Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna'

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Thesis

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in partial fulfilment of requirements for the award of the degree of

Master of Science in Horticulture
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Dedicated
To my
Parents

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Floriculture and Landscape Architecture

Certificate - I

This is to certify that the thesis entitled,"**Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna'"** submitted in partial fulfilment of the requirements for the award of the degree of**Master of Science in Horticulture (Floriculture and Landscape Architecture),** to the**Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir**is a recordof bonafide research work carried out by **Ms. Sadaf Rafiq (Regd. No. 2016-H-123-M)**under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged**.**

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We, the members of the Advisory Committee of **Ms. Sadaf Rafiq (Regd. No. 2016-H-123-M)**a candidate for the degree of **Master of Science in Horticulture (Floriculture and Landscape Architecture)** have gone through the manuscript of the thesis entitled**,"Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna'"**and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

This is to certify that the thesis entitled,"**Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna'"** submitted by**Ms. Sadaf Rafiq (Regd. No. 2016-H-123-M)**to the**Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir**in partial fulfilment of the requirements for the award of the degree of**Master of Science in Horticulture (Floriculture and Landscape Architecture)**was examined and approved by the Advisory Committee and External Examiner on …….

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Title of the Thesis	:	**"Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna'"**

ABSTRACT

The experiment was conducted during 2017- 2018 to standardize the micropropagation protocol of Oriental Lilium hybrid cv. 'Ravenna'. Explants tried included basal and tip segments of bulb scales. Highest culture asepsis was recorded in scale tip segments (63.54%) followed by basal segments (56.46%). Sterilant treatment combination containing carbendazim (200 ppm) for 30 minutes plus mercuric chloride 0.1% for 10 minute followed by ethyl alcohol 70% for 30 seconds was superior to all other treatments in recording highest culture asepsis (77.08%) and also yielded higher explant survival (86.12%). Explant survival was higher in basal segments (88.54%) compared to tip segments (85.52%). Sterilization with only mercuric chloride 0.1% for 10 minutesresulted in highest explant survival (95.48%) but lower culture asepsis (45.83%). Highest culture establishment was recorded in basal scale segments (68.26%) followed by tip scale segments (55.21%). MS medium supplemented with NAA + BAP (0.5 + 2.0 mg l^{-1}) recorded maximum culture establishment (76.17%), highest number of bulblets per explant (5.52) with maximum shoot length (2.20 cm) and leaf number

(3.39). MS medium supplemented with NAA + BAP (1.5 + 2.0 mg l^{-1}) recorded lowest number of days (13.52) to initiation of bulblets.

Highest shoot proliferation (83.33%) along with maximum shoot number (2.41explant^{-1}), shoot length (2.35 cm) and leaf number (5.44) of proliferated shoots was obtained under the treatment combination NAA + BAP (0.5 + 2.0 mg l^{-1}). Rooting of explants was superior with IBA compared to NAA. Lowest number of days to root initiation (17.95) was recorded in MS media fortified with IBA 2.0 mg l^{-1}. Highest rooting of 92.71% along with highest number of primary roots/shoot (12.06) and maximum length of primary roots (3.17 cm) was recorded in MS medium fortified with IBA 1.5 mg l^{-1}. Highest *ex vitro*survival (98.96%) of rooted plantlets during primary hardening in perlite + vermiculite (1:1) mixture was recorded in plantlets, which were rooted in MS media fortified with IBA 1.5 mg l^{-1}.

Keywords: Lilium, micropropagation, tissue culture, bulb scale

Signature of Student Signature of Major Advisor

Dated: _____ Dated:_____

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Sadaf Rafiq

Place: Shalimar, Srinagar

Dated:

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LIST OF ABBREVIATIONS

Abbreviation	Full form
BAP	6-Benzylamino Purine
BA	6-Benzyladenine
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KT	Kinetin (6-Furfurylamino purine)
LH	Lactalbumin hydrolysate
LS	Linsmaier and Skoog's (965) medium
MS	Murashige and Skoog's (1962) medium
NAA	Naphthalene acetic acid
PGRs	Plant growth regulators
psi	Pounds per square inch
TDZ	Thidiazuron
w/v	Weight per volume
v/v	Volume per volume
µm	Micro molar (concentration)

Chapter-1

INTRODUCTION

Lilium is a genus of herbaceous flowering plants growing from bulbs. It is a monocot belonging to the family Liliaceae. The genus *Lilium* consists of about 80 species (2n=2x=24) and has been classified into 7 sections. Many other plants have "lily" in their common name but are not related to true lilies like calla lily, day lily, belladona lily etc. *Lilium* is native to the Northern hemisphere. It is found widely distributed over the temperate and subtropical zones of the northern hemisphere including China, South Canada, Siberia and extends upto Florida in USA. In India, *Lilium* is found in Nilgiri hills and in the Himalayan region. Lilies have been gracing the gardens in various parts of the world for over 3000 years. Lilies are tall perennials ranging in height from 60–180 cm. The flowers are large, often fragrant and come in a wide range of colors including white, yellow, orange, pink, red and purple. Markings include spots and brush strokes. The plants are late-spring or summer-flowering. Flowers are borne in racemes or umbels at the tip of the stem, with six tepals spreading or reflexed, to give flowers varying shapes from funnel shape to a "Turk's cap". Naturally most cool temperate species are deciduous and dormant in winter in their native environment.

Lilium is one of the leading cut flowers of the world. It has become commercially important due to its bold, beautiful and fascinating form of flowers, long vase life and capacity to rehydrate after long transportation. Bulbs are produced commercially for use in the cut-flower and potted-plant industries. They are also used in herbaceous borders, woodlands and shrub plantings and as a patio plant. Lilies are one of the most demanded flowers in India. Each year hundreds of container loads of *Lilium* bulbs are imported to India by many traders for cut flower production by thousands of farmers/growers across India, to meet the domestic and export market.

Lilium is propagated by both sexual and asexual means. Cultivars are propagated by vegetative means to maintain genetic purity, while sexual method is used for the development of new cultivars. The natural vegetative propagules are small bulblets, either produced above ground on the stems (bulbils) or underground on bulb scales (Kumar *et al*., 2006). Most commercially grown cultivars are propagated through scaling, a technique which produces 3-4 bulbs from each bulbscale depending upon bulb scale size and variety. Though a bulb under ideal conditions may yield anywhere between 50 to 100 bulblets, this rate is far too low to meet the present day demand for planting material. Also, reduced vigour of bulbs with repeated cycles of vegetative propagation is reported which may be due to accumulation of soil borne diseases (Van Aartrijk and Blom-Bamhoom, 1983).

Numerous *Lilium* hybrid groups and forms have been developed through intra and inter specific hybridisation. In the first decade of 20th century in Japan and the US, first crosses within the section Sinomartagon were produced, resulting in the development of Asiatic *Lilium* hybrids. Asiatic hybrids remained the main product for more than 30 years (1970-2000). Meanwhile the oriental hybrids originating from crossing of species within Archelirion section became an important hybrid group in 1990s. Intersectional hybrids were developed due to improvement in pollination and embryo rescue techniques and polyploidization methods. First intersectional hybrid group LA (Longiflorum-Asiatic) was developed by intersectional/interspecific hybridisation between hybrids of different sections (the Longiflorum and the Asiatic group). Similarly other intersectional crosses mostly triploid hybrids have been bred which include LO (Longiflorum × Oriental), OT (Oriental × Trumpet) and OA (Oriental × Asiatic) hybrids. Besides the development of seven main hybrid groups (O, A, L, T, LA, LO and OT), a number of other species have been inter-bred successfully. The demand for new and improved cultivars is on the rise. Every year different companies all over the world develop hundreds of new cultivars for meeting the

growing demand of new cultivars at national and international levels. Intra and interspecific hybridisation or intersectional hybridisation followed by growing the seed to develop bulbs and their further multiplication is the usual procedure for developing of new cultivars. Scaling is fast method of multiplication of bulbs but the speed is nonetheless relatively slow and introduction of newly bred cultivars still requires a long period of time (Langens-Gerrits and De Klerk, 1999).

Tissue culture of Lily was started in the late 1950's (Robb, 1957). The successful use of tissue culture techniques for rapid propagation has been reported in various species and cultivars of *Lilium* 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 i*n vitro* scale culture (Bahr & Compton, 2004). *In vitro* adventitious bud regeneration from scales of *Lilium* depends on factors such as auxin, cytokinins (Varshney *et al.,* 2000), sucrose concentration (Jeong, 1996) and light treatment (Kumar *et al.,* 2005). In ornamentals, bulblets are produced using tissue culture techniques in tulip (Kuijpers and Langens-Gerrits, 1996), lily (Bahr and Compton, 2004), *Narcissus* (Staikidou *et al*., 2005), *Hyacinthus* (Takayama *et al*., 1991), *Muscari* (Saniewski and Puchalski, 1987), *Hippeastrum* (Ilczuk *et al*., 2005) and iris (Van der Linde and Schipper, 1992).

Micropropagation has the potency to produce large number of high quality plantlets in a short period of time (George *et al*., 2008). Basically, micropropagation in lily is just like scaling but carried out *in vitro* on an artificial nutrient medium. Also bulblets produced *in vitro* are preferable propagules that can be easily handled, transported and stored and they do not require an extensive acclimatization procedure after transfer to soil (Thakur *et al*., 2006). In micropropagation of *Lilium*, many tissues can be used but bulb scales are the favorite explants (Van Aartrijk and Van der Linde, 1986; Bahr and Compton, 2004; Han *et al*., 2005). Scales of lily bulbs are swollen petioles. Lily scale fragments cultured *in vitro* regenerate bulblets consisting of scales that may or

may not carry a leaf blade (Jasik and De Klerk, 2006). On the bulb scale explants, bulblets regenerate and after 8–10 weeks, these bulblets can be used for further propagation. Individual scales of bulblets, or parts thereof, are used as explant for the next propagation cycle and a cycle can be repeated every 8–10 weeks. Micropropagation offers large scale bulblet production throughout the year under controlled environmental conditions. Using tissue culture, from one large bulb, about one million small bulblets can be obtained in 2 years (Langens-Gerrits, 2003). Mass production and fast regeneration of uniform, disease-free plant material in tissue culture is a necessity for the breeding and culture of lilies. However, to make tissue culture a commercially relevant production system, production protocols need to be developed separately for each crop and cultivar.

Growers in Kashmir valley have opted for *Lilium* cultivation on a large scale. A number of cultivars imported from Holland in recent years have been grown for cut flowers. The number of Lilium growers in the valley is increasing because of higher returns from Lilium cultivation through cut flower production. To reduce the import of Lilium bulbs from European countries, fast multiplication of better performing cultivars is needed. This is possible only through tissue culture.

There is a need to develop our own cultivars to cater to local and regional market. To open up avenues for the development of new cultivars in *Lilium*, *in vitro* mutagenesis is the standard option. To achieve that, development of *in vitro* protocol for regeneration from scale is a pre-requisite. Therefore present study has been proposed with the following objectives:

i. To standardize explant sterilization protocol for *in vitro* establishment of *Lilium*.

ii. To standardize micropropagationprotocol of *Lilium*.

Chapter- 2

REVIEW OF LITERATURE

The literature available on the"Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. Ravenna"is reviewed here under various sub headings.

2.1 Explant type

A plant organ or a piece of tissue used to initiate a culture is known as explant and is the basic unit for starting *in vitro* culture. Many parts of the lily have regenerative capabilities, including stems or shoot tips (Sheridan, 1968; Bigot, 1974), leaves (Niimi and Onozawa, 1979), petals (Takayama and Misawa, 1979) and bulb scales (Robb, 1957; Hackett, 1969; Allen and Fernald, 1973; Stimart and Ascher, 1978). Bulb scales have been found most productive explant for *in vitro* propagation of Lilium by many researchers.

Allen and Fernald (1973) used bulb scale technique to demonstrate that a single lily bulb can generate over 1,000 plantlets. Stimart and Ascher (1978) speculated that 8,000 or more bulbs could be produced from 100 Easter lily scales with a slightly different bulb scale method.

Bulb scale segments of Lilium Oriental hybrid were used as explant by Simmonds and Cumming (1976), Stimart and Ascher (1978) and Chen (1983). They got success in establishment of the callus cultures using different growth regulators.

Takayama and Misawa (1979) found bulbscales of intact or *in vitro* produced bulblets of Liliumas the best explants for bulblet formation on MS agar medium containing NAA (1.0 mg/l) and BA (1.0 mg/l). Takayama and Misawa (1979) theorized that approximately 12.5 billion *Lilium speciosum* or 3 trillion *Lilium auratum* plants could be obtained in one year from a medium-sized bulb.

Takayama and Misawa (1980) found bulb scales of *Lilium auratum*Lindl. as the best explants for culture establishment, when MS agar medium was supplemented with 1.0 mg/l of NAA and BAP each. Novak and Petru (1981) used bulbscales from *in vitro* raised bulblets of *Lilium* cv. Crimson Beauty and found maximum multiplication rate on Linsmaier and Skoog medium supplemented with BAP (5 µM) and NAA (1 µM).

Chung *et al*. (1984) and Lee *et al*. (1994) found bulbil scales as promising explants for *in vitro* multiplication of *Lilium longiflorum* and *Lilium elegans* hybrids.Priyadarshi and Sen (1992) found young bulbscales of *Lilium longiflorum*Thunb. as the best explants for culture establishment and reported direct regeneration of bulblets from the adaxial surface of the explant in MS agar medium.

A continuous system of mass propagation of bulblets of two cultivars of Asiatic *Lilium*was achieved through *in vitro* scale formation (secondary explants) on MS medium (Varshney *et al*., 2000).

Han *et al*. (2005) cultured bulb scales of *Lilium* Oriental hybrid cv. Casa Blanca on MS medium supplemented with cytokinins (kinetin, BA and TDZ). Mirici *et al*.(2005) reported that the most widely used explant source of *in vitro* propagation of *Lilium* is bulb scale segments.

Amaury-M *et al*.(2007) observed that for bulblet induction in *Lilium maculatum* var. bukosanense, five scales from aseptic bulbs were used. Azadi and Khosh-Khui (2007) attempted *in vitro* scale culture of *Lilium ledebourii* (Baker), using bulbs from three harvesting seasons (spring, summer and winter).

Kumar *et al*. (2007) used bulb scales isolated from pre cooled bulbs of two lily hybrids 'Rosalta' and 'Marco Polo' to study the influence of growth regulators on *in vitro* plantlets formation and growth.

An experiment was conducted on *in vitro* propagation of *Lilium* at Biotechnology Centre, Udheywalla, SKUAST- Jammu. The explants used were bulb scales (Pandey *et al*., 2009).

Bulb scales and leaf segments were used as explants for *in vitro* production of *Lilium* Asiatic hybrid 'Prato' (El-Naggar *et al*., 2012). Basal segments of the scales of *Lilium longiflorum*measuring about 1cm x 1 cm were aseptically cut and each segment with the dorsal side in contact with the medium was placed in test tubes containing 15-20 ml of the culture medium (Mir *et al*., 2012).

In vitro propagation of *Lilium longiflorum* was carried out to explore its potential for micropropagation and callogenesis using meristem and twin scales explants. (Ali *et al*., 2013).*Lilium candidum* bulb scales were cultured on MS medium (Burun *et al.,* 2013).

Sindhu *et al*. (2016) conducted an experiment on *in vitro* propagation of Asiatic *Lilium* cv. Pollyanna using bulb scales as the explants.

Bhandari and Aswath (2018) carried out an effective protocol for *in vitro* culture of *Lilium longiflorum* T. cv. Pavia. Three different types of explants like leaves, outer and inner bulb scales were used. Among the three explants; inner scales performed better than other explants across all growth parameters.

2.2 Surface sterilization of explant

Simmonds and Cumming (1976) used 70 per cent ethanol for few seconds to surface sterilize the *Lilium*bulbscales followed by 10 per cent Javex treatment for 30 minutes.

Gupta *et al*. (1978) surface sterilized the bulbscales of *Lilium longiflorum*Thunb. in 0.2 per cent $HgCl_2$ solution for 15 minutes.

Stimart and Ascher (1978) used 95 per cent ethanol for 15 minutes followed by 0.5 per cent sodium hypochlorite treatment for 20 minutes for the surface sterilization of bulbscales of *Lilium longiflorum*Thunb.

Niimi and Onozawa (1979) disinfected the leaves of*Lilium rubellum* Baker with 70 per cent ethanol for one minute followed by 5 per cent calcium hypochlorite treatment for 5 minutes.

Van Aartrijk and Blom-Barnhoorn (1981) disinfected the bulb scales of *Lilium* with 1 % NaOCl solution for 30 minutes.

Novak and Petru (1981) suggested the use of 4% Sodium hypochlorite solution for 30 minutes to achieve surface sterilization of bulbscales and ovaries of *Lilium* oriental hybrid cv. Crimson Beauty.

Niimi (1984) surface sterilized the leaves, stem and flower buds of *Lilium rubellum* Baker by swabbing them with cotton wool containing 70% ethanol, followed by 6% sodium hypochlorite solution treatment for 15 minutes. Bulbscales were disinfected with 0.1 % $HgCl_2$ solution for 30 minutes.

Liu and Burger (1986) disinfected the pedicel sections of easter lily with 1% sodium hypochlorite solution for 10 minutes.

Priyadarshi and Sen (1992) first, washed bulbscales in 5% Teepol for 10 minutes and then immersed in 0.1 % $HgCl_2$ solution for 30 minutes to achieve the surface sterilization of bulb scales of *Lilium longiflorum* Thunb.

Okazaki *et al.* (1994) disinfected the ovaries of *Lilium*with 70% ethanol for 15 minutes.

Curvetto*et al.* (2006)compared traditional methodology of explant and media sterilization of *Lilium*with a protocol that included easily available elements to sterilize materials and culture media, together with addition of hydrogen peroxide (H_2O_2) into the nutrient media as chemical sterilizer. A series of H_2O_2 concentrations (0.005, 0.010, 0.015 and 0.020% p/v) was used to control

contamination during *in vitro* establishment and subsequent cultivation; the explant organogenic response was also examined and compared to the traditional micro-propagation technique. The level of culture contamination was within acceptable limits in all treatments, though it was higher in the H_2O_2 treatments (40%) compared to the traditional methodology (20%).

