Materials And Methods

1 SELECTION OF MATERIAL :

Gloriosa superba L. is a widely distributed species all along the Western ghats. Because of its stable diploid chromosome number no greater fluctuaction either in the morphological character or in the Flower colour (which is a key character) is observed. Secondly this species or in general the entire genus propogates more by undergound organ rather than seeds. Such a stable plant mateial collected from the Western ghat has been under cultivation in the Botanical garden of the department.

This plant having underground tuber emerges with the first shower of mansoon.The habit of the plant is a climber with its leaftip turned into tendril The meristematic region of the shoot is located only in the apical region. There are no lateral axillary buds. If the apical bud is removed the plant stops growing. However, occasionally the apical bud branches. Normally the age of the plant is identified based on the thickness of the shoot which is also determinate. After the mansoon the arial shoot dies down and the horse shoe shaped underground tuber with its terminal eye-bud undergoes hibernation. Thus the plant has only two meristematic parts (i) Apical meristem of the shoot and (ii) eye-bud. Even in case of undergound tubers if the eye-buds are removed it fails to regenerate and dies.

Similar to Gloriosa superba is the Gloriosa lutea, the habit of this plant is more or less same as that of G. superba. This species is not found distributed in the tropical part of India only confined to North Eastern region of the Himalayas. Gloriosa lutea as the meaning is underline in the name of species has a deep lemon yellow coloured flower and unlike Gloriosa superba it is a stable diploid and hence does not show greater variation in the morphology or cytology Lugade and Hegde (1992). This plant which has been collected from the Himalayan ranges is under cultivation in the botanical garden for several years. Similar to that of Gloriosa superba it also yields two meristematic parts. (i) Apical bud of shoot or apical meristem and (ii) Eye-buds of the tuber which is undergound. However the leaf differentiation takes place by leaf meristem and in Gloriosa leaftip is modified in to tendril. The young differentiting leaf and the leaf tip provides meristem. It is felt necessary to mention here that both these species flower during the same period and hence they have synchrony.

2 RAISING THE PLANTS :

Although stocks of G. superba and G. lutea are maintained both in the garden bed as well as in pots, before the mansoon onset the tubers from the pots were removed and they were repotted The earthern pots were initially filled with a mixture of river-bed soil and farm yard manure prepared by the mixing in the proportion of 2:1 respectively.

The healthy tuber with prominent eye-buds were planted retaining only two eye-buds in each pot. With the onset of mansoon shoots started emerging. After they started sprouting they were given a dose of chemical fertilizer Sampurna (having NPK 16+16+16) at the rate of 100 gm per pot, so that a healthy nurished meristematic regions for culture are available.

3 PREPARATON OF CHAMBER FOR INOCULATION :

Before the explants especially apical meristem of shoot were harvested, the inoculation chamber was setup for inoculation. The laminar flow chamber was first surface sterilized with the help of sterile cotton soaked in ethanol. The culture tubes with appropriate medium, sprit lamp, match box, pair of sterilized forceps, raizor blade, whatman filter paper, sterile distilled water, beakers petridishs and 0.1% aqueous solution of Mercuric chloride were kept ready in the chamber. The Ultra Violet lamp was switched on for one and half hour before inoculation.After switching off the Ultra Violet lamp the laminar flow was put on.

4 PREPARATION OF CULTURE TUBES :

The culture tubes and flasks have to be secured from contamination. This was accomplished with the help of special type of a plug which would enable difusion of air without allowing the contaminant to go in. The plugs were prepared with nonabsorbant cotton packed tightly in a musline cloth, so that they can be easily sterilized.

