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RAPID MULTIPLICATION OF ORNAMENTAL BULBOUS PLANTS OF *LILIUM ORIENTALIS* AND *LILIUM LONGIFLORUM*

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Abstract

A protocol for micropropagation of *Lilium orientalis* and *Lilium longiflorum* cv. White Fox has been developed. Effect of different media and sucrose concentrations on shoot formation, root formation and vigour of the plant was observed in this study. Bulb of the plant was used as explant. Among different treatments used for culturing of the plant, the MS medium supplemented with 6-benzylaminopurine (BAP) 3.0 mg/L was found to be the best for shoot initiation from scales of the bulb. After that plants were transferred to different media for multiple shooting. Out of different concentrations used the medium with 0.1 mg/L BAP + 0.1mg/L NAA and 6% sucrose increased frequency of shoot formation up to 100%. An average of about 10 ± 3.94shoots/explants; well-developed roots and bulblet formation were obtained in this medium. Rooted plants were hardened-off in a greenhouse and normal plants with beautiful flowers were produced.A completely randomized design was used for the experiment withfive replicates. The data was analysed by applying one way ANOVA and the treatments' means were compared forsignificance by Duncan's New Multiple Range (DMR) test at 0.05% P.

Introduction

At present, 100 species of *Lilium*, belongs to the large family *Liliaceae* are found in the temperate and subtropical zones of the northern hemisphere (Nhut, 1998). *Lilium* is one of the leading cut flowers all over the world. Lilies have become economically important, mainly because of their large, attractive flowers. Bulbs are produced commercially for use in the cut-flower and potted-plant industries. A large number of ornamental hybrids have been developed. They can be used in herbaceous borders, woodland and shrub plantings and as a patio plant. They are important as large showy flowering garden plants. Additionally, they are important culturally and in literature in much of the world. Some species are grown or harvested for the edible bulbs.

L. longiflorum Thunb. cultivars have become very popular in the USA, some European and Asian countries (Nhut, 2003). Oriental lilies are the most expensive among various lily forms, as their bulbs are highly valuable and require a special technology for bulb production program; they have a wide acceptability in floral industry, mainly as cut flowers and potted plants (Kumar *et al*., 2007).

The successful use of tissue culture techniques for rapid propagation has been reported including L. *Longiflorum* (Bacchetta *et al*., 2003) and oriental hybrid lilies (Lian *et al*., 2002). One of the best and most prolific vegetative propagation methods for lilies is *In vitro* scale culture (Bahr & Compton, 2004). *In vitro* adventitious bud regeneration from scales of *Lilium* depends of factors such as auxin, cytokinins(Varshney *et al.,* 2000), sucrose concentration (Jeong, 1996) and light treatment (Kumar *et al.,* 2005).

This work was focused on development of simple and efficient method for *In vitro* micropropagation of *L. orientalis and L. longiflorum* cv. White Fox.

Materials and Methods

Plant material and explants source: Bulbs of two different species of *L. orientalis* and *L. longiflorum* cv. White Fox were used as explants procured from a seed company in Pakistan (Lahore). Bulb scales (Fig. 1a) derived from *In vivo* bulb of *L. orientalis* and *L. longiflorum* were cultured in MS medium supplemented with different concentrations of growth regulators.

Surface sterilization: Bulbs were washed thoroughly under running tap water for 20 minto remove traces of dirt etc., soaked in detergent for five min, rinsed six times with distilled water, and then submerged in 96% ethanol for one min. Using sterile sharp scalpel, blade the roots were trimmed off from the bulbs and the scales were gently separated from the points of attachment. The scales were further sterilized in 70% commercial bleach for 20 min followed by rinsing in sterile distilled water for four times to remove the traces of bleach. Then placed in a 0.1% (w/v) aqueous solution of $HgCl_2$ for seven minutes, and rinsed six times in sterile distilled water.

