Materials and Methods
4.1. SELECTION OF THE MATERIAL

G. superba L. and G. lutea Hort., members of family Liliaceae has been chosen for study of present investigation. G. superba is distributed throughout the tropical India while G. lutea is confined to the North Eastern region of Himalayas. The habit of both these plants is more or less same but in G. lutea, as the meaning is underlying in the name of species, has a deep lemon yellow coloured flowers. Both these species are stable diploids with chromosome number $2n = 22$ and do not show greater variation in the morphology and cytology (Hegde and Lugade, 1992).

G. lutea which has been collected from Shilong and G. superba collected from Sahyadri ranges of Western Ghats are under cultivation in the Botanical Garden of the department for several years. It is felt necessary to mention here that both these species flower during the same period and hence they have more or less synchronous flowering schedule from June to August.

The Gloriosa plants are herbaceous climbers having glabrous leaves, underground, cylindrical bifurcated, and horseshoe shaped tuberous rootstocks with two equal or unequal limbs with terminal eyebuds. It eventually regenerates in to a new plant in the subsequent year. Thus generally entire genus propagates mainly by underground tubers generations to generation where old tubers die and new develop giving rise to new plant in subsequent year. Propagation by seeds which takes place by seed dispersal is rare. Supan et al. (1993) have reported that seed germination percentage in Gloriosa is very poor i.e.30 % and
the new plant developed from seed takes 2 to 3 years to mature and flower by that time. Underground tuber develops. In other words, the new plant that develops from seed cannot come to flowering unless the tubers get matured which is a prerequisite to flower. The meristematic region of the pseudoshoot is only in the apical region. There are no axillary buds. However occasionally apical bud shows branching.

The underground tuber, which undergoes dormancy after monsoon, has got two terminal eye buds, which sprout in the next monsoon. Even in case of underground tubers if the eye buds are removed it fails to regenerate and dies. In present investigation it has been noticed that even if the leaf tip has been modified into a tendril, the basal part of young leaves have got little meristematic activity (plate XVII - C). Considering the poor germination percentage of seeds, embryo culture was also attempted along with the tuber eye buds and young juvenile shoot without leaves. Thus in the present investigation therefore 1) apical meristem, 2) regenerated tubers and 3) embryo have been used as explants.

4.2 RAISING OF THE PLANTS

Stocks of *G. superba* and *G. lutea* are maintained both in the garden bed as well as in pots. Before onset of monsoon, the tubers from the pots were repotted by using a mixture of river bed soil and farm yard manure in the proportion of 2:1 respectively. Shoots started emerging with the onset of
Table 3.
Composition of Murashige and Skoog's (1962) medium

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Ingredient</th>
<th>Conc. In Stock Solution (mg/l)</th>
<th>Volume of Stock in final volume (ml)</th>
<th>Final conc. In medium (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>NH₄NO₃</td>
<td>16500</td>
<td>100</td>
<td>1650.00</td>
</tr>
<tr>
<td></td>
<td>KNO₃</td>
<td>19000</td>
<td></td>
<td>1900.00</td>
</tr>
<tr>
<td></td>
<td>CaCl₂.2H₂O</td>
<td>4400</td>
<td></td>
<td>440.00</td>
</tr>
<tr>
<td></td>
<td>MgSO₄.7H₂O</td>
<td>3700</td>
<td></td>
<td>370.00</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>1700</td>
<td></td>
<td>170.00</td>
</tr>
<tr>
<td>II.</td>
<td>KI</td>
<td>166</td>
<td>5</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td>H₂BO₃</td>
<td>1240</td>
<td></td>
<td>6.200</td>
</tr>
<tr>
<td></td>
<td>MnSO₄.7H₂O</td>
<td>4460</td>
<td></td>
<td>22.30</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄.7H₂O</td>
<td>1720</td>
<td></td>
<td>8.60</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄.2H₂O</td>
<td>5</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>CuSO₄.5H₂O</td>
<td>5</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>CoCl₂.2H₂O</td>
<td>5</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td>III.</td>
<td>FeSO₄.7H₂O</td>
<td>5570</td>
<td>5</td>
<td>27.85</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA</td>
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</tr>
<tr>
<td>IV</td>
<td>Myoinositol</td>
<td>20000</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>100</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine HCl</td>
<td>100</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Thiamin HCl</td>
<td>200</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>400</td>
<td></td>
<td>2.00</td>
</tr>
</tbody>
</table>
mansoon and on sprouting they were given a dose of NPK (16:16:16) @ 100 g. per pot which resulted in healthy well-nourished shoots.