5 PREPARATION OF INGREDIENTS :

i) Macronutrients : The weighed quantity of chemical compounds such as $(NH_4)NO_3$, KNO_3, $CaCl_2$, $2H_2O$, $MgSO_4$, $6H_2O$, KH_2PO_4 were dissolved in double distilled water one after the other in a volmetric flask and volume was made to one litre. All the chemicals were of high purity.

ii) Micronutrients : To prepare 100 X micronutrients the weighed amount of chemical compounds such as $MnSO_4$, $4H_2O$, $ZnSO_4.4H_2O$, H_3BO_3, KI, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$ were dissolved one after the other in about 400 ml distilled water and the volume was adjusted to one litre with distilled water. All the chemical compounds were of analar grade.

iii) Iron stock : (20X) weighed quantity of analytical grade $FeSO_4.7H_2O$ was dissolved in distilled water and the volume was made to 100 ml. The solution was stored in refigerator and added to the medium just before use.

iv) vitamin Stock : 100 X vitamin stock solution was prepared as follows : Approximately 50 ml of double distilled water was taken in 100 ml volumetric flask. The weighed quantity of vitamines Glycine, Nicotinic acid, pyridoxine HCl, thiamine HCl, Myo-inositol were dissolved one after the other and the final volume was made and stored in the deep freeze.

v) Auxin stocks : Various auxins such as 2,4-Dichlorophenoxy acetic acid. IAA,IBA,NAA which were used in culture medium were kept ready in the form of stock solutions. 400 mg of each of auxin weighed seperately and was dissolved in small amount of ethanol and the volume was adjusted to 100 ml with double distilled water so as to make final concentration 1 ml of this stock solution correspoded to 4 mg of particular auxin. They were stored in refrigerator.

vi) Cytokinin Stock : Cytokinin Kinetine was weighed accurately on a monopan balance and was first dissolved in a few drops of 0.1 N NaOH and then the volume was adjusted to 100 ml with double distilled water in a volumetric flask and stored in refrigerator.

vii) Casein Hydrolysate : 5 gm of Caseinydrolysate was dissolved in small quantity of double distilled water and then the volume was adjusted to 100 ml in a volumetric flask. It was stored in a refrigerator.

viii) Preparation of Coconut Milk :The method is essentially based on that of Klein and Klein (1970) and elaborated in the technical bulletin "PLANT CELL CULTURE". 1991-92 by Sigma. There are varieties of coconuts varying age wise as well as variety wise. Based on that the quantity and nature of coconut milk varies. While using the

coconut milk it has been scrupulusly observed to buy from the same stock and of more or less same age. It was brought to the laboratory, throughly washed from outside. The entire coconut with husk at its micropylar end was slashed with a sharp chopper till the central relatively large soft micropyle (eye) is exposed. The water was drained in a clean beaker filtered through a musline cloth to remove solid partical if any. The milk was deproteinized by boiling. When it cooled the protein settled down and the supernetent was filtered through whatman filter paper number 1. The product is then used in various dilutions immediately or stored in deep freeze at 0-4^{o}C. The remaining proteins in coconut water may vary from one lot to other and may result in precipitaton when the product is frozen. However, this precipitation does not affect or diminish the cytokinin like properties of substance present in the endosperm. To avoid any postmortum changes the precausion was taken to use the coconut milk fresh. Different dilutions of 5-20% (V/V) were made directly with the culture medium.

6 METHOD OF PREPARATION OF CULTURE MEDIA :

The media were prepared in the following ways. Required quantity of different stock solutions were taken in to a desired volumetric flask with auxins cytokinins and

then washed thoroughly with sterile distilled water till the last trace of Mercuric chloride was removed. They were then blotted dry with the help of sterile whatman filer paper. With the help of sterilized pair of foreceps the explants were slowly placed into the culture medium embedding the cut end or the distal end of the meristematic region in the medium. Before exposing the culture medium to the outer atmosphere, the mouth of the tube was held near the flame of the spirit lamp to prevent contamination. All the procedures were carried out in the laminar flow chamber.

b) Leaftip tendril culture :

Since the leaftip of the Gloriosa is modified in to tendril young leaves posses active meristem at their tip. The young leaves were first surface sterilized and washed with double distilled water and then 5-8 mm long tips were excised with the help of sterile scissor and was transferred to the tube in the same way as in the case of apical meristem.

c) Culturing of the shoot region below the apical dome :

