Original Research Article

Methodology of Micropropagation of Elite Genotype in Lotus *(Nelumbonucifera)* - Lakshmi

Abstract

The lotus (*Nelumbonucifera*) 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 (*Nelumbonucifera* Lakshmi) 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, andlength of roots were induced on Murashige and Skoog's (MS) basal medium supplemented with various concentrations of BAP mgl^{-1} (0.5, 1, 2, 3, 4). The response of the explant varied with different BAP concentrations. Media containing MS media with 0.5 mgl^{-1} BAP (E_4T_2) achieved the minimum number of days taken for shoot induction in an immature embryo. And the maximum number of shoots was obtained (E_3T_2) in mature embryos supplemented with 0.5 mgl^{-1} BAP, whereas the maximum root length was obtained in (E_3T_2)mature embryos supplemented with 0.5 mgl^{-1} BAP. The best results were obtained in MS medium treated with (T_2) 0.5 mgl-1 BAP.

Keywords: BAP, Embryo, Rhizomes, Shoot tips**,** Plant regeneration, N. *nucifera*

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Introduction

Lotus 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 (Guo, 2009). Taxonomically, lotus belongs to the

genus of *Nelumbo,* which is the only existing genus of the Nelumbonaceae family. The genus consists of just two species. The Asian lotus (*N. nuciferaGaertn.*), which is found in Asia and Oceania; the American lotus (*Nelumbolutea Wild.*), which is mostly indigenous to North America, is another (Shen-Miller*, 2002); (Wang and Zhang, 2004).* The lotus (*Nelumbonucifera*) is also known as 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 (Shen-Miller, 2002).

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 annual harvest 15,000 tons of dry seeds (Zhao, 1999).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. (Ni *et al.,* 1987; Nguyen and Hicks, 2001; Wang and Zhang, 2004).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(De *et al.,2020*). 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. But 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 very 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

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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 (Phillips and Garda, 2019).

Materials and Method

Plant Material

Lotus elite rhizomes genotype "Lakshmi" were planted in the research field at the Department of Floriculture and Landscape Architecture, Tamil Nadu Agricultural University, Coimbatore in FCRD 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, unmature and mature embryos were employed as explants in the current study.

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 500mg 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 was surface sterilized for 3 minutes and 30 seconds with 0.1% $Hgcl_2$ and 70% ethanol. Sodium hypochlorite was the most efficient surface sterilization treatment in *Prunuspersica L.* Batsch, resulting in continuing shoot development and multiplication, with a 50% survival rate at 15% for 5 minutes and a 60% survival rate at 10% for 10 minutes (Al Ghasheem *et al.*, 2018).

Surface Sterilization of embryos

After 18 and 28 days of flower growth, respectively, unmature and mature embryos were retrieved and cleaned with sterile water. The rhizome bud and young embryos have primarily been used as explants in the tissue culture of lotus (Arunyanart and Chaitrayagun, 2005); (Shou *et al.*, 2008; Mahmad *et al.*, 2014; Zhao*et al.*2016).

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By utilizing a sterile knife Extraneous portions were removed, and embryos were separated from the cotyledons. After removing the seed coat, the seeds 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, 1962 approaches were used to prepare the basal MS medium, which was supplemented with BAP (0.5, 1, 2, 3, and 4 mg/l). 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 μ mol 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

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Result and Discussion

Statistical analysis

All the experiments were repeated three times, and there were 30 explants for each treatment. Where necessary, Factorial Complete Randomized Designs (FCRD) were calculated by using AGRES software.

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 *Nelumbonucifera* elite genotype Lakshmi's rhizome, shoot tip, mature embryo and immature embryo.MS media augmented with BAP (2 mg/l) and NAA(0.5 mg/l) which proved most appropriate for shoot induction and proliferation in *Sinningia sp.*(Sharma and Sharma, 2013). The development of shoots began 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 minimum days taken for shoot emergence were observed in the explant of an immature embryo (E_4) (4.77) and the MS media supplemented with BAP 0.5 mgl^{-1} (4.166) (T_2) .And the maximum number of days taken for shoot emergence was observed in the explant of the shoot tip (E_2) (7.55) and MS media without BAP (T_1)(7.66) .Shoot tip explants cultured on MS medium supplemented with 3 mgl^{-1} of BAP showed the greatest response to shoot growth in Petunia (Sabitha *et al.*, 2009). 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_1, T_5E_1 and T_6E_2 recorded the maximum number of days (8.66).Maximum shoot initiation in the minimum number of days in MS media treated with BAP 4 mgl^{-1} and NAA 0.2 mgl^{-1} (Baijanti *et al.*, 2015)

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 number of shoots were achieved in mature embryos (E_3) (5.72) and BAP 0.5 mg^{l-1} (T_2) (5.16), followed by immature embryos (E4) (3.61), shoot tips (E_2) (3.50), and BAP 1 mgl^{-1} (T_3) (4.75) and control

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(T_1). And the lowest number of shoots was achieved in the rhizome (E_1) (3.16), BAP 2 mgl^{-1} (T_4) (3.88), and BAP 3 mgl^{-1} (T_5) (3.88). According to (Misra and Singh, 1999) in *Gladiolus cv.* American beautyrapid multiplication of shoots *in vitro* has achieved through BAP alone. Among the explants and treatments, the lowest and highest number of shoot proliferations were observed in the interactions of E_1T_4 (1.66) 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 (Sharma and Sharma, 2013)who observed that the increasing the concentration of BAP resulted in reduction in number of shoots per explant. In *Narcissus*,(Santos *et al.*, 1998) reported that BAP 4mgl^{-1} gave more of shoots per cultures as compared to the other treatments.

