RESEARCH ARTICLE

In vitro culture of *Nymphaea nouchali* seeds; a conservation approach for a vulnerable species

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Abstract: *Nymphaea nouchali* var. *nouchali* is a non-viviparous species with a slow natural propagation by rhizomes or seeds. The plant is threatened in its natural habitats due to several reasons and is included in the vulnerable category of the National Red List of Sri Lanka. *In vitro* contamination-free culture method was developed to initiate mass propagation of the species. Results were validated through molecular and microscopic studies. Bacterial growth occurred in the seeds disinfected via the standard method using Clorox™. The mature seeds scarified with 75 % H₂SO₄ for 60 seconds gave contamination-free cultures with optimum seed germination. Scanning Electron micrographs of mature seeds showed the rows containing trichomes running between the two poles of seeds and the sclereids between the rows of trichomes to be the potential habitats for bacteria. Light micrographs showed the thick seed coat that causes a physical dormancy. Sulphuric acid treatment was effective in degrading the trichomes completely and the seed coat partially. The highest seed germination (65.5 %) was obtained with seeds cultured/ treated with 75 % H₂SO₄ on the solidified MS medium. The basal stem of the well-grown seedlings *in vitro* gave rise to mini rhizomes. Molecular analysis showed the close genetic relatedness within and among the isolated plant populations where the seeds were collected. The *in vitro* protocol developed in this study can be used for propagation of seedlings of this vulnerable species for maintaining the biodiversity by population enhancement through restoration and introduction into new habitats.

Keywords: Biodiversity, *in vitro* seed culture, *Nymphaea nouchali*, seed dormancy, seed morphology, sulphuric acid scarification.

INTRODUCTION

The genus *Nymphaea* L., (Nymphaeaceae) commonly known as water lilies are aquatic plants distributed in tropics and temperate regions. The genus harbours around 40–50 species and they are phenotypically diverse, mostly due to varying hydrological and edaphic conditions in their surroundings (Polina & Alexy, 2007). These species can be either annuals or perennials with their rhizomes anchored in mud and grown in open water bodies. The water lilies are one of the most eye catching groups of aquatic plants with year-round flowering (Guruge *et al.*, 2016). Thus, they have attracted the attention of the botanists, horticulturists and plant enthusiasts.

Three *Nymphaea* species have been recorded in Sri Lanka viz., *N. pubescens* Willd., *N. rubra* Roxb. ex Andrews and *N. nouchali* Burm. f. (synonym *N. stellata* Willd.) (Dassanayake, 1996; Guruge *et al.*, 2016). Recent taxonomic studies have revealed the occurrence of two varieties of *Nymphaea nouchali* Burm. f. in Sri Lanka, as *N. nouchali* var. *nouchali* and *N. nouchali* var. *versicolour* (Sims) (Guruge *et al.*, 2017). *Nymphaea nouchali* var. *nouchali* (hereafter mention as *Nymphaea nouchali*) is native to southern and eastern parts of Asia (Dassanayake, 1996).

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Nymphaea nouchali has economical, pharmaceutical, social and cultural values. All parts of the plant (seeds, tender leaves, flowers, flower peduncles, and rhizomes) are rich in starch and are edible (Raja et al., 2010). Because of the medicinal properties including antibacterial, antioxidant, antimicrobial and anti-diabetic activities (Raja et al., 2010; Dash, 2013; Lim, 2014) they are widely used in Ayurvedic medicine (Jayaweera, 1982). The social importance of the plant is evident by selecting the blue-flowered variety of *N. nouchali* as the National Flower of Sri Lanka. The plant is populated in aquatic landscapes.

Nymphaea nouchali is a non-viviparous plant having a comparatively low propagation rate vegetatively via rhizomes and sexually by seeds (Yakandawala & Yakandawala, 2011). It is grown in the lowland waterbodies of wet and dry zones of Sri Lanka (Dassanayake, 1996; Guruge et al., 2016). The plant is now threatened in its natural habitats due to anthropogenic activities, drying of waterbodies and the competition posed by the viviparous *Nymphaea × erangae* for habitats, resulting in whipping off some populations completely (Yakandawala & Yakandawala, 2011; Yakandawala et al., 2017). In addition, the compatibility of *Nymphaea × erangae* for hybridization has also posed an additional threat to the genetic purity of native *N. nouchali* (Yakandawala & Yakandawala, 2011).