An experiment was conducted on *in vitro* propagation of *Lilium* at Biotechnology Centre, Udheywalla, SKUAST- Jammu. The explants (bulb scales) were surface sterilized with 0.1% $HgCl_2$ and 2% Bavistin for 7.5 minutes which resulted in 90.62 per cent of aseptic cultures and 97.32 per cent survival of explants (Pandey *et al*., 2009).

The middle scales of bulbs of *Lilium longiflorum* were separated and washed thoroughly under running tap water. The explants were surface sterilized with 70% ethanol for 30 seconds followed by 5% sodium hypochlorite for 10 minutes and washed 5-6 times with sterilized distilled water before culturing (Mir *et al*., 2012).

Liu *et al*. (2012) studied *in vitro* micro-propagation of L A hybrid lily cv. Eyeliner and found that the optimum treatment and disinfection method of bulb scales was soaking in 1:500 carbendazim solution for 30 min, disinfection in 75% alcohol for 10 to 60 s, disinfection in 2% NaClO solution for 15 min.

Sindhu *et al.* (2016) standardized *in vitro* culture media for efficient regeneration of bulb scales of Asiatic *Lilium* cv. Pollyanna. Pre-treatment with carbendazim (0.2%) + 8-HQC (200 ppm) + GA_3 (100 ppm) for 2 hours followed by surface sterilization with 0.1% mercuric chloride for 7 minutes resulted highest healthy cultures (80%).

Explants of *Lilium longiflorum* T. cv. Pavia were exposed to two sterilants: sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) with their varying concentrations; NaClO (2%, 3%, 4% and 5%) and H_2O_2 (0.05%, 0.10%, 0.15% and 0.20%). Among all treatments, NaClO @ 3% was highly effective

over different regimes of H_2O_2 for minimizing contamination (18.66%), low necrosis (2.67%) and maximum (79.13%) survivability (Bhandari and Aswath.,2018).

2.3 Media Composition, Establishment and proliferation of cultures

Simmonds and Cumming (1976) reported maximum callus production from bulbscales in 12 oriental hybrid cultivars of *Lilium*on MS medium supplemented with BAP (5 µM) and 2, 4-D (5 µM).

Stimart and Ascher (1978) recorded maximum number of bulblets from the basal bulb scale sections of *Lilium longiflorum*Thunb. on LS medium modified by Sheridan, supplemented with 0.03 or 0.3 mg/l NAA in continuous darkness at 25˚C.

Gupta *et al*. (1978) obtained maximum number of bulblets per explant when basal bulb scale explants of *L. longiflorum*Thunb. were cultured on MS agar medium supplemented with Adenine sulphate (25.0 mg/l), IAA (1.0 mg/l) and BAP (0.25 mg/l) incubated at 27 ± 1°C in 15 hours photoperiod.

Niimi and Onozawa (1979) induced bulblets from 5 mm leaf segments of *Lilium rubellum* Baker.on MS medium supplemented with 1.0 mg/l NAA and 0.1 mg/l BAP.

Takayama and Misawa (1980) reported maximum number of bulblets from bulbscales obtained from *in vitro* raised bulblets of *Lilium auratum*Lindl. on MS agar medium supplemented with NAA (0.1 mg/l) and Kinetin (1.0 mg/l) at 25˚C in continuous light conditions.

Novak and Petru (1981) regenerated maximum number of bulblets from basal scale portions of *Lilium* oriental hybrid cv. Crimson Beauty on LS medium containing 5 µM BAP, 1 µM NAA. On the same medium, they induced bud differentiation on the ovary and leaf derived explants in continuous dark.

Van Aartrijk and Blom-Barnhoorn (1981) regenerated bulblets from bulb scale explants of *Lilium speciosum* cultivars: Rubrum No-10,Pirate and Connecticut King when MS medium was supplemented with NAA (0.1 -1.0 mg/l) and BAP (0.01-0.10 mg/l). The cultures were incubated at 21 ± 1°C and 16 hours photoperiod.

Leshem *et al*. (1982) regenerated bulblets, roots and callus from ventral side of the bulbscales of *Lilium longiflorum*Thunb. cv. Onsat on LS medium containing 0.2 mg/l NAA in continuous light and/or dark conditions.

Paek and Chun (1982) obtained better bulblet formation on basal bulb scale tissues (4 bulblets per explant) of *Lilium longiflorum*on MS medium supplemented with 0.5 mg/l NAA or 0.5 mg/l NAA and 0.3 mg/l Kinetin.

Takayama and Misawa (1982) devised a scheme for mass propagation of Lilium *in vitro* and used bulbscales for culture establishment on MS medium containing 90 g/l sucrose, 0.1 mg/l NAA and 8 g/l agar at 25˚C in continuous light. He used *in vitro* raised bulblet scales of *Lilium auratum*Lind.,*Lilium speciosum*Thunb. cv. Uchida and *Lilium longiflorum*Thunb. cv. Hinomoto and found maximum multiplication rate on semi-solid MS medium supplemented with 3-10 mg/l Kinetin or BAP. This multiplication rate was increased by transferring the cultures on to the liquid medium without Kinetin.

Takayama and Misawa (1983) obtained highest multiplication rate from bulbscales of *in vitro* raised bulblets of *Lilium*on MS agar medium containing NAA (0.01 mg/l) and Kinetin (10.0 mg/l) with incubation at 25˚C in continuous light followed by transferring of the cultures to MS liquid medium containing NAA (0.1 mg/l) and incubated at 25°C on a rotary shaker (180 rpm) in continuous light.

Chung *et al*. (1984) reported the highest percentage of bulblets formation and maximum number of bulblets per explant from stem tissue explants of *Lilium longiflorum*on MS medium containing NAA (0.1 ppm) and Kinetin (0.1 ppm).

While with petal, ovary and anther tissue explants, the better results were obtained in MS medium with IAA (10.0 ppm) alone or with Kinetin (0.1 ppm).

Niimi (1984) regenerated bulblets from scales, stems and tepals of *Lilium rubellum*Baker on MS agar medium containing NAA (1.0 mg/l) and BAP (0.1 mg/l) and incubated in the dark at 24± 1˚C. He also used unexpanded leaves of *Lilium rubellum* Baker and cultured them for 120 days in darkness on a basal medium supplemented with 1.0 mg/l NAA and 0.1 mg/l BA to obtain bulblets.

Niimi (1985) reported maximum multiplication from *in vitro* raised bulbscales of *Lilium rubellum* Baker on MS agar medium containing NAA (0.05-0.1 mg/l) and/or BAP (0.01 - 0.1 mg/l).

Liu and Burger (1986) found that MS agar medium containing 5 µM BAP and 2 µM NAA resulted in the formation of maximum number of adventitious buds on pedicel sections of Easter Lily, when incubated at 26-28˚C temperature and 16 hours photoperiod.

Niimi (1986) used 5 mm long strips of basal regions of young leaves of six *Lilium* species and cultured on MS agar medium supplemented with NAA (0.05-1.0 mg/l) and BAP (0.01 -0.1 mg/l) under continuous darkness at 24± 1˚C. The effect of NAA and BAP concentration on percentage of leaf segments forming bulblets per explant was found to be species dependent.

Tomita *et al.* (1988) used bulbscale explants of Mid-Century hybrid cv. Kitanohoshi, *Lilium longiflorum* cv. Georgia, *Lilium speciosum* cv. Uchida, *Lilium regale* and *Lilium concolor* cv. Pulchellum.Explants were cultured on MS medium containing various concentrations of NAA and BAP. They found that the optimum concentrations of NAA and BAP for bulblet formation and *in vitro* rooting varied among the species.

Kruczkowska (1989) recorded the highest number of adventitious bulblets from scales obtained from *in vitro* regenerated bulblets on MS medium supplemented with 0.1 mg/l NAA in *Lilium*. He also reported that maximum

number of bulblets were developed when leaf explants were placed with the adaxial sides down on MS agar medium containing NAA (1.0 mg/l) and BAP (0.5 mg/l).

Panizza*et al*. (1990) reported maximum number of bulblets and leaves when the *in vitro* raised and multiplied bulblet scales of *Lilium speciosum* cv. Uchida and *Lilium longiflorum* cv. White American were cultured on LS agar medium supplemented with NAA (0.1 mg/l) and BAP (1.0 or 10 mg/l).

Maesato*et al*. (1991) reported direct bulblet formation from shoot apices of *Lilium japonicum*Thunb. on MS agar medium containing low concentration of NAA or 2, 4-D with incubation at 20˚C.

Dabrowski*et al*. (1992) reported the best bulblet regeneration, when bulbscales of *Lilium* cv. Sonnentiger were cultured on MS medium supplemented with NAA (0.1 mg/l) and BAP or KIN or 2-iP (0.1 mg/l) each.

Gi and Yeh (1992) reported maximum proliferation in Oriental*Lilium* hybrid cv. Casa Blanca when *in vitro* raised protocorms were cultured on MS agar medium supplemented with NAA (0.5 ppm) and BA (4.0 ppm).

Priyadarshi and Sen (1992) supplemented explants with NAA (5.37 µM) or IAA (5.68 µM) and BA (1.1 µM). Callus induction occurred on MS agar medium containing 2,4-D (4.5 µM) and BA(1.1 µM) and found MS agar medium containing NAA (5.37 µM) best for the multiplication of bulblets from callus and bulbscales in *Lilium longiflorum*Thunb.

Mizuguchi*et al*. (1994) obtained the best growth of callus of *Lilium japonicum* Thunb. on MS medium containing NAA (0.01 ppm) and BAP (0.1 ppm) with incubation at 25˚C in continuous light (3000 lux). MS medium without or with low concentrations of NAA and BAP was effective for the bulblet formation from the white callus.

Ishioka and Tanimoto (1994) recorded the highest bulblet differentiation (7.4 bulblets per explant), when leaf segments of *Lilium longiflorum* Thunb. were cultured on MS agar medium supplemented with NAA (10 µM) and BAP (10 µM).

Lee *et al*. (1994) reported maximum number of bulblets from the basal bulb scale explants in *Lilium elegans* hybrid on MS medium. Maesato*et al*. (1994) reported better multiplication when *in vitro* raised bulblet scales of *Lilium japonicum*Thunb. were cultured on MS agar medium supplemented with NAA (0.1 mg/l) and 2-iP (1 mg/l).

Joung (1995) reported best *in vitro*bulbing of *Lilium* oriental hybrid cv. Casa Blanca and *Lilium leichtlinii* var. tigrinum on MS agar medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) from basal bulbscale sections.

Niimi (1995) reported better results of bulblet regeneration from young leaf explants of *Lilium japonicum*Thunb. on MS medium containing NAA (0.1 mg/l) and BAP (0.01 mg/l).

Jeong (1996) studied the effect of NAA and BAP on multiplication using *in vitro* raised bulblet scales of *Lilium concolor* var. Partheneion, *Lilium amabile* and *Lilium callosum*; and found maximum multiplication on MS agar medium containing NAA (0.1 mg/l) and no BAP. In *Lilium concolor*var. Partheneion, 6.4 bulblets/explant were regenerated when supplemented with NAA (0.01 mg/l) and BAP (0.01 mg/l). In *Lilium amabile,* 3.8 ± 2.2 bulblets/explant were regenerated and in *Lilium callosum*, no pronounced effect of NAA and BAP was reported.

Dilta *et al*. (2000) reported *in vitro* effect of NAA and BA on culture establishment and bulblet formation in two hybrids of lily cv. Pollyanna and Star Gazer. Maximum leaves per bulblet were obtained when cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP.

A simple, rapid and cost-effective *in vitro* scheme for mass propagating two cultivars of Asiatic *Lilium* was developed. An average of seven bulblets were formed after 17 days, when 1×1 cm^2 bulb scale segments (explants) were cultured on Murashige and Skoog medium with 3% sucrose and 0.5 μM NAA. On MS medium containing 0.5 Mm NAA and 6 or 9% sucrose, depending on the cultivar, large numbers of bulblets of increased size (3.5–5.0 cm in circumference) were formed under a 16/8 h photoperiod. A continuous system of mass propagation of bulblets was achieved through *in vitro* scale formation (secondary explants) on MS medium supplemented with 23 μM kinetin and 0.5 μM NAA, as well as scale proliferation on MS basal liquid stationary medium. Upon transplantation, all bulblets sprouted, of which 40% flowered in the first season. Under ideal conditions, ca. 9.68×10^5 bulblets can be produced from a single scale segment in one year by following the systematic propagation steps proposed by Varshney *et al*. (2000).

Bahr and Compton (2004) investigated competence for *in vitro* bulblet regeneration for eight diverse *Lilium* genotypes (*Lilium cv.* Citronella, *Lilium* cv. Stargazer, *Lilium* cv. Stones, *Lilium* cv. Lovely Girl, *Liliumpumilum*, *Lilium lancifolium*, *Lilium lancifolium*cv. Orange Star, and *Lilium speciosum* var. rubrum). Explants established from bulb scales were cultured on lily bulblet regeneration medium [Murashige and Skoog medium modified by reducing the NH_4NO_3 to 0.825 g/l and KH_2PO_4 to 0.170 g/l , and adding (per liter) 30.0 g sucrose, 0.1 gmesoinositol, 0.4 mg thiamine-HCl, 80.0 mg adenine sulfate, 6.0 mg/l naphthalene acetic acid, 2.0 ml/l Plant Preservative Mixture (PPM), and 4.5 g/l Agar Gel at pH 5.7 for 8 weeks before transfer to sphagnum peat moss and 4 weeks refrigeration at 5 ˚C in darkness.

Han *et al*. (2005) cultured bulb scales of *Lilium* Oriental hybrid cv. Casa Blanca on MS medium supplemented with cytokinins (kinetin, BAP and TDZ) containing 2.2 μM BAP which was most favorable in the shoot formation from bulb scales. Cutting shoots into small segments (7-8 mm wide × 12-15 mm

length) were cultured on media containing BAP and IAA (indole acetic acid) and the segments regenerated many new shoots and formed shoot clusters. The media supplemented with 4.4 µM BAP and 5.7 µM IAA or 8.9 µM BAP and 0.6-2.9 µM IAA were effective in allowing the proliferation of shoots from shoot segments under light condition. Sucrose and activated charcoal (AC) improved bulblet growth. Bulblet growth was effectively performed on MS medium containing 60.0 g/l sucrose. Bulblet growth was also improved by the supplementation of activated charcoal. The medium with 2.0 g/l AC was most effective for bulblet growth. Normal bulblet growth was stimulated more by the culture of shoots than that of bulb scales. Bulblet weight from shoots reached to an average of over 1100 mg of a bulblet after 3 months in culture on MS medium containing 60.0 g/l sucrose and 2.0 g/l AC. Most of the bulblets produced by this method generated stems with several leaves in the green house, after cold treatment at 5 °C for 3 months.

Kaur *et al*. (2006) developed a low cost protocol for *in vitro* propagation of *Lilium* cv. Toscana through incorporation of cost-effective media components. MS medium supplemented with 0.75 mg/l BAP and 0.5 mg/l NAA was prepared with tapioca granules, table sugar and tap water in different combinations in place of agar-agar, sucrose and distilled water, respectively. Culture medium containing all the cost effective components was found to be the best for *in vitro* establishment of cultures yielding 6.00 bulblets per explant and medium supplemented with tapioca granules as cost effective component was found to be the best for *in vitro* multiplication of bulblets giving 3.70 bulblets per *in vitro* formed bulblet within five weeks from third subculture.

Lilium longiflorum shoots were cultured on ½ MS medium supplemented with 1.0 g/l activated charcoal, 30.0 g/l sucrose and 8.0 g/l agar and Nhut *et al.*(2006) examined the factors influencing the regeneration ability and *in vitro* plantlet growth of *Lilium* sp. and found that number of regenerated shoots were highest on MS medium supplemented with 0.2 mg/l NAA and 30.0 g/l sucrose.

For bulblet induction in *Lilium maculatum* cv. Bukosanense, five scales from aseptic bulbs were used for each treatment and placed in the MS (half-strength) medium, supplemented with sucrose 3 %, agar 0.8 % to ascertain the effect of two plant growth regulators (PGR): α-naphthalene acetic acid (NAA) at 0.0, 1.0, and 2.0 mg/l, in combination with thidiazuron (TDZ) at 0.0 and 0.5 mg/l. Two illuminating conditions (light or darkness) and the presence or absence of activated charcoal (AC) in the medium were also tested. Explants cultured under light and AC did have a favorable effect on bulblet induction. The average number of induced bulblets under light condition was the double (4 per explant) than those induced under darkness, both in presence of AC, irrespective of PGR level. The best treatments were those with (NAA 2.0 mg/l + TDZ 0.5 mg/l), and without PGR, all of them with AC and under light. After 12 weeks of culture, an average of 2.4 well-formed bulblets were obtained per responsive explant. Six months after culture initiation, more than 640 regenerated plantlets from 320 explants, with roots and leaves were acclimatized and transferred to greenhouse conditions with 100 % success of survival(Amaury-M *et al*., 2007).

Azadi and Khosh-Khui (2007) developed a protocol for the micropropagation in different harvesting time of *Lilium ledebourii* (Baker)\\\\\\\\, an endangered rare species endemic to Iran. Among the various treatments tested, the Murashige and Skoog (MS) medium supplemented with 0.1 mg/l naphthalene acetic acid (NAA) + 0.1 mg/l BAP and 6% sucrose in all harvesting seasons proved to be superior to others.

Kumar *et al*. (2007) used the basal portion of inner bulb scale of two lily hybrids cv. Rosalta and Marco Polo which was cultured on MS medium containing 0.5 mg/l NAA or BAP. The presence of 0.5 mg/l NAA showed higher explant regeneration, producing about 3 bulblets per explant as compared to the control.

