation and *in vitro* rooting. To standardize the best medium for shoot multiplication, number of shoots formed on various media after 4 weeks of third subculture was taken as the observation. The length (cm) of the *in vitro* shoots and the number of *in vitro* roots per shoot on each medium were taken as the observations for standardizing the media for shoot elongation and *in vitro* root formation respectively. Determination of the most suitable media for *in vitro* root formation was also based on three separate experiments which analyzed the effect of different concentrations (0.1, 0.15, 0.2, 0.25 mg/l) of IAA, IBA and NAA on number of roots formed per shoot. The observations were taken after 4 weeks of third subculture on each media. Data were subjected to square root transformation (except the shoot elongation data) and analyzed by Univariate Analysis of Variance using SPSS ver. 20.

Significant differences if any between the means were compared by Tukey HSD (Zar, 1999).

Acclimatization of the plantlets

In the present study, hardening was done by culturing the rooted plantlets on reduced concentration (1/10th) of MS salts devoid of any plant growth regulators, agar or sucrose for the first 4 weeks and then on distilled water for the next 4 weeks under controlled conditions of 24 ± 2^{o} C under a 12-hours photoperiod with light intensity of 35 µmol photons $m^{-2}s^{-1}$. The plants were then potted in small plastic cups containing Neopete (Strelings Farm Research Services Pvt. Ltd., India) which were kept in small glass tanks for another 4 weeks before their final transfer to aquaria. The glass tanks contained tap water reaching up to a height of ¼ of the pots.

Results and discussion

Standardization of Surface Sterilization Procedure

It was observed that, when the basal bud explants were treated with 0.1% $HgCl_2$, shorter treatment durations (1.0 min) resulted in contaminated cultures and longer treatment durations (above 1 minute) resulted in scorching of the tissue. Only 2 out of 10 rhizome explants treated with 0.1% $HgCl_2$ for 1 minute produced contamination-free healthy cultures and none of the explants were survived when treated for more than 1 minute. Explant *sterilization using* 0.1 to 0.8% $HgCl_2$ have been reported in *Piper longum* (Sonia and Das, 2002), *Spilanthes acmella* (Haw and Keng, 2003) *and Bacopa monnieri* (Sharma *et al*., 2010).

Table 1. *In vitro* responses shown by the basal bud explants of *N. macrosperma* on MS media supplemented with various concentrations of Cytokinins.

PGR	Conc. (mg/l)	Average no. of days for sprouting	Average no. of buds/ explant	Average length of Shoots (cm)	Average no. of roots/shoot
None	-	3.46±0.22^{b}	1.89±0.18^{b}	12.22±0.66^{d}	2.95±0.08^{b}
BAP	0.5	2.81±0.34^{a}	2.14±0.12^{c}	11.76±1.16^{cd}	2.95±0.08^{b}
BAP	1.0	3.18±0.21^{ab}	3.64±0.17^{d}	10.73±1.81^{c}	2.79±0.11^{a}
BAP	1.5	3.41±0.22^{b}	1.75±0.16^{b}	3.79± 0.68^{b}	2.66±0.11^{a}
BAP	2.0	4.42±0.44^{c}	1.36±0.22^{a}	0.43±0.25^{a}	2.75±0.15^{a}
kinetin	0.5	4.62±0.19^{c}	2.86±0.19^{c}	6.12±0.73^{b}	2.19±0.14^{a}
kinetin	1.0	4.64±0.11^{c}	1.83±0.20^{b}	2.90±0.46^{a}	2.53±0.10^{b}
kinetin	1.5	3.55±0.19^{b}	1.32±0.24^{a}	2.56±1.31^{a}	2.58±0.16^{b}
kinetin	2.0	3.11±0.13^{a}	1.32±0.24^{a}	2.03±1.31^{a}	2.44±0.17^{b}

Means ± SD. Treatment means with different superscripts in the same column of the sameexperiment are significantly different ($p< 0.05$).

Table 2. *In vitro* responses shown by the basal bud explants of *N. macrosperma* on MS media supplemented with various combinations of Cytokinins.

