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LARGE SCALE *IN VITRO* MULTIPLICATION AND HARDENING OF HYBRID LILIUM

By:

Shanu Kataria

Kavita Baberwal

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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173215, HIMACHAL PRADESH, INDIA

CERTIFICATE

This is to certify that the work titled "*IN VITRO* MULTIPLICATION AND HARDENING OF HYBRID LILUM" submitted by "**MS SHANU KATARIA and Ms KAVITA BABERWAL**" in partial fulfillment for the award of degree of **Biotechnology** of Jaypee University of Information Technology,Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. (Mrs.) Hemant Sood
Lecturer

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of Dr. (Mrs.) Hemant Sood, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for this or any degree or diploma to any other university or institute. All assistance and help received during the course of the investigation has been duly acknowledged.

(Shanu Kataria)

(Kavita Baberwal)

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Needless to say, errors and omissions are mine.

Shanu Kataria

Kavita Baberwal

Date :

SUMMARY

There are between 80 to 100 species of lilies (Liliaceae), and most are native to the Northern Hemisphere in Asia, Europe and North America. The bulbs produce leafy stems and large flowers, some of which are strongly scented. Lilies have been treasured throughout history for their beauty and perfume, and they serve as an important cultural symbol.

Few genera of plants are as amenable as Lilium to varied means of propagation. Besides the obvious means, seed, lilies can be increased from daughter bulbs that offset from the main bulb. In scaling, portions of the bulb are detached and induced to form new bulbs. Many kinds of lilies form underground bulb lets on the stem just above the main bulb. Some produce stem bulbils in their leaf axils, and the production of bulbils on the stem can be induced in most lilies.

The tissue culture laboratory has become an excellent tool for the propagation of new and desirable clones for the commercial market. A clone can be increased very rapidly by this method, with a single choice seedling yielding as many as 2 million tissue-culture bulb lets in two years.

Before they were extensively hybridized, lilies were rare and could be difficult to grow.Although the lily has no real pharmaceutical value, it was commonly believed to have magical qualities. Elizabethan-era medical texts recommended using the lily to treat fever, wounds and arthritis. Lily bulbs are starchy and have long been used in cooking in China. They are a frequent ingredient in the local cooking of Shanghai.

Some lilies, especially *Lilium longiflorum*, as well as a few other hybrids, form important cut flower crops. These tend to be forced for particular markets; for instance, *L. Longiflorum* for the Easter trade, when it may be called the Easter lily.

As the plant has high commercial importance so its availability with authencity and long shelf life are prerequisite, i.e. in the present research, we explore all the means for carrying out its large scale multiplication through micro propagation. We have tried many Ms media having different concentration and combination of growth hormones but MS + IBA (1mg/l) + KN (2mg/l) + GA3 (1 mg/ml) is identified as best media for shoot induction and MS + NAA(1

mg/ml) + IAA (1 mg/ml) is identified as best media for root induction in hybrid lily . After their large scale multiplication the actual step for the acceptability of this technique is the amount of survival of these tissue culture plantlets in field conditions so we tried different potting mixtures for hardening of the rooted plantlets and we achieved 99% survival during transplantation. In this way we have developed successful micro propagation technique for large scale production of *hybrid lilies*.

LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
°C	DegreeCelsius
GA3	Gibberellic acid
IAA	Indole acetic acid
Gm	Gram
IBA	Indole -3 Butyric Acid
Kg	Kilogram
NAA	Naphthalene acetic acid
KN	Kinetin
l / lt.	Litre
mg	Milligram
MS media	Murashige and Skoog Media

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CHAPTER 1 –INTRODUCTION

Lilies

The genus *Lilium* belongs to the Liliaceae family which comprises of about 80 species (Comber 1949), and thousands of cultivars (Leslie 1982-2005). The lily species are taxonomically classified into seven different sections based on various morphological and physiological characteristics. The seven sections are Lilium, Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum (Comber 1949; De Jong 1974).

In general, wild species within each section are relatively easy to cross and the hybrids are fertile (McRae 1990; Van Tuyl et al. 2002). Most of the cultivars, however, are interspecific hybrids within the sections (especially Leucolirion, Archelirion and Sinomartagon) and represent the most important cultivated groups which are:

1. The **Longiflorum** hybrids (**L**-genome) which originate from intra- or interspecific hybridization in the Leucolirion section, have trumpet-shaped, pure white flowers, a distinctive fragrance, year- round forcing ability and mostly outward-facing flowers (McRae 1990).

2. The **Asiatic** hybrids (**A**-genome) are derived from interspecific crosses among at least 12 species of the Sinomartagon section (Leslie 1982-2005). Cultivars of Asiatic hybrid lilies have a wide colour variation in their flower tepals (orange, white, yellow, pink, red, purple and salmon) and early to late flowering (Woodcock and Stern 1950). Some

species in this section show resistance to *Fusarium* and viruses (McRae 1998).

3. The **Oriental** hybrids (**O**-genome) are nowadays the most important lily hybrid group. They result from hybridization among five species of the Archelirion section. Generally, Oriental hybrids are late-flowering, with big and showy flowers with a pleasant fragrance (McRae 1998). Most Oriental hybrids show a fair degree of resistance to *Botrytis elliptica* (Barba-Gonzalez et al. 2005). Fig. 1.1 shows Longflorium, Asiatic and Oriental hybrids.

Fig 1.1. Figures from different lily hybrids: (a) Longiflorum hybrids, (b) Oriental hybrids and (c) Asiatic

In recent years, propagation of numerous ornamental plants by tissue culture has become an accepted commercial practice. Current production techniques for *In vitro* propagation of plants have allowed for strong and continued growth within the micropropagation industry.

Lilium is one of the leading cut flowers all over the world (Kumar,2007). It ranks seventh among the cut flowers in the world in terms of auction turnover, and is a very popular pot plant as well.

According to Van Aartrijk *et al.*, (1990), the main constraints in conventional propagation of lilies include the inadequate availability of healthy, disease-free planting material, and slow multiplication rates. The conventional method of propagating lilies involves scaling yields at most three to five bulbs from each scale, depending on the bulbscale size and species/ variety. Thus scaling a bulb yields somewhere between 50 and 100bulblets, far too few to meet the present demand for planting material (Varshney, 2000).

