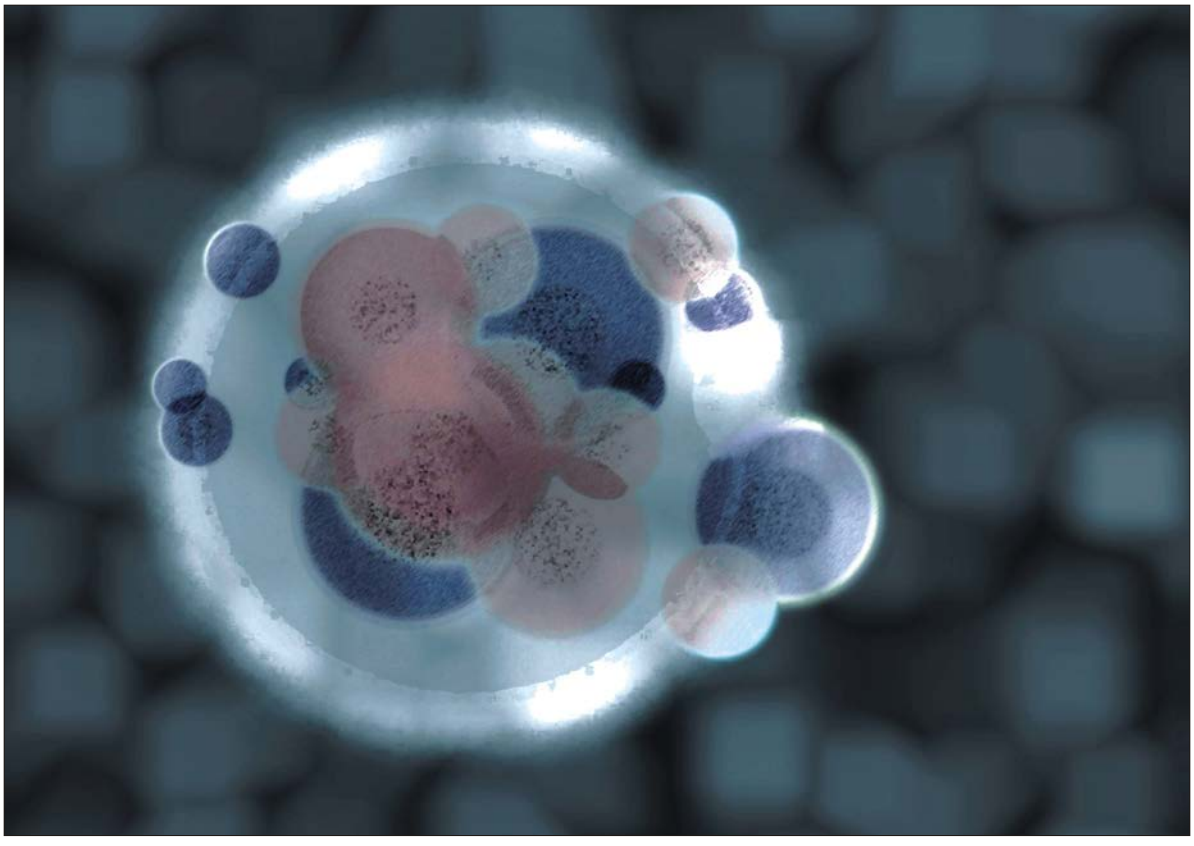

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Research Article
In vitro Culture and Bulblets Induction of Asiatic Hybrid Lily 'Red Alert'