4.3 GLASSWARES AND CLEANING:

All the glasswares taken were of Borosilicate and were first soaked overnight in 10% chromic acid and then in a strong detergent, Laboline or Teepol. They were then rinsed using plenty of tap water and thereafter rinsed with double distilled water. The water was allowed to drain and then dried. The glass wares were variously sterilized either in hot or oven or in autoclave.

4.4 PREPARATION OF CULTURE TUBES

The culture tubes and flasks required to be protected from contamination and this was achieved by special type of plugs. The plugs were prepared using nonabsorbent cotton packed tightly in a muslin cloth that facilitated the air diffusion without any contaminants and were steam sterilized in autoclave. Besides plugs prepared even autoclavable plastic caps were also used wherever necessary.

4.5 PREPARATION OF MEDIA

4.5.1 PREPARATION OF INGREDIENTS FOR MURASHIGE AND SKOOG'S MEDIUM. (M.S.)

1. Macroelements -

For preparation of 1 liter of Murashige and Skoog's medium (Murashige & Skoog, 1962) the required quantity of macronutrients (Qualigen make) such as NH₄NO₃, KNO₃, CaCl₂.2H₂O, MgSO₄.7H₂O, KH₂PO₄ were weighed using
monopan balance and were dissolved sequentially one after the other in 500 ml.
double distilled water in clean beaker by constant stirring. After all salts
dissolved it was transferred quantitatively to 1-liter volumetric flask.

2. Microelements -

The trace elements used in M.S. medium are Mn, B, Zn, Mo, Cu, I and
Co. Stock solution of these salts were made using analytical grade chemicals.
The salts like MnSO₄·4H₂O, H₃BO₃, ZnSO₄·7H₂O, CuSO₄·5H₂O, KI and
CoCl₂·6H₂O were carefully weighed for 100X stock on analytical balance and
dissolved in 50ml. of double distilled water one after the other in a sequence
mentioned in the literature. After all salts went into solution it was qualitatively
transfer to 100ml. volumetric flask and made the volume with double distilled
water.

3. Iron source -

As the availability of Fe reduces at high pH due to precipitation, it was
supplied in a chelated form in combination with EDTA. Hundred ml. of stock of
Iron was prepared. For the preparation of Iron stock, first required quantity of
Na₂EDTA weighed for 20X stock and dissolved in 50 ml. double distilled water
in a beaker. Weighed quantity of FeSO₄·7H₂O crystals were dissolved in the
same and the volume was adjusted to 100 ml. double distilled water.

4. Vitamins -

The weighed quantity of vitamins such as Myoinositol, Nicotinic acid,
Pyridoxine HCl, Thiamine HCl and Glycine were dissolved in 50 ml of DDH₂O
# Table 4.

