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BRIEF COMMUNICATION

Factors influencing shoot multiplication of lotus (*Nelumbo nucifera*)

S.-Y. SHOU*, L.-X. MIAO*, W.-S. ZAI*,**, X.-Z. HUANG* and D.-P. GUO*[1]

*College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, P.R. China**
*Wenzhou Academy of Agriculture Sciences, Wenzhou 325006, Zhejiang, P.R. China***

Abstract

Effect of plant growth regulators, explant size, season of explant collection, temperature (20, 25 and 30 °C) and photoperiod on *in vitro* lotus (*Nelumbo nucifera* Gaertn.) shoot formation and growth were examined. Shoots formation was greatly influenced by growth regulators, explant size and season of explant collection. The maximum number of shoots were induced from bud explants on Murashige and Skoog (MS) medium containing 4.44 μM benzyladenine (BA) + 0.54 μM α-naphthalene acetic acid (NAA). Explants formed by bud of one expanded and one unexpanded leaf, which was collected in spring gave encouraging results of shoot production. Higher temperature favoured shoot induction and subsequent growth was much better at 25 °C compared to that at 20 and 30 °C.

Additional key words: auxins, cytokinins, *in vitro* culture, lotus, photoperiod, plant regeneration, temperature.

Lotus (*Nelumbo nucifera* Gaertn.) is cultivated as an important crop in China, which can be divided into two categories: vegetable lotus and flower lotus. For vegetable lotus, the swollen rhizome is generally used in culinary preparation. In traditional Chinese medicine, stamen, embryo, leaf and flower is also usually used for medicinal purposes (Zhao 1999). The conventional propagation of vegetable lotus is through underground swollen rhizome with a rather low propagation rate. *In vitro* culture methods have been used for multiplication in many plant species, but its application in vegetable lotus is rarely reported possibly because its recalcitrance to regeneration *in vitro* (Zhao 1999). Until now, a protocol for flower lotus regeneration has been reported (Arunyanart 1998, Arunyanart and Chaitrayagun 2005). Here, we report the *in vitro* multiplication of the economically valuable vegetable lotus through shoot proliferation from underground rhizomes.

There are many factors governing the success of *in vitro* regeneration, such as the cultivar differences, the physiological status of explants, developmental stages of explants and plant growth regulators added to media (*e.g.* Nhut 2001, De Bruyn and Ferreira 1992, Guo *et al.* 2005). Although there are a lot of reports on effects of photoperiod and temperature on plant growth *in situ*, studies for their effects on *in vitro* plant growth are still very limited (Vaz *et al.* 2004).

The aim of this study was to investigate *1*) the effect of growth regulators on shoot initiation, *2*) whether *in vitro* shoot formation of lotus depended on the season when the buds were collected, and *3*) the effects of photoperiod and temperature on the *in vitro* growth of plantlets.

Buds from rhizomes of vegetable lotus (*Nelumbo nucifera* Gaertn. cv. Purple Red) were washed thoroughly under running tap water for 60 min, and then soaked in detergent for 5 min, rinsed six times with distilled water and then with 75 % ethanol for 1 min. After three rinses with distilled water, the buds was then dipped into 0.1 % mercuric chloride plus two drops of *Tween 20* for 10 min, and then followed by 2 % sodium hypochlorite for 10 min, and rinsed six times in sterile distilled water and blotted dry on sterilized filter paper.

After removing the sheath, buds *ca.* 1.0 cm were cultured on solidified MS (Murashige and Skoog, 1962) medium containing 20 g dm^{-3} sucrose, and different concentrations (2.22, 4.44, 6.66 and 8.88 μM) of N^6-benzyladenine (BA) with different concentrations of α-naphthalene acetic acid (NAA) for shoot induction (Table 1). The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Cultures were

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Abbreviations: BA - N^6-benzyladenine; MS - Murashige and Skoog (1962) medium; NAA - α-naphthaleneacetic acid.
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[1] Corresponding author; phone: (+86) 571 86971121, e-mail: dpguo@zju.edu.cn

Table 1. Effects of BA and NAA on shoot formation from the rhizome buds of lotus after 4 weeks in culture on MS medium. Means ± SE, $n = 30$. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$.).