Kapoor *et al.* (2008) conducted an experiment in hybrid lilies in which different levels of NAA and BA were used to examine their effect on bulblet

regeneration. The most effective treatment (2 mg/l NAA and 1.5 mg/lBAP) was selected to compare bulblet productivity in different explants in the five hybrid lily cultivars. The bulblet formation was highest (78.64%) in the bulbscale explants. Average number of 3.1 bulblets per explant was recorded in root explants followed by node, bulbscale, leaf and internode. Bulblets formed in the bulb scale explants were the largest with an average fresh weight of 350.6 mg. Node explants of cultivar 'Beartix' produced the greatest number and the largest bulblets. The bulblets stored at 2°C for 30 days in cocopeat survived with 80-82% success.

In vitro bulb scales of seven Asiatic hybrid lily (*Lilium* ×*elegans*Thunb.) lines ('12', '20', '599', '409', '00144', '00150' and '00151'), differing for scale size (large and small), were cultured on MS medium supplemented with 8.8 µM BA and 1.1 µM NAA to induce adventitious shoot formation. After 6 weeks, the frequency of shoot formation was very high in almost all genotypes (>94%); new shoots were obtained from the basal part of bulb scales and became shoot clusters. In the third subculture different combinations of BA (2.2 - 8.8 - 17.6 µM) and NAA (1.1 - 2.2 µM) were used to examine the effect on shoot cluster regeneration. The media supplemented with 2.2 µM BA or 8.8 µM BA and 2.2 µM NAA were the most effective to promote shoot proliferation, shoot length and root growth. Moreover, the medium supplemented with 8.8 µM BA and 2.2 µM NAA was most effective to increase the biomass. In all subcultures, the hybrids '00150', '00144' and '20' showed the highest values for shoot number, fresh weight, dry weight, shoot length and growth index. Going from first to third subculture, shoot cluster cycle method improved adventitious shoot formation and increased the biomass (Cardona Suarez *et al*., 2011).

Liu *et al*. (2012) studied *in vitro* micro-propagation of L A hybrid lily cv. Eyeliner. The optimum medium for bud induction was MS + 0.5 mg/l 6-benzylaminopurine+ 0.1 mg/l naphthalene acetic acid + 90.0 g/l sucrose, at 25°C and in darkness. The optimum medium for bulblet induction was 2MS + 1.0 mg/l

BAP + 0.5 mg/l NAA + sucrose 90.0 g/l + Paclobutrazol (PP333) 2.0 mg/l. The optimum culture condition for bulblet induction was 20°C, 14 h/day lightness + 10 h/day darkness.

Skoric*et al*. (2012) developed a rapid, one-step method for direct leafy and rooted bulblet regeneration of *Lilium martagon* var. Cattaniae Vis. by using seeds as the starting explants for *in vitro* culture initiation. Adventitious bulblets were regenerated from one scale explants on MS (Murashige and Skoog, 1962) medium supplied with various concentrations of plant growth regulators. The most efficient medium for multiplication was MS medium supplemented with 0.2 mg/l BAP and IAA ranging from 0.25 to 2.0 mg/l. On average, five bulblets were obtained per explant after six weeks in culture.

El-Naggar*et al*.(2012) established a protocol for *in vitro* production of *Lilium* Asiatic hybrid cv. Pratousing two explants for shoot proliferation and organogenesis. Explants were cultured on Murashige and Skoog (MS) basal medium supplemented with benzyl adenine (0.25, 0.5, 1.0 and 2.0 mg/l) and naphthalene acetic acid (0.25, 0.5 and 1.0 mg/l). Callus was formed over the bulb scales before shoot organogenesis occurred, while shoot organogenesis occurred directly from the leaf segments without callus formation. It was found that the bulb scales gave higher percent of shoot regeneration than leaf segments when used as explants and was 96.67 and 64.67%, respectively. BA at 0.5 mg/l gave the highest percentage of shoot formation, shoot height and the lowest number of days to proliferation, while BA at 2.0 mg/l caused a delay in shoot organogenesis and reduced shoot height in both explants.

For shoot induction and multiplication in *Lilium longiflorum*, scales of bulblets were inoculated on MS medium supplemented with different concentrations of BA (Benzyl adenine) 0.0–2.0 mg/l individually or in combination with 0.0–2.0 mg/l NAA (á- naphthalene acetic acid). The shoots induced were sub cultured onto the fresh MS medium with same plant growth regulators after every four weeks (Mir *et al*., 2012).

In vitro propagation of *Lilium longiflorum* on MS medium supplemented with 2 mg/l of BAP was found to be best for shoot formation from meristem, with 84% shoot formation within 12 days of meristem inoculation while in case of twin scales, 88% shoot initiation response was obtained on MS medium containing 1.5 mg/l of BAP with 0.5 mg/l of NAA after 12 days of inoculation. Maximum shoot multiplication response was obtained at 3 mg/l of Kinetin with an average of 4.8 shoots per culture after 8.2 days of inoculation (Ali *et al*., 2013).

Aslam *et al*. (2013) developed a protocol for micropropagation of *Lilium orientalis* and *Lilium longiflorum* cv. White Fox. It was observed that among different treatments used for culturing of the plant, the MS medium supplemented with 6-benzylaminopurine (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 shoot multiplication. Out of different concentrations, medium supplemented 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 ± 3.94 shoots/explants were obtained.

Lilium candidum bulb scales were cultured on MS medium containing different doses and combinations of NAA, BA, Kn and 2iP. Maximum bulblet formation (88.2%) was observed in MS supplemented with 0.1 mg/l NAA + 0.01 mg/l BA and the average number of bulblets per explant was 2.9 (Burun *et al.,* 2013).

Jamwal *et al*. (2016) carried out *in vitro* propagation experiments to study the effect of media, genotype and explant on micropropagation of three cultivars (Prato, Brunello and Dreamland) of Asiatic *Lilium* hybrids. MS basal medium supplemented with 1.0 mg/l NAA induced best culture establishment and bulblet multiplication in all the three cultivars.

Sindhu *et al*. (2016) conducted an experiment on *in vitro* propagation of Asiatic *Lilium* cv. Pollyanna. MS medium supplemented with 0.2 mg/l NAA and

1.0 mg/l BAP was the most effective combination for initiation and shoots proliferation.

Ghanbari*et al*.(2017)investigated establishment of bulb explants of *Lilium ledebourii*Bioss on MS (Murashige and Skoog) mediumwith three concentrations which include: medium lacking the growth regulators, medium with NAA (0, 0.5, 1, 1.5 mg/l) and finally a medium with BAP (0, 0.5, 1, 1.5, 2 mg/l). Only in one treatment i.e., hormone free media bulbing and rooting have been observed. The largest number and percentage of bulb and also the largest number and percentage of root were obtained from the medium lacking the growth regulators.

Huong *et al*.(2017)conducted an experiment for optimizing micropropagation protocol of *Lilium*. The results indicated that *in vitro* type 1 Lily scales (near the basal stem) cultured on MS medium supplemented with 60 g/l saccharose, 0 g/l glucose, 0.5 mg/l BAP, 0.1 mg/l NAA, 100 ml/l coconut water, 5.5 g/l agar and 1.0 g/l activated charcoal in full dark conditions was the best with the highest regeneration rate of bulb scale (3.68 bulblets/slice).

Biswas *et al*. (2017) attempted to lower the cost of the *in vitro* micropropagated plants of different varieties of Asiatic *Lilium* and developed an efficient low cost medium "KFA and KFA plus" (Flyash" as the main source of inorganic constituent; Patented), which could replace the widely used expensive Murashige and Skoog's medium. The comparison was done on four major criteria: % bud break, % shooting, % rooting and cost. When cultured on KFA and KFA plus, 70% bulblet formation was observed in KFA and 86.6% bulblet formation in KFA plus as compared to 83.3% in MS medium supplemented with IAA (0.8mg/l) + BAP (1.5mg/l). Healthy response and an average of 1.6 bulblets/explant were obtained. Cost of media was reduced 10 times by using KFA plus as culture media as compared to MS ready media (Hi media, India).

For *Lilium martagon*, the multiplication rate, in term of bulblets/explant, was best obtained with the use of WPM salts, with a temperature of 18°C and a

sucrose concentration of 30 g/l; *Lilium pomponium* propagated better in the presence of MS salts supplemented with 30 g/l of sucrose and 0.5 mg/l of IBA, at 24°C with a photoperiod of 16 hours of light. Results showed that in order to obtain large bulbs,it is preferable to use salts, at a temperature of 24°C, with high IBA and sucrose concentration. The *in vitro* growth in darkness in *Lilium pomponium* ensured higher bulb weight; after 6 months of *ex vitro* growth, it was possible to obtain a minimum of 57% to a maximum of 84.5% of plant survival, in a very good growth condition (Mascarello*et al*., 2018).

Bhandari and Aswath (2018)recorded highest bulblet induction (4.66) and maximum bulblet diameter (7.85 mm) of *Lilium longiflorum* T. cv. Pavia in Murashige and Skoog (MS) medium supplemented with BAP (0.5 mg/l) and NAA (0.3 mg/l). The maximum length of shoots (38.33 mm) and fresh weight (239.51 mg) were recorded with BAP (1.0 mg/l) and NAA (0.1 mg/l).

2.5 Rooting of *in vitro* shoots

Simmonds and Cumming (1976) obtained *in vitro* rooting of the callus derived bulblets of *Lilium* oriental hybrid on MS agar medium supplemented with NAA (0.5 µM).

Stimart and Ascher (1978) reported best rooting *in vitro*of *Lilium longiflorum* Thunb., when MS medium was supplemented with 0.3 mg/l NAA and cultures were incubated at 25˚C in dark or alternative dark and light.

Takayama and Misawa (1979) obtained *in vitro* rooting of bulblets of *Lilium speciosum*on MS medium containing NAA (0.01 -1.0 mg/l).

Novak and Petru (1981) obtained rooting of bulblets of*Lilium* oriental hybrid cv. Crimson Beauty on LS medium without hormones or with 1-5 µM NAA.

Liu and Burger (1986) reported best rooting of the pedicel derived shoots of *Lilium longiflorum* on MS medium containing IAA (10 µM) or IBA (10 µM).

Tomita *et al*. (1988) reported that *Lilium* species depend on appropriate concentration of NAA and BA in medium for *in vitro* rooting.

Maesato*et al*. (1991) found that shoot apices derived bulblets of *Lilium japonicum* Thunb. developed better roots on MS medium containing NAA (2.66 µM or 5.36 µM). Dabrowski*et al*. (1992) reported best rooting of bulblets of *Lilium* cv. Sonnentiger on MS medium supplemented with NAA (0.1 mg/l).

Gi and Yeh (1992) found maximum rooting in bulblets on hormone free MS medium in *Lilium* cv. Casa Balanca. Priyadarshi and Sen (1992) obtained the best *in vitro* rooting of the callus derived plantlets/bulblets of *Lilium longiflorum* Thunb. on MS agar medium containing NAA (5.37 µM).

Mizuguchi *et al*. (1994) obtained better rooting of the callus derived bulblets when MS medium was supplemented with 1 ppm NAA in *Lilium japonicum*Thunb.

Mizuguchi and Ohkawa (1994) reported optimum growth and rooting of bulblets of *Lilium japonicum*on MS medium supplemented with NAA (0.1 ppm) and BAP (0.01 ppm).

Tapioca supplemented MS medium containing 1.0 mg/l NAA was significantly better than all the other modified media giving 86.62% *in vitro* rooting, 2.86 average root number and 4.60 cm average root length in *Lilium* cv. Toscana (Kaur *et al*., 2006).

The individual bulblets of *Lilium* were separated out and transferred for rooting in MS medium supplemented with NAA (1.0 mg/l). This gave 97.32 per cent rooted cultures with maximum root length of 1.66 cm (Pandey *et al*., 2009).

The optimum medium for rooting culture in L A hybrid cv. Eyeliner was ½ MS + 0.8 mg/l NAA + 3.0 g/l activated charcoal, 20°C, and 14 h/day lightness + 10 h/day darkness (Liu *et al*., 2012).

The *in vitro* produced bulblets of *Lilium longiflorum* were separated and individual bulblets were transferred to MS media supplemented with 0.5, 1.0, 1.5 and 2.0 mg/l IBA or NAA after 30 days of third sub-culture. Maximum rooting (93%), root number (19) and root length (5.2 cm) was recorded with 2.0 mg/l NAA(Mir *et al*., 2012).

The best *in vitro* rooting response of *Lilium longiflorum* was obtained on MS medium supplemented with 1.5 mg/l of NAA. An average of 5.2 roots were formed after 12 days of inoculation with an average root length of 1.76 cm (Ali *et al*., 2013).

Aslam *et al*. (2013) observed that *Lilium orientalis* and *Lilium longiflorum* cv. White Fox had well-developed root and bulblet formation on the medium supplemented with 0.1 mg/l BAP + 0.1mg/l NAA and 6% sucrose.

Best rooting of *in vitro* derived bulblets of three cultivars (Prato, Brunello and Dreamland) of Asiatic *Lilium* hybrids was recorded in modified MS medium supplemented with 1.0 mg/l NAA and 90 g/l sucrose(Jamwal *et al*., 2016).

Sindhu *et al*. (2016) achieved best rooting (100%) in Asiatic *Lilium* cv. Pollyanna on half strength MS medium supplemented with 1.0 mg/l IBA.

Biswas *et al*. (2017) observed healthy rooting in 70% bulblets of Asiatic *Lilium* cultured on KFA plus medium supplemented with same concentration of PGR as compared to 50% rooting in MS medium.*In vitro* Lily bulblets became healthy and bigger, formed roots in MS medium supplemented with 0.2 mg/l NAA (Huong *et al*., 2017).

The maximum numbers of basal roots (5.83 /bulblets) of *Lilium longiflorum* T. cv. Pavia were obtained with 0.3 mg/l NAA. However, growth regulator combination of BAP (1.0 mg/l) and NAA (0.3 mg/l) recorded maximum(49.00 mm) length of basal roots (Bhandari and Aswath., 2018).

2.6 Hardening

The commercial worth of any micropropagation system depends upon the ease and efficiency with which the plantlets can be established in soil. The transfer of plantlets from the culture to soil requires meticulous and careful stepwise procedure.

Simmonds and Cumming (1976) found that callus derived plantlets of*Lilium* hybrids could he readily transferred into soil and the losses incurred during transplanting/transfer were only 5 per cent.

Novak and Petru (1981) reported that the rooted bulblets of *Lilium* oriental hybrid cv. Crimson Beauty were directly transferable into soil.

Takayama and Misawa (1983) suggested that *in vitro* produced bulblets of *Lilium auratum* should be exposed to 5˚C to break dormancy before transplanting into soil.

Liu and Burger (1986) suggested that the rooted plantlets of *Lilium longiflorum*Thunb. can be transplanted in the potting soil and grown in the green house.

Gong *et al*. (1996) reported 80-90% survival rate of tissue cultured plantlets of *Lilium* hybrid, when transplanted in the potting mixture of sand, peat and humus soil (1:1:1 v/v).

For hardening of *in vitro* rooted bulblets of *Lilium* cv. Toscana; coco peat, peat moss and coco peat in combination with peat moss were found to be at par giving 100% survival(Kaur *et al*., 2006).

The bulblets of *Lilium ledebourii* (Baker) Boiss, at the end of the culture period were given cold treatment at 4°C for 2-8 weeks at a 2-weeks interval and then transplanted to a potting mixture of sand, leaf mold and peat moss (1:1:1 v/v). The best emergence rate (90%) was achieved at 8 weeks cold treatment for winter harvested bulbs(Azadi and Khosh-Khui., 2007).

After achievement of sufficient number of roots, the rooted plantlets of *Lilium longiflorum*were taken out of the culture vessels, washed with water to remove the adhering agar and treated with 0.2% bavistin (Carbendazim) for 10 minutes and were finally transferred to the pots containing cocopeat or soilrite:soil. Potted plantlets were covered with transparent polythene bags to ensure high humidity and watered every 3- 4 days with half strength MS salt solution for 2 weeks. Polythene bags were gradually perforated after 4 weeks and were removed after 8 weeks in order to acclimatize plants to field conditions. After 8 weeks, acclimatized plants were transferred to greenhouse, where ambient conditions of temperature and humidity were maintained (Mir *et al*., 2012).

Upon rooting, about 200 plantlets of *Lilium martagon* var. Cattaniae were successfully hardened in the green house with a 95% survival rate. Preliminary experiments indicated that the plantlets from the greenhouse could be successfully used for field cultivation (Skoric*et al*., 2012).

Well developed *in vitro* plants of *Lilium longiflorum* were shifted for hardening on different media (sand, silt, peat, sand+ peat and sand+ silt+ peat in the ratio of 1:1, 1:1:1 respectively). Maximum hardening response (100 %)was recorded in Peat (Ali *et al*., 2013).

The potting mixture of vermiculite + FYM (1:1) proved to be the best for survival of transplanted plantlets of three cultivars (Prato, Brunello and Dreamland) of Asiatic *Lilium* hybrids (Jamwal *et al*., 2016).

Chapter – 3

MATERIALS AND METHODS

The present studies entitled "**Standardization of micropropagation protocol of Oriental *Lilium* hybridcv. 'Ravenna' "** was carried out in the Plant Tissue Culture Laboratory of The Division of Floriculture and Landscape Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, during 2017 and 2018. Salient features of the experiments carried out in present investigation involving the study of different types of explants, media, and hormonal regimes for *in vitro* culture in Lilium are summarized in Table 3.1. Materials used and details of methodologies followed in the foregoing study are presented in the following sections.

3.1 MATERIALS

3.1.1 Stock Plants

Mature, flowering sized bulbs of Oriental LiliumHybrid cv. 'Ravenna' were used as explant source in the current study (Plate-1). Bulbs were stored in trays filled with moist coco-peat and kept in store during winter.

3.1.2 Chemicals

The major and minor elements, sucrose, agar, organic compounds like amino acids, vitamins and plant growth regulators required for preparation of media during the present investigation were procured from Hi-media, India.

3.1.3 Glassware

The borosilicate glassware used in the experiments was procured from Borosil. Glassware was washed with detergent followed by thorough washing with tap water. The glassware was then dried in hot air oven at 100 ˚C for 24 hours before use.

