International Journal of Environment and Climate Change

Volume 13, Issue 10, Page 1909-1915, 2023; Article no.IJECC.105376
ISSN: 2581-8627
(Past name: British Journal of Environment & Climate Change, Past ISSN: 2231–4784)

Methodology of Micropropagation of Elite Genotype in Lotus (*Nelumbo nucifera*) Genotype Lakshmi

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJECC/2023/v13i102848

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/105376

Original Research Article

Received: 23/06/2023
Accepted: 29/08/2023
Published: 01/09/2023

ABSTRACT

The lotus (*Nelumbo nucifera* Gaertn.) is an aquatic plant grown extensively throughout Asia, mostly for commercial and ornamental purposes. It is mostly propagated via rhizomes. A potent and reproducible plant regeneration system for lotus has been developed in this work employing rhizomes, shoot tips from the mother plant, and mature and immature embryos collected from eighteen and twenty-six-day-old aseptically fertilized seed. Shoot induction, number of shoots, and length of roots were induced on Murashige and Skoog's (MS) basal medium supplemented with various concentrations of 6-Benzylaminopurine (BAP) mgL^{-1} (0.5, 1, 2, 3, 4). The response of the explant varied with different BAP concentrations. The most favorable outcomes were achieved using MS medium treated with 0.5 mgL^{-1} 6-Benzylaminopurine (T_2).

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Keywords: 6-benzylaminopurine (BAP); embryo; rhizomes; shoot tips; plant regeneration; Nelumbo nucifera.

1. INTRODUCTION

Lotus (*Nelumbo nucifera* Gaertn., Nelumbonaceae) was one of the first plants discovered in the globe and one of the few plants that survived the ice age. According to historical records, lotus cultivation originated in Asia, was brought to northern Australia, Japan, and Europe, and was developed as an industrial crop in China and grown across Asia for its high decorative, culinary, and therapeutic value [1]. Taxonomically, lotus belongs to the genus of *Nelumbo* Adans., which is the only existing genus of the Nelumbonaceae family. The genus consists of just two species. The Asian lotus (*N. nucifera* Gaertn.), which is found in Asia and Oceania; the American lotus (*Nelumbo lutea* Wild.), which is mostly indigenous to North America, is another [2,3]. The lotus (*Nelumbo nucifera*) is also known as the Sacred lotus, Indian lotus, East Indian lotus, Oriental Lotus, Lily of the Nile, Bean of India, and Sacred Water Lily. It represents purity, beauty, majesty, grace, fertility, wealth, richness, knowledge, and serenity because of its significance in the religions of Buddhism and Hinduism [2].

China is the world's leading producer and consumer of lotus. The area under cultivation of rhizome lotus in China has reached 0.2 million/ ha, and can harvest 3 million tons of fresh rhizome per year, and that the area of seed lotus is 20,000 ha with an annual harvest of 15,000 tons of dry seeds [4]. All the plant parts of *N. nucifera* with high economic importance, both as a decorative bloom and as a source of herbal medicine, are consumed as food or used for medicinal purposes, including the rhizome, nodes, roots, seed, young shoot, leaf, stalk, petal, stamen, and pericarp [5,6,3]. In general, lotus is commonly propagated through the rhizome and seed, but the low efficiency of propagation is not enough to meet the needs of production. On an average 35000-40000 rhizomes and10-12 kg seeds are required to produce seedlings sufficient for one hectare of land [7].

The normal propagation rate is quite modest and largely depends on the quality of the water environment and the weather conditions where it is cultivated. However the problem is continuous vegetative propagation from the rhizomes or shoots can lead to cultivar degeneration. In some cultivar types, seed is not or fully developed. Because, dense of petals is not allowing plants to seed set.