## Composition of White's medium (1963)

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Ingredient</th>
<th>Conc. In Stock Solution (mg/l)</th>
<th>Volume of Stock in final volume (ml)</th>
<th>Final conc. In medium (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>KNO₃</td>
<td>8000</td>
<td>100</td>
<td>80.00</td>
</tr>
<tr>
<td></td>
<td>Ca(NO₃)₂·4H₂O</td>
<td>28800</td>
<td></td>
<td>288.00</td>
</tr>
<tr>
<td></td>
<td>MgSO₄·7H₂O</td>
<td>73700</td>
<td></td>
<td>737.00</td>
</tr>
<tr>
<td></td>
<td>Na₂SO₄·10H₂O</td>
<td>46000</td>
<td></td>
<td>460.00</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>6500</td>
<td></td>
<td>19.00</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA</td>
<td>267</td>
<td></td>
<td>65.00</td>
</tr>
<tr>
<td></td>
<td>MgSO₄·4H₂O</td>
<td>150</td>
<td></td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>MnSO₄·H₂O</td>
<td>75</td>
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<td>0.75</td>
</tr>
<tr>
<td></td>
<td>MoO₃</td>
<td>0.01</td>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>CuSO₄·5H₂O</td>
<td>0.1</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Ferric Citrate</td>
<td>50</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>II</td>
<td>Nicotinic acid</td>
<td>50</td>
<td>10</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine HCl</td>
<td>10</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Thiamin HCl</td>
<td>10</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>300</td>
<td></td>
<td>3.00</td>
</tr>
</tbody>
</table>
and volume was made to 100 ml. This stock solution was stored at 0° C. in Deepfreeze whenever not in use.

4.5.2 PREPARATION OF INGREDIENTS FOR WHITE'S MEDIUM.

1. Macro and micro elements stock:

For preparation of 1 liter of White's medium (White, 1963) the required quantity of ingredients of analytical grade such as Ca(NO$_3$)$_2$, 4H$_2$O, KCl, ZnSO$_4$.7H$_2$O, H$_3$BO$_3$, Na$_2$SO$_4$, MnSO$_4$.4H$_2$O, NaH$_2$PO$_4$.2H$_2$O, KI, CuCl$_2$.2H$_2$O, Na$_2$MoO$_4$.4H$_2$O, MgSO$_4$.7H$_2$O and Ferric citrate were carefully weighed for 100X stock on analytical balance and dissolved in 100 ml. double distilled water one after the other in a sequence mentioned in the text. After all salts went into solution it was qualitatively transfer to 1 liter volumetric flask and volume was made with double distilled water.

2. Vitamin stock:

The weighed quantity of vitamins such as Nicotinic acid, Pyridoxine HCl, Thiamine HCl and Glycine were dissolved in 50 ml of DDH$_2$O and volume was made to 100 ml. This stock solution was stored at 0° C. in Deepfreeze whenever not in use.

4.6. OTHER SUPPLIMENTS

1. Auxin stocks -

The stock solutions of various auxins used during experimentation were prepared so as to get 1 mg of auxin per ml. of the stock. Various auxins used for
the studies are 2, 4-D, IBA, IAA. Stock solutions of these auxins were prepared by dissolving 100 mg. each of them separately using few drops of Dimethyl sulfoxide for 2, 4-D and 1 N NaOH for IBA and IAA and making the final volume to 100 ml. with DDH₂O.

2. Cytokinin stocks -

Various cytokinins like kinetin, Benzyl Aminopurine and adenine sulfate were accurately weighed with the help of monopan analytical balance and were dissolved in a few ml. of 1 N HCl and volume was made up to 100 ml with DDH₂O. One ml. of this stock solution corresponded to 1 mg. of that particular cytokinin.

In certain experiments, the nutrient media were supplemented with following adjuvants:

3. Casein hydrolysate:

Casein hydrolysate of Hi-Media was taken and weighed with the help of monopan analytical balance. 500 mg. Of casein hydrolysate was weighed and dissolved in sterile double distilled water in 100 ml. volumetric flask and volume was made.

4. Agar agar-

High purity bacteriological agar (Hi-Media Make) was used throughout the experimentation in the concentration of 6 - 8 g 1⁻¹ as per the requirement of the media.
5. Phenylalanine stock -

100 mg of phenylalanine was dissolved in a few drops of ethanol and final volume was made to 100 ml.

6. Na-acetate stock -

100 mg of Na-acetate salt was weighed accurately using analytical monopan balance and was dissolved in few milliliters of sterile DDH₂O in a 100 ml. volumetric flask and the final volume was made.