Oriental Lilium hybrid cv. “Ravenna”

Plate-1: Lilium cultivar selected for the investigation

3.1.4 Culture media

Murashige and Skoog (1962) media was used during the course of present investigation (Table 3.2).

3.2 METHODS

3.2.1 Preparation of stock solutions

Different types of stock solutions of macro elements, micro elements and organics were prepared and stored in reagent bottles for use. Usually four types of stock solutions (A-D) are made for Murashige and Skoog (1962) basal medium. However, for longer storage, seven types of stock solutions (A, B, C, D, E, F, and G) of Murashige and Skoog (1962) are generally prepared in The Plant Tissue Culture Laboratory of The Division of Floriculture and Landscape Architecture and same was followed in the current investigation (Table-3.3). During the entire investigation period, 200 ml of each stock solution was prepared each time which was sufficient for making 20 litres of medium. For making one litre of medium, 10 ml of each stock solution was added to 600 ml of double distilled water in a beaker and final volume made to one litre by adding double distilled water.

Seven stock solutions were used in the preparation of MS medium. Stock solution (A/B/D) containing only one chemical was prepared by dissolving required quantity of each chemical (quantity of chemical required for making 20 litres of medium) in 150 ml of double distilled water and final volume made to 200 ml with double distilled water. Thus stock solution with strength of 100 X was obtained. Stock solution containing more than one chemical (C/E/F/G) was prepared by weighing the required quantity of each chemical (quantity of chemical required for making 20 litres of medium) separately and dissolved to the last particle in double distilled water followed by mixing them together slowly with continuous stirring. Final volume of 200 ml of the mixture was made by addition of double distilled water. Stock solutions were stored in corning reagent bottles at 4^{o}C.

The required quantity of cobalt chloride was too small (0.5 mg) to be weighed properly on the available digital balance. This quantity was multiplied by 100 and the quantity obtained (50 mg) was dissolved in 100 millilitres of water. Then one millilitre of this solution was added to stock solution C and final volume of 200 ml was made by adding double distilled water. Similar method was used for adding required quantity of cupric sulphate and thiamine to their respective stock solutions.

For the preparation of the iron stock solution (stock solution F), required quantities of $FeSO_4.7H_2O$ and Na_2EDTA were dissolved separately in 75 ml double distilled water. Na_2EDTA solution was heated over hot plate and stirred to dissolve it completely. It was then added to $FeSO_4.7H_2O$ solution gently and stirred well to get a homogeneous light yellow solution. Final volume (200 ml) of the mixture was made by adding double distilled water and stored in an amber-coloured reagent bottle.

Table 3.1: Summary of experiments conducted for the development of micro propagation protocol in Oriental Lilium Hybrid cv. 'Ravenna'

Name of experiment		Design	Factors with level of each factor	Table No.
Sterilization	Culture asepsis and explant survival as affected by sterilants and explant type	CRD factorial	Sterilants (10) Explants (2)	3.4
Establishment	Establishment of explants as effected by PGR's	CRD factorial	PGR combinations (8) Explants (2)	3.5
Proliferation	Shoot proliferation in explants as influenced by PGR's	CRD factorial	PGR combinations (6) Explants (2)	3.6
Rhizogenesis	Rhizogenesis as influenced by PGR's	CRD factorial	PGR combinations (8) Explants (2)	3.7
Hardening	*Ex vitro* survival of rooted plantlets as influenced by PGR's	CRD factorial	PGR combinations (8) Explants (2)	3.7

Table 3.2: Chemical composition of Murashige and Skoog (1962) medium

Components	Chemical formula	Quantity (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride, dihydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	370
Potassium phosphate, monobasic	KH_2PO_4	170
Micronutrients		
Potassium iodide	KI	0.83
Boric Acid	H_3BO_3	6.2
Manganese sulfate, tetrahydrate	$MnSO_4.4H_2O$	22.3
Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6
Sodium Molybdate, dihydrate	$Na_2MoO_4.2H_2O$	0.25
Cupric Sulfate, pentahydrate	$CuSO_4.5H_2O$	0.025
Cobaltous chloride, hexahydrate	$CoCl_2.6H_2O$	0.025
Ethylenediaminetetraacetic acid disodium salt	$Na_2EDTA.2H_2O$	37.3
Ferrous sulphate, heptahydrate	$FeSO_4.7H_2O$	27.8
Organic supplements		
Myo inositol	$C_6H_{12}O_6$	100.00
Glycine	$C_2H_5NO_2$	2.00
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine hydrochloride	$C_8H_{11}NO_3.HCl$	0.5
Thiamine hydrochloride	$C_{12}H_{17}CIN_4SO.HCl$	0.1

Table 3.3: Composition and preparation of stock solutions for Murashige and Skoog(1962) medium

Stock	Components	Chemical formula	Qty. mgl^{-1}	Qty. for 20 litres	Qty. of stock solution	Conc. of stock solution
A	Ammonium nitrate	NH_4NO_3	1650	33.00 g	200 ml	100 X
B	Potassium nitrate	KNO_3	1900	38.00 g	200 ml	100 X
C	Potassium iodide	KI	0.83	16.6 mg	200 ml	100 X
	Boric acid	H_3BO_3	6.2	124 mg		
	Potassium phosphate, monobasic	KH_2PO_4	170	3.4 g		
	Molybdic Acid (Sodium Salt)•$2H_2O$	Na_2MoO_4. 2 H_2O	0.25	5 mg		
	Cobaltous chloride hexahydrate	$CoCl_2.6H_2O$	0.025	0.5 mg		
D	Calcium chloride, dehydrate	$CaCl_2 2H_2O$	440	8.8 g	200 ml	100 X
E	Magnesium sulphate, heptahydrate	$MgSO_4.7H_2O$	370	7.4 g	200 ml	100 X
	Zinc sulphate, heptahydrate	$ZnSO_4.7H_2O$	8.6	172 mg		
	Manganese Sulfate, tetrahydrate	$MnSO_4.4H_2O$	22.3	446 mg		
	Cupric Sulfate pentahydrate	$CuSO_4.5H_2O$	0.025	0.5 mg		
F	Ethylenediaminetetra acetic acid disodium salt	$Na_2EDTA.\ 2H_2O$	37.3	746	200 ml	100 X
	Ferrous Sulfate heptahydrate	FeSO4.$7H_2O$		556 mg		
G	Glycine	-----	2.00	40 mg	200 ml	100 X
	Nicotinic acid	-----	0.5	10		
	Pyridoxine HCl	-----	0.5	10		
	Thiamine HCl	-----	0.1	2		

3.2.2 Preparation of stock solutions of growth regulators

Basal medium was supplemented with different types of growth regulators. Small quantity of Stock solution (50 ml) of each growth regulator was prepared on weekly/monthly basis and stored in refrigerator at $4^{o}C$. Stock solutions of benzyl-aminopurine (BAP), kinetin, naphthalene-acetic-acid (NAA) were prepared by dissolving 50 mg of each growth regulator in 25 ml of 0.50 N NaOH and then volume was made to 50 ml with double distilled water. However, 2, 4-D, indole-3-aceticacid (IAA) and indole-3-butyric-acid (IBA) were prepared by dissolving 50 mg of each growth regulator well with some alcohol drops and then volume was made to 50 ml with double distilled water.

3.2.3 Preparation of culture media

The required quantity of sucrose (usually 30 g/l) and myo-inositol (100 mg/l) was dissolved in 500 ml of double distilled water in a beaker. 10 ml of each stock solutions (A, B, C, D, E, F, G) were poured into the beaker. Required quantity of growth regulators was added as per the treatment requirement. Final volume of the medium was made with the addition of double distilled water. The pH of medium solution was adjusted at 5.7 by adding 1N NaOH or 1N HCl drop wise with a micropipette. Care was taken that electrode does not come in direct contact with the walls of beaker and bulb of electrode remains completely immersed in the solution. The medium was slightly heated in microwave oven and then required quantity of agar was added, stirred well with scalpel or stirrer and again heated in microwave oven to boil so as to dissolve the agar completely. The medium was allowed to cool for few minutes and subsequently dispensed into culture vessels. Non-absorbent cotton plugs were used to plug the culture vessels. The mouth of culture vessels along with cotton plug was covered with newspaper using rubber bands.

3.2.4 Sterilization of culture media

Culture media in test tubes/flasks was sterilized by autoclaving in a vertical autoclave at a temperatureand pressure of 121°C and 1.05 kg cm^{-2} (15 psi), respectively for 20 minutes. This high temperature not only kills bacteria and fungi, but also their heat-resistant spores. Flasks and test tubes were placed in autoclave, lid of the autoclave was closed and electricity switched on. Vacuum valve was closed. Pressure indicator lamp (green lamp) switches off when required pressure (15 psi) is developed inside the autoclave chamber. Time for autoclaving was counted from the switching off of the pressure indicator lamp of the autoclave. After autoclaving the test tubes and flasks for the required time, electric supply of the autoclave was switched off and exhaust valve opened to release the pressure slowly. Autoclaved media was allowed to cool overnight at room temperature and used when required but within 10 days.

3.2.5 Transfer area and aseptic manipulations

All the aseptic manipulations like surface sterilization, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laboratory using laminar air flow cabinet where pure air is forced out continuously so that no contaminant is allowed to enter the cabinet during working time. Pure air is generated by forcing the air to pass through HEPA filter which removes dust and other potential contaminants. The working table of laminar air flow chamber was first surface sterilized with absolute alcohol and then by ultraviolet light for 30 minutes. The forceps, scalpel and petri-dishes were first steam sterilized in an autoclave at 121^{o}C for 20 minutes. These instruments were further sterilized at the start of inoculation by pouring some alcohol over them in the laminar hood followed by flaming. During inoculation forceps and blades were flame sterilized several times. Hands were cleaned and wiped with Hi-Care disinfectant gel before working.

3.2.6 Culture incubation

The cultures were generally incubated at 24±1°C in an air conditioned culture room with a 16/8 hour light/dark regime and a light intensity of 3500 lux.

3.2.7 Collection of explants

Scales of bulbs stored in coco-peat under normal storage conditions were collected from the healthy bulbs of Oriental Lilium Hybrid cv. 'Ravenna'. The bulbs were placed in a beaker and put under running tap water for removal of adhering dirt and any contamination in the laboratory and then the explants were isolated. After that the explants were placed in clean flasks containing distilled water before further processing.

3.3 Standardization of experiments

Different types of experiments were conducted during the present investigation aimed at the development of a protocol for micropropagation of Oriental *Lilium* Hybrid cv. "Ravenna". Details regarding the methodologies adopted are given in the sections that follow.

3.3.1 Explant surface sterilization and inoculation

Explant surface sterilization is the first vital step for the initiation of cultures *in vitro.* Final steps in surface sterilization were performed under the aseptic conditions of laminar flow hood. Knives and forceps were flame sterilized before use. While making incisions, knives and forceps were flame sterilized as and when thought necessary to avoid spread of contamination between different parts of the explants. Details of surface sterilization procedures for different explants are given in the following sections.

3.3.1.1 Bulb scales

Bulbs brought to the laboratory were washed with running tap water several times to remove any adhering dirt or dust particles. The outer diseased and damaged scales were discarded. Healthy outer scales were selected, removed and

given a vigorous shake for 30 minutes in Tween-20 surfactant solution (few drops) fortified with required concentration of fungicide concentration. The surfactant along with the fungicide was washed off under running tap water followed by a final washing with single distilled water. After initial cleaning the explants were brought to laminar air flow hood for treatment with other surface sterilants. The bulb scales after treatment with sterilants were given final 3 rinses with sterile water. Then basal and tip segments were isolated from scales and placed on the medium for establishment conforming to their original polarity. The details regarding the composition of various sterilant formulations and duration of application are given in the Table 3.4.

Table 3.4: Composition of sterilant formulations and duration of application for surface sterilization of various explants

S_1	:	Mercuric chloride 0.1% for 10 minutes
S_2	:	Mercuric chloride 0.1% for 20 minutes
S_3	:	Sodium hypochlorite 1.0% for 10 minutes
S_4	:	Sodium hypochlorite 1.0% for 20 minutes
S_5	:	Mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds
S_6	:	Mercuric chloride 0.1% for 20 minutes followed by ethyl alcohol 70% for 30 seconds
S_7	:	Sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds
S_8	:	Sodium hypochlorite 1.0% for 20 minutes followed by ethyl alcohol 70% for 30 seconds
S_9	:	Carbendazim (200 ppm) for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds
S_{10}	:	Carbendazim (200 ppm) for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds

3.3.1.2 Observations

Data on following parameters were recorded during the course of investigation.

1. Per cent asepsis after four weeks of culture.
2. Percent survival after four weeks of culture.

3.3.2 Explant establishment

Bulb scale segment explants were tried for standardization of establishment and proliferation experiments. Surface sterilized bulb scale segments were placed on establishment and proliferation media containing different growth regulator combinations (Table 3.5).

Table 3.5: Growth regulator combinations used for establishment of bulb scales of Lilium

S. No.	Growth regulators	Concentration (mg/l)
1	NAA + BAP	1.5 + 1.5
2	NAA + BAP	2.0 + 1.5
3	NAA + BAP	0.5 + 2.0
4	NAA + BAP	1.0 + 2.0
5	NAA + BAP	1.5 + 2.0
6	NAA + BAP	2.0 + 2.0
7	NAA	0.5
8	NAA	1.0

3.3.2.1 Observations

Data on per cent establishment was recorded at the end of first cycle of incubation after four weeks. During the establishment, days to initiation of bulblets, number of bulblets per explant, length of established shoots (cm) and leaf number of established shoots was also recorded.

3.3.3 Shoot proliferation

Standardization of propagation through axillary shoot proliferation was initiated, once sufficient number of shoots was obtained *in vitro* from establishment cultures. Microshoots of uniform size were separated from the clumps under aseptic conditions and placed in test tubes individually containing proliferation media (Table 3.6).

Table 3.6: Growth regulator combinations used for proliferation studies of established shoots of Lilium

S. No.	Growth regulators	Concentration (mg l^{-1})
1.	NAA + BAP	1.5 + 1.5
2.	NAA + BAP	2.0 + 1.5
3.	NAA + BAP	0.5 + 2.0
4.	NAA + BAP	1.0 + 2.0
5.	NAA + BAP	1.5 + 2.0
6	NAA + BAP	2.0 + 2.0

3.3.3.1 Observations

Following parameters were recorded during the course of proliferation experiments. Data related to these parameters were recorded at the end of 6 week proliferation cycle.

1. Per cent proliferation
2. Length of proliferated shoots (cm)
3. Leaf number of proliferated shoots
4. Number of shoots explant^{-1}

3.3.4 Standardization of rhizogenesis

Rooting of microshoots was standardized on rooting media containing different concentrations of auxins (Table 3.7). Uniform shoots were separated from uniformly aged clumps under laminar hood. The shoots were transferred to test tubes/flasks containing root development media.

Table 3.7: PGR combinations employed for *in vitro* rooting of microshoots

S. No.	Growth regulators	Concentration (mg l^{-1})
1.	NAA	0.5
2.	NAA	1.0
3.	NAA	1.5
4.	NAA	2.0
5.	IBA	0.5
6.	IBA	1.0
7.	IBA	1.5
8.	IBA	2.0

3.3.4.1 Observations

Data on following parameters were recorded after 4 weeks culture on rooting media.

1. Per cent Rooting
2. Days to root initiation
3. Number of primary roots shoot^{-1}
4. Length of primary roots (cm)

3.3.5 Hardening

3.3.5.1 Media

One media formulation was employed in the present study for *ex-vitro* hardening of the rooted plantlets of Lilium. Media used for hardening of rooted plantlets consisted of perlite and vermiculite in the ratio of 1:1 (v/v). Data on percent survival during primary hardening was recorded after 4 weeks growth on hardening media.

3.3.5.2 Sterilization of hardening media

Equal volumes of perlite and vermiculite were mixed thoroughly and moistened with distilled water. It was then packed in white cloth bags (about 2 kg/bag) and autoclaved at 121°C and 1.05 kg cm^{-2} (15 psi) for 1 hour. The media was allowed to cool and stored until use. Media was dispensed into 200 ml polypropylene glasses under the sterile conditions of a laminar air flow chamber.

3.3.5.3 Transfer of rooted plantlets

Rooted plantlets of Lilium were transferred from *in vitro* rooting media into hardening containers under sterile conditions of a laminar air flow chamber**.** Each *in vitro* developed plantlet was washed with distilled water to remove the traces of agar gel and planted in the media filled polypropylene glasses. Plants were watered with double distilled water.Eachcontainer was covered with another polypropylene glass i.e., one inverted on the other and the joint at the rims was

sealed with a strip of parafilm to maintain proper humidity around the transferred *in vitro* developed plant.

3.3.5.4 Acclimatization

Hardening Polypropylene containers containing rooted plantlets for hardening were placed in the growth chamber under 16/8 hours of light/dark regime. After the plants showed signs of establishment (growth of new leaf), small holes were made into the bottom of the upper inverted glass after 10 days of transfer. The holes were made larger after signs of establishment of the plant. Latter on the upper inverted glass was permanently removed. Observation on *ex vitro* survival per cent of plantlets after 4 weeks were recorded during this transition phase.

3.4 Statistical analysis

Statistical analysis of the data collected for different parameters during the present investigation was subjected to analysis of variance for completely randomized design with four replications (Gomez and Gomez, 1983). To satisfy model assumptions for analysis of variance, percentage data was subjected to angular or square root transformation as suggested by Steel and Torrie (1980).

Chapter - 4

EXPERIMENTAL FINDINGS

The results obtained during the present investigation entitled, "Standardization of micropropagation protocol of Oriental Liliumhybridcv. 'Ravenna' ", are presented in the following sections of this chapter.