Evidence for natural hybridization among *Nymphaea* species has been reported elsewhere in the world, where different karyotypes ranging from $2n = 28$ to 84 has been observed, generating uncertainty in species identification (Raja et al., 2010; Nierbauer et al., 2014). This creates problems for conservation of the species. Hybridization of natives with alien species has been reported to support the evolution of invasiveness and is identified as a major threat to the extinction of native species, where the island flora is thought to be more vulnerable (Veitch & Clout, 2002; Ellstrand & Schierenbeck, 2006; Yakandawala & Yakandawala, 2011). Due to these reasons the native *N. nouchali* has been recognised under the vulnerable category in the National Red List (MOE, 2012), highlighting the importance of conserving the pure populations.

Mass propagation of *N. nouchali* through tissue culture is a promising method to overcome the limitations associated with conserving this vulnerable species. *In vitro* cultures of *Nymphaea* have been reported in different species; *Nymphaea* 'Daubeniana', a highly

viviparous species, using leaves (Jenks et al., 1990), *Nymphaea* hybrid 'James Brydon', using rhizome tips (Lakshmanan, 1994), and *N. alba*, using seeds (Sumlu et al., 2010) in different countries. Fernando et al. (2016) reported heavy contamination rates occurred in the leaf explants of *Nymphaea* at different maturity stages. Our attempts made for inducing somatic embryogenesis in the leaf explants of *N. nouchali* were not successful in demonstrating the recalcitrance of this non-viviparous species.

Among the *in vitro* culture methods, seeds have a direct application for the conservation of endemic or threatened plant species. Thus, in the present study emphasis was given to establish the contamination free culture protocol with the aim of mass propagating this vulnerable species for conservation purpose. Molecular and microscopic evidence were used for validating the results obtained in this research.

METHODOLOGY

Plant materials

Mature pods of *N. nouchali* were collected from the plant populations existing in natural habitats of marshy lands in Sri Lanka, after careful observation for the unique morphological characters in the petals, stamens, stigmatic heads, and leaf lamina of the species, as described by Yakandawala & Yakandawala (2011) and Guruge et al. (2017). The seeds obtained from the burst opened pods were soaked for three days to facilitate sedimentation of seeds by fermenting the outer pulp. After removing the debris, the seeds were isolated and washed thoroughly with soap water, followed by tap water for 30 min.

Basal culture medium

Murashige and Skoog medium (1962), supplemented with 20.0 g/L sucrose and 100.0 mg/L Myo-Inositol (w/v), was used as the basal culture medium unless otherwise mentioned. The pH was adjusted to 5.8. Solidified media with agar (6.0 g/L; w/v) were used unless otherwise mentioned. Media were sterilised by autoclaving at 121 °C, 15 Pa for 15 min (HVP 50, Hirayama, Saitama, Japan). Petri plates (90 × 10 mm) containing 25 mL of culture medium were used for inoculating the seeds. The cultures were maintained at 28 °C in light supplied with CFL (6500 K) with a photoperiod of 16/8 h.

The seeds were sterilised under the laminar flow cabinet and treated with different chemicals mentioned in the following experiments.

Effect of concentrations and duration of Clorox™

Seeds treated with 70% ethanol for 1 min were subjected to ten sterilisation protocols with five Clorox™ concentrations; 10, 30, 50, 70 or 100 % (v/v), each with two exposure times, 10- or 20-min. Experiment was repeated three times. A total of 375 seeds were used for each treatment.

Effect of concentrations and duration of H₂SO₄

After disinfecting with 70 % ethanol for 1 min and 10 % Clorox™ for 10 min, seeds were treated with five concentrations of H₂SO₄, 10, 25, 50, 75, and 100 % (v/v) for five exposure times 15, 30, 60, 90, 180 s. Untreated seeds were used as the control. Experiment was repeated three times and a total of 450 seeds were used for each treatment.

Sixty microliters of Tween were used as the surfactant in the Clorox™ solution. After treating with each disinfectant, the seeds were washed thoroughly with distilled water three times by agitating each for 2 min.