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

In accordance with Table 3, the longest length of shoot was reported in immature embryo E_4 (8.09) and BAP 0.5 mgl^{-1} (T2), while the lowest length of shoot was recorded in shoot tip E_2 (4.18) and BAP 3 mgl^{-1} (T_5) (5.38), followed by T6 (5.49). An immature embryo (E_4) (8.09) had the longest shoot from this explant, followed by a mature embryo (E_3) (8.08). E_3T_2 (9.70) and E_1T_5 (3.10) had the greatest and 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 (Son *et al.*, 2011) in gerbera and (Krishnamurthy, 2000) in tuberose.

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 shoot percentage was obtained in immature embryo (E_4) (6.11), mature embryo (E_3) (6.00), and BAP 0.5 mgl^{-1}, 1 mgl^{-1} (T_2, T_3) (6.16), followed by

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BAP 3 mgl^{-1} and control (T_4, T_1) (5.08) (Table 4), and the lowest shooting percentages were found in rhizome (E_1) (3.61) and BAP 3mg^{l-1} (T_5) (4.33), followed by BAP 4mgl^{-1} (T_6) (4.41). 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 (2.66) and E_1T_3 and E_1T_4 (3.66).

Conclusion

In this work, *N. nucifera.*Lakshmi rhizome, shoot tips, and embryos were used to demonstrate a very effective technique of shoot induction. Tissue culture was an efficient approach for producing a large number of plantlets with a high multiplication rate in a short period of time. Among these BAP concentrations, MS media supplemented with 0.5 mgl^{-1} BAP showed the shortest number of days required for shoot induction (E_4T_2) in an immature embryo and the greatest number of shoots obtained (E_3T_2) in mature embryos, with the greatest root length obtained in (E_3T_2) mature embryos.

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.

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

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	T1(Control)	7.3333	8.6667	8.3333	6.3333	7.6667
2	T2(BAP 0.5mgl^{-1})	3.6667	5.6667	4.0000	3.3333	4.1667
3	T3(BAP 1 mgl^{-1})	5.0000	7.0000	7.3333	4.6667	6.0000
4	T4(BAP 2 mgl^{-1})	7.3333	7.6667	6.3333	4.0000	6.3333
5	T5(BAP 3 mgl^{-1})	8.6667	7.6667	6.4333	5.6667	7.0000
6	T6(BAP 4 mgl^{-1})	8.0000	8.6667	6.6667	4.6667	7.1000
	Mean	6.6667	7.5556	6.4444	4.7778	
	Treatment	E		T		E x T
	SEd	0.36641		0.44876		0.89753
	CD (0.05)	0.73676		0.90235		1.80470

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

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	T1(Control)	4.6667	4.3333	6.3333	3.6667	4.7500
2	T2(BAP 0.5mgl^{-1})	5.0000	4.3333	7.3333	4.0000	5.1667
3	T3(BAP 1 mgl^{-1})	3.3333	4.6667	7.0000	4.1000	4.7500
4	T4(BAP 2 mgl^{-1})	1.6667	4.7667	5.3333	3.0000	3.0833
5	T5(BAP 3 mgl^{-1})	2.0000	3.0000	4.0000	3.3333	3.0833
6	T6(BAP 4 mgl^{-1})	2.3333	2.3333	4.3333	3.6667	3.1667
	Mean	3.1667	3.5000	5.7222	3.6111	
	Treatment	E		T		E x T
	SEd	0.38690		0.47385		0.94771
	CD (0.05)	0.77796		0.95280		1.90559

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Table 3.Effect of BAP on the length of micro shoots on the rhizome, shoot tip, mature embryo, and immature embryo

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	T1(Control)	4.8333	4.2333	9.5000	8.4000	6.7917
2	T2(BAP 0.5mgl^{-1})	5.2667	5.0000	9.7667	8.6333	7.1167
3	T3(BAP 1 mgl^{-1})	4.9667	4.0000	8.0667	7.8667	6.2250
4	T4(BAP 2 mgl^{-1})	3.5333	4.6000	7.5333	8.2000	5.9667
5	T5(BAP 3 mgl^{-1})	3.1000	3.7333	7.0000	7.7000	5.3833
6	T6(BAP 4 mgl^{-1})	4.0000	3.5333	6.6667	7.7667	5.4917
	Mean	4.2833	4.1833	8.0889	8.0944	
	Treatment	E		T		E x T
	SEd	0.32594		0.39919		0.79838
	CD (0.05)	0.65537		0.80267		1.60533

