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STANDARDIZARION OF *IN VITRO* MICRO PROPAGATION OF *LILIUM LONGIFOLIUM*, THUMB, CV. EDEN.

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Abstract

Lily is one of the most important commercial cut flower species and one of the three major bulb crops in the commercial market. The Present investigation aimed at developing *in vitro* micro propagation of *Lilium longiflorum* Thumb, cv, 'Eden' by developing bulblets *in vitro* and their *in vivo* germination, For the induction of bulblets four media (MS1, MS2, MS3, MS4) were compared which had 3% or 6% sucrose and 2 mg/l BAP or 5mg/l BAP, Kinetin being constant 1 mg/l. Both MS2 and MS4 media were appropriate for shoot proliferation and when these shoots after subculturing were kept in media for 65 days tiny bulblets developed. Bulblets with 8.0 to 14mm in diameter can be stored in refrigerator (4^0C) or in cold storage for a year in saw dust powder, after which they can be grown in soil +FYM (1.1) where they produced normal plants. The evaluation involving 600-700 bulbs gave a figure of 80-90% transplantation.

Key words: Lilium, micropropagation, bulblet.

Introduction

Lily plant is one of the most important commercial cut flower species and one of the three major bulb crops in the commercial market because of its large size and colourful and fascinating flowers (Robinson and Fisooza bady 1993). It is an attractive economic flowering plant used as cut flowers or grown in pots. (Pobudkiewicz and Treder 2006, Younis *et al* 2014). Lily is a member of the genus Lilium consist of 110-115 species, and can be planted under various climatic zones.

In vitro protocol produced fast mass production and speedy regeneration of uniform plant material and so has been recognized as a necessity particularly for future breeding and commercial utilization of the Lily species (Muhammad *et al* 2013). Tissue culture techniques are used for fast propagation of some species of the genus lilium as oriental hybrid (Lian *et al* 2002) and Lilium asciatic hybrid (Lian *et al* 2003, Taka *et al* 2018). The use of bulb scale as primary explants for micro propagation of Lilium species has been reviewed (Ascher 1978, Takayama and Misawa, 1979) and an experimental evaluation of the potential and limitation of Lilium species has also been successfully achieved by podicel (Liu and Burger,1986).

The present investigation was conducted in order to optimize an *in vitro* propagation protocol due to increase commercial interest of Lilium species since vegetative propagation does not make up for the number of bulb.

Material and Methods

The bulbs were collected from senescent flowering plants of *Lilium longiflorum*. Bulbs were washed with soap, rinsed with tap water and then disinfected according to the following scheme; External scales were removed from the bulbs, the middle and internal scales were washed again with tap water and disinfected by immersion for 20 min. in 3% sodium hypochlorite solution (2% active chlorine), containing two drop of surfactant Tween 20, followed by treatment for 30 minutes with a solution consisting of 0.1% (w/v) Benomil. (Benlate pm 50% w/w, methyl 1-butyl carbamoyl 2-benzymidazol. carbamate) and 0.03% (w/v) streptomycin.

Disinfected bulbs were cultured on (Murashige an Skoog,1962) medium with 3% or 6% sucrose and supplemented with different growth regulators at different

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concentration giving the following media compositions.

Table :

MS1-Sucrose 3% BAP 2.0mg/l+Kn 1.0mg/l

MS2-Sucrose 6% BAP 2.0mg/l+Kn 1.0mg/l

MS3-Sucrose 3% BAP 5.0mg/l+Kn 1.0mg/l

MS4-Sucrose 6% BAP 5.0mg/l+Kn 1.0mg/l

The media were gelled with 0.8 percent agar (Qualiegens, Bombay) and the pH of the medium was adjusted to 5.8 and autoclaved at 1:1 kg/cm^3 and 121 ^{0}C for 15 min. The cultures were incubated at 25$\pm$2^{0}C under 16 hours fluorescent light supplemented with incandescent bulbs (3000 lux). The explants were placed vertically into culture media. For each culture medium 20-30 explants were used. Glass growth tubes each containing 20ml of solid media were used for the initial phases of the cultures and for subsequent proliferation phase glass bottles containing 50 ml of medium were used.