4.1 Culture asepsis

Two types of bulb scale explants of oriental Lilium hybrid including basal and tip scale segments were sterilized with various sterilants individually or in combination. Main effect of sterilants on culture asepsis averaged over the explant type was found non-significant (Table 4.1). Among the individual sterilization treatments, S_2 (0.1% mercuric chloride for 20 minutes) resulted in maximum culture asepsis of 54.17% followed by S_4 (1.0% sodium hypochlorite for 20 minutes) with 48.96% and minimum of 43.75% with S_3 (1.0% sodium hypochlorite for 10 minutes). Combined treatment of sterilants gave better results. Maximum culture asepsis of 77.08% and 71.88% were obtained with S_9 (Carbendazim 200ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) and S_{10} (carbendazim 200ppm for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) respectively. Both these treatments differed significantly from one another and were superior to all other sterilant treatments.

Main effect of explant type averaged over sterilants was significant. Maximum culture asepsis of 63.54% was recorded with tip segments and minimum of 56.46% with basal segments. Interaction effect between explant type and sterilants was non-significant.

Table 4.1: Influence of various sterilant treatments and explant type on culture asepsis (%) in Oriental Lilium hybrid cv. 'Ravenna'

Sterilant treatments		Explant type		Mean
		Scale Base* (E_1)	Scale Tip* (E_2)	
S_1 :	Mercuric chloride 0.1% for 10 minutes	41.66 (6.53)	50.09 (7.14)	**45.83** **(6.84)**
S_2 :	Mercuric chloride 0.1% for 20 minutes	52.08 (7.28)	56.25 (7.57)	**54.17** **(7.42)**
S_3 :	Sodium hypochlorite 1.0% for 10 minutes	39.58 (6.36)	47.92 (6.99)	**43.75** **(6.68)**
S_4 :	Sodium hypochlorite 1.0% for 20 minutes	43.75 (6.68)	54.17 (7.43)	**48.96** **(7.06)**
S_5 :	Mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	58.33 (7.70)	66.67 (8.23)	**62.50** **(7.96)**
S_6 :	Mercuric chloride 0.1% for 20 minutes followed by ethyl alcohol 70% for 30 seconds	66.67 (8.22)	72.92 (8.60)	**69.79** **(8.41)**
S_7 :	Sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	56.25 (7.56)	64.59 (8.10)	**60.42** **(7.83)**
S_8 :	Sodium hypochlorite 1.0% for 20 minutes followed by ethyl alcohol 70% for 30 seconds	64.57 (8.09)	66.67 (8.23)	**65.62** **(8.16)**
S_9 :	Carbendazim (200 ppm) for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	72.92 (8.60)	81.25 (9.07)	**77.08** **(8.83)**
S_{10} :	Carbendazim (200 ppm) for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	68.75 (8.35)	75.00 (8.72)	**71.88** **(8.53)**
Mean		**56.46** **(7.54)**	**63.54** **(8.01)**	

C.D $_{(P\leq 0.05)}$

Explant type (E)	**:**	**0.10**
Sterilant treatments (S)	**:**	**0.23**
E × S	**:**	**NS**

*Data recorded after 4 weeks of culture

Figures in the parenthesis are square root transformed values of percentage data.

4.2 Explant Survival

Explant survival data gives an idea about the harmful effects of sterilants on the establishment and growth of explants. Aseptic cultures obtained after sterilization were used to study the damaging effect of sterilants on their further growth. Sterilant treatments had a significant effect on explant survival. Sterilants when used individually yielded higher explant survival as compared to combined sterilant treatments. Data presented in Table 4.2 reveals that maximum explant survival of 95.48 % was recorded with S_1 (Mercuric chloride 0.1% for 10 minutes) followed by S_2 (Mercuric chloride 0.1% for 20 minutes) with 91.86 %. Both these treatments differed significantly from all other treatments and combined treatments of sterilants resulted in reduction of explant survival. Minimum (81.25%) explant survival was recorded with S_8 (Sodium hypochlorite 1.0% for 20 minutes followed by ethyl alcohol 70% for 30 seconds) followed by S_{10} (Carbendazim 200 ppm for 30 minutes followed by sodium hypochlorite 1.0 % for 10 minutes followed by ethyl alcohol 70% for 30 seconds) with 82.92 %. Both these treatments differed significantly from all other treatments. Sterilant combinations which yielded highest aseptic cultures recorded moderate explant survival to the tune of 86.12% and 82.92% with S_9 (Carbendazim 200 ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) and S_{10} (Carbendazim 200 ppm for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds), respectively.

Explant type had a significant effect upon explant survival. Basal scale segments recorded maximum explant survival (88.54%) and minimum of 85.52% was observed with scale tip segments. Interaction effect between explant type and sterilants was found non-significant.

Table 4.2: Influence of various sterilant treatments and explant type on explant survival (%) in Oriental Lilium hybrid cv. 'Ravenna'

	Sterilant treatments	Explant type		Mean
		Scale Base* (E_1)	Scale Tip* (E_2)	
S_1	Mercuric chloride 0.1% for 10 minutes	97.22 (9.91)	93.75 (9.73)	**95.48 (9.82)**
S_2	Mercuric chloride 0.1% for 20 minutes	93.65 (9.73)	90.08 (9.54)	**91.86 (9.64)**
S_3	Sodium hypochlorite 1.0% for 10 minutes	90.63 (9.57)	87.08 (9.38)	**88.85 (9.48)**
S_4	Sodium hypochlorite 1.0% for 20 minutes	86.46 (9.35)	83.62 (9.20)	**85.04 (9.27)**
S_5	Mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	90.97 (9.59)	87.74 (9.42)	**89.36 (9.51)**
S_6	Mercuric chloride 0.1% for 20 minutes followed by ethyl alcohol 70% for 30 seconds	85.42 (9.30)	84.97 (9.27)	**85.19 (9.28)**
S_7	Sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	85.56 (9.30)	82.89 (9.16)	**84.22 (9.23)**
S_8	Sodium hypochlorite 1.0% for 20 minutes followed by ethyl alcohol 70% for 30 seconds	82.89 (9.16)	79.62 (8.98)	**81.25 (9.07)**
S_9	Carbendazim (200 ppm) for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	88.42 (9.46)	83.82 (9.21)	**86.12 (9.33)**
S_{10}	Carbendazim (200 ppm) for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds	84.17 (9.23)	81.67 (9.09)	**82.92 (9.16)**
	Mean	**88.54 (9.46)**	**85.52 (9.30)**	

C.D $_{(P\leq 0.05)}$
Explant type (E) : 0.06
Sterilant treatments (S) : 0.14
E × S : NS

*Data recorded after 6 weeks of culture
Figures in the parenthesis are square root transformed values of percentage data.

4.3 Explant establishment

Data regarding the effect of growth regulators on establishment of basal and tip scale segments in Oriental Lilium hybrid cv. 'Ravenna', is presented in Table 4.3

Growth regulator treatment combinations had a significant effect on the explant establishment. Maximum culture establishment of 76.17 % was recorded in medium supplemented with NAA + BAP (0.5 + 2.0 mg/l) and minimum of 50.00 % with NAA + BAP (2.0 + 2.0 mg/l). Increasing the NAA concentration from 0.5 to 2.0 mg/l resulted in reduction in explant establishment from 76.17 to 50.00 % when BAP concentration was kept constant at 2.0 mg/l.

Explant type had a significant effect upon explant establishment. Basal scale segments recorded maximum explant establishment (68.26%) and minimum of 55.21% was observed with scale tip segments.

Interaction effect between explant type and sterilants was found significant. Highest culture establishment (83.58%) was recorded with basal scale explants when cultured on medium supplemented with NAA + BAP (0.5 + 2.0 mg/l) and lowest of 43.75% with tip scale segments cultured in medium fortified with NAA + BAP (2.0 + 2.0 mg/l).

4.4 Days to initiation of bulblets

Data regarding influence of plant growth regulator combinations on days to initiation of bulblets in basal and tip scale explants is presented in Table 4.4

Perusal of data reveals that both growth regulator combinations and explant type had a significant influence on the number of days to initiation of bulblets. Least number of days (13.52) to initiation of bulblets was recorded in explants under T_5 (NAA 1.5 + BAP 2.0 mg/l) which was statistically at par with 13.95, 14.25 and 14.64 days recorded under T_3, T_1 and T_8 treatment combinations respectively. Bulblet initiation was delayed under T_6 (2.0 mg/l NAA + 2.0 mg/l BAP) where maximum number of days to bulblet initiation (17.00) was recorded.

Table 4.3: Influence of growth regulator combinations and explant type on culture establishment (%) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	68.75 (8.35)	66.67 (8.23)	**67.71 (**8.29)
T_2	NAA + BAP	2.0 + 1.5	54.17 (7.43)	62.50 (7.97)	**58.34 (**7.70)
T_3	NAA + BAP	0.5 + 2.0	83.58 (9.20)	68.75 (8.35)	**76.17 (**8.77)
T_4	NAA + BAP	1.0 + 2.0	72.92 (8.60)	50.00 (7.14)	**61.46** (7.87)
T_5	NAA + BAP	1.5 + 2.0	64.59 (8.10)	45.84 (6.84)	**55.21 (**7.47)
T_6	NAA + BAP	2.0 + 2.0	56.25 (7.57)	43.75 (6.69)	**50.00 (**7.13)
T_7	NAA	0.5	75.00 (8.72)	54.17 (7.43)	**64.58 (**8.07)
T_8	NAA	1.0	70.84 (8.48)	50.00 (7.14)	**60.42 (**7.81)
	Mean		**68.26 (**8.30)	**55.21 (**7.47)	

C.D $_{(P\leq 0.05)}$
Explant type (E) **:** 0.06
Growth regulators (T) **:** 0.12
E × T **:** 0.16
*Data recorded after 4 weeks of culture
Figures in the parenthesis are square root transformed values of percentage data.

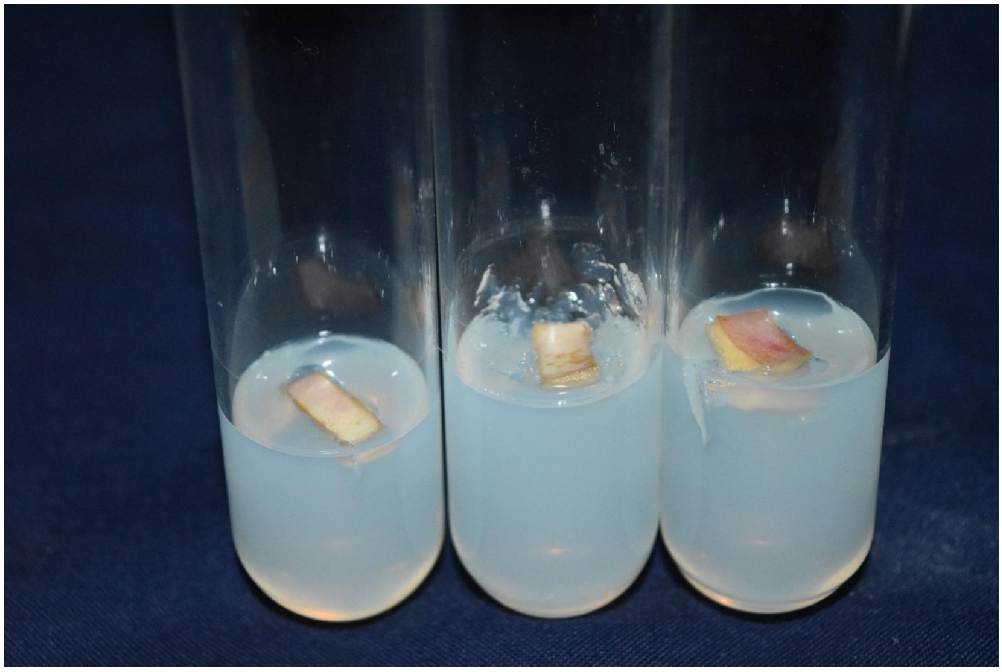
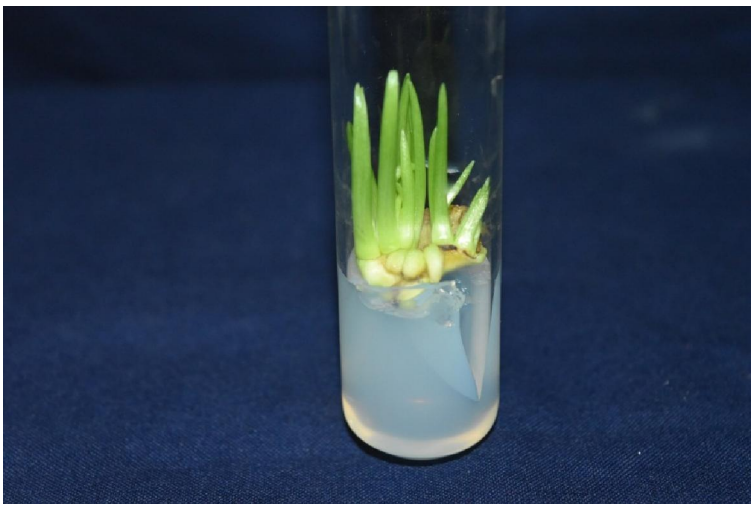
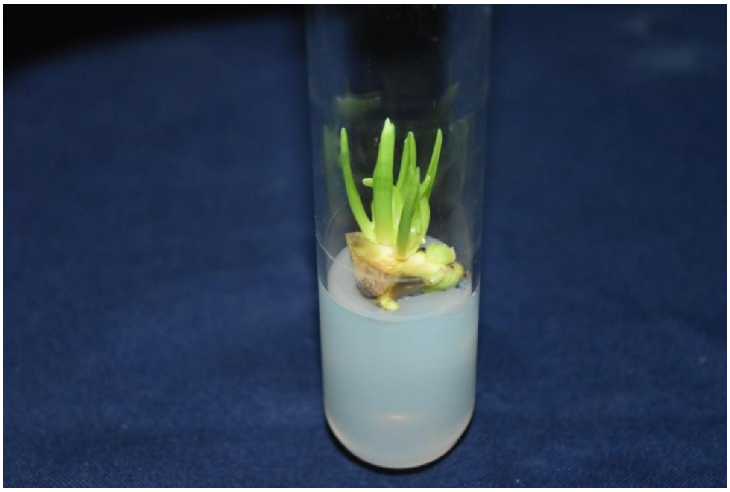


Plate-2: Bulb scale explant in establishment medium



Establishment of basal scale segments
(NAA + BAP: 0.5 + 2.0 mg/l)



Establishment of tip scale segments
(NAA + BAP: 0.5 + 2.0 mg/l)

Plate-3: Culture establishment of bulb scale segments

Table 4.4: Influence of growth regulators and explant type on days to initiation of bulblets in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T1	NAA + BAP	1.5 + 1.5	13.37	15.12	**14.25**
T2	NAA + BAP	2.0 + 1.5	14.75	16.37	**15.56**
T3	NAA + BAP	0.5 + 2.0	13.29	14.62	**13.95**
T4	NAA + BAP	1.0 + 2.0	14.00	15.62	**14.81**
T5	NAA + BAP	1.5 + 2.0	12.75	14.29	**13.52**
T6	NAA + BAP	2.0 + 2.0	16.37	17.62	**17.00**
T7	NAA	0.5	15.12	17.37	**16.25**
T8	NAA	1.0	13.58	15.70	**14.64**
	Mean		**14.15**	**15.84**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.60

Growth regulators (T) : 1.21

E × T : 1.43

*Data recorded after 4 weeks of culture

This treatment was significantly different from all other treatments except T_7 (0.5 mg/l NAA).

Explants derived from basal scale segments developed bulblets in an average of 14.15 days which was significantly earlier than 15.84 days recorded for explants from tip scale segments.

The interaction between explant type and growth regulator combinations was significant. Lowest number of days to initiation of bulblets (12.75) was recorded with basal scale segments when treated with T_5 and highest (17.62 days) with tip scale segments when treated with T_6.

4.5 Number of bulblets per explant

Data regarding influence of plant growth regulator combinations on number of bulblets per explant in basal and tip scale explants is presented in Table 4.5

There was a significant effect of growth regulators on the number of bulblets per explant. Explants in T_3 growth regulator combination developed an average of 5.52 bulblets which was significantly higher than all other treatments. It was followed by T_5 with 4.81 bulblets/explant and T_6 with 4.37 bulblets/explant. Minimum number of bulblets (3.27/explant) was observed in T_8 (1.0 mg/l NAA) followed by T_2 (NAA 2.0 + BAP 1.5 mg/l) with 3.40 bulblets/explant. Both these treatments were at par with each other.

Explant type had a significant influence on the bulblet number. Basal scale segments produced maximum (4.91) bulblets/explant compared to tip scale segments with 3.51 bulblets/explant.

Effect of the interaction of explant type and growth regulator combinations on number of bulblets per explant was significant. Highest bulblet number of 6.97 was recorded in basal scale segments under T_3 combination and lowest of 2.79 in tip scale segments when treated with T_8.

Table 4.5: Influence of growth regulators and explant type on number of bulblets per explant in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	4.38	3.58	**3.98**
T_2	NAA + BAP	2.0 + 1.5	3.36	3.44	**3.40**
T_3	NAA + BAP	0.5 + 2.0	6.97	4.08	**5.52**
T_4	NAA + BAP	1.0 + 2.0	4.92	3.79	**4.35**
T_5	NAA + BAP	1.5 + 2.0	5.87	3.75	**4.81**
T_6	NAA + BAP	2.0 + 2.0	5.33	3.41	**4.37**
T_7	NAA	0.5	4.75	3.29	**4.01**
T_8	NAA	1.0	3.75	2.79	**3.27**
	Mean		**4.91**	**3.51**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.31

Growth regulators (T) : 0.63

E × T : 0.89

*Data recorded after 4 weeks of culture

4.6 Length of established shoots (cm)

Length of established shoots was used to study the role of exogenous growth hormones on the explant growth. This parameter improved significantly under the influence of growth regulators. Data regarding influence of plant growth regulator combinations on length of established shoots (cm) in basal and tip scale explants is presented in Table 4.6

Length of established shoots was recorded maximum (2.20 cm) on medium containing NAA + BAP (0.5 + 2.0 mg/l) which was significantly superior to 2.11 cm and 2.08 cm obtained on medium fortified with NAA + BAP (1.0 + 2.0 mg/l) and NAA + BAP (2.0 + 1.5 mg/l), respectively. Minimum length of established shoots (1.65 cm) and (1.68cm) was recorded on medium containing NAA (0.5 mg/l) and NAA + BAP (2.0 + 2.0 mg/l), respectively. Both these treatments were at par with each other.