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

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	T1(Control)	6.0000	4.3333	4.0000	6.0000	5.0833
2	T2(BAP 0.5mgl^{-1})	4.0000	6.3333	7.0000	7.3333	6.1667
3	T3(BAP 1 mgl^{-1})	3.6667	7.0000	7.3333	8.3333	6.1667
4	T4(BAP 2 mgl^{-1})	3.6667	5.6667	6.6667	4.3333	5.0833
5	T5(BAP 3 mgl^{-1})	1.6667	4.3333	5.6667	5.6667	4.3333
6	T6(BAP 4 mgl^{-1})	2.6667	4.6667	5.3333	5.0000	4.4167
	Mean	3.6111	5.3889	6.0000	6.1111	
	Treatment	E		T		E x T
	SEd	0.40635		0.49768		0.99536
	CD (0.05)	0.81707		1.00071		2.00141

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Fig. 1. Effect of BAP on number of shoots regenerated from the rhizome, shoot tip, mature embryo, and immature embryo

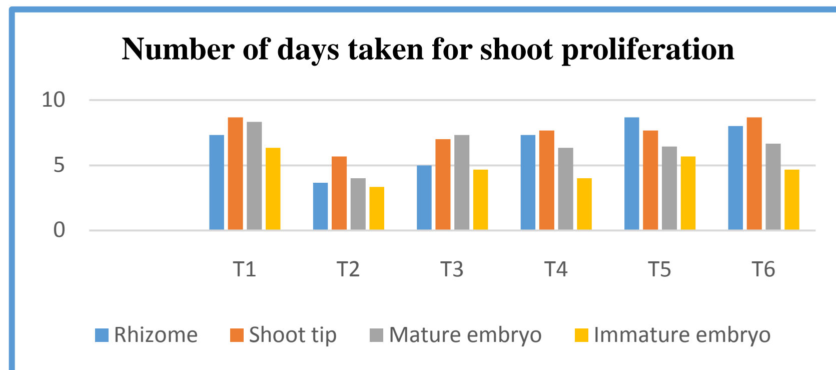


Fig. 2. Effect of BAP on the length of micro shoots on the rhizome, shoot tip, mature embryo, and immature embryo

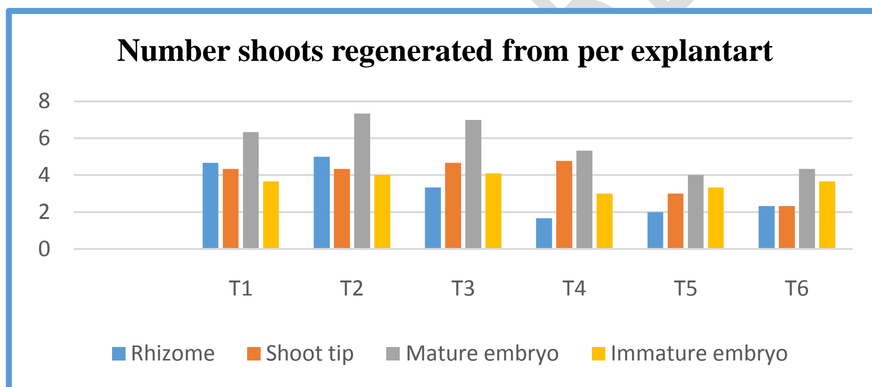
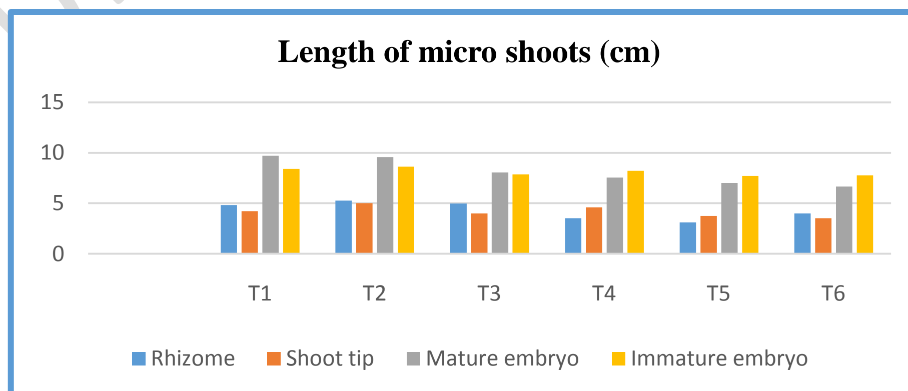


Fig. 3. Effect of BAP on the shoots formation percentage from the rhizome, shoot tip, mature embryo, and immature embryo



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

B) Shoot proliferation from Shoot tip of *N. nucifera* Lakshmi in MS media containing 2 mgl^{-1} BAP

C) Shoot proliferation from mature embryo of *N. nucifera* Lakshmi in MS media containing 0.5 mgl^{-1} BAP

D) Shoot proliferation from immature embryo of *N. nucifera* Lakshmi in MS media containing 1 mgl^{-1} BAP