PGR	Conc. (mg/l)	Average no. of days for sprouting	Average no. of buds/ explant	Average length of the shoots (cm)	Average no. of roots/shoot
BAP,kinetin	0.5, 0.5	3.30±0.60^{abc}	2.50±0.17^{d}	4.60±1.17^{d}	2.52±0.19^{e}
BAP,kinetin	0.5, 1.0	2.88±0.18^{abc}	2.35±0.25^{c}	4.56±0.58^{d}	2.30±0.10^{cde}
BAP,kinetin	0.5, 1.5	2.70 ± 0.25^{a}	2.77±0.15^{ef}	4.54±1.45^{d}	2.23±0.23^{cd}
BAP,kinetin	1.0, 0.5	2.97±0.13^{abc}	3.37±0.21^{g}	3.70±0.55^{cd}	2.38±0.17^{de}
BAP,kinetin	1.0, 1.0	3.59 ± 0.46^{c}	3.37±0.21^{g}	2.87±0.32^{c}	2.31±0.23^{cde}
BAP,kinetin	1.0, 1.5	3.43±0.85^{abc}	2.87±0.25^{f}	2.75±0.32^{c}	2.12±0.12^{bc}
BAP,kinetin	1.5, 0.5	3.57±0.33^{bc}	2.32±0.21^{cd}	1.67±0.26^{b}	1.87±0.14^{a}
BAP,kinetin	1.5, 1.0	2.76±0.19^{ab}	2.19±0.14^{bc}	1.30±0.26^{ab}	1.97±0.14^{a}
BAP,kinetin	1.5, 1.5	3.06±0.43^{abc}	1.99±0.17^{ab}	0.54± 0.23^{a}	1.84±0.15^{ab}

Means ± SD. Treatment means with different superscripts in the same column are significantly different ($p< 0.05$).

But in the present study, $HgCl_2$ was found as not suitable for producing axenic cultures from the explants. Therefore, various concentrations of commercial bleach for various durations were tried for the purpose. Out of the various concentrations of commercial bleach and various durations of treatment tried to obtain axenic cultures, treatment with 15% concentrated solution of commercial bleach (0.79% Sodium hypochlorite as active ingredient) for 20 minutes followed by a quick dip in 70% ethanol produced 80% contamination-free explants. When the treatment duration and bleach concentration decreased from the optimum level, rate of contamination was steadily increased; whereas scorching of the tissue was observed with increase in bleach concentration and treatment duration. Reports on explant sterilization using the appropriate concentration of commercial bleach are available on *Ludwigia repens* (Ozturk *et al.*, 2004), *Phyla nodiflora* (Ahmed *et al.*, 2005), *Rosa damascene* (Nikbakht *et al.*, 2005) and *Limnophila aromatica* (George *et al.*, 2014). Present result also support the use of alcohol in combination with commercial bleach for surface sterilization of explants as advocated by Bonga (1982), Jenks *et al.* (2000) and Maridass *et al.* (2010).

Table 3. Effect of various types of auxins and their concentration in the medium on *in vitro* root formation in *N. macrosperma*.

Conc. of PGR (mg/l)	Average no. of roots/shoot on IAA	Average no. of roots/shoot on IBA	Average no. of roots/shoot on NAA
0	2.95±0.08^{c}	2.95±0.08^{bc}	2.95±0.08^{d}
0.10	2.72±0.09^{b}	2.95±0.08^{bc}	2.07±0.11^{b}
0.15	2.83±0.14^{bc}	3.10±0.32^{c}	2.19±0.12^{b}
0.20	2.32±0.11^{a}	2.84±0.13^{b}	2.38±0.14^{c}
0.25	2.30±0.10^{a}	2.55±0.10^{a}	1.87±0.14^{a}

Treatment means with same superscripts in the same column belong to the same homogenous sub group.

Standardization of Culture initiation medium

Shoot initials without any callus formation were clearly visible on all the basal bud explants cultured on PGR-free MS basal medium within 4-6 weeks of explant inoculation, which subsequently developed into normal shoots after 6-8 weeks of culture initiation.