BA [μM]	NAA [μM]	Shoot length [cm]	Fresh mass [g shoot^{-1}]	Number of shoot [bud^{-1}]	Number of leaves [bud^{-1}]	Frequency of shoot initiation [%]
4.44	2.69	4.0 ± 0.04 f	0.8 ± 0.06 b	3.3 ± 0.16 b	3.5 ± 0.08 b	50.1 ± 0.35 b
4.44	5.37	4.1 ± 0.01 e	0.6 ± 0.06 e	2.4 ± 0.14 cd	2.7 ± 0.05 bc	36.7 ± 0.25 c
4.44	8.06	4.7 ± 0.05 c	0.6 ± 0.10 ef	2.1 ± 0.11 d	2.4 ± 0.11 c	26.4 ± 0.15 de
4.44	10.74	5.2 ± 0.03 a	0.5 ± 0.06 fg	1.7 ± 0.09 de	2.0 ± 0.09 cd	23.3 ± 0.21 e
4.44	0.00	3.1 ± 0.05 h	1.2 ± 0.05 a	4.2 ± 0.12 a	4.7 ± 0.13 a	60.0 ± 0.17 a
4.44	0.54	4.7 ± 0.02 c	1.2 ± 0.06 a	3.5 ± 0.05 b	4.5 ± 0.12 a	63.0 ± 0.22 a
0.00	0.54	4.5 ± 0.03 d	0.4 ± 0.06 g	1.4 ± 0.08 e	2.6 ± 0.09 bc	13.5 ± 0.18 f
2.22	0.54	5.1 ± 0.05 b	0.7 ± 0.05 cd	2.7 ± 0.10 c	3.0 ± 0.14 b	46.7 ± 0.44 b
6.66	0.54	4.0 ± 0.05 f	0.8 ± 0.06 bc	3.0 ± 0.13 bc	3.2 ± 0.10 b	40.8 ± 0.12 bc
8.88	0.54	3.8 ± 0.03 g	0.6 ± 0.09 e	2.3 ± 0.09 cd	2.4 ± 0.07 c	33.5 ± 0.26 cd

