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Xia Yu, Jiajing Sheng, Lingling Zhao, Ying Diao, Xingwen Zheng, Keqiang Xie, Mingquan Zhou, Zhongli Hu

In vitro plant regeneration of lotus (*Nelumbo nucifera*)

Abstract: In this study, an efficient and reproducible plant regeneration system for lotus (*Nelumbo nucifera* Gaertn.) was established using shoot apical meristems from the buds and one-week-old aseptically germinated embryos as explants. Multiple shoot clumps were induced on Murashige and Skoog (MS) basal medium supplemented with various combinations of N^6-Benzylaminopurine (6-BA) and α-Naphthalene acetic acid (NAA). The maximum response was obtained with 2.22 μM 6-BA, and produced 21.33 shoots per explant after four weeks. After five subcultures, multiple shoot clumps were transferred to MS basal medium supplemented with various combinations of 3-Indolebutyric acid (IBA), NAA and sucrose for root induction. After four weeks, plantlets with well-developed roots were achieved on MS basal medium supplemented with 0.54 μM NAA and 30 g/L sucrose with 100% rooting rate. The successfully acclimated plantlets were transferred to pots with the addition of 2 g/L $KMnO_4$ into the soils, and finally fertile plants with much bigger leaves were obtained in the greenhouse. The survival rate was 97.33%.

Keywords: Shoot apical meristem, Multiple shoot clumps induction, Root induction, Plant regeneration

DOI 10.1515/biol-2015-0016
Received June 23, 2014; accepted August 24, 2014

***Corresponding author: Zhongli Hu:** State Key Laboratory of Hybrid Rice, College of Life Science, Wuhan University, Luojia Hill, Wuhan, Hubei, 430072, P.R. China, E-mail: huzhongli@whu.edu.cn
Xia Yu, Jiajing Sheng, Lingling Zhao, Ying Diao: State Key Laboratory of Hybrid Rice, College of Life Science, Wuhan University, Luojia Hill, Wuhan, Hubei, 430072, P.R. China
Xingwen Zheng, Keqiang Xie: Guangchang White Lotus Research Institute, Guangchang, Jiangxi, 344900, China
Mingquan Zhou: Lotus Center Wuhan University, Wuhan 430072, China

Abbreviations:

MS - Murashige and Skoog (1962) medium;
6-BA - N^6-Benzylaminopurine;
NAA - α-Naphthalene acetic acid;
IBA - 3-Indolebutyric acid;
2,4-D - 2,4-dichlorophenoxy acetic acid

1 Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is a perennial aquatic plant, commonly growing in lakes, moorland and ponds. In China, lotus is mainly cultivated as an important economic crop for its edible rhizome and seeds for more than 2000 years [1]. Besides, the embryo, leaf, and flower of lotus are used as the herbal medicines [2], which have strong bioactive ingredients, such as alkaloids, flavonoids, antioxidants, antisteroids, antipyretic, anticancerous, antiviral and anti obesity properties [3-6]. Lotus is also planted as an ornamental plant for its beautiful flowers. The flowers of lotus symbolize the spiritual ideal, closely associated with Buddhism [7]. Because of the associated high socio-economic value, lotus has been developed for more than three hundreds cultivars. In general, lotus is commonly propagated through the rhizome, but the low efficiency of propagation is not enough to meet the needs of production [8]. Therefore, it is very important to design an efficient method to improve the efficiency of propagation in lotus.

In vitro culture is a rapid propagation method for plant breeding, and a large number of sterile and genetically stable plants can be obtained in a short period. Hence, there are obvious advantages *in vitro* culture compared with the traditional propagation methods. Until now, several studies have reported successful in vitro propagation of crops, such as maize, wheat, *etc*. [9, 10], however, there are few reports describing in vitro culture of the lotus plant. Somatic embryogenesis from buds of lotus was reported by Arunyanart and Chaitrayagun [11], although the frequency was low. Shou *et al*. [8] reported a protocol

for lotus *in vitro* propagation, but the multiplication rate was still low. Therefore, it is necessary to develop an efficient and rapid vitro propagation system for lotus. In this study, a rapid and direct plant regeneration system for lotus was established by using shoot apical meristems from the buds and one-week-old aseptically germinated embryos as explants, which was of great significance to commercial cultivar propagations and scientific research.

2 Experimental Procedures

2.1 Plant materials

The lotus cultivar "Tai-Kong lotus 36" was planted in lotus research center, Wuhan University. The buds from the growing rhizomes (Fig 1A) and dormant embryos from seeds (Fig 1B) of "Tai-Kong lotus 36" were used as explants in this study.