Therefore, it is particularly important to design an efficient method to improve the efficiency of propagation in lotus. Micro propagation through tissue culture is an appealing solution to these problems and would also support future molecular studies of lotus and transgenic breeding. Plant *in vitro* culturing is the extensively used aseptic regeneration of cells, tissues, organs, or complete plants in a controlled laboratory environment for plant propagation, virus eradication, transgenic plant breeding, and the preservation of uncommon plant genotypes or cells. The basal medium of a plant tissue culture system provides all of the nutrients, energy, and water required for explant development, while the incubation systems provide optimum light and temperature conditions [8]. On that the aim of this investigation is to standardize the explants and medium for shoot induction.

2. MATERIALS AND METHOD

2.1 Plant Material

Lotus elite rhizomes genotype “Lakshmi” was planted in the research field at the Department of Floriculture and Landscape Architecture, Tamil Nadu Agricultural University, Coimbatore, India in Factorial Completely Randomized Design with three replications during the month of February 2023. In the Department of Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, the tissue culture work was carried out *viz*., rhizome, shoot tip, immature and mature embryos were employed as explants in the current study.

2.1.1 Surface Sterilization of rhizome and shoot tip

Explants of lotus rhizome and shoot tip were washed with normal water until the dirt was removed, then immersed in 500 mg carbendazim to avoid fungal infection. The rhizome and shoot tip were removed after 10–15 minutes of soaking in carbendazim and rinsed with 2-3 drops of Tween 20. The rhizome surface was sterilized for 3 minutes and 30 seconds with 0.1% $HgCl_2$ and 70% ethanol.

2.1.2 Surface Sterilization of embryos

After 18 and 28 days of flower growth, respectively, immature and mature embryos were collected and cleaned with sterile water. The rhizome bud and young embryos have primarily been used as explants in the tissue culture of lotus [9-12].

By utilizing a sterile knife Extraneous portions were removed, and embryos were separated from the cotyledons. After removing the seed coats, the cotyledons were properly washed three times under running tap water, then soaked in tween 20 (2-3 drops) for 2 minutes and rinsed three times with distilled water. Surface-sterilized seeds were immersed in 75% ethyl alcohol for 30 seconds, 0.1% (w/v) NaOCl for 5 minutes under sterile conditions, and thoroughly washed three times with sterile distilled water.

Classic Murashige and Skoog [13] approaches were used to prepare the basal MS medium, which was supplemented with BAP (0.5, 1, 2, 3, and 4 mgL^{-1}). All of the media were adjusted to pH 5.8 using 1 M NaOH and 1 M HCl before autoclaving at 121°C for 20 minutes. All cultures were incubated at 25 ± 2°C for 16 hours with a slight intensity of 40 µmol m^{-2} s^{-1} given by fluorescent lamps.

List 1. Details of the experiments

Experiment 1	Number of explants	4
	Number of replications	3
Experiment 2	Number of treatments	6
	Number of replications	3

3. RESULTS AND DISCUSSION

3.1 Statistical Analysis

All the experiments were repeated three times, and there were 30 explants for each treatment. Where necessary, Factorial completely randomized design (FCRD) was calculated by using AGRES software.

3.1.1 The effect of BAP on the number of days taken for shoot proliferation from the rhizome, shoot tip, mature embryo, and immature embryo

Aside from different sterilization and germination stages, the same experimental approach was applied for *Nelumbo nucifera* elite genotype Lakshmi's rhizome, shoot tip, mature embryo and immature embryo. MS media augmented with BAP (2 mgL^{-1}) and NAA (0.5 mgL^{-1}) which proved to be the most appropriate for shoot induction and proliferation in *Sinningia sp.* [14]. The development of shoots began from four days to one week after inoculation in MS medium supplemented with BAP mgL^{-1} (0.5, 1, 2, 3, 4), as shown in Table 1.