Optimisation of culture conditions for seed germination

Germination of the seeds treated with acid was further tested by culturing them onto three different media. After disinfecting the seeds with 70 % ethanol for 1 min followed by 10 % Clorox™ for 10 min as mentioned above, the seeds were treated with 25, 50 and 75 % (v/v) H₂SO₄ for 1 min. MS basal medium supplemented with 0.5 mg/L 2, 4-D and 2 mg/L BAP solidified with 6.0 g/L agar, the same medium in liquid form and the Albert's solution (2.22 g/L), were tested for seed germination. The responses were compared with the untreated seeds. The experiment was repeated three times and a total of 360 seeds were used for each treatment.

Data analysis

Factorial experiment with completely randomised design (CRD) was used. Observations were made using a stereo

microscope in weekly intervals. The data were analysed using SAS statistical package (SAS, 1999). Chi-square or maximum likelihood ANOVA was conducted using the Proc CatMod procedures of PC-SAS to analyse counted data. Treatment means were compared using SE, 95 % confidence intervals or orthogonal contrast coefficients where appropriate (Compton, 1994).

Microscopic analysis

Freshly isolated, acid treated, contaminated and germinated seeds were fixed separately in FAA (50 % ethanol + 10% formaldehyde + glacial acetic acid, 18:1:1) for 72 h and dehydrated with a graded ethanol series, 30, 50 and 70 % (v/v) for 2 h and stored in 70 % ethanol, until morphological and histological observations were made. Microscopic analysis was done for a sample of 10 seeds from each category.

Morphological observations were made using a scanning electron microscope (SEM; Carl Zeiss EVO LS 10, Germany). For the histological analysis, the samples were further dehydrated with 90, 95 and 100 % ethanol and embedded in paraffin wax. Sections (4–5 µm) were obtained using a rotary microtome (Microteck, Germany) and stained with 5 % Naphthol Blue Black (Sigma-Aldrich, Lyon, France) for 5 min at 60 °C. Slides were observed under the light microscope (Optika B-500, Ponteranica, Italy) and photographed (Optika B-500, Ponteranica, Italy).

Genetic Diversity analysis

Samples collected from three locations; Chilaw, Puttalam, and Kurunegala, were analysed using Random Amplified Polymorphic DNA (RAPD) markers to assess the genetic variation of, within, and among isolated populations of *N. nouchali*. The RAPD technique was selected to assess the three populations due to the characteristics of the test; low-cost, rapidity, simplicity, quantity of DNA required, use of universal primers that work in any genome, high potential to detect polymorphism (Goulart *et al.*, 2005), and non-availability of genetic information of the species. The standard CTAB method was used with several modifications to extract the Genomic DNA from leaves (Doyle & Doyle, 1990; Priya *et al.*, 2017). Ten primers from the series of OPK (2, 4, 5, 7, 8, 10, 11, 12, 14, 16, 18, and 20) were initially tested and five were selected (4, 8, 10, 14, 18) based on the polymorphism among the samples.

Five primers (Table 1) were used, and each primer was repeated three times. PCR Amplification was carried out in volumes of 20 μ L containing 50–100 ng of genomic DNA, 200 μ M each of dNTPs, 2.5 μ M $MgCl_2$, 1X buffer and 2.5 units of GoTaq Polymerase (Promega Technologies, USA) and 16.5 ng/ μ L of random 10-mer primer (Operon Technologies, California) with a Simpli Amp™ Thermal Cycler (Applied Biosystems, USA) with an initial denaturation step at 94 °C for 1 min, 45 cycles at 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. A final extension step at 72 °C for 5 min was also included. The RAPD products were subjected to gel electrophoresis on 1.2 % agarose gel with a ladder of 1 kb DNA (BIORIN GmbH) and photographed using gel documentation apparatus (QUANTUM CX5, GmbH). RAPD assays for each primer were repeated three times and bands were scored as presence (1) or absence (0) for each primer across all genotypes. The estimated genetic distances between populations and a dendrogram were obtained using dominant diploid data from the program POPGENE, version 1.32, according to the method as per Nei (1972, 1978) based on unweighted pair group method with arithmetic mean (UPGMA) algorithm (Yeh, 1999).