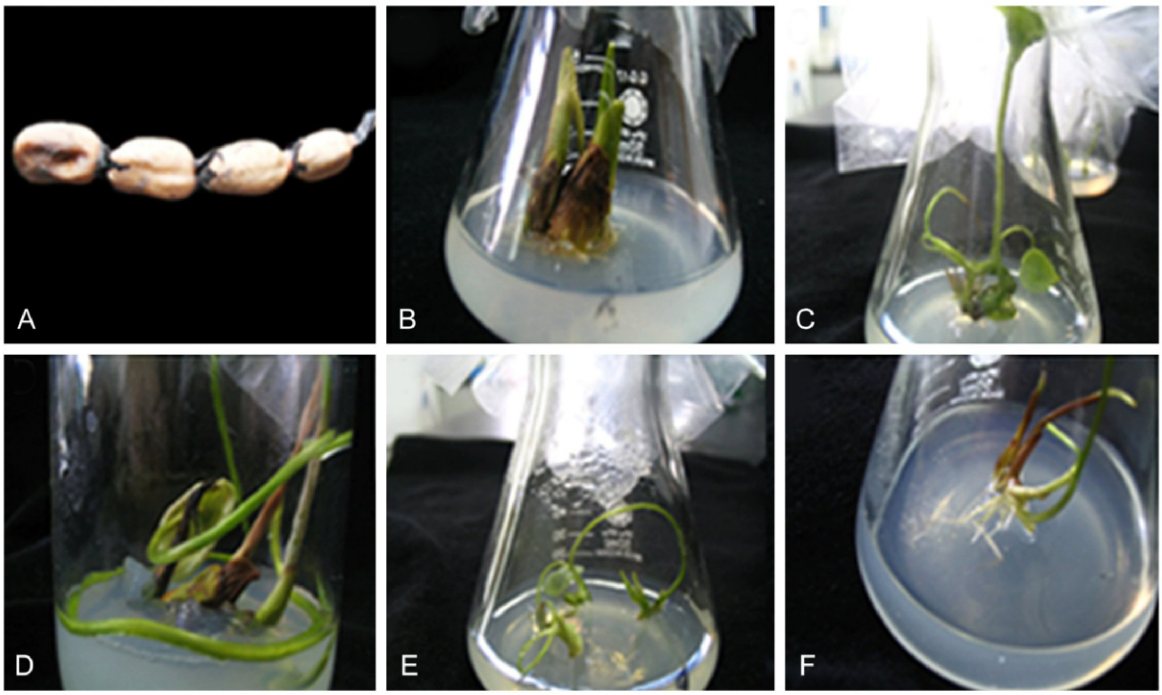

Fig. 1. Shoot development from *in vitro* buds of lotus: *A* - explant of swollen rhizome; *B* - buds cultured for 3 weeks; *C*,*D*,*E* - plantlets grown for 2 weeks; *F* - shoots rooted for 2 weeks.

incubated at 25 ± 1 °C with a 16-h photoperiod at an irradiance of 40 μmol m^{-2} s^{-1} supplied by white fluorescent lamps. For each treatment, one explant per flask was cultured and the experiment was repeated three times. There were at least 30 explants for each treatment. The number of shoots produced per explant, the number of leaves produced per explant, frequency of shoot initiation, shoot length and fresh weight of shoot were counted 4 weeks after culture.

Buds with different size were cut off from rhizomes or buds from different season all year round were collected to test the capacity of different explants to produce shoots *in vitro*. The explants were placed on MS medium containing 8 g dm^{-3} agar, 30 g dm^{-3} sucrose, 4.44 μM BA, and 0.54 μM NAA for 4 weeks.

Shoots derived from buds were cultured on MS medium containing 0.5 - 2.0 μM NAA, 0.2 % activated charcoal, with or without 0.1 μM BA for 1 week, then transferred to growth regulator free medium for 4 weeks (Table 4). All cultures were incubated as mentioned above and for each treatment, 30 explants were cultured and the experiment was repeated three times. Data were recorded after 4 weeks in root induction.

After 4 weeks, shoots from culture initiation were

Table 2. Effect of explant size (buds with expanded or unexpanded leaves) on shoot formation. Means ± SE, n = 30. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$).

Explant	Number of leaves [bud^{-1}]	Number of shoots [bud^{-1}]
Bud	2.00 ± 0.07 c	2.50 ± 0.11 d
Bud + 1 unexp. leaf	3.12 ± 0.12 b	3.49 ± 0.18 c
Bud + 1 exp. leaf	3.46 ± 0.16 b	4.00 ± 0.24 b
Bud + 1 unexp. + 1 exp. leaf	4.45 ± 0.26 a	5.32 ± 0.21 a
Bud + 2 unexp. leaves	3.22 ± 0.18 b	3.65 ± 0.16 c
Bud + 2 exp. leaves	2.04 ± 0.09 c	2.68 ± 0.14 d

Table 3. Effect of month of explant collection on frequency of shoot initiation. Means ± SE, n = 30. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$).

Month	Survival rate [%]	Frequency of shoot formation [%]
January	12.2 ± 0.21 d	83.4 ± 0.31 bc
February	27.8 ± 0.15 a	86.3 ± 0.26 ab
March	18.1 ± 0.12 b	90.4 ± 0.32 a
April	9.8 ± 0.16 de	90.1 ± 0.20 a
May	9.2 ± 0.20 e	83.8 ± 0.12 bc
June	11.5 ± 0.23 d	80.7 ± 0.25 c
July	11.0 ± 0.06 d	70.6 ± 0.17 d
August	14.7 ± 0.19 c	70.0 ± 0.11 d
September	11.6 ± 0.18 d	66.2 ± 0.27 de
October	11.4 ± 0.15 d	63.3 ± 0.18 de
November	11.3 ± 0.17 d	60.5 ± 0.38 e

removed and individual shoot was excised aseptically. These shoots were subcultured on fresh MS medium supplemented with 4.44 μM BA and 0.54 μM NAA. Shoots were incubated in different growth chambers and kept at 20 ± 1, 25 ± 1, or 30 ± 1 °C, all at different photoperiods of 12, 14 or 16 h (40 μmol m^{-2} s^{-1}). Each treatment consisted of at least 25 flasks, each inoculated with one plant. The number of leaves and formed shoots per explants were recorded after 4 week culture.