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Abstract

Background and Objective: Asiatic of lily is an important hybrid for trade in the international market because of its colour, beauty and long vase life. The objective of study was to establish a micropropagation protocol for Asiatic hybrid lily using bulb scales for continuing *in vitro* cultures. **Methodology:** Sterilized treatments were investigated at three concentrations (0.05, 0.1 and 0.2%) of $HgCl_2$ (MC). The MS medium supplemented with various concentrations (0, 0.5, 1, 1.5 and 2.0 mg L^{-1}) of BA (6-Benzylaminopurine) and 2ip (isopentyl adenine) was used for *in vitro* scales multiplication. For bulblets induction, 0.5 mg L^{-1} of both BA and thidiazuron (TDZ) each alone or combined with α-naphthalene acetic acid (NAA) at 1.0 and 2.0 mg L^{-1} were examined. Different parameters were statistically analyzed using randomized complete block design. **Results:** The highest percentage of both free contaminated explants and survival (88.89 and 77.77%, respectively) were clearly appeared when 10% of sodium hypochlorite and mercuric chloride at 0.1% were used. Using MS (Murashige and Skoog, 1962) culture medium supplemented with different concentrations of BA or 2ip had a significant promotion effect on the number of bulbscales/explant which can induce adventitious bulblet formation and use for micropropagation. MS culture medium supplemented with TDZ at 0.5 mg L^{-1}+NAA (2.0 mg L^{-1}) was favored for obtaining the highest numbers of shoots, leaves and bulblets induced/explant (9.33, 4.67 and 9.33, respectively). IAA at 0.5 mg L^{-1} was favored for rooting percentage, number of roots as well as number of formed bulblets/shootlet (100%, 2.5 and 3.33, respectively). Most of plants were easily grow, normally acclimatized to the green house conditions and 98% of them were survived. **Conclusion:** Tissue culture technique is suitable propagation method for producing great new bulbs of Asiatic hybrid lily plant.

Key words: *Lilium* hybrid, micropropagation, bulblets formation, rooting and hardening off, Asiatic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lilium is one of ornamental plants belongs to the family *Liliaceae*. It is a very popular as cut flower and pot plant in floral industry[1]. Asiatic of lily is an important hybrid for trade in the international market because of its colour, beauty and long vase life.

Conventional propagation method is not suitable for propagation of lily where, using seeds is not a practical method because of slow growth of the bulbs until flowering and at large scale propagation it is difficult to obtain a large number of bulbs in a short time[2]. Moreover, there is a need to develop mass propagation methods to obtain disease- free of this important ornamental plant species for florist trade where, repeated cycles of vegetative propagation caused accumulation of soil diseases subsequently, decreased vigor of bulbs[3]. Pelkonen and Kauppi[4], Varshney *et al*.[5], Nhut *et al*.[6] mentioned the successful studies on *in vitro* propagation of *Lilium* species through direct or indirect regeneration where, Varshney *et al*.[5] could obtain 9.68×10^5 bulblets from a single scale segment of lily Asiatic hybrids in 1 year. Therefore, tissue culture technique is suitable propagation method for new lily genotypes[2].

In vitro studies as different culture media with different types and concentrations of growth regulators have been reported for lilies. The ratio of cytokinin with auxin has great influence on plant morphogenesis and has determined the formation of roots and shoots[7,8].

Cytokinin levels are generally known to promote formation of buds and shoot differentiation in tissue cultured organs[9-11]. It act as cell division mediating factors such as shoot development and plant meristem activity as well as the arrangement of parenchyma cells and procambium necessary to the size of the apical meristem. BA is the most effective cytokinin for shoot regeneration from explants of Asiatic *Lilium*[11]. The important role of BA concentration in culture medium for the transition from juvenile to vegetative adult phase in *Lilium formolongi* 'White Aga' *in vitro* was mentioned[12]. Induction of shoot formation using BAP and TDZ was also revealed[13].

For micropropagation of tiger lily var. Flore Pleno, bulbils shown to be good choice to produce bulblets, shoots and roots was greatly affected by the concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) in culture medium[14].

The objective of the present study was to establish an *in vitro* culture protocol and bulblets formation induction of *Lilium* Asiatic hybrid 'Red Alert' using different cytokinins (BA, 2ip and TDZ) and auxins (NAA, IBA and IAA) at various concentrations.

MATERIALS AND METHODS

This study was carried out at Tissue culture Technique Laboratory of Ornamental Plants and Woody Trees Department, Agricultural and Biological Researches Division, National Research Center (NRC) and Tissue Culture and Germplasm Conservation Research Laboratory, Horti. Res. Institute, Agri. Res. Center (ARC), Egypt during years 2016 and 2017 to establish *in vitro* culture protocol for Asiatic hybrid lily 'Red Alert'.