4.7. PREPARATION OF COCONUT WATER

The composition of coconut milk has been studied extensively from which it has become clear that it contains a number of amino acids, several vitamins, sugars, sugar alcohols, growth regulators and proteins. It is noteworthy to mention here that as all above ingredients in coconut milk tend to vary from age to age and variety to variety. It has been scrupulously observed that during experimentation, coconuts of more or less same age and from same stock were used. Further method of preparation of coconut milk is essentially based on that of Klein and Klein (1970) and elaborated in the technical bulletin 'Plant cell culture, 1991-92 by SIGMA. The coconuts brought from the local source were washed thoroughly from outside and chopped at their micropylar end with a sharp chopper till the large micropyle (eye) is exposed. The water was drained out and filtered through a double layered muslin cloth so as to remove any solid particles. Then it was deprotenised by boiling. When it is cooled in sterile chamber the proteins settled down and supernatant was filtered through a sterile
Whatman filter paper No.1. Such deprotenised coconut water was then used either immediately after dilution to 5 - 20% (v/v) or stored in deep freeze, at 0-4°C. Remaining proteins in coconut milk may undergo precipitation when it is frozen. However, this precipitation does not supposed to diminish the cytokinin like properties of the substance.

4.8. METHOD OF PREPARATION OF CULTURE MEDIA

Required quantities of different stock solutions were taken in to a volumetric flask with desired quantity of macro elements, micro elements, Iron, vitamin stocks, auxins, cytokinins, coconut water and precursors (wherever necessary). Small amount of DDH₂O was added to it followed by the desired quantity of sucrose and it was shaken continuously until sucrose went into the solution. Volume was adjusted using DDH₂O and pH was adjusted to 5.8 - 5.9 before autoclaving.

The broth consisting of Macronutrients, Micronutrients vitamins, sugar and precursors (wherever necessary) were heated in a beaker on a hot plate until boiling and required quantity of agar was added with constant stirring so as to avoid agar coagulates. This broth was then distributed in to the culture tubes and conical flasks depending upon the requirement and plugged with cotton plugs and sterilized at 15 lbs. pressure for 30 min. After autoclaving, the test tubes were kept in inclined position and allowed to cool so as to form slants. On subsequent day this solidified medium was inoculated holding under aseptic conditions. The cultures were subcultured every fifth week.
4.9. PREPARATION OF INOCULATION CHAMBER

Before excising the explants the inoculation chamber was set up in the following way:

1. The laminar flow chamber (Micro Filt Make) was first surface sterilized with the help of sterile cotton soaked in rectified spirit.

2. Other materials: All the tools used for the work were of surgical grade. The culture tubes with slants, presterilized surgical tools, spirit lamp, beakers with 0.1% aqueous solution of HgCl₂ as a surface sterilent and sterile DDH₂O were kept ready in the chamber. The UV lamp was switched on for one and half-hour prior to inoculation. After switching off the UV lamp the laminar flow was put on.

4.10. PREPARATION OF EXPLANT FOR CULTURE:

As explained earlier different species of *Gloriosa* raised in the Botanical Garden of the Department were watered late in summer. Due to high temperature and humidity, late in summer the tubers started sprouting. In the following way the explants were harvested at various stages of growth.

4.10.1. APICAL MERISTEM CULTURE:

*Gloriosa* has got only two meristematic regions viz. apical meristem and tuber eybuds. It has been already mentioned that on cutting apical meristem plant growth ceases. This constraint was overcome by cutting the explant mostly
only after branching. This facilitated the further growth of plant as well as provided meristematic material for inoculation.

The healthy apical buds from such a plant were chosen and excised with a sharp razor blade. These were brought to the laboratory. The well-differentiated leaves were removed carefully without disturbing the meristem. They were then washed with mild detergent followed by distilled water and after surface sterilization inoculated on to the slants as described earlier in aseptic conditions. While inoculating, the care was taken to embed the cut portion in to the medium.

4.10.2. REGENERATED TUBER CULTURE

The cormlets developed in the cultures were separated under aseptic conditions and cultured on variously supplemented MS medium in similar way and were provided light and temperature regime as described else where.