Explants in experiments were arranged in a randomized complete block design. All measurements were subjected to analyses of variance (*ANOVA*). The significance of differences was evaluated by Duncan's multiple range test.

Cytokinins are generally considered as a critical factor for *in vitro* shoot production and there are many reports that BA exhibits beneficial effect over other cytokinins for shoot multiplication (Dantu and Bhojwani 1987, De Bruyn and Ferreira 1992, Rao and Purohit 2006). Previous studies also have shown that for optimal shoot initiation incorporation of both a cytokinin and an auxin in the medium is necessary. In the present experiment, shoots were successfully produced from bud explants using MS medium supplemented with BA and NAA (Table 1). Shoot multiplication rate was higher with the addition of BA compared to those without BA but the number of shoots was lower on the media supplemented with NAA in comparison with control. The medium containing 4.44 μM BA was most effective for shoot formation (Table 1). Fresh mass per shoot and shoot length decreased as concentrations of BA increased to 6.66 and 8.88 μM. Buds cultured on medium containing 4.44 μM BA and 0.54 μM NAA gave rise to healthy shoots (Fig. 1B). Plantlet height was apparently promoted by the addition of NAA, and inhibited by BA (Table 1).

For all explants used, buds with 1 expanded leaf and 1 unexpanded leaf formed more shoots, and more leaves in shoots than others. Buds with leaf primordia were shown to be easy to produce shoots than buds without leaf primordia. However, buds with two expanded leaves yielded almost the same shoots as those without leaf (Table 2). These results indicate that explant size plays significant role in shoot formation and growth (Salehi and

Table 4. Effect of NAA and BA on rooting of formed shoots. Means ± SE, n = 30. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$).

NAA [μM]	BA [μM]	Rooting rate [%]	Number of roots [explant^{-1}]
2.69	0	46.7 ± 0.23 b	7.1 ± 0.5 e
5.37	0	60.1 ± 0.32 a	9.5 ± 0.9 a
8.06	0	37.2 ± 0.15 c	6.1 ± 0.5 f
10.74	0	26.8 ± 0.26 d	2.2 ± 0.3 h
2.69	0.44	46.0 ± 0.31 b	7.1 ± 0.4 e
5.37	0.44	56.7 ± 0.17 a	9.2 ± 0.7 b
8.06	0.44	33.4 ± 0.21 cd	4.5 ± 0.6 g
10.74	0.44	24.5 ± 0.12 e	2.0 ± 0.3 h

Table 5. Effect of temperature and photoperiod on *in vitro* growth of plantlets on MS medium containing 4.44 μM BA and 0.54 μM NAA after 4 weeks culture. Means ± SE, n = 30. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$).

Temperature [° C]	Photoperiod [h]	Number of leaves [bud^{-1}]	Number of shoots [bud^{-1}]
20	12	1.2 ± 0.09 b	1.3 ± 0.09 b
20	14	1.3 ± 0.11 b	1.6 ± 0.12 b
20	16	1.2 ± 0.05 b	1.5 ± 0.05 b
25	12	3.5 ± 0.08 a	4.6 ± 0.11 a
25	14	3.5 ± 0.13 a	4.7 ± 0.14 a
25	16	3.4 ± 0.12 a	4.8 ± 0.13 a
30	12	3.0 ± 0.10 a	4.1 ± 0.09 a
30	14	3.2 ± 0.07 a	4.2 ± 0.08 a
30	16	3.0 ± 0.06 a	4.1 ± 0.07 a

Khosh-Khui 1997). Additionally, explant age appeared to play an important part in frequency of shoot formation. Elder explant seemed to be less competent for shoot initiation than younger ones. A significant difference in capability of shoot initiation found among explants may be also due to the different degree of their sensitivity towards growth regulators (Sasaki 1979, Nhut *et al.* 2001).