Explant source and surface sterilization: Bulbs (5-7 cm in diameter) of *Lilium* were collected from commercial nursery, were washed to remove mud and dirt under running tap water for 1 h. The scales were gently excised from the points of attachment using sterile scalpel blade and surface sterilized in ethanol 70% (v/v) for 30 sec, then rinsed in three tested concentrations (5, 10 and 15%) of sodium hypochlorite (Clorox) for 7 min. After that, the inner scales were washed with sterilized distilled water 3 times, then each section sterilized treatment was rinsed in three concentrations (0.05, 0.1 and 0.2%) of $HgCl_2$ (MC) solution (w/v) for 10 min and finally rinsed 3 times in sterile water.

Culture establishment stage: After surface sterilization, clean bulb scales as explants were cultured in jars containing 25 mL MS free of hormones[15] supplemented with 3% sucrose and 0.7% agar. The pH of the medium was adjusted to 5.6-5.8 then autoclaved at 121°C and 15 psi for 15 min. Free contaminated and survived explant were recorded at the end of this stage (1 month).

***In vitro* culture conditions:** The *in vitro* cultures during all stages were placed in the incubation room at 23+2°C and 70% relative humidity under 16 h photoperiod and 1.5 kilo lux light intensity provided by cool, white, fluorescent lamps.

Proliferation stage: After establishment, the obtained bulblets were separated and subcultured on MS medium supplemented with various concentrations (0. 0.5, 1, 1.5 and 2.0 mg L^{-1}) of BA and 2ip for *in vitro* scales multiplication. Scales of *in vitro* formed bulblets could be used further for *in vitro* scale formation and used as secondary explants.

Micropropagation and *in vitro* bulblets induction: For this stage, 0.5 mg L^{-1} of both BA and thidiazuron (TDZ) each alone or combined with α-naphthalene acetic acid (NAA) at 1.0 and 2.0 mg L^{-1} were examined. The characteristic features of

regenerated plantlets were observed such as number of shootlets/explant, number of leaves/shootlet, number of bulblets/shootlet, rooting (%), number of roots/shootlet and length of roots (mm).

Rooting stage: Individual bulblets were transferred to rooting media contained three types of auxins (NAA, IBA and IAA) at three concentrations (0.5, 1.0 and 1.5mg L^{-1}) which were tested for rooting ability (%), number and length of roots as well as the obtained bulblets diameter and number.

Hardening off: The rooted plantlets were removed from culture jars and washed with tap water, then immersed in Benlate solution (1.0 g L^{-1}) as a fungicide for 5 min. The plantlets were transferred to pots containing peat moss, peat moss+sand (1:1) or peat moss+perlite (1:1), covered with transparent polyethylene pages for 2 weeks and gradually they removed in the greenhouse. Survival percentage of plantlets (%) was determined after 8 weeks.

Statistical analysis: All experiments were repeated twice and the average of data recorded for different parameters statistically analyzed using randomized complete block design with 10 replicates per treatment. The LSD test at 5% for comparison among means was used according to methods of Steel and Torrie[16].

RESULTS AND DISCUSSIONS

Culture establishment: A protocol for scale explants sterilization was developed to obtain sufficient number of *Lilium* for *in vitro* culture establishment (Table 1 and Fig. 1). The highest percentage of both free contaminated explants and survival (88.89 and 77.77%, respectively) were clearly appeared when 10% of sodium hypochlorite and mercuric chloride of 0.1% were used. Increasing the concentration of these sterilants to the maximum ones (15 and 2%) led to decreasing the contamination percent and subsequently highest percentage of aseptic cultures (70.37 and 92.59%, respectively) but caused killing most of explants and led to the lowest survival percentage (55.55 and 37.03%, respectively). Confirmed results were recorded by Singh and Tiwari[17] on jackfruit who attributed the phytotoxic for the survival of the explants to use of heavy metal of mercury. Nesi *et al.*[18] found that using 1.25% NaOCl (15 min) and rinsing 2 times with sterilize distilled water was suitable for Asiatic hybrid lily.