One of the best and most prolific vegetative propagation method for lilies is *in-vitro* scale culture (Jeong, 1996; Varshney *et al.*, 2001; Bahr & Compton, 2004). *In vitro* adventitious bud regeneration from scales of *Lilium* depends on factors such as concentrations of plant growth regulators employed, (Jeong, 1996; Varshney *et al.*, 2000), concentrations of sucrose (Jeong, 1996; Varshney *et al.*, 2000; Kumar *et al.*, 2005), light treatment (Varshney *et al.*, 2000; Lian *et al.*, 2002b; Kumar *et al.*, 2005), scale position or kind of explant (Jeong, 1996; Varshney *et al.*, 2000). The current study reports developing a micropropagation of regime for *L. regale* in which regeneration, followed by production of adventitious shoots on a large scale was optimized, and it is envisaged to yield showy, attractive flowers. The use of tissue cultured plant material can rationalize lilium cultures because direct planting into pots without special manipulation for a large harvest of bulbs is possible. Therefore micropropagation by means of *in-vitro* techniques is of great interest in order to speed up the propagation rate and to reduce the need for bulblets. The objective of this work was to define an efficient protocol for the micropropagation of lilium, cv. Regale, by the manipulation of Benzyl-3-amino purine

(BAP) concentrations, type of culture medium and sectioning of the axillary buds used as explants, aiming at the maximization of the multiplication rate in order to make this a feasible mode of propagation.

Use of shoot primordia and embryoid techniques have also been advocated for macro-propagation of the lilies (Tanaka *et al.*, 1991; Sugiura, 1994). A shoot primordium culture gives rise to virus-free tissue given that it is obtained from the apical meristem tip. Successful shoot primordia production in *L. longiflorum* via apical meristem tip culture has been reported (Tanaka *et al.*, 1991). Shoot primordia production of the Oriental Hybrid Lily and the Asiatic Hybrid Lily from the meristem tip via protoplasts culture has also been reported (Sugiura, 1993). Also, embryoids do offer an alternative for lily propagation, especially given relatively lower labour costs compared to that for the shoot primordia. Minimal protocol is required for the embryoids techniques thus eliminating the bulk of labour cost in propagation (Sugiura, 1994). Embryoids are not only suitable for long term storage in super-freezers (Sakai, 1991), with no regeneration difficulties, but are also easy to transport in the from of artificial seeds (Murashige, 1978; Yakuwa and Oka, 1987).

Nonetheless, an important drawback in plants regenerated from tissue culture is accompanying mutations that occur with a consequence of gradual deterioration of genetic purity of the crop. We therefore focused on possible somaclonal mutations arising out of embrioids and shoot primordia, and assessed their commercial viability as potential systems for lily propagation.

Tissue culture is commonly cited as a method of producing virus-free plants, but the method of doing this is much more complex than most people assume. When plant tissue is initiated into culture, it is tested with an enzyme-linked immunosorbent assay test for the presence of common lily viruses. If virus is present, it can be eliminated by "meristemming," a process in which tiny pieces of rapidly growing meristem tissue are removed, cultured, and repeatedly tested. Obtaining truly virus-free tissue in this way can take more than a year of repeated procedures. When the resulting plants leave the laboratory, of course, they can quickly become reinfected, so breeding virus-tolerant lilies remains a high priority.

The material used to initiate a lily tissue culture usually comes from bulb scales, but stem segments with an internode, or flower buds, can also be used. To avoid sacrificing a precious plant, the bulb may be carefully dug, leaving the stem intact in the ground, where it usually produces stem bulblets. Tissue cultures of endangered wild species have been produced from unopened flower buds.

The donor plant should be one that is unlikely to have been exposed to virus. The material to be cultured should be kept fresh and packed dry to prevent fungal growth.

Once the material reaches the laboratory, it is surface sterilized in a 10 percent solution of household bleach for 20 to 30 minutes. It is then rinsed and cut into segments (called "explants") approximately 5 millimeters (0.25 inch) square; the bottom half of the scale is used for this, the portion closest to the basal plate forming bulblets most readily. The explants are then placed in their culture vessels in a medium, wrapped in plastic, and stored at 21°C (70°F). Often a considerable percentage of the new cultures fails because of contamination by soil organisms. Those that survive produce visible bulblets within four to six weeks.

The medium in which the explants grow is a solution of basic fertilizer nutrients and a few complex vitamin-like compounds. Often, synthetic hormones are added; most lily media contain 0.03 milligrams per liter of naphthalene acetic acid (NAA) , a synthetic auxin. Sucrose (table sugar) supplies the energy for growth. The medium is gelled with agar or a proprietary agent such as Gelrite. The most important factor in the medium is the concentration of sugar, which supplies all the energy that a normally growing plant would derive from photosynthesis. (Tissue culture takes place in the dark.) Lilies need higher levels of sugar than many other tissue-cultured plants. Sucrose is added at the rate of 45 to 60 grams per liter of medium; during the latter stages, when bulblets are being produced, this may be increased to as much as 90 grams per liter.

The cultures are grown in complete darkness at a temperature of 20° to 24°C (68° to 75°F). They produce few if any leaves, which maximize production of new bulblets as the energy is directed to that end. After the culture has been initiated, it is repeatedly tested for virus until the technicians are reasonably sure it is virus-free. Then the multiplication stage starts, during which

cultures are maintained in very active growth. Under optimal conditions, these cultures can be "subcultured" or divided into multiple new cultures every 8 to 10 weeks.

Subculturing takes place when the cultures have produced small clumps of bulbs 0.5 to 1 centimeter (0.25 to 0.5 inch) in diameter. These clumps are separated (a process called "singulation") and replated onto fresh medium. After 8 to 10 weeks, each forms a new clump.

Alternatively, the-small bulbs may be dissected into their individual scales. This produces more bulb lets but requires a longer cycle. Some varieties can also be subcultured by chopping the small clumps of bulbs into pieces about 1 millimeter square.

The last phase of tissue culture micro propagation of lilies is the rooting and bulbing stage, during which the subculture is made to produce as large a single bulblet as possible. The bulbing medium contains no growth hormones (because multiplication is not desired) and has an increased concentration of sucrose at 60 to 90 grams per liter. A period of 10 to 16 weeks is required to produce a high-quality bulblet.

Large single bulblets are the easiest to grow after they are removed from culture. Some laboratories produce small clumps of bulbs in the last stage, but the grower must invest extra labor in dividing these before planting, and losses in the first year are usually higher. Bulblets originating in the sterile environment of the tissue culture laboratory must be scrupulously protected from infection, especially by fungi, in early stages of growth. Timing is also critical: varieties requiring a vernalization period before spring planting must not be delivered too late.

When the bulblets are removed from the culture vessels, they must be washed thoroughly in running water, removing all traces of agar. The bulblets are then dipped in a fungicide to protect against soil-borne diseases, such as Fusarium and Cylindrocarpon. A combination of TBZ (Mertect) and captan has been very effective.

The bulblets are then packed in plastic bags, using a well-aerated medium with a low moisture level; a mixture of dry sphagnum peat and moist vermiculite has proven ideal. The bulblets and their roots must be kept under optimal conditions, neither too wet nor too dry. Pack only a limited number in each bag, and ensure that there is good aeration. The bags are stored for a minimum of 8 weeks (for Asiatic lily) to 12 weeks (for Oriental lily) before planting. In the

spring they are planted in sterilized greenhouse beds, or in trays of pasteurized soil that are also kept in the greenhouse.