Significantly maximum length of established shoots (2.05 cm) was recorded in basal scale segments and minimum (1.81 cm) in tip scale segments.

The interaction effect between explant type and growth regulator combinations was significant. Maximum length of established shoots (2.34 cm) was recordedwith basal scale explants when treated with NAA + BAP (0.5 + 2.0 mg/l) and minimum (1.61 cm) with tip scale segments when treated with NAA (0.5 mg/l).

4.7 Leaf number of established shoots

Leaf number of established shoots was also used to study the role of exogenous growth hormones on the explant growth. This parameter also improved significantly under the influence of growth regulators. Data regarding influence of plant growth regulator combinations on leaf number of established shoots in basal and tip scale explants is presented in Table 4.7.

There was reduction in leaf number with the increase in NAA concentration in the media. Maximum leaf number of established shoots (3.39)

was recorded with NAA + BAP (0.5 + 2.0 mg/l) followed by 2.99 with NAA + BAP (2.0 + 1.5 mg/l) and 2.96 with NAA + BAP (1.0 + 2.0 mg/l). Minimum leaf number (1.90) was recorded with NAA (0.5 mg/l) followed by 2.14 with NAA (1.0 mg/l).

Significantly maximum leaf number of established shoots (3.15) was recorded in basal scale segment explants and minimum leaf number of established shoots (2.24) was recorded in tip scale segment explants.

The interaction effect between explant type and growth regulator combinations was found significant. The highest leaf number of established shoots (3.94) was recorded with basal scale segment explants when treated with T_3 and lowest (1.77) with tip scale explants when treated with T_7.

Table 4.6: Influence of growth regulators and explant type on length of established shoots (cm) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	2.07	1.82	**1.94**
T_2	NAA + BAP	2.0 + 1.5	2.22	1.95	**2.08**
T_3	NAA + BAP	0.5 + 2.0	2.34	2.06	**2.20**
T_4	NAA + BAP	1.0 + 2.0	2.32	1.89	**2.11**
T_5	NAA + BAP	1.5 + 2.0	2.14	1.77	**1.95**
T_6	NAA + BAP	2.0 + 2.0	1.64	1.71	**1.68**
T_7	NAA	0.5	1.69	1.61	**1.65**
T_8	NAA	1.0	1.95	1.71	**1.83**
	Mean		**2.05**	**1.81**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.03

Growth regulators (T) : 0.06

E × T : 0.08

*Data recorded after 4 weeks of culture

Table 4.7: Influence of growth regulators and explant type on leaf number of established shoots in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	2.92	2.24	**2.58**
T_2	NAA + BAP	2.0 + 1.5	3.31	2.66	**2.99**
T_3	NAA + BAP	0.5 + 2.0	3.94	2.84	**3.39**
T_4	NAA + BAP	1.0 + 2.0	3.60	2.33	**2.96**
T_5	NAA + BAP	1.5 + 2.0	3.54	2.17	**2.86**
T_6	NAA + BAP	2.0 + 2.0	3.39	2.05	**2.72**
T_7	NAA	0.5	2.03	1.77	**1.90**
T_8	NAA	1.0	2.45	1.84	**2.14**
	Mean		**3.15**	**2.24**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.18

Growth regulators (T) : 0.37

E × T : 0.49

*Data recorded after 4 weeks of culture

4.8 Shoot proliferation

Same growth regulator treatment combinations except two, which were used for explant establishment, were tried for further proliferation of established scale segment cultures. Established cultures were divided into individual shoots and put in fresh media supplemented with NAA + BAP. Two treatments T7 (NAA 0.5 mg/l) and T8 (NAA 1.0 mg/l) were discarded because callusing and rooting of some cultures was observed during establishment stage in these treatments. Data regarding proliferation parameters was recorded 4 weeks after putting the individual shoots in proliferation media. Effect of growth regulator treatments and explant type had a significant influence on culture proliferation (Table 4.8). It is evident from the perusal of Table 4.8 that growth regulator treatments significantly improved culture proliferation percentage. Maximum culture proliferation (83.33%) was observed with T_3 (NAA 0.5 + BAP 2.0 mg/l) followed by T_2 (NAA 2.0 + BAP 1.5 mg/l) with 77.08% culture proliferation. Both these treatments differed significantly from one another and from all other treatments. Minimum culture proliferation of 61.46% was observed with NAA + BAP (2.0 + 2.0 mg/l) followed by 66.67% with NAA + BAP (1.5 + 2.0 mg/l). All treatments differed significantly from one another.

Explant type also had a significant influence on culture proliferation. Highest culture proliferation (75.35%) was recorded with basal scale segments and lowest (68.40%) with tip scale segments.

The interaction effect between explant type and growth regulator combinations was found non-significant.

Table 4.8: Influence of growth regulator combinations and explant type on culture proliferation (%) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	70.83 (57.35)	66.67 (54.74)	**68.75 (56.04)**
T_2	NAA + BAP	2.0 + 1.5	81.25 (64.37)	72.92 (58.62)	**77.08 (61.50)**
T_3	NAA + BAP	0.5 + 2.0	87.50 (69.54)	79.17 (62.83)	**83.33 (66.18)**
T_4	NAA + BAP	1.0 + 2.0	77.08 (61.40)	70.84 (57.35)	**73.96 (59.37)**
T_5	NAA + BAP	1.5 + 2.0	70.84 (57.35)	62.50 (52.23)	**66.67 (54.79)**
T_6	NAA + BAP	2.0 + 2.0	64.59 (53.47)	58.34 (51.24)	**61.46 (52.36)**
Mean			**75.35 (60.58)**	**68.40 (56.17)**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 1.40

Growth regulators (T) : 2.42

E × T : NS

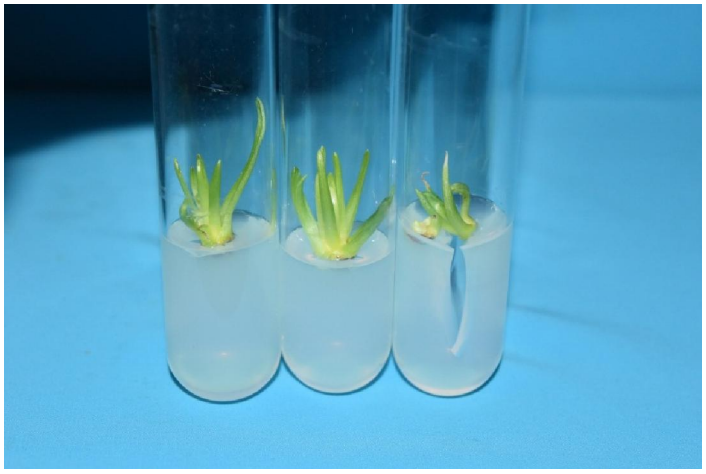
*Data recorded after 4 weeks of culture

Figures in the parenthesis are arcsine transformed values of percentage data.

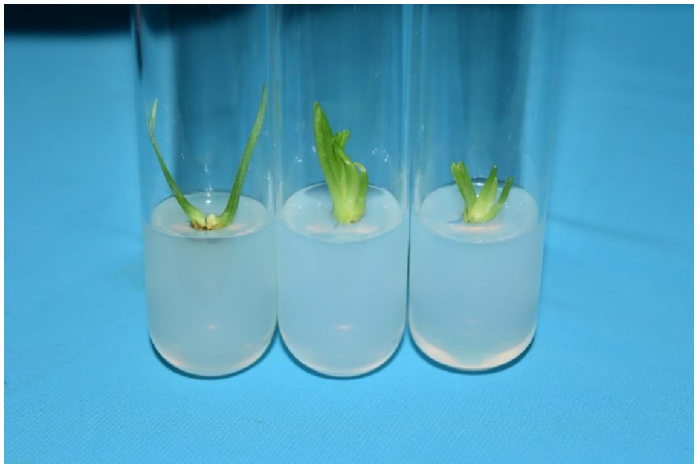
Direct rooting of basal scale segments in establishment medium (NAA=1.0 mg/l)

Direct rooting of tip scale segments in establishment medium (NAA=1.0 mg/l)

Plate-4: Direct rooting of Explants in establishment medium



Shoot proliferation in basal segments
(NAA + BAP: 0.5 + 2.0 mg/l)



Shoot proliferationin tip segments
(NAA + BAP: 0.5 + 2.0 mg/l)

Plate-5: Proliferation in bulb scale segments

4.9 Number of shoots/explant

Number of shoots/explant was significantly affected by growth regulator combinations and explant type. Data regarding influence of plant growth regulator combinations on number of shoots/explant in basal and tip scale explants is presented in Table 4.9

Highest number of shoots/explant 2.41 and 2.28 was recorded under T_3 (NAA 0.5 + BAP 2.0 mg/l) and T_2 (NAA 2.0 + BAP 1.5 mg/l) treatment combinations, respectively. Both the treatment combinations were at par with each other but significantly different from others. The lowest number of shoots per explant 1.62 and 1.81was recorded under T_6 (NAA 2.0 + BAP 2.0 mg/l) and T_5 (NAA 1.5 + BAP 2.0 mg/l) treatment combinations respectively. Both these treatments differed significantly from rest of the treatments.

Explant type had a significant influence on this parameter. Highest number of shoots/explant (2.37) was recorded in basal scale segments and lowest (1.74) in tip scale segments.

The interaction effect between explant type and growth regulator combinations was significant. Highest number of shoots/explant (2.90) was recorded with basal scale segments when treated with T_3 and lowest (1.58) with tip scale segments when treated with T_6.

4.10 Length of proliferated shoots (cm)

Data regarding influence of plant growth regulator combinations on length of proliferated shoots in basal and tip scale explants is presented in Table 4.10

Regarding growth regulator combinations, highest mean length of proliferated shoots 2.35 cm and 2.19 cm was recorded under T_3 (NAA 0.5 + BAP 2.0 mg/l) and T_2 (NAA 2.0 + BAP 1.5 mg/l) treatment combinations respectively. Both these treatment combinations were significantly different from each other's. The lowest mean length of proliferated shoots (1.68cm) was recorded under

T_6(NAA 2.0 + BAP 2.0 mg/l) followed by 1.87 cm recorded under T_5 (NAA 1.5 + BAP 2.0 mg/l).

Mean length of proliferated shoots was significantly influenced by explant type. The highest length of proliferated shoots (2.21 cm) was recorded in basal scale segments and lowest (1.87 cm) in tip scale segments.

The interaction between explant type and growth regulator combinations was found significant. The highest length of proliferated shoots (2.59 cm) was recorded with basal scale segments when treated with T_3 and lowest (1.63 cm) with tip scale segments when treated with T_6.

4.11 Leaf number of proliferated shoots

Data regarding influence of plant growth regulator combinations and explant type on the leaf number of proliferated shoots in basal and tip scale explants is presented in Table 4.11

Regarding growth regulator combinations, highest leaf number of proliferated shoots 5.44 and 4.93 was recorded under T_3 (NAA 0.5 + BAP 2.0 mg/l) and T_2 (NAA 2.0 + BAP 1.5 mg/l) treatment combinations, respectively. Both these treatment combinations were significantly different from each other's. The lowest leaf number of proliferated shoots 3.97 and 4.43 was recorded under T_6 (NAA 2.0 + BAP 2.0 mg/l) and T_5 (NAA 1.5 + BAP 2.0 mg/l) treatment combinations, respectively. Both the treatments were at par with each other.

Average number of leaves/proliferated shoot was significantly affected by explant type. The highest mean leaf number/proliferated shoot (5.07) was recorded in basal scale segments and lowest (4.32) was recorded in tip scale segments. The interaction between explant type and growth regulator combinations was non-significant.

Table 4.9: Influence of growth regulator combinations and explant type on number of shoots/explant in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	2.65	1.72	**2.18**
T_2	NAA + BAP	2.0 + 1.5	2.70	1.86	**2.28**
T_3	NAA + BAP	0.5 + 2.0	2.90	1.92	**2.41**
T_4	NAA + BAP	1.0 + 2.0	2.34	1.72	**2.03**
T_5	NAA + BAP	1.5 + 2.0	2.00	1.62	**1.81**
T_6	NAA + BAP	2.0 + 2.0	1.67	1.58	**1.62**
	Mean		**2.37**	**1.73**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.07

Growth regulators (T) : 0.12

E × T : 0.17

*Data recorded after 4 weeks of culture

Table 4.10: Influence of growth regulator combinations and explant type on length of proliferated shoots (cm) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	2.35	1.91	**2.13**
T_2	NAA + BAP	2.0 + 1.5	2.43	1.94	**2.19**
T_3	NAA + BAP	0.5 + 2.0	2.59	2.12	**2.35**
T_4	NAA + BAP	1.0 + 2.0	2.23	1.85	**2.04**
T_5	NAA + BAP	1.5 + 2.0	1.94	1.79	**1.87**
T_6	NAA + BAP	2.0 + 2.0	1.74	1.63	**1.68**
	Mean		**2.21**	**1.87**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.08

Growth regulators (T) : 0.13

E × T : 0.19

*Data recorded after 4 weeks of culture

Table 4.11: Influence of growth regulator combinations and explant type on leaf number of proliferated shoots in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA + BAP	1.5 + 1.5	4.88	4.40	**4.64**
T_2	NAA + BAP	2.0 + 1.5	4.93	4.94	**4.93**
T_3	NAA + BAP	0.5 + 2.0	5.77	5.12	**5.44**
T_4	NAA + BAP	1.0 + 2.0	5.13	4.38	**4.76**
T_5	NAA + BAP	1.5 + 2.0	5.12	3.74	**4.43**
T_6	NAA + BAP	2.0 + 2.0	4.57	3.37	**3.97**
	Mean		**5.07**	**4.32**	

C.D $(P \leq 0.05)$

Explant type (E) : 0.28

Growth regulators (T) : 0.48

E × T : NS

*Data recorded after 4 weeks of culture

Rhizogenesis

Proliferated cultures were divided and healthy individual shoots of uniform size obtained following division were put in fresh rooting media fortified with different concentrations of auxins.

4.12 Days to root initiation

Growthregulatortreatments and explant type had a significant effect on the duration of root initiation in micro-shoots of oriental Lilium hybrid cv. Ravenna (Table 4.12).

Regarding growth regulators, lowest days to root initiation (17.95) was recorded under T_8 (IBA 2.0 mg/l) which was statistically at par with 18.60 and 19.80 days recorded under T_7 (IBA 1.5) and T_4 (NAA 2.0 mg/l) treatments, respectively. The highest days to root initiation (24.25) was recorded under T_1 (NAA 0.5 mg/l) which was statistically at par with 23.45 and 22.95 days recorded under T_2 (NAA 1.0 mg/l) and T_5 (IBA 0.5 mg/l) treatments, respectively.

Explant type had a significant effect on root initiation. The lowest days to root initiation (20.24) were recorded in basal scale segments and highest days to root initiation (22.18) were recorded in tip scale segments. However, the interaction between explant type and growth regulators was found non-significant.

4.13 Rooting percentage

Data regarding the effect of various auxin concentrations on rooting percentage in Oriental Lilium hybrid cv. ‘Ravenna’ has been presented in Table 4.13

Growth regulator treatments significantly influenced by root percentage of micro-shoots. There was an increase in the rooting of shoots with the increase in the concentration of auxins either IBA or NAA. However, IBA treatments recorded more rooting percentage as compared to the NAA treatments. Maximum rooting (92.71%) was recorded in plantlets under T_7 (IBA 1.5 mg/l) followed by

T_6 (IBA 1.0 mg/l) with 88.33%. Lowest rooting (65.63%) was recorded with NAA (2.0 mg/l) followed by 73.96 % and 76.04 % with NAA (0.5 mg/l) and NAA (1.0 mg/l), respectively. All these treatments were significantly different from each other and rest of the other treatments.

In terms of different explants, highest mean rooting (84.12) percentage was recorded in basal scale segments and the lowest rooting (77.55) percentage in tip scale segments.

The interaction between explant type and growth regulators was significant, however the maximum rooting (97.92) percentage was recorded with basal scale segments when treated with T_7 and lowest (64.59) with tip scale segments when treated with T_4.

Table 4.12: Influence of various growth regulator combinations and explant type on days to root initiation in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA	0.5	25.40	23.10	**24.25**
T_2	NAA	1.0	23.95	22.95	**23.45**
T_3	NAA	1.5	21.33	21.18	**21.25**
T_4	NAA	2.0	20.38	19.23	**19.80**
T_5	IBA	0.5	24.13	21.78	**22.95**
T_6	IBA	1.0	22.45	20.48	**21.46**
T_7	IBA	1.5	20.48	16.73	**18.60**
T_8	IBA	2.0	19.38	16.53	**17.95**
	Mean		**20.24**	**22.18**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 1.37

Growth regulators (T) : 2.73

E × T : NS

*Data recorded after 4 weeks of culture

Table 4.13: Influence of various growth regulator combinations and explant type on rooting (%) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA	0.5	75.00 (8.72)	72.92 (8.60)	**73.96 (8.66)**
T_2	NAA	1.0	77.08 (8.84)	75.00 (8.72)	**76.04 (8.78)**
T_3	NAA	1.5	83.33 (9.18)	79.17 (8.95)	**81.25 (9.07)**
T_4	NAA	2.0	66.67 (8.23)	64.59 (8.10)	**65.63 (8.16)**
T_5	IBA	0.5	87.50 (9.41)	77.08 (8.84)	**82.29 (9.12)**
T_6	IBA	1.0	93.75 (9.73)	82.92 (9.16)	**88.33 (9.45)**
T_7	IBA	1.5	97.92 (9.94)	87.50 (9.41)	**92.71 (9.68)**
T_8	IBA	2.0	91.67 (9.63)	81.25 (9.07)	**86.46 (9.35)**
	Mean		**84.12 (9.21)**	**77.55 (8.85)**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.08

Growth regulators (T) : 0.16

E × T : 0.23

*Data recorded after 4 weeks of culture

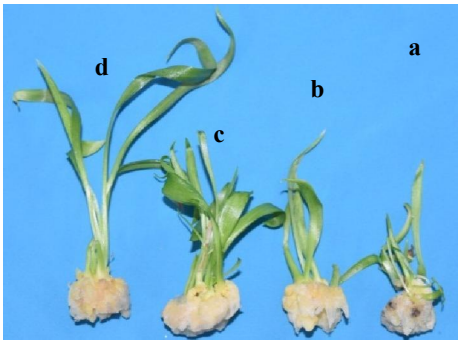
Figures in the parenthesis are square root transformed values of percentage data.