***In vitro* proliferation:** The effect of 2 types of cytokinins (BA and 2ip) at different concentrations (0.0, 0.5. 1.0, 1.5 and 2.0 mg L^{-1}) on *in vitro* proliferation of *Lilium* hybrid Asiatic is shown in Table 2 and Fig. 1. It is obvious that the MS culture medium supplemented with different concentrations of BA or 2ip had a significant promotion effect and caused increasing the number of bulbscales/explant which can induce adventitious bulblet formation and use for micropropagation of lilis (A process was known "Scaling" as mentioned by McRae[19]). It is clear from data that the number of bulbscales was gradually increased with increasing the concentration of 2ip in the culture medium until 1.5 mg L^{-1} which caused the greatest bulb scales number (6.55) followed by 1.0 mg L^{-1} (5.56). These results were agreed with those by Uranbey[20] on *Muscari aucheri*, detected that increasing the concentration of BAP and 2-ip had a positive effect on bulblet production. For

Table 1: A protocol for scale explants sterilization under effect of different concentrations of sodium hypochlorite (Clorox) and mercuric chloride (MC)

	Characters							
	Survival (%)				Aseptic cultures (%)			
MC (A)/ Clorox (B)	0.05	0.10	0.20	Mean (B)	0.05	0.10	0.20	Mean (B)
5%	100	100.00	55.55	85.18	11.11	33.33	17.78	40.74
10%	100	77.77	33.33	70.37	11.11	88.89	100.00	66.67
15%	100	44.44	22.22	55.55	22.22	88.89	100.00	70.37
Mean (A)	100	74.07	37.03		14.81	70.37	92.59	
LSD 5%	A = 13.02	B = 13.02	A×B = 22.55		A = 19.2	B = 19.23	A×B = 33.31	

Table 2: Effect of cytokinin type and concentration on *in vitro* proliferation of *Lilium* Asiatic hybrid (As mean of two subcultures)

		Cytokinin								
		BA concentration (ppm)				2ip concentration (ppm)				
Characters	Control (0.0)	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	LSD at 0.05
Number of bulb scales/explant	2.00	3.17	2.00	3.84	3.00	3.89	5.56	6.55	4.44	0.45
Number of shootlets/explant	3.82	3.58	3.09	4.31	3.02	3.45	2.40	2.83	3.50	1.36

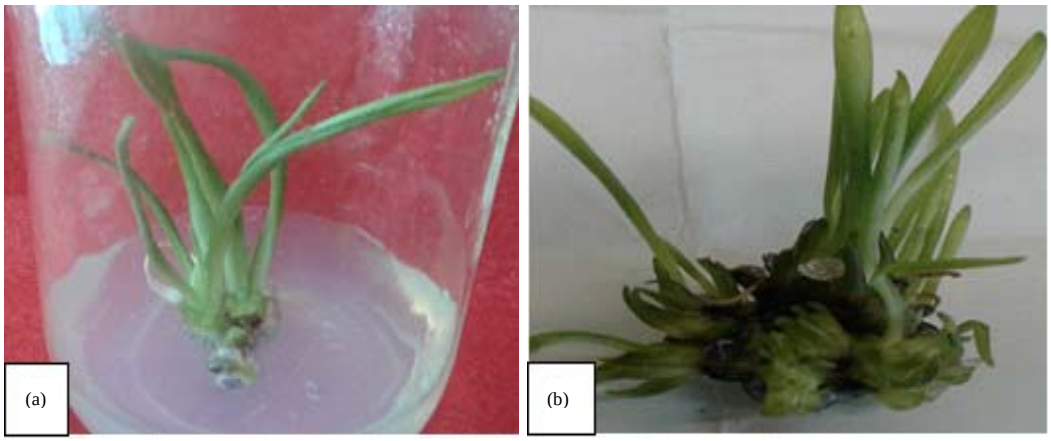

Fig. 1(a-b): *In vitro* multiplication of *Lilium* Asiatic hybrid (a) Cultures after 1 month on MS free hormones and (b) Bulblet scaling at 1.5 mg L^{-1} of 2ip