Culture media and conditions: Bulb scales were cultured on MS media (Murashige & Skoog, 1962) supplemented with different concentrations of hormones i.e. BAP (1-3) mg/L, TDZ (18-22µm) and 1.5g/L phytagel to induce shoots. The medium was freshly prepared and dispensed in 100mL glass test tubes. The pH of all the media was adjusted to 5.5-5.7 using 1.0 N of HCl or 1 N KOH and then autoclaved at 121°C for 20 min. The cultures were incubated at 22±2°C in a culture room with 2000 lux of light irradiance provided by cool fluorescent lamps and were exposed to a photoperiod of 16 h light and 8 h dark.

Shoot induction, multiplication and bulblet formation: MS medium with 3% sucrose was supplemented with different concentrations of different phythormones alone or in combinations to find the best concentration. Among the all tested combinations these with 6-benzylaminopurine BAP, (1-3mg/L) and with thidiazurone, TDZ, (18 and 20µM) to induce shoots from bulb scales were selected for further study. In order to induce multiple shoots and formation of bulblets, shoot clusters were cut longitudinally into 5 to 7 mm segments, and cultured on MS liquid media containing BAP + NAA (0.1-0.5mg/L. respectively), with different concentrations of sucrose ranging from (2-9%). For each treatment, one explant per test tube was cultured and the experiment was repeated five times. Data were recorded after three weeks in shoot induction and six weeks in shoot multiplication experiments.

Fig. 1. Micropropagation of *Lilium elegans* hybrid and *Lilium longiflorum* hybrid: (a) Scale used as explants. (b) Shoot initiation in BAP1.0 mg/L from scale culture of *Liliumelegans*. (c) Shoot initiation from scale culture of *Lilium longiflorum* sp. in BAP 1.0 mg/l. (d) Bud originated from upper part of the scale of *Lilium longiflorum* with initiation of shoot and root in BAP 3mg/l. (e) Shoot initiation from basal part of the scale of *Lilium elegans* in BAP 3.0 mg/l. (f) Multiple shooting on MS medium supplemented with 0.1 mg/l BAP + 0.1 mg/l NAA + 6% Sucrose. (g) Bulblet formation on MS medium + 0.1mg/l BAP +0.1mg/l NAA and 6% Sucrose. (h) Plantlet formed on MS medium + 0.1mg/l BAP + 0.1mg/l NAA. (i,j) Acclimatization of mericlones in green house. (k) Flowering plant of *Lilium elegans*. (l) Flowering plant of *Lilium longiflorum* (White Fox).

Root initiation: Roots were initiated in the same medium which was used for shoot formation. An average of the number of plants formed, shoot length, root length, effect of sucrose onpercentage of shoot formation and bulblet formation was observed after 20 days of culture, respectively.

Hardening stage: Plantlets produced were cleaned, dipped in 2% Benlate solution for one minute and then transferred to different types of potting media, which were sand, soil and vermicompost (1:1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week. Plantlets were acclimatized in the greenhouse for eight weeks under 50% shade and mist spray irrigation. The percentage of plant survival, plant height (cm) attained and numbers of leaves per plant were recorded after eight weeks of culture.

Statistical analysis: The experiment was planned under completely randomized design (CRD) with five treatments with 5 replication. The data thus generated were analysed through one way analysis of variance (ANOVA) and the treatments' means were compared for significance by Duncan's New Multiple Range (DNMR) test at 0.05% P using Co- Stat computer software.

Results and Discussion

Effect of BAP and TDZ on shoot regeneration from *Lilium* bulb scales: As evident in the Table 1, cultures inoculated in BAP 3.0 mg/L showed highest rate of shoot regeneration of 92% (Fig. 1e) along with roots with many root hairs (Figs. 1f, g, h) (Table 1). Concentrations of TDZ (18, and 22µM) did not response towards shoot regeneration process. The promotive effect of BAP was also noticed by Takayama & Misawa (1982) that tested the effect of NAA, BAP and Kinetin on organ formation from bulb scales and observed that BAP has a stronger physiological effect on organ formation both for shoot formation and root formation. Han *et al*., (2004) used bulb scales of *L. longiflorum* cv. Georgia and cultured these on MS medium with BA (benzyladenine) to induce shoots. After 6 weeks, the frequency of shoot formation was very high (more than 92.3%) on the media with 2.2µM BA was most effective in inducing shoots from bulb scales.The highest results for shoot induction (98.33%) and number of shoots (22.07) were observed from same cormel sprout in Gladiolus on MS medium containing BAP 4 mg L-1 by Memon *et al*., (2013). Sivanesan *et al*., (2012) observed that BA was found to be the most effective cytokinin for multiple shoot induction among the other cytokinins used in the study. It is clearly evident from the work of other scientists that BAP has showing tremendous results among all other cytokinins. This is cleared from the done experiment and from results of the work carried out by other researchers.