Rooting of basal scale shoots

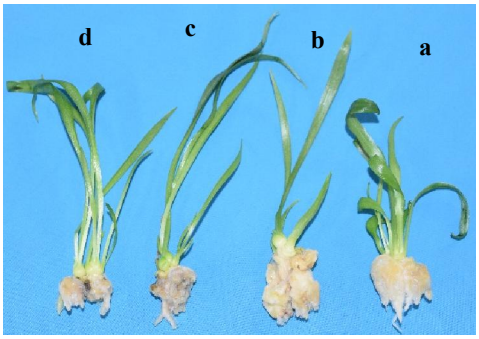
(NAA: 1.5 mg/l)

Rooting of tip scale shoots

(NAA: 1.5 mg/l)



a) NAA: 0.5 mg/l
b) NAA: 1.0 mg/l
c) NAA: 1.5 mg/l
d) NAA: 2.0 mg/l



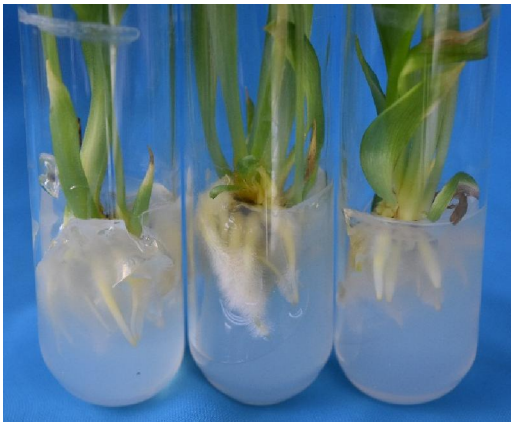
a) NAA: 0.5 mg/l
b) NAA: 1.0 mg/l
c) NAA: 1.5 mg/l
d) NAA: 2.0 mg/l

Plate-6a: Rhizogenesis with NAA

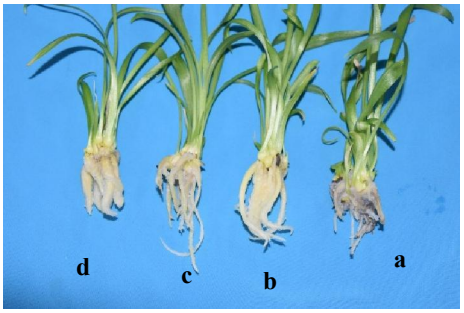
Rooting of basal scale shoots

(IBA: 1.5 mg/l)

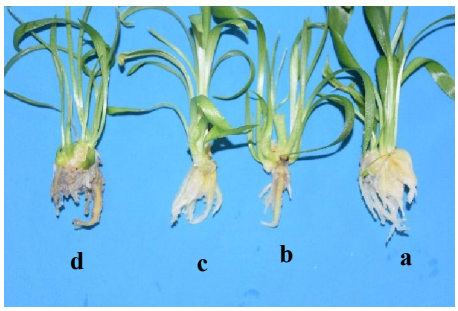
Rooting of tip scale shoots



(IBA: 1.5 mg/l)



a) IBA: 0.5 mg/l
b) IBA: 1.0 mg/l
c) IBA: 1.5 mg/l
d) IBA: 2.0 mg/l



a) IBA: 0.5 mg/l
b) IBA: 1.0 mg/l
c) IBA: 1.5 mg/l
d) IBA: 2.0 mg/l

Plate-6b: Rhizogenesis with IBA

4.14 Number of primary roots/shoot

Data regarding the effect of various auxin concentrations on number of primary roots/shoot in Oriental Lilium hybrid cv. 'Ravenna' has been presented in Table 4.14

Growth regulator treatments had significant influence on the number of primary roots/shoot. Root number increased with increase in auxin concentration upto 1.5 mg/l but decreased at highest concentration (NAA or IBA @ 2 mg/l). Maximum primary root number/shoot (12.06) was recorded in medium supplemented with 1.5 mg/l IBA followed by 10.86 with 2.0 mg/lIBA. Both these treatments differed significantly from each other. Minimum primary root number/shoot of 7.29 was recorded in media fortified with NAA (0.5 mg/l) followed by 7.98 with NAA (1.0 mg/l).

Explant type had a significant influence on primary root number/shoot which was recorded maximum (10.02) with basal scale segments and minimum (9.08) with tip scale segments.

The interaction effect between explant type and growth regulators was significant. Maximum number of primary roots/shoot (13.40) was recorded in basal scale segments when treated with T_7 and lowest (7.03) with tip scale segments when treated with T_1.

4.15 Length of primary roots (cm)

Data regarding the effect of various auxin concentrations on length of primary roots (cm) in Oriental Lilium hybrid cv. 'Ravenna' has been presented in Table 4.15

Regarding length of primary roots, IBA was found superior to NAA in improving the root length. Length of roots under IBA (1.5 mg/l) was significantly higher (3.17 cm) than rest of the treatments. This was followed by root length of 2.81 cm and 2.74 cm recorded with 2.0mg/lIBA and 1.0 mg/l IBA, respectively. Lowest root length of 0.68 cm was recorded under treatment NAA (0.5 mg/l)

followed by 0.75 cm root length with NAA (1.0 mg/l). Both these treatments were at par with each other.

Significant effect of explant type was observed on the length of primary roots. Maximum length of primary roots (2.17 cm) was recorded in basal scale segments and minimum (1.65 cm) in tip scale segments.

The interaction effect between explant type and growth regulators was significant. Maximum length of primary roots (3.57 cm) was recorded with basal scale segments when treated with T_7 and lowest (0.59 cm) with tip scale segments when treated with T_1.

4.16 Hardening

Data related to the per cent survival during primary hardening of rooted plantlets in Oriental Lilium hybrid cv. 'Ravenna' is presented in Table 4.16

Significant influence of growth regulators and explant type was noticed on the plantlet survival during primary hardening. IBA treated plantlets had higher survival as compared to NAA treated plantlets. Plantlet survival was observed highest (98.96 %) followed by (95.83 %) and (94.79%) in MS media fortified with IBA (1.5 mg/l), IBA (2.0 mg/l) and IBA (1.0 mg/l), respectively. All these treatments were at par with each other. The lowest survival of (80.21 %) followed by (82.29 %) and (83.33%) was recorded by the plantlets rooted in MS media fortified with NAA (0.5 mg/l), NAA (2.0 mg/l) and NAA (1.0 mg/l), respectively. All these treatments were at par with each other.

In terms of different explant types, highest plantlet survival (93.49 %) was recorded in basal scale segments and lowest (84.63 %) in tip scale segments. However, the interaction between explant type and growth regulators was non-significant.

Rooted plantlets were hardened in media containing perlite and vermiculite (1:1) under culture room conditions.

Table 4.14: Influence of various growth regulator combinations and explant type on number of primary roots/shoot in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA	0.5	7.55	7.03	**7.29**
T_2	NAA	1.0	8.30	7.65	**7.98**
T_3	NAA	1.5	11.15	9.68	**10.41**
T_4	NAA	2.0	9.78	8.80	**9.29**
T_5	IBA	0.5	8.85	8.53	**8.69**
T_6	IBA	1.0	10.05	9.55	**9.80**
T_7	IBA	1.5	13.40	10.73	**12.06**
T_8	IBA	2.0	11.08	10.65	**10.86**
	Mean		**10.02**	**9.08**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.45

Growth regulators (T) : 0.91

E × T : 1.28

*Data recorded after 4 weeks of culture

Table 4.15: Influence of various growth regulator combinations and explant type on length of primary roots (cm) in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA	0.5	0.77	0.59	**0.68**
T_2	NAA	1.0	0.84	0.66	**0.75**
T_3	NAA	1.5	1.33	1.02	**1.17**
T_4	NAA	2.0	1.86	1.49	**1.67**
T_5	IBA	0.5	2.51	2.10	**2.30**
T_6	IBA	1.0	3.22	2.27	**2.74**
T_7	IBA	1.5	3.57	2.77	**3.17**
T_8	IBA	2.0	3.28	2.35	**2.81**
	Mean		**2.17**	**1.65**	

C.D $_{(P\leq 0.05)}$

Explant type (E) : 0.08

Growth regulators (T) : 0.15

E × T : 0.21

*Data recorded after 4 weeks of culture

Table 4.16: Influence of various growth regulator combinations and explant type on percent survival during primary hardening in Oriental Lilium hybrid cv. 'Ravenna'

Treatment	Growth regulators	Concentration (mg/l)	Explant type		Mean
			Scale Base* (E_1)	Scale Tip* (E_2)	
T_1	NAA	0.5	87.50	72.92	**80.21**
			(9.41)	(8.59)	**(9.00)**
T_2	NAA	1.0	89.58	77.08	**83.33**
			(9.52)	(8.83)	**(9.17)**
T_3	NAA	1.5	93.75	83.33	**88.54**
			(9.73)	(9.17)	**(9.45)**
T_4	NAA	2.0	89.58	75.00	**82.29**
			(9.52)	(8.71)	**(9.11)**
T_5	IBA	0.5	93.75	83.33	**88.54**
			(9.73)	(9.18)	**(9.45)**
T_6	IBA	1.0	97.92	91.66	**94.79**
			(9.94)	(9.62)	**(9.78)**
T_7	IBA	1.5	100.00	97.92	**98.96**
			(10.05)	(9.94)	**(10.00)**
T_8	IBA	2.0	95.83	95.83	**95.83**
			(9.83)	(9.83)	**(9.83)**
Mean			**93.49**	**84.63**	
			(9.72)	**(9.23)**	

C.D $_{(P\leq 0.05)}$

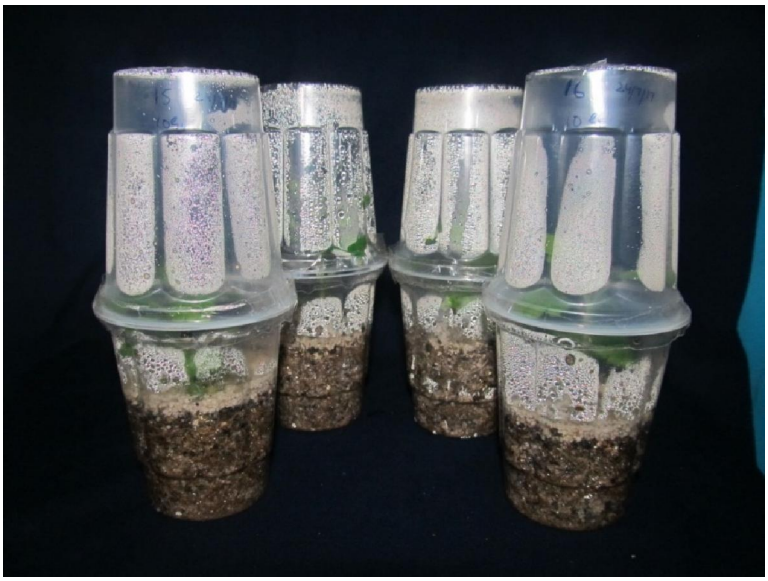
Explant type (E) : 0.18

Growth regulators (T) : 0.37

E × T : NS

*Data recorded after 8 weeks of culture

Figures in the parenthesis are square root transformed values of percentage data



Hardening of rooted plantlets at 1 week

Hardening of rooted plantlets at 2 weeks

Plate-7: Hardening of rooted plantlets

Plate-8: Hardened rooted plantlets

Chapter- 5

DISCUSSION

The results obtained from the foregoing study "Standardization of micropropagation protocol of Oriental Lilium hybrid cv. 'Ravenna'" are discussed in this chapter in light of the available literature.

5.1 Culture asepsis and survival of bulb scale segments

Choosing the correct explant is essential if the desired outcome of any tissue culture procedure is to be achieved with minimal delays, besides proper sterilization of the explants is the pre-requisite step leading to the development of a successful protocol for *in vitro* propagation. Various sterilants were used to improve the culture asepsis in *Lilium* during the present investigation so as to make more explants available for conducting various experiments.

In the current study, bulb scale segments were surface sterilized with various sterilants including mercuric chloride, sodium hypochlorite and ethyl alcohol individually or in combination. Effect of sterilants and explant type upon the culture asepsis and subsequent survival of basal and tip scale segments of oriental Lilium hybrid was observed significant (Table 4.1 and 4.2). Among the single sterilant treatments, S_2 (0.1% mercuric chloride for 20 minutes) showed better performance, yielding 54.17% aseptic cultures followed by S_4 (1.0% sodium hypochlorite for 20 minutes), while the performance of S_3 (1.0% sodium hypochlorite for 10 minutes) was very poor yielding minimum aseptic cultures. Increase in sterilant treatment duration from 10 to 20 minutes resulted in increased culture asepsis. Combined treatments of explants with two or more sterilants have been found very effective for sterilization of underground buds/explants in comparison to treatment with single chemical (Rather, 2010). Treatment of underground Lilium bulb scales with different sterilants significantly increased the culture asepsis. Maximum aseptic cultures (77.08%) were obtained with S_9 (Carbendazim 200 ppm for 30 minutes followed by mercuric chloride 0.1% for 10

minutes followed by 70% ethyl alcohol for 30 seconds). The results indicated that passing explants through fungicidal treatment followed by mercuric chloride and ethyl alcohol is indispensable for obtaining higher culture asepsis in bulb scale explants of *Lilium*. Mercuric chloride is highly antimicrobial with action against both fungi and bacteria. Concentrated ethanol is a powerful disinfectant that kills majority of fungal and bacterial spores through instant dehydration.

Results obtain in the present studies are in conformity with several workers who used carbendazim, mercuric chloride, sodium hypochlorite and ethyl alcohol to achieve the desired level of culture asepsis and survival *in vitro*. Taha *et al.* (2018) reported maximum culture asepsis in Asiatic Lilium hybrid cv. Red Alert with the combined use of 10% sodium hypochlorite and 0.1% mercuric chloride. Liu *et al.* (2012) obtained higher culture asepsis in LA Hybrid cv. eyeliner with combined treatment of carbendazim, 75% alcohol and sodium hypochlorite. Mir *et al*. (2012) obtained optimum better culture asepsis by surface sterilizing middle scales of bulbs of *Lilium longiflorum* with 70% ethanol for 30 seconds followed by 5% sodium hypochlorite for 10 minutes. Pandey *et al*. (2009) surface sterilized the explants (Lilium bulb scales) with 0.1% $HgCl_2$ and 2% Bavistin for 7.5 minutes and obtained 90.62% aseptic cultures. Priyaadarshi and Sen (1992) obtained maximum survival of explants after washing them in 5% teepol for 10 min and then in 0.1% $HgCl_2$ for 30 min. Gupta *et al*. (1978) surface sterilized the bulb scales of *Lilium longiflorum* in 0.2 per cent $HgCl_2$ solution for 15 minutes.

Two types of bulb scale explants were used. Highest culture asepsis was observed in tip scale segments compared to basal scale segments, which recorded minimum. Lilium scales are attached to basal plate of the bulb from which roots develop. Basal segment of scale is more close to roots and basal plate of the bulb, while the tip segment or terminal portion of bulb scale is away from basal plate and roots. Close proximity to basal plate and roots may be the cause of lower culture asepsis of basal segment explants.

Explant survival data is used to give an idea about the harmful effects of sterilants on the establishment and growth of explants (Rather, 2010). Aseptic cultures obtained after sterilization were used to study the damaging effects of sterilants on their further growth. Explant survival was highest with single sterilant treatments compared to combined sterilant treatments. Maximum explant survival of 95.48% was observed with S_1 (0.1% mercuric chloride for 10 minutes) and minimum with S_4 (1.0% sodium hypochlorite for 20 minutes). Sodium hypochlorite or its combination with other sterilants resulted in higher explant lethality and yielded minimum explant survival, in comparison to mercuric chloride and its combinations. Combined sterilant treatments resulted in reduction of explant survival. Among the combined treatments, S_5 (mercuric chloride 0.1% for 10 minutes followed by 70% ethyl alcohol for 30 seconds) and S_9 (Carbendazim 200 ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by 70% ethyl alcohol for 30 seconds) recorded better explant survival of 89.36 and 86.12%, respectively. These two treatments also recorded higher culture asepsis as well. These results are in conformity with Rather *et al.* (2014) who also recorded reduced explant survival under combined treatments in comparison to single sterilant treatments with underground buds of herbaceous peony. Aslam *et al.* (2013) used a combined sterilization treatment regime including 96% ethanol, 70% commercial bleach and 0.1% $HgCl_2$ for developing micropropagation protocol of *Lilium orientalis* and *Lilium longiflorum* cv. White Fox.

Overall results indicated that basal scale segments survived better than tip scale segments which may be due to difference in their maturity. Basal segment of the scale is more mature and thicker than tip segment which is thin and tender. Tender explants show more phytotoxic effects of sterilants compared to mature ones and hence less survival.

5.2 Culture establishment

In the current study, culture establishment of Oriental Lilium hybrid cv. 'Ravenna' was studied in terms of percent establishment, days to initiation of bulblets, number of bulblets per explant, length of established shoots and leaf number of established shoots in basal and tip scale segment explants. All the eight plant growth regulator combinations tested during the investigation resulted in successful culture establishment. MS media fortified with NAA + BAP (0.5 + 2.0 mg/l) proved best for culture establishment in terms of establishment percentage, number of bulblets per explant, length of established shoots and leaf number of established shoots, both in basal and tip scale segments (Table 4.3, 4.5, 4.6 and 4.7). Early bulblet initiation was observed with NAA + BAP (1.5 + 2.0 mg/l) taking 13.52 days, which was statistically at par with treatment NAA + BAP (0.5 + 2.0 mg/l) with 13.95 days (Table 4.4).