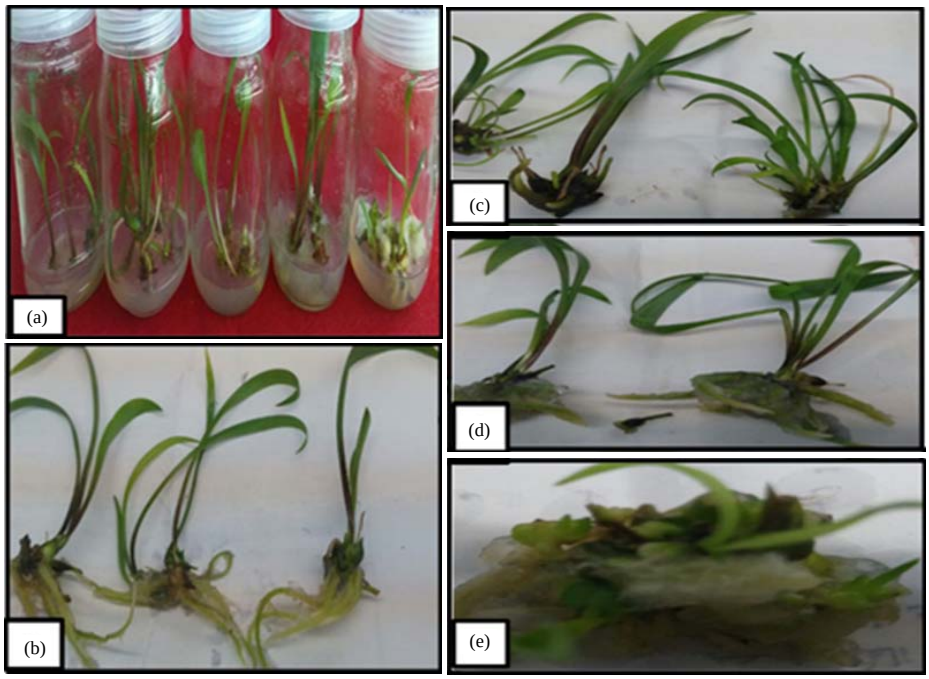

Fig. 2(a-e): (a) *In vitro Lilium* Asiatic hybrid affecting by various concentrations of BA and TDZ alone or supplemented with NAA, (b) Control treatment (MS free hormones), (c) BA0.5+NAA 1 ppm, (d) TDZ 0.5+NAA1 ppm and (e) TDZ0.5+NAA 2 ppm

shoot multiplication, the optimum treatment of for increasing the number of shootlets formed per explant to the highest value (4.31) was 1.5 mg L^{-1} of BA but, the other concentrations of two cytokinins that were used led to decrease of this value comparing with control (MS free hormones). Similar results were obtained in study by El-Naggar *et al.*[21] on *Lilium* 'Prato' who found that the highest number of shoots was obtained at 1.0 and 2.0 mg L^{-1} of BA. The role of BA as synthetic cytokinin in plant growth and development in *Lilium species* was attributed to its effect the formation of adventitious shoots as revealed by Takayama and Misawa[22] and Maesato *et al.*[23].

Micropropagation and bulblets induction: The bulbscales and shootlets resulting from previous stage were used as explants for secondary shootlets and bulblets induction under effect of BA and TDZ at 0.5 mg L^{-1} alone or in combination with NAA (1.0 or 2.0 mg L^{-1}). The results in Table 3 and Fig. 2

Table 3: *In vitro* secondary shoots, rooting behaviors of *Lilium* Asiatic hybrid and bulblets induction affecting by various cytokinins

Treatments (mg L^{-})	Characters: Number of shootlets/ explant	Number of leaves/ shootlet	Number of bulblets/ shootlet	Rooting (%)	Number of roots/ shootlet	Length of roots (mm)
Control (MS free hormones)	1.33	2.50	2.50	100.00	2.67	31.67
BA 0.5	1.00	2.20	2.83	42.78	1.00	15.33
BA 0.5 +NAA 1	1.67	2.83	2.17	100.00	2.67	32.00
BA 0.5 +NAA 2	6.67	5.67	7.67	100.00	2.50	11.50
TDZ 0.5	1.33	2.33	5.33	64.87	2.00	9.33
TDZ 0.5 +NAA 1	6.67	4.67	6.00	100.00	8.00	15.33
TDZ 0.5 +NAA 2	9.33	4.67	9.33	0.00	0.00	0.00
$LSD_{0.05}$	1.40	1.08	1.18	3.12	0.96	3.17