The mean minimum days taken for shoot emergence were observed in the explant of an immature embryo (E_4) (4.78) and the minimum mean value was in MS media supplemented with BAP 0.5 mgL^{-1} (4.17) (T_2). The mean maximum number of days taken for shoot emergence was observed in the explant of the shoot tip (E_2) (7.55) and the maximum mean value was in MS media without BAP (T_1) (7.67). Shoot tip explants cultured on a MS medium supplemented with 3 mgL^{-1} of BAP showed the greatest response to shoot growth in *Petunia* sp. [15]. Among the interactions, the immature embryo (E_4) and BAP 0.5 mgL^{-1} (T_2) showed the minimum number of days (3.33) taken for shoot emergence, whereas T_1E_2, T_5E_1 and T_6E_2 recorded the maximum number of days (8.6). The maximum shoot initiation in the minimum number of days in a MS media treated with BAP 4 mgL^{-1} and NAA 0.2 mgl^{-1} was found by Baijanti et al. [16].

3.1.2 The effect of BAP on the number of shoots regenerated from the rhizome, shoot tip, mature embryo, and immature embryo

In accordance with Table 2, the highest mean value number of shoots was achieved in mature embryos (E_3) (5.72) and BAP 0.5 mgL^{-1} (T_2) (5.16), followed by immature embryos (E4) (3.61) and shoot tips (E_2) (3.50), and with BAP 1 mgL^{-1} (T_3) and control (T_1) with a value of 4.75. The lowest mean value number of shoots was achieved in the rhizome (E_1) (3.16), and with BAP 2 mgL^{-1} (T_4) and BAP 3 mgL^{-1} (T_5), both with a value of 3.08. According to [17] in *Gladiolus cv.* 'American Beauty' rapid multiplication of shoots *in vitro* has been achieved through BAP alone. Among the explants and treatments, the lowest and highest number of shoot proliferations was observed in the interactions of E_1T_4 (1.67) and E_3T_2 (7.33). Lotus shoot formation decreased when BAP concentration increased. A study shown that BAP concentration is inversely related to the number of shoot forms. These results are in agreement with the findings of [14] who observed that increasing the concentration of BAP resulted in reduction in number of shoots per explant. In *Narcissus* sp. [18] reported that BAP 4mgL^{-1}

gave more of shoots per cultures as compared to the other treatments.

3.1.3 The effect of BAP on the length of micro shoots (cm) on the rhizome, shoot tip, mature embryo, and immature embryo

In accordance with Table 3, the longest mean value of length of the shoot was reported in immature embryo E_4 (8.09 cm) and BAP 0.5 mgL^{-1} (T2) (7.12 cm), while the lowest mean value of length of shoot was recorded in shoot tip E_2 (4.18) and BAP 3 mgL^{-1} (T_5) (5.38 cm), followed by T_6 (BAP 4 mgL-1) (5.49). Thus, an immature embryo (E_4) (8.09 cm) had the longest mean value of shoots from this explants, followed by a mature embryo (E_3) (8.08 cm). Results of the interaction from explants and treatments were E_3T_2 (9.77 cm) and E_1T_5 (3.10 cm) had the greatest and the lowest shoot interaction durations, respectively.

According to Table 3, comparing all the explants with treatments, the highest length was observed in mature and immature embryos. Results pertaining to the micro shoots length indicated that lower levels of BAP produce more shoot length as compared to higher levels. A similar observation was recorded by [19] in *Gerbera* sp. and [20] in tuberose plants.

3.1.4 The effect of BAP on the shoot's formation percentage from the rhizome, shoot tip, mature embryo, and immature embryo

The proportion of shoot development varied depending on the explants and treatments used.

The highest mean value of shoot percentage was obtained in immature embryo (E_4) (6.11%) and mature embryo (E_3) (6.00%), and BAP 0.5 mgL^{-1} and 1 mgL^{-1} (T_2, T_3) (6.17%), followed by BAP 3 mgL^{-1} and control (T_4, T_1) (5.08%) (Table 4). The lowest mean value of shooting percentages were found in rhizome (E_1) (3.61%) and BAP 3mgL^{-1} (T_5) (4.33%), followed by BAP 4mgL^{-1} (T_6) (4.42%). The maximum interaction percentage of shoot formation showed up from explants and treatments in E_4T_3 (8.33%), while the lowest interaction percentage was shown in E_1T_5 (1.66%), followed by E_1T_6 (2.67%) and E_1T_3 and E_1T_4 (3.67%).