Table 1. Effect of different concentrations of BAP and TDZ on shoot and root formation.

Plant hormones		Frequency of shoot regeneration (%)	No. of shoots/explant	No. of roots/explant
BAP (mg/L)	TDZ (µM)			
1	-	85	1.0 ± 0.244	-
2	-	58	1.0 ± 0.141	-
3	-	92	1.0 ± 0.268	7.0 ±0.17
-	18	47	1.0 ± 0.248	-
-	22	50	1.0 ± 0.424	-

No. of test tubes cultured = 10, each value is mean of five replicate with standard error (mean ± S. E)

Effect of BAP + NAA on shoot multiplication of regenerated plants: Previously regenerated plantlets were transferred to MS medium supplemented with different concentrations of BAP + NAA for multiplication of shoots. Best results were obtained in 0.1mg//L BAP + 0.1mg/L NAA with 90% of frequency of shoot regeneration (Table 2). Along with shoot multiplication, this medium also induced highest number of root formation (Table 2). Length of roots formed was also highest in the same medium (Fig. 2B). Similar concentration of BAP + NAA was also reported by Azadi and Khi-Kosh (2007), they tested the various treatments to induce multiple shooting in *Lilium In vitro* regenerated plants, the MSmedium supplemented with 0.1 mg/L NAA + 0.1 mg/L BA in all harvesting seasons proved to be superior to others. Naz *et al*., (2011) noticed that maximum shoot formation response i.e., 90% was shown by medium MS+TDZ 18µmol and 80% in MS+ BAP 2 mg/l. MS+ BAP 2 mg/l appears to be the better medium for the micropropagation of*Riccinuscommunis*as compared to other media combinations applied. It is noticed that these concentrations of bothauxins and cytokinins (BAP 0.1 + NAA 0.1) mg/L enhanced multiplication rate in *Lilium* plants otherwise all other moderate and higher concentrations of these hormones would not show better results.

Table 2. Effect of different concentrations of BAP + NAA on multiple shoot formation.

Plant hormones		Frequency of shoot regeneration (%)	No. of shoots/explant	No. of roots/explant
BAP (mg/L)	NAA (mg/L)			
0.1	0.1	90	8.0 ±2.51[a]	10.0 ±3.73[a]
0.2	0.4	50	1.0 ±0.59[b]	1.0 ±0.07[d]
0.3	0.6	40	1.0 ±0.73[b]	1.0 ±0.13[d]
0.4	0.8	55	1.0 ±0.11[b]	3.0 ±0.91[b]
0.5	0.5	60	1.0 ±0.12[b]	2.0 ±0.45[c]

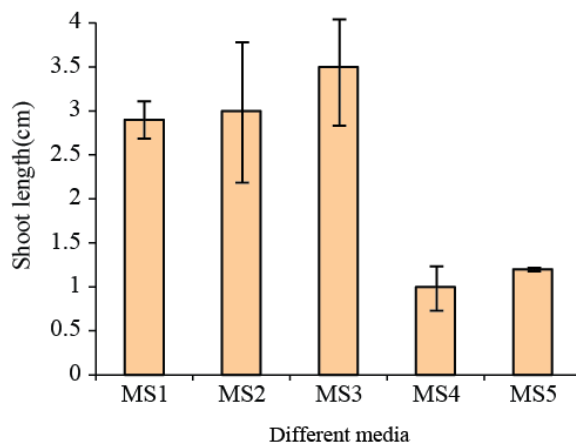
No. of test tubes cultured = 10, each value is mean of five replicate with standard error (mean ± S. E). Means within a column not sharing a common superscript differ significantly ($p<0.05$) according to Duncan's multiple range test