Uses

Many species are widely grown in the garden in temperate and sub-tropical regions. Sometimes they may also be grown as potted plants. A large number of ornamental hybrids have been developed. They can be used in herbaceous borders, woodland and shrub plantings, and as patio plants. Some lilies, especially *Lilium longiflorum* form important cut flower crops. These may be be forced for particular markets; for instance, *L. longiflorum* for the Easter trade, when it may be called the Easter lily.

Lilium bulbs are starchy and edible as root vegetables, although bulbs of some species may be very bitter. The non-bitter bulbs of L. *lancifolium*, *L. pumilum*, and especially *L. brownii* and Lilium davidii var unicolor cotton are grown on a large scale in China as a luxury or health food, and are most often sold in dry form. They are eaten especially in the summer, for their perceived ability to reduce internal heat. They may be reconstituted and stir-fried, grated and used to thicken soup, or processed to extract starch. Their texture and taste draw comparisons with the potato, although the individual bulb scales are much smaller. *Yuri-ne* (lily-root) is also common in Japanese cuisine, especially as an ingredient of chawan-mushi (savoury egg custard).

The "lily" flower buds known as *jīnzhēn* ("golden needles") in Chinese cuisine are actually from the daylily Hemerocallis fulva. This plant – despite its looks – is actually more closely related to asparagus than to true lilies.

Lilies are used as food plants by the larvae of some Lepidoptera species including the Dun-bar.

Propagation and growth

Lilies can be propagated in several ways;

by division of the bulbs

by growing-on *bulbils* which are adventitious bulbs formed on the stem

by scaling, for which whole scales are detached from the bulb and planted to form a new bulb

by seed; there are many seed germination patterns, which can be complex

by micropropagation techniques;commercial quantities of lilies are often propagated *in_vitro* and then planted out to grow into salable sized plants

Fertilization barriers and hybrid sterility

The species of *Lilium* possess a wide range of ornamental traits as well as resistances to fungal and virus diseases. Interspecific hybridization is the most important method in *Lilium* breeding to produce cultivars from distantly related species (Van Tuyl and Lim 2003). Most of the lily cultivars have an interspecific hybrid origin, basically between diploids (2n = 24) and, more rarely, between tetraploid parents. Breeding of lilies for new cultivars is time consuming. They need 2 to 3 years from sowing of seeds to first flowering. Vegetative propagation takes another 3 - 5 years. Because of their heterozygous genome structure they are maintained as clones (Booy et al. 1998).

It is desirable to combine or introgress some important horticultural traits from species of different sections into a single cultivar. In *Lilium* it is very difficult to obtain F1 interspecific hybrids due to pre- and post fertilization barriers. Nearly all the F1-hybrids between Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA) are sterile. This sterility is due to irregular chromosome paring between the parental genomes during meiosis (Asano 1982; Lim et al. 2000; Barba-Gonzalez et al. 2004). For successful interspecific hybridization, pre- and postfertilization barriers can be overcome by the use of various *in vitro* pollination and embryo rescue techniques respectively (Van Tuyl et al. 1991). Successful interspecific crosses in *Lilium* are depicted in Fig. 1.2 as a crossing polygon (Lim et al. 2007) Somatic chromosome doubling of the F1 hybrids using colchicine or oryzalin can induce allotetraploids in which homologous pairing can restore fertility (Van Tuyl and De Jeu 1997). However, this method could not contribute much to introgression breeding due to the formation of the so-called "permanent hybrids" as their progenies never segregate for parental characters due to autosyndatic chromosome pairing (Ramanna and Jacobsen 2003; Van Tuyl and Lim 2003).

On the contrary, intergenomic recombination may occur in sexual polyploids induced through the formation of 2*n* gametes (Ramanna et al. 2003; Ramanna and Jacobsen 2003).

Interestingly, some F1 LA and OA hybrids produce functional 2*n* gametes in reasonable frequencies. When such 2*n* gametes are used for generating back cross progenies they have the potential to generate genetic variation as has been shown in BC1 progenies of Oriental × Asiatic (Barba-Gonzalez et al. 2005a) and Longiflorum × Asiatic hybrids and cultivars (Zhou 2007; Zhou et al. 2008). 2*n* gametes occur occasionally in interspecific hybrids of *Lilium* (Van Tuyl et al. 1989) and, as has been demonstrated in other plants, they result from abnormal meiosis (Mok and Peloquin 1975; Ramanna 1979; Lim et al. 2001a). In lilies the BC1 progenies resulting from functional 2*n* gametes hybridize and sometimes their homoeologous chromosomes pairs and recombine at certain level (Lim et al. 2001a; Barba-Gonzalez et al. 2004; Zhou 2007). This may result in the assembly of complementary characters within a single genotype in a back cross breeding program.

Toxicology

Many varieties of lily are extremely toxic to cats, causing acute renal failure even in small amounts. This is particularly true in the case of Easter lily plants, though other *Lilium* and the related Hemerocallis can also cause the same symptoms.

Diseases and Pests:

Botrytis - is a fungus disease, which affects the leaves of lilies, caused by excessive moisture and warm temperatures. The first signs can be brown spots on the leaves. In severe cases the whole leaf and stem can become infected and the whole plant decay and collapse. Injury to plants, like frost or hail will make it easier for Botrytis spores to enter the leaf; spraying is strongly advised very soon after injury. The disease is not carried by the bulb so it will not affect flowering the following year. In early stages of infection, if possible remove noticeable spotted leaves. Spraying is highly advisable, and is only effective when foliage is dry. A copper spray can be used or natural remedies such as a baking soda mixture (1 tbsp. per gallon of water)

sprayed weekly on the foliage during wet periods. Good air circulation will help prevent a outbreak. Planting lilies some distance apart will also control infection. In the fall clean up and burn dead stems and leaves.

Basal Rot - This fungus invades the bulb through the roots and basal plate. The symptoms on the growing plants are usually premature streaky yellowing of the foliage. The disease can become present in warm moist soils. As a preventive, avoid over-watering during warm summer months and provide good drainage. As for infected bulbs, you can remove the infected scales, dip the bulbs in a fungicide solution.

Blue Mold - Because lilies have a high sugar content, bruising or mechanical injury can cause a penicillin mold to form on the injured part of the bulb. This is harmless to the bulb and can be carefully removed. The bulb can be dusted with a fungicide powder and planted as usual.

Virus Diseases - Lily viruses are transmitted largely by aphids. Visible evidence of virus could be all or some of the following; Irregular mottling and flecking on the leaves, reduction in plant size and height, distorted growth, color-breaking in the flowers and leaves, brown ring patterns on bulb scales.

A few tips to help control viruses are: Destroy clumps of lilies that show severe infection, insuring that all bulbs and scales are discarded. Remove plants showing infection early in the season. Avoid planting lilies next to other host plants like Tulips or lilium tigrinum (also known as the Tiger Lily). Control aphid infections with the use of insecticides.