Time taken for bud release was decreased with subsequent sub culturing in the same medium as well as upon transfer to PGR-containing media. Statistical analysis showed that, number of days taken for axillary bud release, number of shoots formed per explant, and length of the shoot vary significantly ($p< 0.05$) with varying concentrations/combinations of cytokinin in the MS media (Table 4). It took an average of 3.46 days for bud release on PGR-free MS basal medium. When the explants were cultured on MS medium supplemented with 0.5 mg/l BAP, the average time taken for bud release was reduced to 2.81 days. With increase in the concentration of BAP in the medium from 0.5 mg/l, there was a gradual increase in the average duration for bud break (Table 1). Among the various concentrations of kinetin tried, 2.0 mg/l alone showed earlier sprouting when compared to the PGR-free MS basal medium (Table 1). Except 1.0 mg/l BAP + 1.0 mg/l kinetin and 1.5 mg/l BAP + 0.5 mg/l kinetin all other combinations of BAP and kinetin showed early sprouting of the axillary buds (Table 2). Among the various media tried, sprouting of the axillary buds occurred at the earliest in an average of 2.7 days after inoculation on MS medium supplemented with a combination of 0.5 mg/l BAP and 1.5 mg/l kinetin (Table 2).

Efficiency of combination of BAP and kinetin for culture initiation was also reported in *Avicenia marina* (Al-Bahrany and Al-Khayri, 2003), *P. longum* (Sonia and Das, 2002) and *Dioscorea rotundata* (Adeniyi *et al.*, 2008). Among the two types of cytokinins used in the present study, earlier shoot bud initiation was observed with BAP-containing media (Table 1).

Table 4. ANOVA Effect of various concentrations/combinations of PGRs on culture initiation, shoot multiplication, shoot elongation and root formation in *N. macrosperma*.

Experiment	Sum of Squares	df	Mean Square	F	Sig
Effect of various concentrations of BAP on culture initiation[1]:					
Treatment	8.483	4	2.121	23.333*	.000
Error	2.272	25	.091		
Total	10.755	29			
Effect of various concentrations of kinetin on culture initiation[1]:					
Treatment	11.961	4	2.990	97.423*	.000
Error	0.767	25	.031		
Total	12.729	29			
Effect of various concentrations of BAP on shoot multiplication[1]:					
Treatment	30.733	4	7.683	255.352*	.000
Error	1.354	45	.030		
Total	32.087	49			
Effect of various concentrations of kinetin on shoot multiplication[1]:					
Treatment	15.760	4	3.940	87.587*	.000
Error	2.024	45	.045		
Total	17.784	49			
Effect of various concentrations of BAP on shoot elongation:					
Treatment	1141.989	4	285.497	256.276*	.000
Error	50.131	45	1.114		
Total	1192.12	49			
Effect of various concentrations of kinetin on shoot elongation:					
Treatment	1724.295	4	181.074	281.978*	.000
Error	28.897	45	0.642		
Total	753.192	49			
Effect of various concentrations of BAP on root formation[1]:					
Treatment	0.631	4	0.158	13.057*	.000
Error	0.544	45	0.012		
Total	1.175	49			
Effect of various concentrations of kinetin on root formation[1]:					
Treatment	3.030	4	0.758	41.785*	.000
Error	0.816	45	0.018		
Total	3.846	49			
Effect of various combinations of BAP and kinetin on culture initiation[1]:	6.099	9	1.678	3.792*	.000
Treatment	0.272	50	.179		
Error	15.033	59			
Total					
Effect of various combinations of BAP and kinetin on shoot multiplication[1]:					
Treatment	24.695	9	2.744	70.140*	.000
Error	3.521	90	.039		
Total	28.216	99			
Effect of various combinations of BAP and kinetin on shoot elongation:					
Treatment	959.973	9	106.664	215.361*	.000
Error	44.575	90	.495		
Total	1004.548	99			
Effect of various combinations of BAP and kinetin on root formation[1]:					
Treatment	9.991	9	1.110	41.775*	.000
Error	2.392	90	.027		
Total	12.383	99			
Effect of various concentrations of IAA on root formation[1]:					
Treatment	3.512	4	0.878	73.825*	.000
Error	0.535	45	.012		
Total	4.047	49			
Effect of various concentrations of IBA on root formation[1]:					
Treatment	1.693	4	0.423	14.571*	.000
Error	1.307	45	.029		
Total	3.001	49			
Effect of various concentrations of NAA on root formation[1]:					
Treatment	6.990	4	1.748	116.478*	.000
Error	0.675	45	.015		
Total	7.665	49			

Efficiency of BAP over kinetin on culture initiation has been reported in many plants including *Psidium guajava* (Loh and Rao, 1989), *Piper* spp. (Bhat *et al.*, 1995), *Citrus* spp. (Maggon and Singh, 1995), *Ocimum sanctum* (Patnaik and Chand, 1996), *B. monnieri* (Tiwari *et al.*, 1998) and *P. nodiflora* (Ahmed *et al.*, 2005).