Table 4: Effect of different auxins (NAA, IBA and IAA) at various concentrations (0.0, 0.5, 1 and 1.5 mg L^{-1}) on rooting and bulblets formation of *Lilium*

Auxin (mg L^{-1})	Characters: Rooting (%)	Number of roots/ shootlet	Length of roots (mm)	Bulblet diameter (cm)	Number of bulblets/shootlet
Control (0.0)	0.00	0.00	0.00	0.40	1.67
NAA (0.5)	35.52	2.33	1.00	0.50	2.33
NAA (1.0)	66.33	2.00	1.23	0.33	3.00
NAA (1.5)	44.00	1.33	1.30	0.27	2.33
IBA (0.5)	88.65	1.90	1.23	0.37	2.33
IBA (1.0)	100.00	2.00	1.83	0.40	2.67
IBA (1.5)	100.00	2.17	1.50	0.50	2.00
IAA (0.5)	100.00	2.50	1.83	0.37	3.33
IAA (1.0)	100.00	2.50	1.50	0.37	2.67
IAA (1.5)	100.00	2.00	2.50	0.23	2.33
$LSD_{0.05}$	2.46	0.99	0.60	0.20	1.20

shows that MS culture medium supplemented with TDZ at 0.5 mg L^{-1}+ NAA (2.0 mg L^{-1}) was favored for obtaining the highest numbers of shoots, leaves and bulblets induced per explant (9.33, 4.67 and 9.33, respectively) comparing with control which caused the lowest ones. Meanwhile, this treatment caused non *in vitro* rooting but, lowering the concentration of NAA to 1.0 mg L^{-1} supplemented to cytokinin (BA or TDZ) had promotion effect on rooting percentage, number and length of formed roots. Using BA at 0.5 mg L^{-1} plus NAA at 1.0 or 2.0 mg L^{-1} resulted in the highest percentage of rooting (100%) as control treatment. In this trend, Sam *et al.*[24] reported that bulblets also were formed from young shoot on MS supplemented with 0.5 mg L^{-1} NAA. The highest number of bulblets per explant was recorded from longitude-divided bubbles on MS medium containing 0.5 mg L^{-1} NAA and 0.2 mg L^{-1} BAP.

It seems from results that high response of bulblets formation was induced by adding NAA at 2.0 mg L^{-1} to the cytokinin, especially TDZ followed by BA at 0.5mg L^{-1} (Table 3). Similar finding by Niimi[25], who mentioned that NAA at 0.05 and 0.1 mg L^{-1} stimulated bulblet formation in *Lilium rubellum* and the addition of BA had little effect. In another study, Maesato *et al.*[23] reported that supplementation of medium with cytokinins, alone or in combination with NAA, induced lower response than that supplemented with NAA alone because the excised bulbscales have sufficient endogenous cytokinin-like substances which could attain required balance for organ regeneration when supplemented with auxins.

Rooting and bulblets formation: The significant responses of rooting ability and bulblets formation to different auxins (NAA, IBA and IAA) at various concentrations (0.0, 0.5, 1.0 and 1.5 mg L^{-1}) are shown in Table 4. The data recorded that IAA at concentration 0.5 mg L^{-1} was favored treatment for rooting percentage, number of roots as well as number of formed bulblets/shootlet (100%, 2.5 and 3.33, respectively). It was noticed that increasing the concentration of IBA or IAA to 1.0 or 1.5 mg L^{-1} gave the same results for rooting percentage which was in highest percent (100%) comparing with control (MS free hormones) and other treatments. Also, the low concentration of NAA (0.5 mg L^{-1}) had promotion effect on both number of roots/shootlet and the diameter of formed bulblets. The longest roots were obtained with 1.5 mg L^{-1} of IAA. The role of auxins in promoting rooting also have been detected for *Lilium auratum*[26] and *Lilium nepalense*[27]. The efficient of IAA was less than NAA for root induction in *Lilium longiforum*[28]. Kongbangkerd *et al.*[29] showed that high