Explants collected in March and April showed much higher survival rate and multiplication frequency, while explants collected in November and December exhibited the lowest survival rate and multiplication frequency compared to those collected from other seasons (Table 3). The reason for this difference may be that active meristematic cells in explant collected in spring are easier to initiate shoot in media supplemented with growth regulators, the dormant buds, on the other hand, are comparatively difficult to form shoot.

Most of shoots developed roots in the media within 4 weeks of culture. The rooting rate of explants was dependant on NAA concentrations supplemented in the media (Table 4). The root number increased with the NAA concentration from 2.69 to 5.37 μM, and then decreased when NAA concentration higher than 8.06 μM. The supplement of 0.44 μM BA showed a slight influence on rooting.

The growth of plantlet, representing by the number of leaves and shoots, was more profoundly affected by temperature than photoperiod (Table 5, Fig. 1). Plantlets grew well at 25 and 30 °C, producing significantly higher numbers of leaves and shoots than at 20 °C. The highest shoot and leaf formation was obtained at 25 °C, therefore this temperature was most suitable for plantlet growth. Both the number of leaves and shoots per explant were not significantly affected by photoperiod under the same temperature treatment (Table 5).

In conclusion, our protocol for lotus *in vitro* propagation has been demonstrated to be a very efficient procedure, which can be commercially practicable, although there are still problems such as the relatively low multiplication rates compared to that of other plants.

References

Arunyanart, S., Chaitrayagun, M.: Induction of somatic embryogenesis in lotus (*Nelumbo nucifera* Gaertn.). - Sci. Hort. **105**: 411-420, 2005.

Arunyanart, S.: *In vitro* culture of lotus (*Nelumbo nucifera* Gaertn.). - J. jap. Soc. hort. Sci. **67** (Suppl.): 257, 1998.

Arunyanart, S., Soontronyatara, S.: Mutation induction by γ- and X-ray irradiation in tissue cultured lotus. - Plant Cell Tissue Organ Cult. **70**:119-122, 2002.

Dantu, P.K., Bhojwani, S.S.: *In vitro* propagation and corm formation in gladiolus. - Gartenbauwissenschaften **52**: 90-93, 1987.

De Bruyn, M.H., Ferreira, D.I.: *In vitro* corm production of *Gladiolus dalenii* and *G. tristis*. - Plant Cell Tissue Organ Cult. **31**: 123-128, 1992.

Guo, D.P., Zhu, Z.J., Hu, X.X., Zheng, S.J.: Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of stem mustard (*Brassica juncea* var. *tsatsai*). - Plant Cell Tissue Organ Cult. **83**: 123-127, 2005.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 472-497,1962.

Nhut, D.T., Le, B.V., Fukai, S., Tanaka, M., Tran Thanh Van, K.: Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of *Lilium longiflorum* via young stem culture. - Plant Growth Regul. **33**: 59-65, 2001.

Rao, M.S., Purohit, S.D.: *In vitro* shoot but differentiation and plantlet regeneration in *Celastrus paniculatus* Willd. - Biol. Plant. **50**: 501-506, 2006.

Salehi, H., Khosh-Khui, M.: Effect of explant length and diameter on *in vitro* shoot growth and proliferation rate of miniature roses. - J. hort. Sci. **72**: 673-676, 1997.

Sasaki, H.: Physiological and morphological studies on development of vegetable crops. VI. Effect of several auxins, cytokinins and cytokinin ribosides on the adventitious bud formation of lettuce hypocotyls tissue *in vitro*. - J. jap. Soc. hort. Sci. **48**: 67-72, 1979.

Vaz, A.P.A., Figueiredo-Ribeiro, R.C.L., Kerbauy, G.B.: Photoperiod and temperature effects on *in vitro* growth and flowering of *P. pusilla*, an epiphytic orchid. - Plant Physiol. Biochem. **42**: 411-415, 2004.

Zhao, Y.W.: Aquatic Vegetables in China. - China Agriculture Press, Beijing 1999.