Lily Beetle - Long prevalent in Europe, the lily beetle (Lilioceris lilii) has been reported in eastern North America. The larvae and adult beetles feed on the leaves of lilies. The larva is a yellow grub with a dark head, covering itself in dark, slimy excrement. The adult is up to 8 millimetres (0.25 inch) long and bright scarlet with black legs and antennae. Both life stages have voracious appetites and soon devour entire plants. The eggs are laid on the underside of the foliage. The following controls are effective: Spray plants with contact and systemic insecticides; both are effective. Drench soil with a soil insecticide to kill the mature larvae that live just under the soil surface in winter. Also, avoid transporting infested soil to other sites. Catch adult beetles

between the fingers and smash them. The lily beetle has only appeared in a few places on this continent, and with care, it should be possible to prevent any lasting infestation.

As plants are commercially very important and their consumption in market is quite high, so in order to meet the market demand their large scale multiplication and good shelf life are the significant characteristics required in plants and flowers. So this study is carried out with the following objectives:

OBJECTIVES:

- To carry out large–scale in vitro multiplication in solid and liquid medium
- *In vitro* root induction
- Hardening of *in vitro* grown plants and final transplantation to field conditions

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CHAPTER 2: REVIEW OF LITERATURE

2.1 General micropropagation

In-vitro propagation comprises of various stages: selection of explants; aseptic culture establishment; multiplication of propagules; rooting and acclimatization of plantlets. Conservation through vegetative propagation is slow and time consuming but tissue culture offers an alternative tool for rapid multiplication and conservation of disease free propagules in a short period.

2.2 Bulblet production from node explant grown *in vitro* in hybrid lilies:

An attempt was made to regenerate bulblets from *ex vitro* node explants of hybrid lilies in the year 2007. Node sections (3-4 mm) isolated from the middle part of the stem in hybrid lilies were cultured on Murashige and Skoog (MS) medium supplemented with several combinations of NAA and BA. Growth regulator-free medium was not effective in inducing bulblet regeneration. A significant increase in the percent of explant producing bulblets and number of bulblets per explants was observed when 2 mg/l NAA was used in combination with 1.5 or 2 mg/l BA. The heaviest bulblets were obtained with 2 mg/l NAA in combination with 1.5 mg/l BA after 90 days of culture.The cultivar Apeldoorn produced greater number of bulblets whereas more weighty bulblets were produced in the cultivar Beartix. 1 or 2 mg/l IBA was most effective in producing roots. The rooted bulblets were hardened with 80-82% survival success after 30 days of transfer in the pots.

2.2.1 Culture conditions:

The medium was adjusted to pH 5.8, followed by addition of 0.8% (w/v) Agar. The cultures without growth regulators served as control. Three replications with 10 explants in each were

maintained for each treatment. The explants were cultured in Erlenmeyer flask (100 ml) containing 30 ml of medium and closed by nonabsorbent cotton plugs. The cultures were maintained under 16/8 h light/dark photoperiod provided with cool-white fluorescent lamps (50 μmol m-2 s-1) at a temperature of 25±2 oC. The *in vitro* produced bulblets were separated and individual bulblet was transferred to MS medium supplemented with 0.5, 1, 1.5 and 2 mg/l IBA after 30 days

The data wereanalyzed statistically using factorial completely randomized design consisting of two factors each with three replications. The first factor (A) was cultivars (five levels) and the second factor (B) was MS medium with growth regulators (five levels). The rooted bulblets were taken out of the culture vessels, washed with water to remove the adhering agar and treated with 0.2% bavistin (Carbendazim, a fungicide) for 30 min and were transferred to the pots (4" diameter) containing cocopeat. The hardening and acclimatization procedures were followed as described by Kumar et al., (2007). When the leaves were dried, the bulblets were removed from the pots, washed thoroughly and dried at room temperature.

2.2.2 Materials and Methods:

The explants were surface sterilized with 5% sodium hypochlorite for 8-9 min and washed 3-4 times with sterilized distilled water before culturing. Nodal sections (3-4 mm) were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 100 mg/l meso-inositol and in all possible combinations of 0, 1 and 2 mg/l NAA and 0, 1.5 and 2 mg/l BA.

2.3 Acclimatization of Asiatic hybrid lilies under stress conditions after propagation through tissue culture:

Bulb scales were separated from the bulbs, washed properly in running water and with 5% liquid detergent. Explants were dipped in 70% alcohol for one minute and then surface-sterilized with 0.1% HgCl2 for 15 min. Washing was done at least three times at 5 min intervals, with sterilized double distilled water. The explants were inoculated in the Murashige and Skoog's9 medium supplemented with 1 mg/l indole-3-acetic acid (IAA) + 0.5 mg/l 6-benzyladenine (BA) + 10 mg/l adenine sulphate (AdS). The pH of all the media used was adjusted to 5.8 before autoclaving at

1.08 kg/cm2 for 15 min. The cultures were incubated at 26 □2□C under 3 klux light through fluorescent tubes, for 16 h photoperiod (50–60 mol m–2 s–1).

2.4 IN VITRO POLLEN SELECTION FOR HEAT-TOLERANCE IN LILIES

Heat-tolerance is an important character for lily bulb and flower production under subtropical and tropical conditions. Since a gametophytic-sporophytic genetic overlap has been demonstrated in many plant species, pollen selection at high temperatures might be an efficient method to achieve heat tolerance.

2.4.1 Materials and Methods

Bulbs of one accession of L. formosanum, one accession of L. neilgherrense and 14 accessions of L. longiflorum (Table 1) were planted in pots in a greenhouse of CPRO-DLO in April and flowered in June and July. Fresh pollen was collected from the plants and directly used. Pollen was germinated on a modified BK-medium (Brewbaker and Kwack 1963). As a modification, the component H3BO3 was 3- times as high as in the BK-medium and supplemented with 10% of sucrose and solidified with 0.5% phytagel.

Flower buds with pedicle of the L. longiflorum cultivar Indian Summer were cut from the plants in the greenhouse two to four days before anthesis. The complete flowers were sterilized in 70% ethanol for 3 min, and commercial bleach containing 2% NaOCl for 30 min, and subsequently rinsed three times, i.e. 5, 10 and 20 min, in sterile distilled water. After rinsing, petals and anthers were dissected with a scalpel and the pistil, style, ovary and pedicle were placed vertically in a 20 cm test tube partly filled with medium. Tubes were closed with cotton plugs covered by a double layer of alluminium foil.Tubes were placed in a climate room with a light intensity of 12 Wm 2 , a photoperiod of 16hrs and a temperature of 25C. A MS-medium (Murashige and Skoog 1962) supplemented with 8% of sucrose,solidified with 0.4% phytagel and a pH of 6.0 was used to culture the flower bud

2.4.2 Result

Development of a bioassay for heat-tolerance of pollen

The analysis of variance of the pollen tube length measurements (Table 2) and the pollen germination measurements (Table 3) show a highly significant temperature and genotype effect. Also a smaller but significant interaction between cultivar and temperature is present, which means that not all the accessions react the same at different temperatures.