Fig. 1. Mother plants of *Nymphoides macrosperma*

Fig. 2. Multiple shoot formation on MS medium containing 1.0 mg/l BAP.

Standardization of the medium for shoot multiplication

In the present study, BAP was more effective than kinetin on shoot multiplication also. Among the various media tried, shoot multiplication was highest on MS medium supplemented with 1mg/l BAP with an average of 3.64 shoots per explant (Table 1; Figure 2); but with the same level of kinetin in the MS medium, the average shoot number was 1.83 only. Among the various concentrations of kinetin tried, maximum shoot multiplication was observed with 0.5 mg/l kinetin with an average of 2.86 shoots per explant (Table 1). Even though, BAP and kinetin combinations produced better multiplication when compared to the shoot multiplication obtained with 0.5 to 2.0 mg/l kinetin alone and 0.5 mg/l and 1.5 mg/l BAP alone, 1.0 mg/l BAP in combination with 0.5 to 2.0 mg/l kinetin was not better than 1.0 mg/l BAP alone on shoot multiplication. Among the various combinations of BAP and kinetin tried, shoot multiplication was maximum and the same (an average of 3.3.7 shoots per explant) on 1.0 mg/l BAP in combination with 0.5 mg/l kinetin and 1.0 mg/l BAP in combination with 1.0 mg/l kinetin (Table 2). BAP promotion of shoot multiplication has been reported in several plants such as *P. guajava* (Loh and Rao, 1989), *Piper* spp. (Bhat *et al.*, 1995), *Citrus* spp. (Maggon and Singh, 1995), *O. sanctum* (Patnaik and Chand, 1996) and *B. monnieri* (Tiwari *et al.*, 1998). Superiority of BAP over other cytokinins for multiple shoot formation has also been reported in many fruit plants (Lundergan and Jainic, 1980), *Acorus calamus* (Anu *et al.*, 2001) and *Myriophyllum aquaticum* (Smitha *et al.*, 2005). However, further increase in the concentration of BAP or kinetin above the optimum levels (1.0 mg/l for BAP and 0.5 mg/l for kinetin) reduced the rate of shoot multiplication (Table 1). Similar observations on the inhibition of shoot multiplication by cytokinins beyond the optimum level have been reported by Ahuja *et al.* (1982) in *Catharanthus roseus*, Tiwari *et al.* (1998) in *B. monniera*, Wang *et al.* (2004) in *Scripus robustus*, Espinosa *et al.* (2006) in *Prunus serotina* and Pandeya *et al.* (2010) in *Clitoria ternatea.*

Fig. 3. Shoot elongation on PGR-free MS medium.

Shoot elongation

Analysis of variance (Table 4) showed that, the average shoot length varies significantly with various concentrations of BAP or kinetin or various combinations of BAP and kinetin in the media ($p < 0.05$). Multiple shoots formed on MS media

containing 0.5 and 1.0 mg/l BAP elongated considerably (11.76 cm and 10.73 cm respectively) in the same media (Table 1). It was observed that, the average length of the *in vitro* shoots (primary branch bearing terminal leaf and secondary branches from the node) decreased with increase in the concentration of BAP in the media (Table 1). On MS medium supplemented with 0.5 mg/l kinetin, average length of the *in vitro* shoots was 6.12 cm; and a gradual decrease in average shoot length was observed with increase in the concentration of kinetin also. Even though the shoots produced were smaller when compared to those formed on media containing 0.5 mgl^{-1} BAP or 1.0 mgl^{-1} BAP alone, shoot elongation was satisfactory on media containing 0.5 mgl^{-1} BAP in combination with 0.5 to 1.5 mgl^{-1} kinetin (4.60 to 4.54 cm; Table 2). Therefore, the present observation is contradictory to the findings of Preece *et al.* (1987), Pijut *et al.* (1991) and Ozturk *et al.* (2004) who have observed pronounced inhibition in elongation and growth of shoots in woody plants, *Pinus strobus* and *L. repens* respectively on media Thidiazuron (TDZ) and BAP. Among the various media used in the present study, greatest elongation of the *in vitro* shoots was observed on plant growth regulator-free MS basal medium with an average length of 12.22 cm (Table 1; Figure 3). This observation supports the opinion of Singha and Bhatia (1988), Fasolo *et al.* (1989), Preece and Imel (1991) and Ozturk *et al.* (2004) that, presence of growth regulators is not essential for the growth of *in vitro* developed shoots.