4.10.3. SEED AND EMBRYO CULTURE

Gloriosa seeds are having prolonged dormancy and seed germination percentage is very poor. In order to see whether the seed is able to germinate, the harvested seeds were dried and stored in secured manner. Such seeds were soaked overnight in running tap water so as to remove the inhibitory principles which otherwise inhibit the germination. After 4th day the seeds were surface sterilized with 0.1% HgCl₂, washed thoroughly with sterile distilled water and
inoculated in to the culture tubes containing variously supplimented MS or White's medium.

For embryo culture, the seeds were processed as above and on surface sterilization the embryos were dissected out carefully using sharp surgical blades and separated from cotyledon. They were inoculated into the variously supplemented MS or White's medium aseptically without disturbing their integrity. Same technique was followed in embryo rescuing technique. The photographs were taken at different growth stages using Nicon Camera and Kodak Gold coloured film.

4.11. TEMPERATURE AND LIGHT REGIME

All cultures (except those which required dark period) were maintained under optimum light and temperature conditions. The light source was accomplished with a bank of fluorescent tubes supplemented with incandescent lamp. This light source was maintained in such a way that at the point of incidence the cultures received 2000 lux. Temperature regime was maintained at 25 ± 2 °C. through out the experiment period and cultures were given photoperiod of 16 h. light and 8 h. dark.

4.12. PLANTLET ROOTING AND HARDENING

The well developed plantlets were transferred carefully to the liquid media using a presterilized paper boat. Upon vigorous rooting these plants were taken for hardening in a sterilized mixture of soil : sand : compost (2:1:1).
Initially they were kept in humid chamber and watered with dilute Hoagland solution for one week. They were later transferred to the field condition.

4.13. HISTOLOGICAL STUDIES

For the histological studies calli were fixed in F.A.A. prepared by mixing Formalin, glacial acetic acid and 70% ethanol mixed in proportion of 5:5:90 at various intervals. To study the proembyonic nature of callus, small portions of calli containing single cells with dense cytoplasm and prominent nuclei were separated by physical means (Nomura and Komamine, 1985) and identified by using acetorcein. For further histological studies, calli fixed in FAA were dehydrated through ethanol xylol series and embedded in 52 - 54°C paraffin wax according the procedure described by Johansen (1940). Serial sections were cut at 8 -10 um on rotary microtome. After deparaffining and hydration, the slides were stained in haematoxyline for 10 -20 minutes and stain differentiation was done with acidified water. The slides were mounted in DPX. Photography was carried out by using MFAK's system of JENEVAL carlzeiss microscope at suitable magnifications and culture photography was done with Nicon camera using NP-55 black and white film and coloured film of KODAK.

4.14. CALLUS SUBCULTURE

Suitability of any medium is indicated by regeneration and callus formation. To harvest callus on large scale, the shoot meristem explants were allowed to calluse and on sufficient growth, the fragments of the calli were subcultured on the same media but supplemented with various concentrations of
cytokinins, auxins, sugars and precursors of the colchicine. To determine the
effect of above ingredients on callus biomass, the subcultured calli in replicates
were harvested after 4 weeks time and parameters like fresh weight, dry weight
and moisture percentage were noted for each replicate. The data were analysed
using appropriate statistical methods.

4.15. DETECTION AND QUANTIFICATION OF COLCHICINE

4.15.1 METHOD OF QUALITATIVE DETECTION BY TLC

a) Preparation of Extract:

For Thin Layer Chromatographic detection of the alkaloid, method
followed was that of Cleark (1970). In this method, the calli were sampled,
crushed separately in 10 % Acetic Acid in ethanol till it was turned in to fine
paste and was transferred to a clean evaporating dish. The traces of sample were
collected by several washes with crushing medium. The sample was filtered
through a several layered muslin cloth and centrifuged at 100g. The supernatant
was condensed on a water bath till it was reduced to a small volume of 2 ml. The
alkaloid from the condensed medium was precipitated by adding drop by drop
liquid ammonia till all the alkaloids got precipitated. Then it was centrifuged at
3000 rpm so as to sediment the precipitated alkaloid. The supernatant was then
discarded. The sedimented precipitate was washed repeatedly with 1 %
Ammonia solution and dissolved in ethanol. This ethanolic extract was then
loaded on TLC plate prepared with using Silica gel-G with binder of Merck
Dermstat. Mixture of N-Butanol, citric acid and water (prepared by mixing 870
ml. Butanol + 4.8 g of citric acid in 130 ml water) was used as a solvent system. After running the TLC it was oven dried and sprayed with *Dragendorff's Reagent, which gave orange spots wherever alkaloid was present.