2.5 BULB GROWTH IN LILY REGENERATED *IN VITRO*

High quality, *in-vitro* produced bulblets should grow rapidly and flower as fast as possible after transfer to soil. Fast growth occurred in large bulblets and in bulblets that formed a stem with several leaves instead of one or two leaf-bearing scales. Since stem formation occurred more often in large bulblets, we concluded that bulblet growth *in vitro* is an important factor for rapid growth of lily bulblets after planting. During *in vitro* culture, growth of the bulblets depended on sucrose concentration. Another important factor was the size of the explant: on a large explant, large bulblets regenerated. The presence of the explant was necessary during the whole regeneration period. When bulblets were excised from the explant after 5–6 weeks, and cultured onto the same medium, growth ceased. We studied changes in carbohydrate content during regeneration *in vitro* as influenced by explant size and sucrose concentration. We concluded that the contact area of the explant with the medium plays a key role in determining bulblet weight. Interestingly, the heaviest bulblets were regenerated on actively growing explants that accumulated starch.

2.6 Interspecific crosses of lily by in vitro pollinated ovules

Because of pre- and post-fertilization barriers, successful interspecific crosses have not been reported for *Lilium* using Asiatic hybrid as the mother to cross with Oriental or *Lilium longiflorum*. Using an in vitro ovular pollination method, where pollen tubes directly penetrate isolated ovules, this study demonstrated that 1) the Brewbaker and Kwack agar plate

containing sucrose (10%), with the isolated ovules being placed ca. 1 mm apart from the pollen, is optimal for pollination, 2) interspecific crossing barriers do not occur during penetration of the pollen tubes into the micropyle, 3) micropylar penetration can be obtained in all kinds of intra- and inter-specific cross combinations of lily cultivars, 4) multiple penetration occurs, in which the penetrating pollen tubes pass the embryo sac without entering the synergid cells, 5) a small amount of pollen tube penetrates the synergid cells in the A x O cross, and 6) completion of pollen tube penetration (8-12 h after germination) is much earlier than the division of the generative cell in sperm cells (17 h after germination), which may be the primary cause for the failure in fertilization.

Culture Media

The BK (Brewbaker and Kwack, 1963) medium for in vitro pollination contained (in 1 L of double distilled water) 5 g Difco agar, 0.3 g H_3BO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $KNO_3$and 0.3 g $Ca(NO_3)_2 \cdot 4H_2O$, adjusted to pH 6. With KOH, sucrose at 5 to 12% was included. The ovule culture medium consisted of full strength Murashige and Skoog (1962) basal salts and vitamins, and was supplemented with 0.4% phytagel, 6% sucrose, and 0.01 mg/l NAA at pH 5.8.

Plant Materials

The cultivars from three different groups, namely Oriental hybrids (O)-Stargazer, Asiatis hybrids (A)-Connecticut King, and *Lilium longiflorum* (L)-Gelria, were used to perform interspecific crosses (A x O, O x A, O x L, L x O, A x L, L x A) and Oriental hybrids (O)-Stargazer, Casa Blanca, Asiatis hybrids (A)-Connecticut King, Orlito, and *Lilium longiflorum* (L) - Gelria, Indian Summer, were used to perform intraspecific crosses (O x O, L x L and A x A). Pollen from Stargazer was used for the time course studies of generative cell division and pollen tube penetration. The combination Mont Blanc x Stargazer (A x O) was used to check whether the pollen tube contents could be transferred into the synergid cells. The pollen and ovules were obtained from flowers collected from the greenhouse one day after anthesis. Greenhouse temperatures varied from 15°C at night to 20-25°C during the day with a summer peak of 30-35°C.

2.7 In vitro bulblet formation from leaf segments of lilies, especially *Lilium rubellum* Baker

Bulblets were induced from 5-mm lily leaf segments, cultured on Murashige and Skoog's mineral salts medium (1962) together with α-naphthalene acetic acid (NAA) 1 mg/l, 6-benzylaminopurine (BA) 0.1 mg/l and several organic elements.

Bulblet formation was affected by several factors, especially the time of excision and the origin of the explant; bulblets developed well in leaf explants excised before flowering, and mainly in those excised within 15 mm of the leaf base. However, bulblets were seldom induced in explants excised after flowering, regardless of their origin.

NAA was almost indispensable for bulblet formation, but BA had a slight stimulatory effect only when NAA was also present. Sucrose concentration and light also affected bulblet regeneration.

2.8 In vitro flowering of *Lilium rubellum* Baker

Scales excised from *in vitro* cultured bulblets of *Lilium rubellum* were cultured on MS medium supplemented with 0.044 or 4.4 μM BA in the dark for 300 days, the culture period of which was divided into three stages, with temperatures in each stage as follows: 25 °C in stage 1 (days 0–60); 25 °C or 8 °C in stage 2 (days 61–210); 20 °C in stage 3 (days 211–300). Numbers of bulblets per scale and bulblets with elongated stem (the transition from juvenile to vegetative adult phase) depended on the culture condition. The highest number of bulblets and the percentage of bulblets with elongated stems were obtained in scales cultured in medium with 4.4 μM BA at 25 °C in stage 2. On the other hand, BA concentration (0.044 and 4.4 μM) and/or temperatures (25 and 8 °C) in stage 2 influenced flowering in the bulblets with elongated stems (the transition from vegetative adult to flowering phase). Flowering occurred in bulblets with elongated stems when bulblets on scale were cultured in medium with 4.4 μM BA at 8 °C in stage 2, whereas no flowering occurred in bulblets cultured in any other culture conditions. In conclusion, the concentration of BA affects the transition from juvenile to vegetative adult phase, and the exposure of *in vitro* developed bulblets to low temperature is indispensable to initiate the flowering process.

2.9 Propagation of *Lilium* hybrids. I. Dependence of bulblet production on time of scale removal and growth substances

Production of bulblets from bulb scales of 12 *Lilium* Oriental hybrid clones indicated that an approximately threefold range in regenerative capacity existed within this group. The cultivar 'Empress of India' was the highest producer and provided 10 bulblets per bulb scale during a six-month period. This could provide an estimated yield of 500 bulblets per bulb. Propagation from bulb scales of most of the clones was equally effective with scales which were removed from the plants either immediately after cold treatment of the dormant bulbs, at the cessation of flowering, or at the cessation of growth of the aerial stems (post-senescence). Only 'Empress of India' and 'American Eagle' deviated, in that they produced fewer bulblets at the post senescence stage. When bulb scales were removed for propagation immediately after flowering, and treated with a combination of BAP and NAA (both 5 μM), bulblet production of the low producing clones was significantly increased, while that of the high producing 'Empress of India' was significantly decreased. These effects were less pronounced when the bulb scales were removed at the post-cold stage and virtually absent at the post senescence stage.