RESULTS AND DISCUSSION

Being an aquatic plant, contamination is the most critical challenge in any *in vitro* culture method. The explants collected from *N. nouchali* grown in marshy habitats naturally contain endogenous contaminants apart from heavy population of exogenous ones. Fernando *et al.* (2016) reported that the main contaminants in different leaf stages of *Nymphaea* are different; fungi in mature leaf stages and bacteria in immature leaf stages limit establishing *in vitro* cultures. Seeds of *N. nouchali* are enclosed in the pods; however, mature pods collected for culture initiation are partially dehiscent at the time of explant collection, exposing the seeds to the heavily contaminated water in the marshy lands. Furthermore, the seed extraction protocol *via* fermentation may also cause the accumulation of heavy bacterial populations on the seed coat. Therefore, establishment of contamination free seed cultures is an essential step to be optimised, aiming at mass propagation technique for the species.

Effect of sterilisation agent for initiating contamination free seed cultures

Effect of Clorox™

In the factorial experiment, an interaction effect was not observed in tested factors of Clorox™

concentration and the exposed time duration. Therefore, they were considered as major factors. Clorox™ concentration ($\chi^2=130.36$; $p < 0.0001$) and the duration ($\chi^2 = 4.7$; $p < 0.0302$) were effective in controlling the contamination (Figure 1). The lowest contamination rate (39.2 %) was observed in seeds sterilised with 100 % Clorox™. It indicated the less effectiveness of the concentrated Clorox™ in disinfection.

Tested concentrations affected significantly on the germination of both contaminated and non-contaminated seeds ($\chi^2 = 62.01$; $p < 0.0001$ and $\chi^2 = 56.05$; $p < 0.0001$, respectively). However, the seed germination was significantly lower in 100 % Clorox™ (13.9 %; $\chi^2 = 12.15$; $p < 0.0005$) compared to 50 % Clorox™. Longer duration (20 min) for Clorox™ was effective in seed sterilisation (47.7%; $\chi^2 = 4.7$; $p < 0.0302$) but negatively affected on seed germination (2.03 %; $\chi^2 = 13.02$; $p < 0.0003$), compared to the 10 min duration.

Figure 1: Effect of Clorox™ concentration on sterilization of *Nymphaea nouchali* var. *nouchali* seeds. Means with different letters are significantly different at $p < 0.05$ at 95 % confidence level; $n = 750$

Results indicated that 70 % ethanol and Clorox™ at any concentration is not sufficient for disinfection of seeds, leaving some bacteria in the hidden habitats of the seed surface. After culturing the seeds into the nutrient medium, remaining microbes were multiplied and subsequently contaminated the cultured seeds. Results further revealed that the germination frequency is higher in contaminated seeds than non-contaminated ones. The bacteria available on the surface of contaminated seeds may act on degradation of the hard seed coat to remove the physical dormancy, thus triggering their germination. The dormancy imposed by the physical barrier of the seed coat limits the gas exchange and passage of water (Penfield, 2017), limiting the germination. Thick seed coat in *N. nouchali* may contribute to the

morphophysical dormancy (Dalziel *et al.*, 2018) that can be removed by bacterial degradation under natural conditions. Seed dormancy has been reported in genus *Nymphaea* (Smits *et al.*, 1995; Dalziel *et al.*, 2018). Sumlu *et al.* (2010) reported that seed germination was observed only in the scarified seeds of *N. alba*, with hard seed coat by cutting it mechanically. In the present study a few seedlings obtained from non-contaminated seeds may be due to the removal of dormancy through the scarification occurred in the seed preparation process. Therefore, attempts were made in this study to test the hypothesis by scarifying the seeds with a strong acid.

Effect of H_2SO_4

Sulphuric acid was effective in establishing the contamination free cultures. Interaction effect was not observed among the tested factors of acid concentration and the exposed time duration to the acid. Therefore, they were considered as major factors. The H_2SO_4 concentrations tested were effective in disinfection ($\chi^2 = 365.10$; $p < 0.0001$), germination ($\chi^2 = 68.57$; $p < 0.0001$) and gave rise to the non-contaminated seedlings ($\chi^2 = 125.03$; $p < 0.0001$) (Figure 2a). Among the tested concentrations, a complete disinfection was observed in seeds treated with 50–100 % acid. The concentrations of 10 and 25 % acid, reduced the contamination rate over the control of untreated.

The greatest germination (6.3 %) was observed in the seeds treated with 75 % acid that gave 100 % disinfection. Among the seedlings derived from the seeds treated with 10 % and 25 % acid, both contaminated and non-contaminated seedlings were observed. Although 100 % H_2SO_4 was effective in complete disinfection of the seeds, none of them germinated indicating the loss of seed viability in the concentrated acid. Observations revealed that treatment with 75 % acid was effective on both purposes, i.e. complete eradication of contaminants and to remove the physical seed dormancy through scarification.