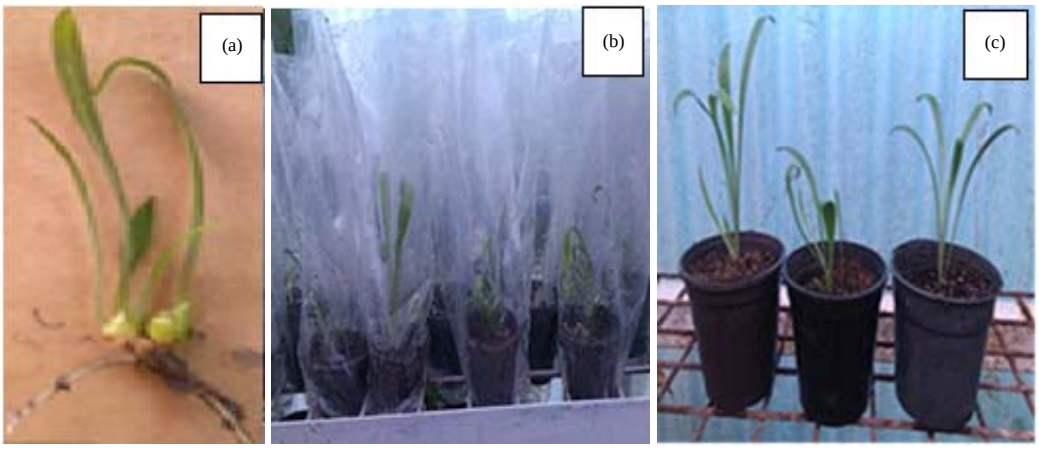

Fig. 3(a-c): *Lilium* hardening off (a) Rooted plants, (b) Transplanting of *Lilium* to greenhouse and (c) Normal grown plants after hardening off process

concentration of NAA caused thickening of the short roots. Skoric *et al.*[30] observed that addition of IAA instead of NAA did not induce root formation in *L. martagon* var. *cattania* and most of the shoots were rooted on medium with higher NAA (1-2 mg L^{-1}) concentration, had short thick roots and some callus tissue at the base of the scale.

In these results, it seems that all tested auxin concentrations promoted the number of formed bulblets except for high concentration of IBA (1.5mg L^{-1}) which caused the lowest value (2.0 bulblets) with no significant difference between this value and that of control (1.67). Moreover, increasing the auxin concentration above 1.0 mg L^{-1} led to decreasing bulblets number/shootlet. The confirmed finding by Skoric *et al.*[30], who recorded the decrease of bulblets formation in *Lilium martagon* var. *cattania* by the increase of NAA concentration. Ghanbari *et al.*[31] mentioned that the highest number and percentage of bulb and also the largest number and percentage of roots were obtained from the medium lacking the growth regulators and suggested that the available endogenous hormones in explants is the most significant factor for bulblets formation.

Hardening off: The obtained plantlets with adequate number of roots were removed from *in vitro* jars and transferred to plastic pots containing mixture peat moss, peat moss+sand (1:1) or peat moss+perlite (1:1), covered with transparent polyethylene pages for 2 weeks and gradually they removed in the greenhouse. Most of plants were easily grow normally acclimatized to the green house conditions (Fig. 3). The survival percentage of plantlets was about 98% after 8 weeks. In this respect, Panwar *et al.*[32] found that when the *in vitro* raised *Lilium polyphyllum* D. Do nex Royle plantlets with roots were transferred to plastic glass containing sterile soil and vermiculite in 1:1 ratio inside the greenhouse for 1 month and then shifted to plastic glass containing compost-enriched soil, the plants were finally transferred to field with 85% success.

CONCLUSION

It can be concluded that MS culture medium supplemented with TDZ at 0.5 mg L^{-1}+NAA (2.0 mg L^{-1}) was favored for obtaining the highest numbers of shoots, leaves and bulblets induction. The low concentration of NAA (0.5 mg L^{-1}) had promotion effect on both number of roots/shootlet and the diameter of formed bulblets. Most of plants were survived and easily acclimatized to the greenhouse conditions.

SIGNIFICANT STATEMENT

This study discovers the possible *in vitro* propagation of Asiatic hybrid lily and the synergistic effect of TDZ at 0.5 mg L^{-1}+ NAA (2.0 mg L^{-1}) that can be beneficial for obtaining the highest numbers of shoots, leaves and bulblets induction. Thus, a new theory on these consecutive propagation stages may be arrived at tissue culture laboratories for florist trade.

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