2.10 Propagation of *Lilium* hybrids. II. Production of plantlets from bulb-scale callus cultures for increased propagation rates

A method has been developed for the large-scale propagation of lilies from callus cultures. Bulb scales, being available at all times, are an ideal source of explants. Callus was induced throughout the annual cycle of all the 12 Oriental hybrid cultivars tested, by treating the bulb scales with a combination of 5 μM BA and 5 μM 2,4-D. Once initiated, this callus grew vigorously on media lacking exogenous growth substances. Callus induction by BA and 2,4-D, and the subsequent growth of such callus in the absence of exogenous growth substances, occurs within a broad range of cultivars and species within the genus. The callus could be maintained on a medium lacking ammonium salts, but the maximum growth rate was obtained at 10 mM NH_4NO_3. At low (0.2 mM) or zero levels of NH_4NO_3, light as compared with darkness was inhibitory to growth.

The callus maintained its capacity for organogenesis. Maximum production of plantlets was obtained in continuous light on agar medium with 0.5 μM NAA.

Potentially, the successive use of liquid and agar cultures can produce 6×10^{12} plants per year from 1 g (dry wt.) of callus. Plantlets derived from callus were consistently diploid and could be readily transferred to soil.

2.11 Bulblet-productivity of explants from scales, leaves, stems and teplas of *Lilium rubellum* Baker

The number of bulblets per cultured expiant was greatest in the expiants of leaves, i.e. 2.2 bulblets, followed in decreasing order by scale-, stem- and tepal-expiants. Bulblets formed in the expiants of stems were heaviest, 135 mg on average. The expiants of leaves were excellent for regenerating many bulblets per expiant, while those of stems produced the heaviest bulblets.

2.12 Long term in vitro storage of lily: effects of temperature and concentration of nutrients and sucrose

Methods for long-term preservation of lily germplasm were examined. In vitro regenerated bulblets of 10 lily (Lilium L.) genotypes (Asiatic hybrids, Oriental hybrids, L. longiflorum and L. henryi) were stored for 28 months at -2C and 25 C on four different media: 1/4 or full strength Murashige and Skoog nutrients with 9% (w/v) or 6% sucrose. Sprout growth, bulb growth, and viability were determined. The combination of 1/4 strength MS nutrients and 9% sucrose gave the highest reduction in sprout and bulb growth, the highest viability and the highest percentage of regrowth after 28 months of storage. At 25 C, all lily genotypes survived 28 months of storage under these conditions. At -2 C, Asiatic and Oriental hybrids survived 28 months of storage, whereas genotypes of L.longiflorum and L. henryi survived 6 months of storage, but died during prolonged storage.

Material and Methods

Plant material The cultivars used for the experiment were: 'Avignon','Connecticut King', 'Enchantment', 'Esther', 'Mont Blanc' (Asiatic hybrids), 'Casa Blanca', 'Star Gazer' (Oriental hybrids), 'Gelria', 'Snow Queen' (L. longi-florum) and L. henryi (CPRO-number 72122). The

genotypes were established in in vitro conditions by PermX Multiplant, a former commercial tissue culture company in Wageningen.

2.13 Bulblet regeneration from ex vitro root explant in lily hybrid

The influence of growth regulators on in vitro bulblet formation from ex vitro roots was studied in asiatic and oriental hybrids of Lilium. The root segments (3–4 mm) isolated from the middle zone of 2–3 cm ex vitro root were cultured on Murashige and Skoog (MS) medium containing 1 or 1.5 mg/dm3 naphthalene acetic acid (NAA) and/or benzyladenine (BA). Bulblets were not produced in the presence of NAA and BA alone. A significant increase in the per cent explants producing bulblets was observed with 1.5 mg/dm3 NAA and 1 mg/dm3 BA. Maximum number of bulblets and average fresh weight per bulblet was observed with 2 mg/dm3 NAA and 1.5 mg/dm3 BA after 90 days of culture. No differences were found among cultivars in bulblet regeneration of explant or bulblet number although more weighty bulblets occurred in cv. Apeldoorn. About 82% bulblet survival was recorded in coco peat after 30 days

CHAPTER 3: MATERIALS AND METHODS

3.1 Micropropagation of *lilium:*

3.1.1 Collection of Plant material

3.1.2 Preparation of explants

3.1.3 Surface Sterilization

3.1.4 Media Preparation

3.1.5 Preparation of modified MS media

3.1.6 Cleaning of glassware

3.1.7 Culture conditions

3.1.8 Inoculation of prepared explant

3.1.9 *In vitro* shoot multiplication on prepared MS media

3.1.10 Induction of *in vitro* rooting on prepared MS media

3.2Selection of plant material and establishment of axenic cultures

The hybrid lily plants were procured from the *AdarshPushpa Kendra,* Chail, H.P.,India and planted in a pot in a polyhouse at the experimental area of the Deptt. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat. Axillary shoot tips of pot grown plants were surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4-5 washings in sterile water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

3.3 Cleaning of Glassware

All the glassware to be used was washed with a dilute solution of labolin with the test tube brush and then rinsed thoroughly with tap water. Next it was wiped with 70% ethanol. The glassware with contaminated cultures was first autoclaved for 15 minutes at 121 lbs. and the molten media was disposed off. The glassware was then cleaned as mentioned above before reusage.

3.4 Preparation of media and incubation of cultures

Modifications of MS media supplemented with different concentrations and combinations of IBA, KN, GA_3, NAA and BAP were prepared (Murashige and Skoog 1962). The pH of the media was adjusted to be between 5.5 - 5.7 using 0.1N HCl and 0.1N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media were autoclaved at 121°C and 15 lb/in^2 pressure for 15-20 minutes. The autoclaved media were kept in the Laminar Air Flow hood for 1-2 days before inoculations to screen for inherent contamination. One set of cultures was incubated at 16hr light /8hr dark cycle at 25 ± 2^0 C in plant tissue culture chamber. The other set of cultures was incubated at low temperature 15 ± 2^0 C with the same light and dark cycle. Data were collected on days to multiple shoot formation, per cent shoot apices with multiple shoots, and number of shoots per explant. The data were analyzed for test of significance. The cultures were subcultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth, followed by subculturing on the rooting media prior to transfer to the potting mixtures for the acclimatization.

3.5 Cost-effective media for micropropagation

Once a MS medium supplemented with auxins and cytokinins was found suitable for *in vitro* shoot multiplication and root induction of lily cultures, modifications were made so as to cut down on the cost factor of the media. The alternatives adopted were replacing the sucrose with table sugar, distilled water with the normal tap water, use of autoclavable poly-bags and omission of agar-agar, to see the effect on shoot proliferation and growth. Data were recorded for all parameters as given above and statistically analyzed.