Effect of different concentrations of sucrose on shoot multiplication and bulblet formation: Out of different concentrations used, 0.1mg/L BAP + 0.1mg/L NAA + 6% of sucrose showed 100% of frequency of shoot regeneration, 10.0 ± 3.94 shoots/explants mentioned (Table 3). Joshi & Dhar (2009) used different concentrations of sucrose observed that, of the different concentrations of sucrose tested, 4.5% (w/v) sucrose showed significantly ($p<0.01$) higher percentage regeneration (70.8±4.2% and 79.2±4.2% of regeneration on callus and bulblet scale explants, respectively). In this study, medium with 6% sucrose shown outstanding performance for overall growth of the plant described in different figures, such as in case of highest frequency of shoot multiplication (Fig. 1f), highest shoot length (Fig. 2A), bulblet formation (Fig. 1g). But the highest sucrose concentration for initiation and improvement of bulblet growth reported by Han *et al*., (2005) is 9 g/L.

Table 3. Effect of different concentrations of BAP + NAA and percentage of sucrose on multiple shoot in, and bulblet formation.

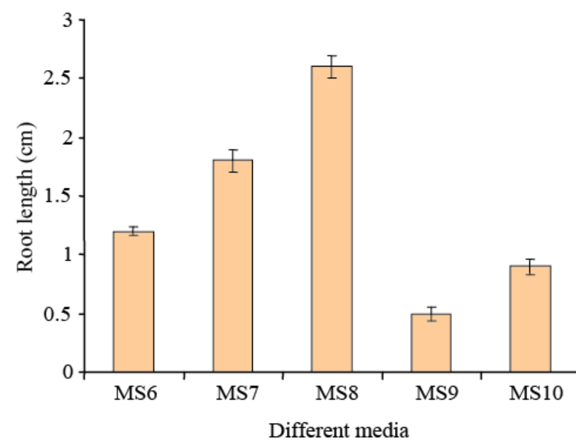
Plant hormones		Sucrose (%)	Frequency of shoot regeneration (%)	No. of shoots/explant	Frequency of bulblet formation (%)	No. of roots/explant
BAP (mg/L)	NAA (mg/L)					
0.1	0.1	6	100	10.0 ± 3.94^a	90	13.0 ± 5.48^a
0.2	0.4	9	50	1.0 ± 0.11^b	80	1.0 ± 0.02^d
0.3	0.6	3	40	1.0 ± 0.18^b	-	1.0 ± 0.09^d
0.4	0.8	2	55	1.0 ± 0.08^b	-	3.0 ± 0.23^b
0.5	0.5	8	60	1.0 ± 0.06^b	60	2.0 ± 0.17^c

No. of test tubes cultured = 10, each value is mean of five replicate with standard error (mean ± S. E). Means within a column not sharing a common superscript differ significantly ($p<0.05$) according to Duncan's multiple range test



MS1= (0.4BAP + 0.8NAA) mg/L + 2% Sucrose
MS2= (0.3BAP + 0.6 NAA) mg/L +3% Sucrose
MS3= (0.1BAP + 0.1NAA) mg/L + 6% Sucrose
MS4= (0.5BAP + 0.5NAA) mg/L + 8% Sucrose
MS5= (0.2BAP + 0.4NAA) mg/L + 9% Sucrose

Fig. 2A. Effectof different concentrations of hormones and sucrose on shoot length (cm).



MS6= (0.4BAP + 0.8NAA) mg/L
MS7= (0.3BAP + 0.6 NAA) mg/L
MS8= (0.1BAP + 0.1NAA) mg/L
MS9= (0.5BAP + 0.5NAA) mg/L
MS10= (0.2BAP + 0.4NAA) mg/L

Fig. 2B. Effect of different concentrations of hormones on root length (cm).

Acclimatization of mericlones: Survival frequency of plantlets under *ex vitro* conditions on soil was 90%. Rooted plants were hardened-off in a greenhouse for 2 months, and normal flowering plants were produced (Figs. 1 i, j, k, l).

Conclusion

The present study demonstrates a simple and efficient method for high frequency direct shoot regeneration from scales of 2 *Lilium* species. The system is rapid, starting with the initiation of tissue culture. Such a high regeneration frequency would be useful for mass propagation and multiplication of this valuable plant in Pakistan.

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