Fig. 4. *In vitro* root formation on MS medium containing 0.15mg/l IBA.

Fig. 5. Plantlets transferred to pots for acclimatization.

Rooting of the in vitro shoots and acclimatization of the plantlets

100% the *in vitro* shoots formed on media containing various concentrations and combinations of BAP and kinetin were rooted on the same media within 4-6 weeks of explant inoculation. Analysis of variance showed significant differences ($p< 0.05$) between the average root numbers on media containing various concentrations of BAP or kinetin or various combinations of BAP and kinetin (Table 4). An average number of 2.95 healthy roots were formed on PGR-free MS basal medium as well as MS medium containing 0.5 mg/l BAP (Table 1). The average root number on media containing 1.0 mg/l BAP was 2.79, which according to the Post Hoc test was at par with the average root numbers on media containing 1.5 mg/l BAP and 2.0 mg/l BAP. An average number of 2.19 to 2.58 roots were formed on media containing 0.5 to 2.0 mg/l kinetin (Table 1). Average root number ranged from 1.84 to 2.84 on media containing various combinations of BAP and kinetin (Table 2). These results suggest that, the plantlets formed on the shoot multiplication media can directly be planted into pots and no separate rooting attempt is necessary for the micropropagation of *N. macrosperma*. Root regeneration from shoots regenerated on media containing cytokinin was reported in *B. monnieri* (Tiwari *et al.*, 1998), *S. acmella* (Haw and keng, 2003), *L. repens* (Ozturk, 2004) and *M. aquaticum* (Smitha *et al.*, 2005). The present result is contradictory to the reports on

inhibition of root formation on cytokinin-containing media in apple cultivars (Fasolo *et al*., 1989), Eastern redbud (Yunsita *et al*., 1990) and silver maple (Preece *et al*., 1991). Hutteman and Preece (1993) have opined that, rooting of regenerated shoots is difficult because of a "carry over effect" from cytokinins in the shoot proliferation medium. But in this study it was found that, presence of BAP or kinetin in the shoot regeneration medium did not inhibit root development.

Fig. 6. Fully developed plants inside the tank.

As the use of auxins for *in vitro* root formation has been advocated by many workers (Al-Bahrany and Al-Khayri, 2003; Sharma *et al*., 2010, Tiwari *et al*., 1998; Dissanayake *et al*., 2007), studies were also made to analyze the *in vitro* rooting response of *N. macrosperma* on media containing various type of auxins (IAA, IBA and NAA) at various concentrations (0.1, 0.15, 0.2, 0.25 mg/l). Statistical analysis of the data revealed that, the average number of roots per shoot vary significantly ($p < 0.05$) with varying concentrations of IAA, IBA or NAA in the MS medium. Of the three types of auxins tried for root formation from the *in vitro* shoots of *N. macrosperma*, number of roots per shoot was highest with IBA followed by IAA and then NAA (Table 3). Among the various media tried, highest root number (3.10 roots per shoots) was observed on MS media supplemented with 0.15 mg/l IBA (Table 3; Figure 4). This result is supported by the findings of Al-Bahrani and Al-Khayri (2003) who have reported that IBA is the most effective auxin in enhancing root number in *A. marina* in comparison to NAA and IAA. They have also reported that, the concentration of IBA, NAA or IAA in the medium affects the elongation of the *in vitro* roots. According to Sharma *et al.* (2010), IBA supplemented MS medium produced maximum number of roots in *B. monnieri*. Similar reports are also available on the superior effect of IBA on *in vitro* root formation in *A. calamus* (Anu *et al.,* 2001) and *Cryptocoryne wendtii* (Dissanayake *et al*., 2007). However, in the present study, the average number of roots per shoot was decreased with increase in the concentration of IBA from 0.15 mg/l in the MS medium. 95% of the plantlets were survived after the hardening procedures and established well in the aquatic tanks.

Conclusion

To our knowledge, the present study is the first report for *in vitro* propagation of *N. macrosperma*. The protocol developed here will be useful for large scale propagation to meet the market demands as well as for *ex situ* conservation of this endangered and endemic aquatic plant.

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