The composition of Media was based on Murashige & Skoog (1962) medium divided into different stocks (Annexure I).The Stock Solutions were prepared and kept in the refrigerator. Each salt of the stock solution was dissolved separately in distilled water so as to avoid precipitation. The Chemicals used were of reliable grade and were obtained from reliable firms like Sigma Aldrich. Each Stock solution of MS Media was added one by one to prepare the desired medium. After addition of sucrose (30g/l), growth hormones, desired volume was made with distilled water and the pH of the medium was adjusted to 5.6-5.7 by using pH meter. Then, agar-agar (9g/l) was added and dissolved by boiling the medium till it became homogenous. The

medium was finally dispensed in 250 ml culture jars for culturing. These were closed with the jar caps and autoclaved at a pressure of 15 psi, 121 ºC for 20 minutes for proper sterilization of culture medium. This medium was allowed to solidify and was used after 2 days of preparation. In all 10 different combinations of media was prepared using different concentrations of appropriate growth hormones like KN, BA, IBA, NAA, 2,4-D for each of the experimental setups.

3.6 Tap Water

Tap water was used instead of distilled water for the media formulation. This reduced the cost of the media by omitting the cost incurred during the distillation process. The tap water was sterilized during the autoclaving of the media and hence posed no contamination problems. The plantlets were strong, showed rapid multiplication and on comparison the growth observed in both types of media was similar.

3.4 Surface sterilization of explants

Shoot apecies of field grown plants were washed with sterile water to remove dirt and debris and surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4-5 washings in sterile water. Surface sterilized seeds were kept in autoclavable petri plates having filter papers in culture room at 25oC by maintaining optimum moisture(60-70%). Data will be collected for % survival of sterilized explants.

3.5 Multiplication of shoot cultures in static and liquid media

For the purpose of shoot multiplication, we tried 10 different combinations and concentrations of

KN, IBA and GA3. Two sets of cultures were tried where in one set MS+ GH + sucrose 3% (w/v) + agar-agar 0.8% (w/v) and other set have all other components except agar. One set of cultures was incubated at 16/8hr light/dark cycle at 25±1^0 C in plant tissue culture chamber. The other set of cultures was incubated at low temperature 15±20 C with the same light and dark cycle. Data were collected on days to multiple shoot formation, number of shoots per explants and per cent shoot apices with multiple shoots. The data were analyzed for test of significance. The cultures were sub cultured after every 4 weeks on shoot proliferation media for approximately 2 months, so as to obtain good growth, and highly proliferated shoots.

3.6 Induction of roots in shoots

Individual shoots were excised from the parent cultures and transferred onto MS media supplemented with different concentrations and combinations of IBA,IAA for root induction. The cultures were incubated under the same culture conditions as mentioned above. Data for days to root initiation, number of roots/shoot and per cent rooting were recorded and analyzed for test of significance.

3.8 Hardening and acclimatization of plantlets

Once the initial formation of roots could be seen, the rooted shoots were gently removed from the culture vessels, washed under running tap water, treated with 0.5% Bavistin and transferred to pots containing different combination and concentrations of sand, soil, vermiculite, perlite and coco peat in the glasshouse conditions for acclimatization and hardening.

3.8.1 Vermiculite

Vermiculite is a micaceous mineral that is expanded in a furnace, forming a lightweight aggregate. Handled gently, vermiculite provides plenty of air space in a mix. Handled roughly, vermiculite compacts and loses its ability to hold air. Vermiculite holds water and fertilizer in the potting mix. It also contains calcium and magnesium and has a near-neutral pH.

3.8.2 Perlite

Perlite is an amorphous volcanic glass that has relatively high water content, typically formed by the hydration of obsidian. In horticulture perlite can be used as a soil amendment or alone as a medium for hydroponics or for starting cuttings. It is sterile and pH-neutral. When added to a soil mix, perlite can increase air space and improve water drainage. Its pieces create tiny air tunnels that allow water and air to flow freely to the roots. Perlite will hold from three to four times its weight in water, yet will not become soggy. It helps prevent water loss and soil compaction.

3.8.3 Coco peat

Coco peat, also known as coir pith, coir fiber pith, coir dust, or simply coir, is made from coconut husks, which are byproducts of other industries that use coconuts. Raw coconuts are washed, heat-treated, screened and graded before being processed into coco peat products of various granularity and denseness, which are then used for horticultural and agricultural applications and as industrial absorbent. It is used as a soil additive. It holds water well, re-wets well from dry, holds around 1000 times more air than soil and is mixed with sand, compost and fertilizer to make good quality potting soil. Once the plants are transferred, they are kept covered with jars and poly bags for 10 – 15 days for preservation purposes and after that are exposed by uncovering for a period of 10 – 15 mins every day for the first few days during early mornings or late evenings. The exposure time is gradually increased over time.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Establishment of surface sterilization

4.1.1Sterilization of explants:

Sterilization is a term referring to any process that eliminates (removes) or kills all forms of life, including transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) present on a surface, contained in a fluid, in medication, or in a compound such as biological culture media.Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is required. Mercuric chloride is seldom used as a plant sterilant today, as it is dangerous to use, and is difficult to dispose of. Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired.

The explants were subjected to bacterioside and fungiside agents for different time durations. Bavistin and $HgCl_2$are the most commonly used agents.

Bavistin	**$HgCl_2$**
2 min	2 min
2 min	1.5 min
5 min	2 min
1 min	30-40 sec
2 min	1 min
3 min	2 min

Table 4.1: Time durations

Bavistin	$HgCl_2$
2 min	1 min

Table 4.1.1: Best results

4.2 Media preparation: Different permutations and combinations of growth hormones were tested for shoot multiplication where cytokinins not only gave good no of shoots but good growth . Ga3 helps in elongation of shoots and kn increases their number(Table4.2 fig.)

Table 4.2 : Different concentration and combinations of hormones tried for shoot multiplication in Hybrid Lily

GA3(mg/ml)	NAA(mg/l)	IBA(mg/l)	KINETIN(mg/l)	Shoots/ Explant	No. of roots per explant
0	0	0	0	NA	NA
1	1	0	1	4	1
1	1	1	0	5	5
0	1	0	2	9	7
2	1	2	0	17	4
1	0	2	1	22	9

1	0	1	2	29	7
1	0	3	1	20	9
0	0.1	2	2	11	9
2	0.1	0	0	12	7
1	0.1	1	2	15	8
1	2	3	1	10	9
0	0	3	0	18	8
1	3	0	0	14	6

4.3 ***Comparative Growth Differnce*** There was a difference seen in the no.of shoots obtained on static media and that on liquid media. The results that we found on them are illustrated below :

Table 4.3. Comparative growth difference between Static and Liquid cultures

MS+GROWTH HORMONES: IBA and KN	No of shoots formed	No of rhizoids
Static	29	10
Liquid	35	16

4.4 *In vitro* rooting on prepared MS media

The Rootable shoots which were excised and put in separate culture jars containing MS Media enriched with rooting growth hormones IBA of different concentrations were observed after 1-2 week(s) and the best results for number of roots and root length was seen in media containing (MS + NAA 1mg/ml + IBA 1mg/l + Sucrose 30 g/l + agar-agar 9 g/l).