The duration that the seeds were exposed to the acid was also affected on disinfection ($\chi^2 = 148.17$; $p < 0.0001$), germination ($\chi^2 = 12.66$; $p < 0.0131$) and giving rise to the non-contaminated seedlings ($\chi^2 = 10.89$; $p < 0.0279$) (Figure 2b). The lowest contamination rate, the greatest germination rate and the greatest frequency of the non-contaminated seedlings among the germinated were observed in the seeds treated for 60 and 180 seconds. Based on this observation, acid treatment for 60 seconds duration was selected as the optimum.

Figure 2: Effect of H_2SO_4 on establishing the contamination free cultures and germination of *Nymphaea nouchali* var. *nouchali* seeds (a) acid concentration; control, without acid treatment $n = 450$; other treatment $n = 2250$; (b) duration $n = 1800$; means with different letters are significantly different at $p < 0.05$ at 95% confidence level

Sulphuric acid has been used in sterilisation protocols very rarely in other crops; 1 N acid for 1 minute in *Citrus sinensis* buds (Niedz *et al.*, 2002) and undiluted acid for 1 minute in *Phaseolus vulgaris* seeds (Malik & Saxena, 1991). However, this is the first report of using it in sterilisation of the seeds of genus *Nymphaea*.

Higher germination frequency resulted with elevated concentrations of acid revealed that the scarification is effective with high concentrations, indicating the loss of morphological or physical dormancy. Use of H_2SO_4 for scarification has also been reported in other aquatic seeds viz., *Astragalus vulnerariae*, where 40 % H_2SO_4 was used for 15 minutes (Dilaver *et al.*, 2017). Zero germination observed in the seeds treated with 100 % acid indicates the loss of viability by exposing the embryo to the concentrated acid through the cracks made by extensively degraded seed coat. The higher germination rate observed in the seeds treated with 75 % acid revealed that the scarification made at this concentration, be the optimum level for weakening the seed coat to facilitate permeability, and thereby to eliminate the physical dormancy. Weakened or cracked seed coat acts as the site for water entry as reported in

other crops viz., *Stylosanthes humilis* (Chaves *et al.*, 2017) and *Aspalathus linearis* (Kelly & Van Staden, 1985). The lower contamination rates observed in 50 % and 25 % acids revealed that the concentrations were detrimental to the bacteria though the trichomes were not degraded.

Optimisation of culture conditions for seed germination and seedling growth

The factorial experiment conducted for further testing the effect of H₂SO₄ concentrations (25, 50 and 75 %) on seed germination in different media revealed the occurrence of interaction ($\chi^2 = 608.41$; $p < 0.0001$). The greatest germination (65.5 %) was obtained by culturing the seeds treated with 75 % H₂SO₄ onto the solidified MS medium (Figure 3).

Figure 3: Effect of culture medium and sulphuric acid concentration on seed germination of *Nymphaea nouchali* var. *nouchali*. Seeds treated with different concentrations of sulphuric acid (0, 25, 50, 75 %) were cultured on to the Albert solution (A) and MS liquid (MSL) and solidified (MSS) media. Means with different letters are significantly different at $p < 0.05$ at 95 % confidence level. $n = 360$

The plants derived from the acid treated seeds showed a normal growth containing a well-grown shoot and the roots (Figure 4a, c) indicating that acid scarification had no adverse effect. The basal stem of the well-grown seedlings gave rise to mini rhizomes. Bacteria was the contaminant in all cultures. Milky jelly-appearance of the contamination suggested the presence of bacterial contamination (Figure 4b).

The results revealed that the physical state of the culture medium and the medium composition has a significant effect on seed germination. Although *N. nouchali* is an aquatic plant, solidified medium favoured

for seed germination. Reason could be the proper gas exchange in the scarified seeds caused for better germination. The composition of the medium, i.e. MS basal medium supplemented with 0.5 mg/L 2, 4-D and 2.0 mg/L BAP, was effective in giving rise to well-grown complete seedlings, containing both shoots and roots with a higher germination rate (≈ 66 %). Seed is a highly available explant material in *N. nouchali* where each pod contains thousands of seeds. Therefore, mass scale plant production is possible through the protocol developed in the study. *In vitro*-derived contamination free seedlings can be further used for producing clones through micropropagation. The mini rhizomes formed in the well-grown seedlings would also serve as an additional source of explant for *in vitro* propagation of this species.