2.2 Multiple shoot clumps induction from buds of lotus

The buds were excised from the rhizomes and then washed thoroughly with tap water. After removing the outer sheath, and the buds were surfaced-sterilized by washing with 75% ethyl alcohol for 30 s, 0.1% (w/v) mercuric chloride ($HgCl_2$) for 7 min under sterile condition, and then rinsed thoroughly with sterile distilled water four times. After the washes, the inner sheath was removed. The shoot apical meristem was excised and directly cultured on 16 different regeneration media consisting of Murashige and Skoog (MS) basal medium [12] supplemented with various combinations of N^6-Benzylaminopurine (6-BA; 0, 2.22, 4.44, 6.66 μM) and α-Naphthalene acetic acid (NAA; 0, 0.54, 2.7, 5.4 μM). All media contained 30 g/L sucrose, 2 g/L polyvinylpyrrolidone (PVP) and were adjusted to pH 5.8 prior to adding 2.5 g/L Phytagel™ agar (sigma), and finally autoclaved at 121°C for 15 min. After four weeks, multiple shoot clumps were divided and subcultured on the same fresh regeneration medium. All the cultures were incubated with 12 h of cool-white fluorescent light (40 $\mu molm^{-2}s^{-1}$ of photosynthetically active radiation) at 27 ± 2°C.

2.3 Multiple shoot clumps induction from dormant embryos of lotus

After removing the seed coat, seeds were initially washed thoroughly under running tap water three times, and then soaked in detergent for 2 min and rinsed with distilled water three times. The surface-sterilized seeds were soaked in 75% ethyl alcohol for 30 s, 0.1% (w/v) $HgCl_2$ for 13 min under sterile condition, and then rinsed thoroughly with sterile distilled water three times. Dormant embryos were collected from sterilized seeds, and then cultured

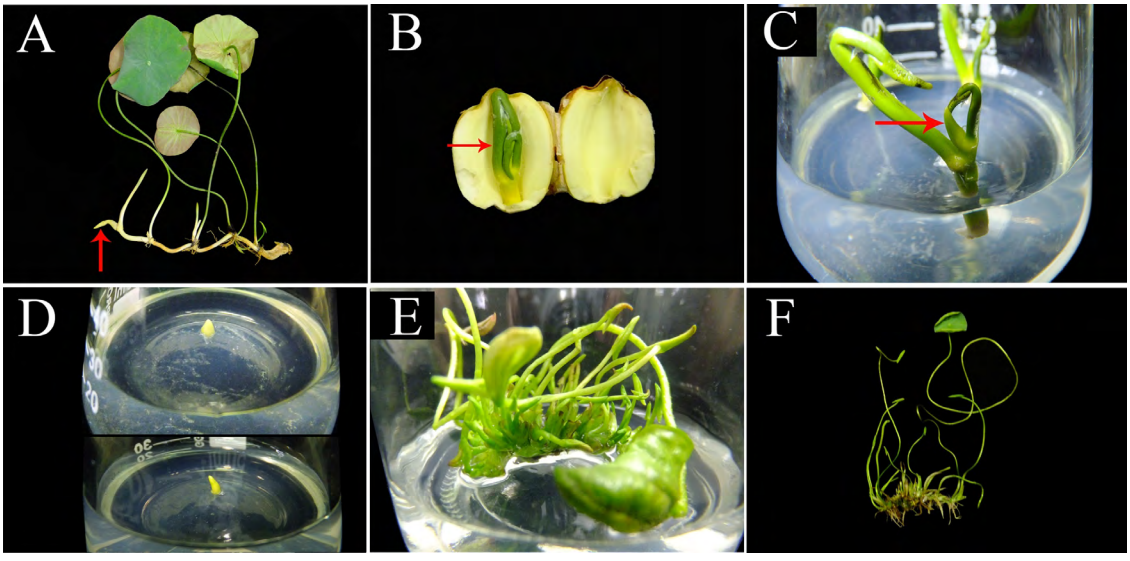

Figure 1: Plant regeneration from shoot apical meristems of two materials in lotus: The bud from growing rhizome (arrow) (A); Dormant embryo (arrow) (B); Germinated embryos after one week on MS basal medium (arrow indicated dynamic shoot apical meristem) (C); Shoot apical meristem cultured on 16 different regeneration media (the upper from the bud and the below from germinated embryo) (D); Multiple shoot clumps developed after four weeks on MS basal medium supplemented with 2.22 μM 6-BA (E); Successfully acclimated plant prior to transplantation outdoors (F).

on germination medium consisting of MS basal medium without plant growth regulators. Similar methods were previously applied in multiple shoot induction of Oat (*Avena sativa* L.) by Zhang *et al*. [13]. After one week, these embryos were germinated (Fig 1C), and the dynamic shoot apical meristem was excised from the embryo, and placed on 16 different regeneration media (Fig 1D). After four weeks, multiple shoot clumps were divided and subcultured on the same fresh regeneration medium, incubated at the same conditions described above.