Similar observations were made by Mir *et al*. (2012) while working with *Lilium longiflorum*. Stimart and Ascher (1978) and Niimi (1985) reported NAA to be essential for the formation and growth of bulblets in scale culture. Van Aartrijk *et al*. (1985) noticed that auxin and its distribution within the tissue are important factors in the process of bud regeneration from lily scales. Higher number of leaves per bulblet may be attributed to the classical properties of cytokinins (Skoog and Miller, 1957 and Murashige, 1974). Thimann and Laloraya (1960) reported that cytokinins activated protein synthesis in buds for incorporation of labelled amino acids thus promoted cell division and cell elongation. Van Aartrijk and Blom-Barnhoorn (1981) regenerated bulblets from bulb scale explants of *Lilium speciosum* cultivars Rubrum No-10, Pirate and Connecticut King when MS medium was supplemented with NAA (0.1 -1.0 mg/l) and BAP (0.01- 0.10 mg/l). Dilta *et al*. (2000) reported *in vitro* effect of NAA and BA on culture establishment and bulblet formation in two hybrids of lily cv. Pollyanna and Star Gazer. Maximum leaves per bulblet were obtained when cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP. Similar results were found

by Novak and Petru (1981) in Lilium Oriental hybrid 'Creamson Beauty' on MS medium containing BAP + NAA (1.0 + 0.1 mg/l).

Explant type had a significant effect on explant establishment. Basal scale segments proved better explants recording higher values for establishment parameters as compared to tip scale segment explants. Lowest number of days to initiation of bulblets (14.15 and 15.84 days), highest number of bulblets per explant (4.91 and 3.51), length of established shoots (2.05 and 1.81 cm) and leaf number of established shoots (3.15 and 2.24) were recorded in basal and tip scale segments, respectively. Between the two explants tried in the investigation, basal scale segment explants performed better than tip scale explants. It may be attributed to optimum maturity and healthy status of basal scale segments. Tip scale segments are usually thin and hence weaker than basal scale segments. Stimart and Ascher (1978) recorded maximum number of bulblets from the basal bulb scale sections of *Lilium longiflorum* Thunb. Superior performance of basal scale segments may also be attributed to the fact that they are close to basal plate of the bulb and contain meristematic cell groups, which develop into adventitious buds. Kahane *et al.* (1992) reported that between bulb scales, axillary buds occur (only a few in each bulb) and most newly generated shoots/bulblets do not originate from these axillary buds but from distinct groups of cells at the abaxial side of scales near the basal plate. Grootaarts *et al.* (1981) observed that at the abaxial side of scales of nerine near the basal plate, there are a number of meristematic cell groups.

5.3 Culture proliferation

In the present study culture proliferation in *Lilium* was gauged in terms of per cent culture proliferation, number of shoots/explant, length of proliferated shoots and leaf number of proliferated shoots. Same growth regulator combinations involving NAA and BAP, which were used in culture establishment, were tried for further culture proliferation except two treatments. Two treatments T_7 (NAA 0.5 mg/l) and T_8 (NAA 1.0 mg/l) were discarded because callusing and

rooting was observed during establishment stage in these treatments. MS- solid media fortified with NAA + BAP (0.5 + 2.0 mg/l) proved best for culture proliferation. Maximum proliferating cultures, highest number of shoots/explant, length of proliferated shoots and leaf number of proliferated shoots (Tables 4.8, 4.9, 4.10 and 4.11) were recorded with this growth regulator treatment combination. Increasing the auxin concentration from 0.5 to 2.0 mg/l in media fortified with 2.0 mg/l BAP, resulted in the reduction of values of various proliferating characteristics like shoot & leaf number, shoot length and percentage of proliferating cultures. Results indicated that low auxin to cytokinin ratio favoured proliferation of Lilium cultures. These results are in conformity with the findings of Mir *et al*. (2012) in *Lilium longiflorum*. Takayama and Misawa (1979) also reported that higher auxin/cytokinin ratio increased root formation whereas lower ratio increased bulblet/shoot formation in Lilium.

Cytokinins are responsible for cell division and stem growth. The mode of action is to release axillary meristems from apical dominance and cytokinins are, therefore widely used in plant tissue culture for shoot multiplication (Taji and Williams, 1996). Cytokinins at appropriate levels are known to suppress terminal bud elongation and promote axillary shoot proliferation. Therefore, arriving at a right cytokinin level for optimum shoot production is a pre-requisite for mass propagation of any specie. A number of workers since classical experiments of Murashige have tried various growth regulator cocktails with diverse results in Lilium. Takayama and Misawa (1983) obtained highest multiplication rate from bulb scales of *in vitro* raised bulblets of *Lilium* on MS agar medium containing NAA (0.01 mg/l) and Kinetin (10.0 mg/l). Niimi (1985) reported maximum multiplication from *in vitro* raised bulb scales of *Lilium rubellum* on MS agar medium containing NAA (0.05 - 0.1 mg/l) and/or BAP (0.01 - 0.1 mg/l). Gi and Yeh (1992) studied the *in vitro* propagation of oriental lily cv. 'Casa Blanca' by shoot tip culture and found maximum proliferation with 0.5 mg/l NAA + 4 mg/l BA. Skoog and Miller (1957) reported that the higher number of leaves might

have produced due to the fact that cytokinins activate DNA synthesis thus enhance cell division. Kawarabayashi and Asahira (1989) reported maximum number of leaves per bulblet in *Lilium* species when cultured on MS medium supplemented with BA (1 mg/l) and NAA (0.1 mg/1). The longer leaf length may be ascribed on the basis of the fact that cytokinins promote cell division (Skoog and Miller, 1957).

5.4 Rooting percentage

Influence of varying concentrations of IBA and NAA on rooting behavior of Oriental Lilium hybrid cv. Ravenna was studied in the current investigation in terms of days to root initiation, rooting percentage, number of primary roots/shoot and length of primary roots. IBA was found superior than NAA. Sindhu *et al*. (2016) also reported better performance of IBA than NAA in rooting of microshoots of Asiatic *Lilium* cv. Pollyanna. Lowest days to root initiation were recorded in MS media supplemented with highest concentration of IBA (2.0 mg/l), while the highest per cent rooting, number of primary roots/shoot and length of primary roots was recorded in medium supplemented with 1.5 mg/l IBA. Rooting percentage and quality are known to improve significantly with the use of auxins particularly IBA. Arbaoui*et al.* (2017)used IBA (0, 0.5, 1.0, 1.5, 2.0 mg/l) for rooting of *in vitro* shoots of *Lilium longiflorum* and found 1.5 mg/l IBA best concentration for various rooting characteristics recording maximum root number and root length with this concentration. Sindhu *et al*. (2016) achieved best rooting (100%) in Asiatic *Lilium* cv. Pollyanna on half strength MS medium supplemented with 1.0 mg/l IBA. They also reported maximum root number and root length with 1.0 mg/l IBA. Kumar and Choudhary (2005)observed 100% rooting in Lilium bulblets when 0.5 mg/l IBA was used in combination with 0.5 mg/l NAA. Fei *et al.* (2008)also reported 100% rooting in bulblets in half-strength MS medium supplemented with 0.1 - 0.5 mg/l IBA.

5.5 Hardening

In vitro raised plantlets are very tender and delicate owing to high humidity in culture vessels (80-100%), controlled temperatures (typically 25 ± 2ºC), low light intensity and hetero or mixo-trophic mode of nutrition (Kozai *et al*., 1997). Main goal of a hardening system is to maintain an atmosphere with low evaporative demand around the tissue cultured plant freshly extricated from a sequestered system. This helps the plant to avoid substantial tissue water deficits before roots start functioning in the hardening module. Towards this end, hardening media plays an important role in *ex vitro* establishment of tissue cultured plants. Standardized rooting media for hardening of tissue cultured plantlets had already been standardized in the Plant Tissue Culture laboratory of the Division, which consists of perlite and vermiculite in the ratio of 1:1. Perlite is a chemically inert material with low bulk density of about 9-11 lbs/cft providing aeration in the root media. However, it has no cation exchange capacity and comparatively lower water holding capacity than vermiculite which has a bulk density of 7-10 lbs/cft and a cation exchange capacity of 19-22 me^{-}_{100g}.Vermiculite and perlite mixes form excellent media with optimum water holding capacity, aeration and drainage. The formulation also holds nutrients in an easily exchangeable form thus allowing the roots to resume normal function significantly earlier than other media. Standardized rooting media resulted in more than 80% *ex vitro* survival of rooted plantlets. Highest *ex vitro* survival of 98.96% was observed in plantlets which came from media fortified with IBA (1.5 mg/l). Plantlets which came from IBA treated cultures had higher survival rate as compared to NAA treated cultures. Higher *ex vitro* survival of IBA treated cultures may be attributed to their better rooting characteristics like root number and root length. Gong *et al*. (1996) reported 80-90% survival rate of tissue cultured plantlets of *Lilium* hybrid, when transplanted in the potting mixture of sand, peat and humus soil (1:1:1 v/v). For hardening of *in vitro* rooted bulblets of *Lilium* cv. Toscana; coco peat, peat moss and coco peat in combination with peat moss were found to be at par giving 100% survival (Kaur *et al*., 2006).

Chapter- 6

SUMMARY AND CONCLUSION

Thefindings in the present investigation entitled "Standardization of micropropagation protocol of Oriental Liliumhybrid cv. 'Ravenna' " is summarized under the following headings.

6.1 Culture asepsis

- Among the individual sterilization treatments, S_2 (0.1% mercuric chloride for 20 minutes) resulted in maximum culture asepsis of 54.17% followed by S_4 (1.0% sodium hypochlorite for 20 minutes) with 48.96% and minimum of 43.75% with S_3 (1.0% sodium hypochlorite for 10 minutes). Combined treatment of sterilants gave better results. Maximum culture asepsis of 77.08% and 71.88% were obtained with S_9 (Carbendazim 200ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) and S_{10} (Carbendazim 200ppm for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) respectively. Both these treatments differed significantly from one another and were superior to all other sterilant treatments.
- Explant type had a significant effect on culture asepsis. Maximum culture asepsis of 63.54% was recorded with tip segments and minimum of 56.46% with basal segments.
- Interaction effect between explant type and sterilants on culture asepsis was statistically non-significant.

6.2 Survival

- Maximum explant survival of 95.48% was recorded with S_1 (Mercuric chloride 0.1% for 10 minutes) followed by S_2 (Mercuric chloride 0.1% for 20 minutes) with 91.86%. Both these treatments differed significantly from all other treatments. Combined treatments of

sterilants resulted in reduction of explant survival. Minimum (81.25%) explant survival was recorded with S_8 (Sodium hypochlorite 1.0% for 20 minutes followed by ethyl alcohol 70% for 30 seconds) followed by S_{10} (Carbendazim 200 ppm for 30 minutes followed by sodium hypochlorite 1.0 % for 10 minutes followed by ethyl alcohol 70% for 30 seconds) with 82.92 %. Both these treatments differed significantly from all single sterilant treatments. Sterilant combinations which yielded highest aseptic cultures recorded higher explant survival to the tune of 86.12% and 82.92% with S_9 (Carbendazim 200 ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds) and S_{10} (Carbendazim 200 ppm for 30 minutes followed by sodium hypochlorite 1.0% for 10 minutes followed by ethyl alcohol 70% for 30 seconds), respectively.

- Explant type had a significant effect upon explant survival. Basal scale segments recorded maximum explant survival (88.54%) and minimum of 85.52% was observed with tip scale segments.
- Interaction effect between explant type and sterilants was found statistically non-significant.

6.3 Establishment

- MS- solid media fortified with NAA + BAP (0.5 + 2.0 mg/l) proved best for culture establishment in terms of explant establishment (76.17%), number of bulblets per explant (5.52), length of established shoots (2.20 cm) and leaf number of established shoots (3.39). The minimum duration to initiation of bulblets (13.52 day) was recordedin media supplemented with NAA + BAP (1.5 + 2.0 mg/l), which was statistically at par with treatment NAA + BAP (0.5 + 2.0 mg/l) with 13.95 days.
- Explant type had a significant influence on various parameters of explant establishment. Basal scale segment proved superior to tip scale

segments and recoded maximum explant establishment (68.26%), number of bulblets per explant (4.91), length of established shoots (2.05 cm), leaf number of established shoots (3.15) and minimum duration to initiation of bulblets (14.15 day).

- Interaction effect between explant type and growth regulators on various parameters of culture establishment was found significant. Maximum explant establishment (83.58%), number of bulblets per explant (6.97), length of established shoots (2.34 cm) and leaf number of established shoots (3.94) was recorded with basal scale segment explants when cultured on media supplemented with NAA + BAP (0.5 + 2.0 mg/l). Minimum duration to initiation of bulblets (12.75 day) was observed with basal scale segments when cultured on media supplemented with NAA + BAP (1.5 + 2.0 mg/l).

6.4 Culture proliferation

- Established cultures obtained during establishment stage were divided and individual shoots were put in growth regulator supplemented media for further multiplication. Growth regulator treatments had a significant influence upon all parameters related to explant proliferation. Highest culture proliferation (83.33%), number of shoots/explant (2.41), length of proliferated shoots (2.35 cm) and leaf number of proliferated shoots (5.44) was recorded on MS media fortified with NAA + BAP (0.5 + 2.0 mg/l). Whereas the lowest culture proliferation (61.46%), shoot number/explant (1.62), length of proliferated shoots (1.68 cm) and leaf number (3.97)was recorded with growth regulator combination of NAA + BAP (2.0 +2.0 mg/l).
- Significant influence of explant type was observed on various culture proliferation. Basal scale segment explants showed superior performance in various culture proliferation parameters. Maximum culture proliferation (75.35%), number of shoots/explant (2.37), length

of proliferated shoots (2.21 cm) and leaf number of proliferated shoots (5.07) was recorded with basal scale segment explants.

- Significant interaction effect of explant type and growth regulatorswas observed on number of shoots/explant and length of proliferated shoots, recording maximum values of 2.90 and 2.59 cm, respectively with basal scale segments when cultured in media supplemented with NAA + BAP (0.5 + 2.0 mg/l). Non-significant interaction effect between explant type and growth regulatorswas observed for culture proliferation (%) and leaf number of proliferated shoots.

6.5 Rhizogenesis

- Healthy individual shoots from proliferated cultures were taken and put in rooting media supplemented with different auxin types (IBA & NAA) and concentrations (05, 1.0, 1.5, 2.0 mg/l). Growth regulators had a significant effect on various rooting parameters. Performance of IBA was superior in comparison to NAA. Highest rooting (92.71%), number of primary roots/shoot (12.06) and length of primary roots (3.17 cm) was recorded in medium supplemented with 1.5 mg/l IBA. However, days to root initiation were found minimum (17.95) with 2.0 mg/l IBA.
- Explant type had a significant effect on various parameters related to rhizogenesis of microshoots. Basal scale segments performed significantly better than tip scale segments. Maximum rooting of shoots (84.12%), number of primary roots/shoot (10.02) and length of primary roots (2.17 cm) was recorded with basal scale segment explants and minimum with tip scale segment explants. However, days to root initiation were found minimum (20.24) with basal scale segments.
- Interaction effect between explant type and growth regulators on all rooting parameters was found significant except days to root initiation.

Maximum rooting (97.92%), number of primary roots/shoot (13.40) and length of primary roots (3.57 cm) was recorded with basal scale segments when cultured on media fortified with 1.5 mg/l IBA.

6.6 Hardening

- Rooted plantlets were transferred into polypropylene glasses filled with rooting media consisting of perlite and vermiculite in the ratio of 1: 1. Highest *ex vitro*survival (98.96%) was observed in plantlets, which came from MS media supplemented with IBA (1.5 mg/l) followed by 95.83%in plantlets which came from media supplemented with IBA (2.0 mg/l). IBA treated plantlets had higher survival rate as compared to NAA treated plantlets. Also plantlets developed from basal scale segment explants had significantly higher survival (93.49%) compared to plantlets from tip scale segments (84.63%)
- Interaction effect between explant type and growth regulators on ex vitro survival of rooted plantlets during primary hardening was found statistically non-significant.

CONCLUSION

I. Combined sterilization of explants with Carbendazim 200ppm for 30 minutes followed by mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 30 seconds resulted in maximum culture asepsis (77.08%) and optimum explant survival (86.12%).

II. Best growth regulator combination for culture establishment was found NAA + BAP (0.5 + 2.0 mg/l), which yielded maximum culture establishment (76.17%), bulblets per explant (5.52), length (2.20 cm) and leaf number (3.39) of established shoots.

III. Proliferation of established shoots was also highest in media fortified with NAA + BAP (0.5 + 2.0 mg/l), recording maximum proliferating cultures

(83.33%), highest number of shoots/explant (2.41), length (2.35 cm) and leaf number (5.44) of proliferated shoots.

IV. Best rooting was recorded in MS media fortified with IBA 1.50 mg/l, recording maximum rooting of microshoots (92.71%), number of primary roots/shoot (12.06) and length of primary roots (3.17 cm).

V. Basal scale segment explants proved better than tip scale segments, showing superior performance in all establishment, proliferation and rooting parameters.

VI. Standardized rooting media (perlite and vermiculite in the ratio of 1:1) resulted in more than 80% *ex vitro* survival of rooted plantlets. Highest *ex vitro* survival of 98.96% was observed in plantlets which came from media fortified with IBA (1.5 mg/l).

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner **Dr. A. Q. Jhon** Ex. DRI and HOD, Division of Floriculture and Landscape Architecture, SKUAST-Kashmir during Viva-Voce examination held on 28-01-2019 have been incorporated in the manuscript entitled **"Standardization of micropropagation protocol of Oriental *Lilium* hybridcv.'Ravenna'"** submitted by **Ms. Sadaf Rafiq (Regd. No. 2016-H-123-M).**

(Dr. Z. A. Rather)
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