After 4 weeks of culture incubation on MS2 and MS4 the adventitious shoots growing between the scales of the explants were split into two pieces for use as secondary explants, the leaves were trimmed to reduce its length to 20-30mm. The shoot clumps obtained on both MS2 and MS4 media were subjected to two different methods of leaf cutting as described by Chow *et al.* (1992 a). Briefly, for severe cutting method all shoots and leaves were cut down, and the remaining amorphous achlorphyllous tissue were then cut to pieces (approximately with similar size) used as inocula for subcultures (4 pieces/bottle of culture). In the order method the inocula had the basal part of leaves and achlorophyllous tissue at their base; these methods were alternatively used and after some subcultures the severe cutting method was selected because it allowed better shoot proliferation. Shoot clumps produced on media MS2 and MS4 were transferred to fresh medium and cultured for longer period (65 days) under the same light and temperature conditions. After 65 days of incubation, tiny bulblets appeared at the base of the shoots Bulblets with 8.0 to 14.5 mm. in diameter, transplanted in pots containing soil +FYM (1:1 w/w) for *in vivo* germination or stored in saw dust powder at 4^{0}C in refrigerator or stored in cold storage for a year, after which they can be grown in soil +FYM (1:1) where they produced normal plants. Prior to transplantation/Storage they were soaked for 30 minutes in fungicide Bavastin (1%) for decontamination.

Results and Discussion

The bulbs cultured indicated the problem of contamination, phenolic exudation, medium discoloration and explant browning which was interrelated and influenced by a number of factors including culture medium, size and age of explants and season, which was to be prevented. Contamination is an everlasting problem in plant tissue culture. Several micro organism have been identified as contaminants, Bacterial contamination is most common (Leifert *et al.* 1991). With respect to their topographical localization contamination may inhabit the surface of the tissue (epiphytic) or live within the tissue (endophytic). The former are for the greater part removed by adequate surface sterilization but for the latter there is no easy treatment. The main obstacle is controlling internal contaminants that is within the tissue. When antibiotics are added via medium they do not reach a concentration sufficiently high to be effective. This is caused by general difficulties is uptake and transport of medium ingredients in tissue cultured plants (De Klerk 2010, De Klerk and Askari 2012). When the antibiotic are omitted after a number of subcultures, the contamination always returns, Addition of antibiotic is however, helpful because they prevent overgrowing of the nutrient medium. Entophytic microorganisms may be beneficial to some extent (Hallmann *et al* 1977) but they may also be inhibitory. (*Long et al*, 1988, Pirllila *et al*, 2008).

In the present investigation we used 3% Sodium hypochlorite solution, 0.1% Benomil and 0.03% Streptomycin to remove external and internal contamination.

For removal of phenolics from the medium, three procedures were followed.

Table 1: Shoot initiation and proliferation in *Lilium longiflorium* bulb cultured on media supplemented with different levels of growth regulators (GR)

Medium (G R mg/l)	Shoot initiation		Shoot proliferation
	No.of shoots per explants		No.of shoots per inoculum after 65 days
	After 4Week	After 12Weeks	
MS1 sucrose3% (2BAP +1 Kn)	None	3.2$\pm$0.12	nd
MS2 sucrose6% (2BAP +1 Kn)	9.1$\pm$0.29	20.6$\pm$0.52	21.3$\pm$0.51
MS3 sucrose3% (2BAP +1 Kn)	None	5.7$\pm$0.12	nd
MS4 sucrose6% (2BAP +1 Kn)	13.0$\pm$0.33	27.5$\pm$0.84	29.2$\pm$1.01

The data are presented as mean $\pm$ S.E. for shoot proliferation the data presented are for the end of fourth and fifth subculture period and are measured across the two culture passage. nd-not determined.