Microscopic analysis

The scanning electron micrographs (SEM) clearly showed the trichomes and sclereids available on the seed coat in fresh seeds. Rows of long trichomes parallel to the long axis of the seed (Figure 4d), and the sclereids arranged in regular transverse rows (Figure 4e) in between the rows are the characteristic features of the *N. nauchali* seeds. This observation is comparable with some species of Mexican *Nymphaea* including *N. ampla*, *N. elegans* and *N. gracilis*, etc. (Bonilla-Barbosa *et al.* 2000), as it provides information on the diversity of the genus *Nymphaea*. The seed coat morphology also demonstrated the reason for the occurrence of heavy contamination in the cultured seeds *in vitro*. The trichomes and the grooved sclereids facilitate safe harbouring of bacteria during sterilisation process causing heavy bacterial contamination after culture initiation. Concentrated H₂SO₄ and 75 % acid degraded those trichomes completely (Figure 4f and 4g) and caused total eradication of microbes from the cultures as shown in Figure 2a, thus eliminating the occurrence of contamination. The 50–10 % acid was not strong enough to degrade the trichomes. Although 50 % acid did not degrade the trichomes, it may be adequately strong to kill the microbes completely, whereas the other two concentrations (10 % and 25 %) were not as effective as such. *In vitro* seed propagation was reported only in some *Nymphaea* species; *N. alba* (Sumlu *et al.*, 2010; Latowski *et al.*, 2014) and *N. lotus* var. *thermalis* (Blidar *et al.*, 2019), which have no trichomes on the seed coats.

Evidence also provided through light micrographs to demonstrate the effect of acid treatment in seed culture. The thick seed coat in fresh seeds (Figure 4h) may cause physical seed dormancy. In the seeds treated

with concentrated acid, the cracks were observed in the seed coat (Figure 4i and 4j). It may allow moving the strong acid (100 %) into the embryo, leading to loss of seed viability, where none of the seeds were germinated as mentioned above. In the seeds treated with 75 % acid, cracks were not visible, thus maintained the seed viability. Degradation of the seed coat occurred to different degrees, with 75–50 % acid concentration causing germination of seeds by removing physical dormancy. In the untreated seeds, heavy bacterial action degraded the seed coat (Figure 4k) to the same degree of different acid treatments, allowing seed germination.

Figure 4: Morphological and microscopic aspects of seed culture of *Nymphaea nouchali* var. *nouchali*. (a-c) Morphological aspect seed germination: (a) non-contaminated (bar = 1 cm) and (b) contaminated seeds (bar = 1 cm); (c) healthy seedlings derived from acid treated seeds (bar = 1.4 mm). (d-g) Scanning electron micrographs; (d) a fresh seed (bar = 100 µm); (e) close view of the seed coat. Note the trichomes (Tr) arranged in lines and sclereids (s) in between those lines (bar = 20 µm). (f) A seed treated with 100 % acid. Note the cracked seed coat (bar = 100 µm) and (g) close view of the seed coat after treating with 100 % acid. Note the complete degradation of the trichomes. (h-k) Histological aspect the testa (T) of the seeds: (h) a fresh seed (bar = 200 µm); (i) a seed treated with 100 % (bar = 200 µm); (j) seed treated with 75 % acid (bar = 200 µm); (k) a contaminated seed (bar = 200 µm).

Genetic diversity of isolated plant populations

Although the study showed that seed culturing is a promising technique for *in vitro* propagation of *N. nouchali*, unawareness of the level of genetic purity of the seedling populations is a barrier for implementing the technique for conservation of true-to-type species. RAPD technique has been used for similar genetic studies for

several species such as *Ensete ventricosum* (Birmeta *et al.*, 2004), *Cedrus atlantica* (Renau-Morata *et al.*, 2005; Mendonça *et al.*, 2014) and *Hevea brasiliensis* (Nakkanong *et al.*, 2008; Liyanage *et al.*, 2014).