Table 1. Effect of BAP on the number of days taken for shoot proliferation from the rhizome, shoot tip, mature embryo, and immature embryo of *Nelumbo nucifera* genotype Lakshmi

S. No	Factors	Number of days taken for shoot proliferation				
	Explant /Treatment	Rhizome (E_1)	Shoot tip (E_2)	Mature Embryo(E_3)	Immature embryo(E_4)	Mean
1	**T_1(Control)**	7.33	8.66	8.33	6.33	**7.66**
2	**T_2(BAP 0.5mgL^{-1})**	3.66	5.66	4.00	3.33	**4.16**
3	**T_3(BAP 1 mgL^{-1})**	5.00	7.00	7.33	4.66	**6.00**
4	**T_4(BAP 2 mgL^{-1})**	7.33	7.66	6.33	4.00	**6.33**
5	**T_5(BAP 3 mgL^{-1})**	8.66	7.66	6.43	5.66	**7.00**
6	**T_6(BAP 4 mgL^{-1})**	8.00	8.66	6.66	4.66	**7.10**
	Mean	**6.66**	**7.55**	**6.44**	**4.77**	
	Treatment	**E**		**T**		**E x T**
	SEd	0.366		0.448		0.897
	CD (0.05)	0.736		0.902		1.804

Table 2. Effect of BAP on the number of shoots regenerated from the rhizome, shoot tip, mature embryo, and immature embryo of *Nelumbo nucifera* genotype Lakshmi

S. No	Factors	Number shoots regenerated from per explant				
	Explant /Treatment	Rhizome (E_1)	Shoot tip (E_2)	Mature Embryo (E_3)	Immature embryo (E_4)	Mean
1	**T_1(Control)**	4.66	4.33	6.33	3.66	**4.75**
2	**T_2(BAP 0.5mgL^{-1})**	5.00	4.33	7.33	4.00	**5.16**
3	**T_3(BAP 1 mgL^{-1})**	3.33	4.66	7.00	4.10	**4.75**
4	**T_4(BAP 2 mgL^{-1})**	1.66	4.76	5.33	3.00	**3.08**
5	**T_5(BAP 3 mgL^{-1})**	2.00	3.00	4.00	3.33	**3.08**
6	**T_6(BAP 4 mgL^{-1})**	2.33	2.33	4.33	3.66	**3.16**

S. No	Factors	Number shoots regenerated from per explant				
	Explant /Treatment	Rhizome (E_1)	Shoot tip (E_2)	Mature Embryo (E_3)	Immature embryo (E_4)	Mean
	Mean	**3.16**	**3.50**	**5.72**	**3.61**	
	Treatment	**E**		**T**		**E x T**
	SEd	0.386		0.473		0.947
	CD (0.05)	0.777		0.952		1.905

Table 3. Effect of BAP on the length of micro shoots on the rhizome, shoot tip, mature embryo, and immature embryo of *Nelumbo nucifera* genotype Lakshmi

S. No	Factors	Length of micro shoots (cm)				
	Explant /Treatment	Rhizome (E_1)	Shoot tip(E_2)	Mature Embryo(E_3)	Immature embryo(E_4)	Mean
1	**T_1(Control)**	4.83	4.23	9.50	8.40	**6.79**
2	**T_2(BAP 0.5mgL^{-1})**	5.26	5.00	9.76	8.63	**7.11**
3	**T_3(BAP 1 mgL^{-1})**	4.96	4.00	8.06	7.86	**6.22**
4	**T_4(BAP 2 mgL^{-1})**	3.53	4.60	7.53	8.20	**5.96**
5	**T_5(BAP 3 mgL^{-1})**	3.10	3.73	7.00	7.70	**5.38**
6	**T_6(BAP 4 mgL^{-1})**	4.00	3.53	6.66	7.76	**5.49**
	Mean	**4.28**	**4.18**	**8.08**	**8.09**	
	Treatment	**E**		**T**		**E x T**
	SEd	0.325		0.399		0.798
	CD (0.05)	0.655		0.802		1.605