TABLE 4.4: *In vitro* root induction on different Combinations and Concentrations of auxins

MS + Growth Hormones (mg/l)		Days to root formation	Avg No. of Roots per explant	Avg root length per explant (cm)
NAA	IAA			
0	0	18-20	2.1	0.3
0	1	14-16	2.7	0.45
0	3	12-14	3.8	0.45
0	4	12-14	4.3	1.5

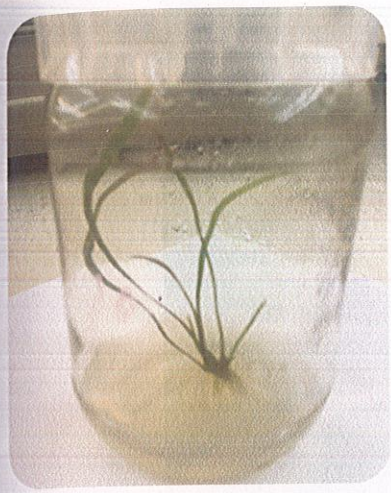
1	1	8-10	5.5	2.1

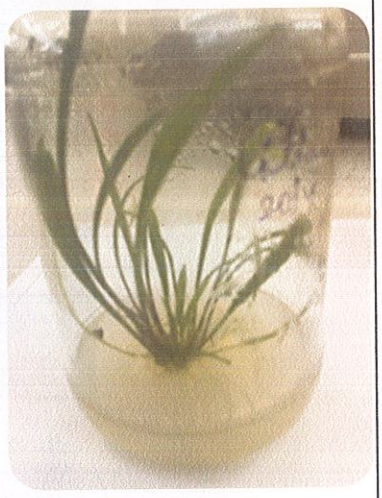
4.5 Hardening of *in vitro* rooted plantlets in glass house

After 5 weeks of growth on rooting medium, roots were well developed and the plantlets from all rooting media was taken out from all culture jars and after the treatment already mentioned, were potted in potting plastic pots containing different potting mixtures. After a week, when the plants showed initial signs of establishment in pots, with the appearance of new leaves, the pots were initially uncovered for overnight. After 2 weeks of transfer to pots, and when the plantlets were totally uncovered, we observed that the potting mixture of sand, soil with perlite, vermicultite, coco peat in ratio 1:1:1 gave us the best results in terms of plant survival and growth.

Table 4.5 : Growth and Survival of hardened plants in different potting mixtures Under glass house conditions.

Potting Mixture (Sand+Soil)	Ratio	No. of Leaves	Percentage Survival of plants(%)
Vermiculite	--	17	25
Perlite	--	18	25
Coco-peat	--	15	50
Perlite+Vermicultie	1:1	18	50
Perlite+coco-peat	1:1	21	75
Vermiculite+coco-peat	1:1	23	50
Perlite+Vermiculite +coco-peat	**1:1:1**	**25**	75

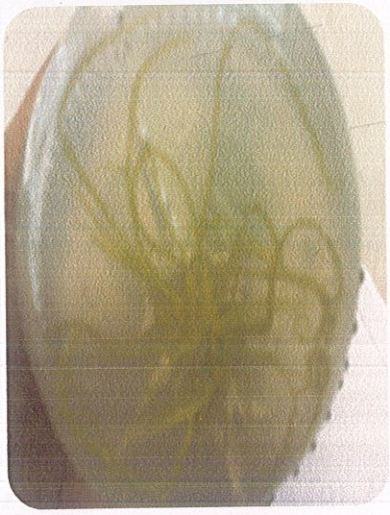


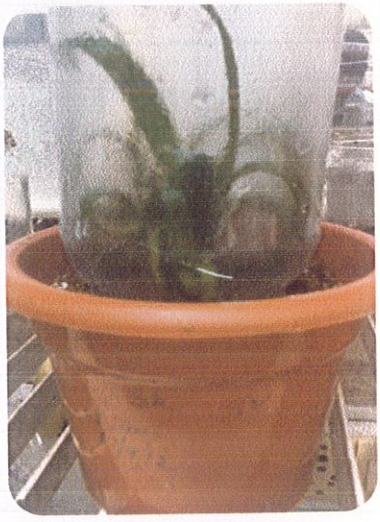


Small shoot apex

Shoot multipliction after 2-3 weeks

After 4-5weeks





Extensive Shoot Formation

***in vitro* root induction**

hardened plant

Complete hardened plant

Figure4.1 : Complete micropropagation outline for Hybrid Lilies

CHAPTER 5 – CONCLUSION

The current study reports the protocols for development of *in vitro* shoot and root cultures with the help of micropropagation.As hybrid lily has high demand so its availability with authencity is a prerequisitive. The same objective has been achieved in the present study. We have applied successful techniques for its micro propagation with maximum 50 shoots proliferating on the MS medium containing KN (2mg/l) + IBA (1mg/l) + GA3(1mg/l) + sucrose 3% (w/v),*in vitro* root induction was achieved best on the MS medium containing NAA(1 mg/ml) + IAA(1 mg/ml) with good growth of plantlets. We have multiplied shoots and micro propagated plantlets and successfully transferred them to green house. So this complete process is used as an alternative to conserve this culturaly,socialy and religiously important cut-flower crop by growing it in large number in lab and then transferring to field. This novel technique of *in vitro* conservation and micro propagation can help in conservation and production of a large number of disease-free, true-to-type plants. Hybrid Lily enjoys a good domestic and international market. So if these micro propagation techniques are transplanted to field for practical purposes at a large scale, this will reduce the burden on the wild for its collection. Beside the strategy evolved would maintain quality and homogeneity of Hybrid Lily.

ANNEXURES

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME
A - 10X	KNO_3	1900	19	}
	$MgSO_4 .7H_2O$	370	3.7	100ml/l
	KH_2PO_4	170	1.70	
B - 20X	NH_4NO_3	1650	33	50ml/l
C - 100X	$CaCl_2 .2H_2O$	440	44	10 ml/l
D - 100X	Na_2EDTA	37.26	3.72	} 10 ml/l
	$FeSO_4 .7H_2O$	27.85	2.28	
E - 100X	KI	0.83	0.083	100 ml/l
F - 100X	H_3BO_3	6.2	0.62	}
	$CoCl_2 .6H_2O$	0.025	0.0025	}
	$ZnSO_4 .7H_2O$	8.6	0.86	}

	$CuSO_4 .5H_2O$	0.025	0.0025	10 ml/l
	$MnSO_4 .4H_2O$	22.3	2.23	
	$Na_2MbO_4 .2H_2O$	0.25	0.025	
G - 100X	m-INOSITOL	100	10	} 10ml/l
	GLYCINE	2	0.2	
H -100X	PYRIDOXINE-HCl	0.5	0.05	}
	NICOTINE ACID	0.5	0.05	} 10 ml/l
	THIAMINE	0.1	0.01	}

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