2.4 Root induction of multiple shoot clumps

After five successive transfers of culture, multiple shoot clumps were transferred to MS basal medium supplemented with various combinations of 3-Indolebutyric acid (IBA; 0, 1.97 μM), NAA (0, 0.54 μM), and sucrose (20, 30, 40 g/L) to develop a root system. All media contained 2 g/L PVP and were adjusted to pH 5.8 prior to adding 2.5 g/L Phytagel, and then autoclaved at 121°C for 15 min. Cultures were incubated at conditions described above.

2.5 Transplantation of *in vitro* culture plantlets

After four weeks, plantlets at approximately 8-15 cm in height were covered with a plastic bag instead of the parafilm. After one week of acclimation, the plantlets were removed from the culture, washed carefully with tap water and transferred to pots in a greenhouse with a photon fluence of 100 mmol × $m^{-2}s^{-1}$ and a 12 h photoperiod at 23 ± 2°C. Before transplantation, the soil in pots were soaked for one week in 2 g/L $KMnO_4$ to kill infectious microbe [14].

2.6 Statistical analysis

All the experiments were repeated three times, and there were 30 explants for each treatment. Where necessary, least square means and analysis of variance (ANOVA, general linear model procedure) were calculated using SPSS (Statistical Product and Service Solutions) software.

3 Results and Discussion

3.1 The effects of various combinations of 6-BA and NAA on multiple shoot clumps induction

Besides different sterilization and germination stage, same experimental method was used for shoot apical meristems from the buds and one-week-old aseptically germinated embryos, which showed same results. In the first week, the shoot apices started to elongate and formed a single short shoot on 16 different regeneration media, respectively. After four weeks, the growth of shoot apical meristems was different (Table 1). The shoot apical meristem formed 4-6 shoots with roots on MS medium supplemented with 0.54 μM NAA, and 3-5 weak and short shoots were formed on MS medium supplemented with the highest concentrations of 6-BA (6.66 μM) and NAA (5.4 μM), which finally died. It seemed that the addition of 6-BA greatly increased the formation of shoots in lotus, but when NAA was combined with 6-BA, NAA significantly inhibited the formation of shoots. The optimal medium for multiple shoot induction was MS medium supplemented with 2.22 μM 6-BA, which obtained 21.33 shoots per shoot apical meristem (Fig 1E), followed by MS medium supplemented with 2.22 μM 6-BA and 0.54 μM NAA (20.00).

Table 1: The effects of various combinations of 6-BA and NAA on multiple shoot clumps induction from shoot apical meristems of two materials in lotus

Number	Plant growth regulators (μM)		Mean number of shoots per explant
	6-BA	NAA	
1	0	0	$5.00^{a,b}$
2	0	0.54	$5.18^{a,b}$
3	0	2.7	$7.81^{a,b,c}$
4	0	5.4	$7.41^{a,b}$
5	2.22	0	21.33^{f}
6	2.22	0.54	$20.00^{e,f}$
7	2.22	2.7	$15.83^{d,e}$
8	2.22	5.4	$7.93^{a,b,c}$
9	4.44	0	$18.30^{e,f}$
10	4.44	0.54	$12.96^{c,d}$
11	4.44	2.7	$10.28^{b,c}$
12	4.44	5.4	$6.44^{a,b}$
13	6.66	0	$15.33^{d,e}$
14	6.66	0.54	$10.08^{b,c}$
15	6.66	2.7	$5.78^{a,b}$
16	6.66	5.4	3.80^{a}

* Mean values marked with the same letter are not significantly different at $P < 0.05$