Table 4. Effect of BAP on the shoot's formation percentage from the rhizome, shoot tip, mature embryo, and immature embryo of *Nelumbo nucifera* genotype Lakshmi

S. No	Factors	Percentage response to shooting (%)				
	Explant /Treatment	Rhizome (E_1)	Shoot tip(E_2)	Mature Embryo(E_3)	Immature embryo(E_4)	Mean
1	**T_1(Control)**	6.00	4.33	4.00	6.00	**5.08**
2	**T_2(BAP 0.5mgL^{-1})**	4.00	6.33	7.00	7.33	**6.16**
3	**T_3(BAP 1 mgL^{-1})**	3.66	7.00	7.33	8.33	**6.16**
4	**T_4(BAP 2 mgL^{-1})**	3.66	5.66	6.66	4.33	**5.08**
5	**T_5(BAP 3 mgL^{-1})**	1.66	4.33	5.66	5.66	**4.33**
6	**T_6(BAP 4 mgL^{-1})**	2.66	4.66	5.33	5.00	**4.41**
	Mean	**3.61**	**5.38**	**6.00**	**6.11**	
	Treatment	**E**		**T**		**E x T**
	SEd	0.406		0.497		0.995
	CD (0.05)	0.817		1.000		2.001

Fig. 1. Shoot proliferation from rhizome of *N. nucifera* Lakshmi in MS media containing 0.5 mgl^{-1} BAP (E_1T_2)

Fig. 2. Shoot proliferation from shoot tip of *N. nucifera* Lakshmi in MS media containing 2 mgL^{-1} BAP (E_2T_4)

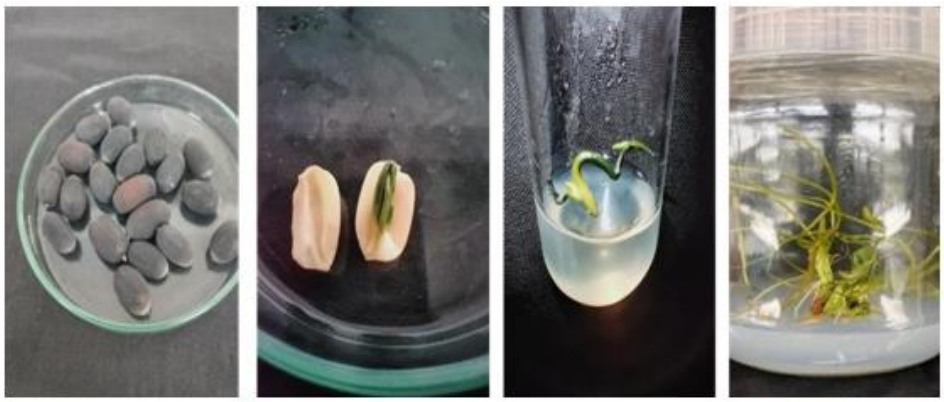

Fig. 3. Shoot proliferation from mature embryo of *N. nucifera* Lakshmi in MS media containing 0.5 mgL^{-1} BAP (E_3T_2)

Fig. 4. Shoot proliferation from immature embryo of *N. nucifera* Lakshmi in MS media containing 1 mgL^{-1} BAP (E_4T_3)

4. CONCLUSION

In this work on propagation of *N. nucifera* genotype Lakshmi the rhizome, shoot tips, and embryos were used to demonstrate a higly effective effective technique of shoot induction. Tissue culture is an efficient approach for producing a large number of plantlets with a high multiplication rate in a short period of time, in this case also. Among these BAP concentrations, MS media supplemented with 0.5 mgL^{-1} BAP showed greatest number of shoots obtained in mature

embryos (E_3T_2), and the greatest microshoots length was obtained in mature embryos (E_3T_2).

5. FUTURE SCOPE

Tissue culture is the ideal approach for promoting ornamental horticulture because it produces a large number of disease-free plantlets in a short period of time, potentially saving the genetic resource from plant deterioration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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