A total of 56 bands were generated with an average frequency of 11.2 bands per primer (Figure 5a). The genetic distance matrix obtained for individuals of three populations showed that the genetic distance among nine genotypes collected from three locations ranged from low to moderate values of 0.0364 to 0.5596 with an average of 0.3404 (Table 2).

This indicates a closer genetic relatedness between the populations. As estimated from the distant matrix, the lowest within-population genetic variation with an average of 0.26 was found in the Chilaw population compared to the 0.30 and 0.31 of the Kurunegala and Puttalam populations, respectively. UPGMA clustering grouped the nine genotypes into two major clusters (A and B; Figure 5b). However, the results indicated high genetic relatedness within the plant populations.

Figure 5: Assessment of genetic diversity in isolated *Nymphaea nouchali* var. *nouchali* plant populations using randomly amplified polymorphic DNA (RAPD). (a) RAPD profile generated for primer OPK 04. Lane 1 - 1kb ladder; Lane 2, 3, 4 - genotypes from Chilaw; Lane 5, 6, 7 - Genotypes from Kurunegala; lane 8, 9, 10 - Genotypes from Puttalam. (b) UPGMA dendrogram showing the relationship among nine genotypes. Note the two clusters generated A and B

Table 1. Banding pattern details of the 10-mer random primers

Primer	Sequence	Total number of bands observed	Number of polymorphic bands	Polymorphism %
OPK 04	5- CCGCCCAAAC-3	10	6	60.00
OPK 08	5- GAACACTGGG-3	9	4	44.44
OPK 10	5- GTGCAACGTG-3	14	9	64.29
OPK 14	5- CCCGCTACAC-3	11	4	36.36
OPK 18	5- CCTAGTCGAG-3	12	7	58.33

Table 2. Genetic similarity/distances among the nine genotypes of *Nymphaea nouchali*

Genotype	1	2	3	4	5	6	7	8	9
Chilaw-01 (1)	***	0.96	0.86	0.80	0.70	0.66	0.75	0.63	0.73
Chilaw-02 (2)	0.04	***	0.86	0.80	0.66	0.66	0.71	0.59	0.73
Chilaw-03 (3)	0.15	0.15	***	0.84	0.70	0.70	0.71	0.59	0.70
Kurunegala-01 (4)	0.22	0.22	0.18	***	0.86	0.71	0.73	0.57	0.64
Kurunegala-02 (5)	0.36	0.41	0.36	0.15	***	0.71	0.73	0.64	0.68
Kurunegala-03 (6)	0.41	0.41	0.36	0.34	0.34	***	0.59	0.61	0.61
Puttalam-01 (7)	0.29	0.34	0.34	0.31	0.31	0.53	***	0.73	0.88
Puttalam-02 (8)	0.47	0.53	0.53	0.56	0.44	0.50	0.31	***	0.79
Puttalam-03 (9)	0.31	0.31	0.36	0.44	0.39	0.50	0.13	0.24	***

Nei's genetic identity (above diagonal) and genetic distance (below diagonal) are shown

The study revealed that a stable level of genetic uniformity existed within and among the three *N. nouchali* populations, suggesting the non-occurrence of gene contamination in these locations by undesirable alien alleles introduced by outcrossing. The close genetic relationship of these populations also suggests that the heterogeneity expected of the *in vitro* seedlings from these populations to be low, hence showing the possibility of using the technique for mass propagation from seeds collected from the isolated habitats.

CONCLUSIONS

Seed morphology is identified as the cause for heavy culture contamination and dormancy of the seeds. The higher germination rate observed in the seeds treated with 75 % acid for 60 seconds revealed that the treatment is optimal for elimination of the microbes by degrading the trichomes. Furthermore, it was effective in eliminating the physical dormancy by partially degrading the seed coat. The protocol developed in this study can be used for mass propagation of *N. nouchali*. As future prospectus, the explants collected from the *in vitro* seedlings can be used for developing clonal propagation techniques

through somatic embryogenesis or micropropagation of the species, which lacks any specialised organs for vegetative reproduction in mass scale. The output of the study would help in habitat restoration and establishing new populations enabling this vulnerable species to conserve for future generations. Further, the mass propagation of seed-derived plants could also be used to supply the demand for ornamental plant industry. This is the first report of using H₂SO₄ as sterilisation agent of the genus *Nymphaea* that enabled successful propagation through seeds *in vitro*.

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Conflicts of Interest

The authors declare no conflict of interest.

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