Phytohormones play an important role in multiple shoot induction [10]. The results showed effects of various combinations of 6-BA and NAA on the induction of multiple shoot clumps in lotus. It is known that cytokinin is very important for shoot organogenesis [15]. Chen and Gao [16] had reported that cell division and differentiation can be promoted by the mixed components of 6-BA and auxin in *Plumbago auriculata*. But in this study, the increasing of NAA had a negative effect on the induction of multiple shoot clumps. Dabauza *et al.* [17] had reported that 6-BA can promote adventitious bud differentiation at a high frequency, but the addition of NAA led to a significant decrease in shoot formation compared to the media without NAA in *Citrullus colocynthis* (L.) Schrad. Maybe NAA weakened the positive effect of 6-BA in multiple shoot clumps induction in some species. So 6-BA alone can greatly induce the formation of shoots in lotus, and 21.33 shoots were obtained on MS medium with 2.22 μM 6-BA after four weeks of culture. When 6-BA increased from 4.44 μM to 6.66 μM, there was a decrease in shoot regeneration, and this finding was in accordance with the other reports [18, 19]. Buathong *et al.* [20] had reported that MS medium supplemented with 50 μM 6-BA successfully produced 8.67 ± 0.77 shoots from embryogenic callus derived from the apical buds of lotus embryos after eight weeks of culture. Shou *et al.* [8] had studied the effect of plant growth regulators, explant size, season of explant collection, temperature and photoperiod on *in vitro* lotus shoot formation, and the maximum number of shoots per bud was 3.5 ± 0.05 after four weeks of culture, that was a low multiplication rate. Arunyanart and Chairrayagun [11] had reported somatic embryogenesis from buds of lotus, and somatic embryos were produced from 33% of the calli on MS medium with 2 μM 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 μM 6-BA after twelve weeks of culture, still the frequency of somatic embryogenesis was low and the period was long. In the present experiment, a large number of shoots can be achieved in a relatively shorter period and with higher multiplication rate.

3.2 The effects of various combinations of NAA, IBA and sucrose on root induction of multiple shoot clumps

After five subcultures, multiple shoot clumps were transferred to MS basal medium supplemented with various concentrations of IBA, NAA and sucrose for root induction. After four weeks, the highest rate of rooting was 100% and the maximum mean number of roots per cluster of shoots was 18.23 on MS basal medium supplemented with 0.54 μM NAA and 30 g/L sucrose. Roots introduced by this medium were thick and strong, which were suitable for transplantation (Table 2). Without plant growth regulators, the number of roots was low, and the roots were thin and short, and the addition of IBA can induce slender roots. Short and brown roots were obtained on MS basal medium supplemented with the mixture of NAA and IBA. It seemed that the addition of IBA or NAA can promote the formation of roots, and NAA was more advantageous than IBA. The media supplemented with NAA were better than the mixture of IBA and NAA, maybe IBA inhibited the function of NAA to some degree. Similar observation was reported by Li *et al.* [21]. Sucrose also played a key role in root induction, and low or higher concentration of sucrose inhibited the formation of roots, and the optimal concentration was 30 g/L.

Table 2: The effects of various combinations of NAA, IBA and sucrose on root induction of multiple shoot clumps in lotus

Number	Plant growth regulators (μM)		Sucrose(g/L)	Rooting rate (%)	Mean number of roots per cluster of shoots
	NAA	IB			
1	0	0	20	14.33^{a}	1.23^{a}
2	0	0	30	52^{d}	$5.63^{b,c}$
3	0	0	40	22.77^{b}	$3.83^{a,b}$
4	0	1.97	20	39.57^{c}	$4.23^{a,b,c}$
5	0	1.97	30	$83.97^{f,g}$	$12.07^{e,f}$
6	0	1.97	40	81.5^{f}	$10.5^{d,e}$
7	0.54	0	20	75.6^{e}	$7.7^{c,d}$
8	0.54	0	30	100^{h}	18.23^{h}
9	0.54	0	40	98.6^{h}	$15.87^{g,h}$
10	0.54	1.97	20	53.5^{d}	$6.03^{b,c}$
11	0.54	1.97	30	85.37^{g}	$14.43^{f,g}$
12	0.54	1.97	40	$84.37^{f,g}$	$12.73^{e,f,g}$

* Mean values marked with the same letter are not significantly different at $P < 0.05$

3.3 Transplantation of *in vitro* culture plantlets derived from shoot apical meristems of lotus

After four weeks, the plantlets approximately 8-15 cm in height with well-developed roots were obtained on MS basal medium supplemented with 0.54 μM NAA and 30 g/L sucrose (Fig. 1F). After one week of acclimation, the successfully acclimated plantlets were planted in pots and were housed in the greenhouse. After three weeks, plants grew well with much bigger leaves in the greenhouse. The survival rate of transplanting plants was 97.33%.

4 Conclusion

In this study, the high-frequency plant regeneration system for lotus was established by using buds and dormant embryos. The regeneration technique based on the buds of lotus is an efficient and rapid way for large-scale production of lotus plants in a relatively short period and with high multiplication rate, which propagates commercial cultivars. On the other hand, the regeneration technique based on the embryos of lotus solves the problem in lotus crossbreeding. This way it is possible to conserve the precious hybrid seeds and expand them in a short time for the further research.

Acknowledgments: Authors declare that this work was supported by the National Science and Technology Supporting Program (No.2012BAD27B01).

Conflict of interest: The authors declared that they have no conflicts of interest to this work.

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