To test whether there is any lateral meristem in the vicinity of apical meristem, shoot portions one cm below the apical meristem but of about one to one and half cm in size were also cultured in the same way as described above. They were first surface sterilized with aqueous mercuric chloride solution, throughly washed with sterile distilled water and then embeded into culture tube maintaining the polarity.

d) Seed Culture :

In Gloriosa seeds are having prolonged dormancy and seed germination percentage is poor. In order to see whether the seed is able to sprout, the seeds harvested in the earlier lifecycle period were dried and stored in a secured container. Such seeds were first soaked in running tap water for a period of 4 days to ensure that colchicine which otherwise known to inhibit the germination is removed. After fourth day they were surface sterilized with mercuric chloride, washed thoroughly with sterile distilled water and inoculated in the culture tube with embryo facing upward.

e) Callus subculture :

Normally the sign of regeneration is that, the explants starts callusing on the medium. To make the subculture of the callus and to study the probability of raising the callus mass the explant or the shoot meristem was allowed to calluse in the MS medium. When it started growing sufficiently at large size the sample of the callus mass was subcultured basically in the MS medium but supplimented with varying concentrations of auxins.

f) Suspension culture :

To study possibility whether the callus cells can seperate out under suspension, a piece of callus mass was sampled from actively growing callus culture and transferred

coconut water (Whenever required). The solution was added with small amount of double distilled water and required quantity of sucrose was dissolved in it by continuous shaking. The volume was adjusted with double distilled water and the desired pH was adjusted with 0.1N HCl or 0.1 N NaOH.

The medium were first heated slowly on a hot plate with constant stirring. while heating and stirring the weighted quantity of agar was slowly added. The heating was carried out till the agar went in to the solution. This medium was then distributed into the culture tubes for autoclaving. The culture tubes were pluged and autoclaved at 15 lb pressure for half an hour. After autoclaving they were kept on wooden stand and allowed to solidify. On subsequent day these plants were incoulated holding under sterile conditions in laminar flow chamber in a way described elsewhere.

It is necessary to mention here that all chemicals, vitamins hormones used of high purity grade and agar used was of Himedia.

7 SELECTION OF EXPLANTS FOR CULTURE :

The shoot meristem was chosen as an explant. About one cm shoot apex was excised from a healthy plant with sterile raizor blade. The leaves were removed and brought to the culture room. The shoot explants were then surface sterilized with mercuric chloride solution for 5 minutes. They

to the liquid broth of a suspension culture(MS medium without agar) in a 250 ml erlenmeyer flask, in a secured way. Each flask contained only 50 ml of the suspension culture medium. The flasks were clamped on rotary shaker and it was agitated at a rate of 60 rpm under dark condition. The periodic sampling were carried out and observed under microscope for cell seperation.

8 TEMPERATURE AND LIGHT REGIME :

It is known that all cultures must receive optimum temperature and light. The culture is airconditioned maintaining the constant temperature of $25^{\circ} \pm 2^{\circ}$ C. The light requirement has been met with by banks of fluroscent tubes supplimented with incandescent lamps. The light sources were held at a constant distance so that at the point of incidence the culture received 2000 lux.

9 CHROMATOGRAPHIC SEPERATION OF COLCHICINE :

a) Preparation of extract :

The alkaloid detection from callus was carried out by paper chromatographic method as per Clark (1970).For this the profusely grown callus mass from the cultures was taken and was washed with sterile distilled water so as to remove the adherent media. Then it was boltted with Whatman filter paper and crushed in 10% glacial acetic acid in ethanol till it was turned into a fine pest. It was transferred to a clean evaporating dish. The traces of sample were transferred by several washes with crushing medium. The sample was filtered through several layers of muslin cloth. The filtrate was then

condensed on a water bath till it reduced to volume of 5 ml. The alkaloid in the condensed medium was then precipitated by adding liquid ammonia drop by drop till all the alkaloid get precipitated. After ensuring that all the alkaloid was precipitated it was centrifuged at about 300 X g. The supernatant was then discarded taking only the precipitate. The precipitate was washed again with 1% Ammonium hydroxide and dissolved in ethanol. This ethanolic extract of alkaloid was then used for paper chromatography and Thin layer chromatography.