B. Preparation of *Dragendorff's Reagent: This reagent was prepared by the method of Harborne (1973).

i) 0.6 g of Bismuth subnitrate was dissolved in 2 ml Conc. HCl and 10 ml water

ii) 6 g Potassium Iodide was dissolved in 10 ml water.

These stock solutions were mixed together with 7 ml Conc. HCl and 15 ml water and then it was diluted to 400 ml.

4.15.2. METHOD OF QUANTIFICATION BY HPLC:

I. PREPARATION OF EXTRACT FOR HPLC:

The callus masses from control and different precursor supplemented media were sampled and washed with distilled water so as to remove the traces of adherent media. Then they were blotted dry and weighed. The fresh weights were noted. These callus masses were crushed in mortar with a pestle in 70% ethanol and extracted several times. It was filtered through Whatman filter paper no. 1. The ethanolic extract was exhausted on water bath. To remove ethanol 10 ml. water was added to aqueous solutions, which allows the resins and oils to separate out. This aqueous solution was filtered through glass wool under reduced pressure and the resinous mass was extracted further with 10 ml portions of distilled water until extraction was complete. The filtrate in the form of aqueous solutions was then shaken with equal amount of petroleum ether.
Here light petroleum was used to separate out and remove the colouring matter and oil. The aqueous solution thus collected is saturated with NaCl to facilitate easy further extraction of the alkaloid. The alkaloid is now extracted with the chloroform twice or more till the extraction was complete. The chloroform solution was dried with anhydrous sodium sulphate. This was then slowly passed through a 15 X 1 cm. column of Aluminum Oxide (BDH poole chromatographic alumina) presaturated with benzene to facilitate the mobility of alkaloid through alumina. The alkaloid was eluted with total 100 ml of chloroform. Chloroform was then removed by evaporation on water bath leaving behind the gummy viscous yellow residue. This residue is taken up in a known quantity of HPLC water and colchicine content was determined by HPLC technique.

II. QUANTIFICATION

a) Preparation of sample:

The residue dissolved in known amount of HPLC water was filtered through milipore micro filter of 0.2 um pore size. The mobile phase used in HPLC analysis was 55 % MeOH (HPLC grade) in 8.6 mM H3PO4 (AR grade) (pH 3.1).

b) Preparation of standard:

For detection of peak of standard colchicine during analysis, working solution was prepared by dissolving 21.7 mg of colchicine in 25 ml mobile phase and again diluting 2 ml from this to 50 ml with mobile phase. This was filtered through above mentioned milipore filter and was used to run the standard.
c) HPLC SPECIFICATIONS


Specifications:

- Column: C 18 Silica Pack capillary column 4.6 x 250 mm.
- Solvent: 55% MeOH prepared in 8.6 mM H3PO4 (pH 3.1)
- Run time: 10 min.

Detection of peaks: UV spectrophotometer 254 & 340 nm.

4.16. HYBRIDIZATION STUDIES

Both the species of Gloriosa under study i.e. G. superba and G. lutea were stable diploid with n = 11. Both these species are morphologically distinct (Miller, 1930; Naraian, 1972) and flower during the same period having synchrony. With a view to producing and studying the hybrids between such a stable diploids and synchronously flowering plants, the hybridization programme was undertaken.

Potted plants of both the species were emasculated when attained flowering stage and bagged to prevent pollination. The receptivity of stigma was recognised by observing secretions on the stigmatic lobes and brushing freshly sampled mature anthers of pollinator plant using forceps carried out pollination. The pollinated flowers were then bagged. Repeating the pollination on subsequent day ensured pollination. During these hybridization studies reciprocal crosses were also made and on maturity of fruits, the seeds were collected, air dried and stored in a secured manner.