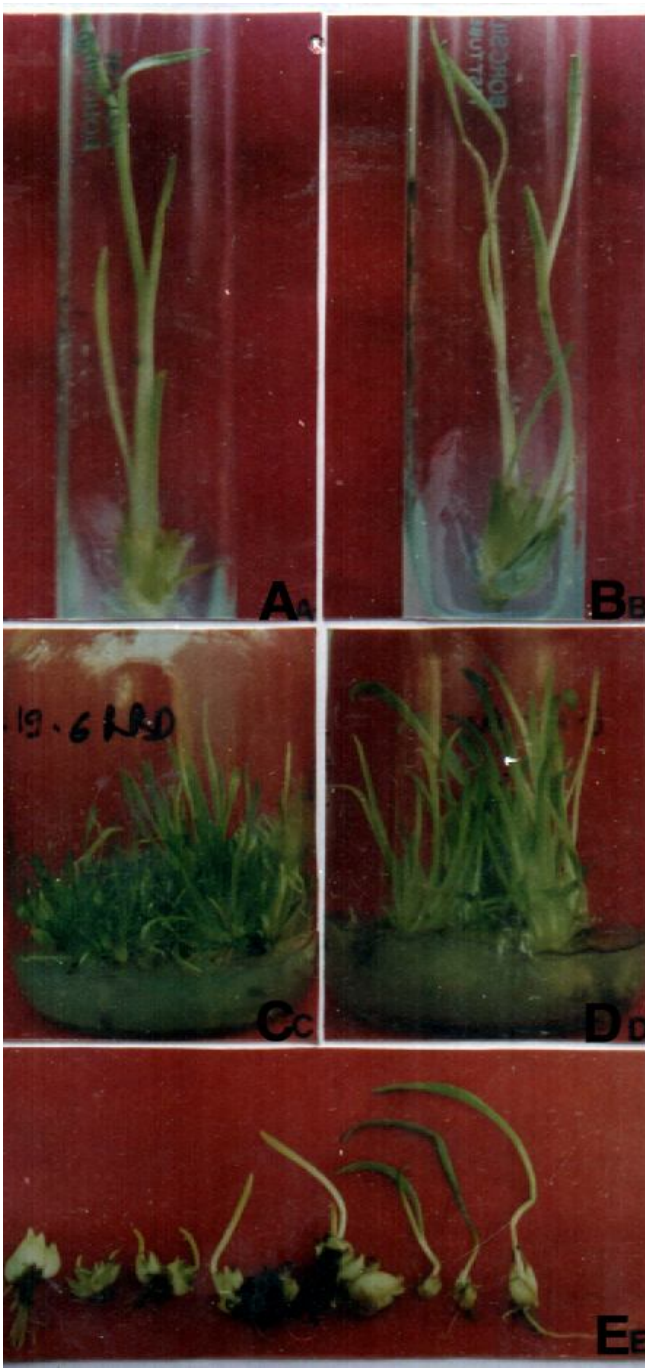

Fig: Sequential phase of the cultures for the production of bulbs.

A: Shoot proliferation in MS2 media after 4 weeks.

B: Shoots proliferation in MS4 media after 4 weeks.

C: Abundant shoot proliferation when shoot clumps were cultured on MS2 media after 45 days.

D: Abundant shoot proliferation when shoot clumps were cultured on MS4 media after 45 days.

E: Different stages of *in vivo* germination of *Lilium bulblets*.

1- Explants after sterilization were placed in liquid medium supplemented with 0.05% PVP (Polyvinyl pyrrolidone) in 250 ml conical flasks, on an automated shaker at a speed of 25 rev/ second for 4,8,12 and 24 hours, respectively. The liquid medium was changed at 2 hours intervals. After the treatment the explants were washed thoroughly with distilled water and inoculated on to agar solidified medium.

2- Repeated subculturing was done to eliminate the effect of phenolics on the explants. Sub culturing was tried after 12, 24 and 48 hours of inoculation.

3- 0.1% PVP and 1% charcoal was added to MS medium and used for inoculation.

Minimum phenolic exudation occurred when explants after sterilization with 0.05% PVP in 250 ml conical flasks, on an automated shaker at a speed of 25 rev /second for 24 hours.

Both 3% sucrose and 6% sucrose were tried it was observed that 6% sucrose gave better result than 3 % sucrose. It was reported that the growth of bulblets depend on the sucrose concentration. (Yamagishi 1998, Kumar *et al*. 2005.).

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