b) Paper Chromatography :

Whatman chromatographic paper No.1 was cut in to four pieces in the mechine direction. These chromatographic strips were buffered with 5% sodium citrate by dipping into the solution and air drying. The solvent used was a mixture of n-butanol aqueous citric acid prepared by mixing 870 ml n-butanol and 4.8 g citric acid in 130 ml water. About 500 to 600 μl sample was loaded on the chromatogram with the help of micropipette. Parallel to that 100 μl standard colchicine solution was also loaded on the chromatogram so as to make final concentration of the standard on the spot 0.4 mg. The chromatogram was run in the ascending manner for about 6 h. After running the chromatogram it was dried at room temperature and sprayed with Dragendroffs reagent. Orange spots developed wherever the alkaloid was present.

c) Thin layer chromatography :

The TLC method followed for seperation was that of Stahl (1969) described for alkaloid by Harborne (1973). Two mm thick strips of glass plates of size 2" X 7" were washed throughly, first with acetone to remove greasy substances., then with detergent ' Laboline and subsequently with distilled water. They were dried in an oven at 100° to 110° C. The slury of silica gel with binder in it (SRL grade) was prepared in water by vigrously shaking for about one and half minute. It was immediately spread over the TLC plate uniformly with the help of applicator. Such a plates were initially air dried and then activated by heating in an oven at 100° to 110° C for 30 min. The extract prepared for paper chromatography was loaded at the bottom of the plate with the help of micro-pipette. Side by side standard alkaloid was also loaded. These plates were kept in corning glass jars containing solvent prepared by mixing methanol and ammonium hydroxide in the proportion of 200 :3.

The jars were tightly covered with glass plate and allowed to run. After reaching the front, the plates were removed and air dried. After spraying with Draggendroff reagent it gave yellowish orange spots where ver alkaloid is present.

d) Preparation of Spray Reagent

Dragendroff's reagent : In this reagent two stock solutions are mixed just before use (a) 8 g of $Bi(NO_3)_3$ $5H_2O$ are dissolved in 20 ml of HNO_3 (Sp.gr.1.18) and (b) 27.2 g of

KI in 50 ml of water. The two solutions are mixed and allowed to stand when KNO_3 crystallizes out, the supernatent is decanted off and made up to 100 ml with distilled water.(Peach and Tracey, 1955).

10 CYTOLOGICAL AND GENETICAL STUDIES :

When the explant shoot meristem started callusing in the medium, the callus mass was subcultured in order to make the chromosome studies and to study the cell division. The portion of callus mass was sampled from culture tube in the morning hours in keeping with its division cycle. It was put to 0.2% colchicine and held at low temperature at 4°C for about 3 hours to achieve chromosome condensation and better chromosome seperation. While the colchicine broke the spindle apparatus, the chilling effect led to the condensation of the chromosomes. After 3 hours pretreatment, sample was removed from the freeze, allowed to attain the room temperature and immediately maccerated on a microslide in 1% aqueous acetocarmine and slides were prepared for microscopic observations. The interesting slides were microphotographed and the plates were prepared.

11 HYBRIDZATION :

With a view to produce and study hybrids between Gloriosa superba and Gloriosa lutea the hybridization programme was undertaken. The potted plants as well as the plants raised in garden beds of both the species, when attained flowring stage and just before the anthesis period were emasculated.

The flowers were kept bagged to prevent pollination. Similarly the flowers from which the anthers are collected were also bagged to prevent foreign pollen contamination. When the stigma came to maturity the receptivity could be readily recognised by the secretion on the sigmatic lobe. From the pollinater flower matured anthers with ripe pollens were collected with the help of forceps and burst open on the surface of the stigma. The flower was then bagged. The pollination was ensured by repeating the process on subsequent day. Reciprocal crosses were made between two species.