II MATERIALS AND METHODS

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A) Karyotype studies :

i) Pretreatment :

The somatic chromosome number was determined from the root tips. To get healthy roots the cuttings of Coleus forskohlii were allowed to keep in a sterile moist fine sand in small pots. After ensuring that the roots are of one inch long, the cuttings were removed by excessively irrigating the pots. This step enabled to harvest the turgid roots. The roots were washed, cleaned before taking them for pretreatment. To obtain proper separation of metaphase chromosomes of Coleus forskohlii 0.2% aqueous colchicine solution was successfully employed. To accomplish better penetration the root tips were given incision with razor blade and vacuated with vacuum pump. The entire container was chilled for 4 hr. in freezer at 8 - 12°C to accelerate the reaction of condensation. After 4 hours root tips were washed thoroughly with distilled water and fixed in farmer's fluid. The farmer's fluid was freshly prepared by mixing 3 parts of absolute ethyl alcohol and 2 parts of glacial acetic acid. The root tips were kept in fixative for 48 hr. After ensuring fixation the root tips were transferred to 70% ethanol and stored.

ii) Preparation of slides for microscopic examination :

The pretreated root tips stored in 70% ethanol were transferred to 45% propionic acid. After several changes of

propionic acid, to ensure complete removal of ethanol, the root tips were hydrolysed in 1 N HCl at 60°C for 4-5 minutes. They were then washed thoroughly in distilled water and transferred to freshly prepared 2% propiono orcein. The meristematic portion of the root tips were carefully secured on the microslide and squashed in a drop of propiono orcein by putting the coverglass. Inverted technique was employed to get uniform unicellular spreading of the tissue. Excess of stain was squeezed out by pressing the slide with thumb holding inverted on blotting paper. The slide was gently tapped with the rubber end of the pencil. It was sealed with bee's wax (prepared by mixing molten paraffin wax and bee's wax in a proportion of 1:1). The sealed slides were stored overnight to deepen the intensity of staining so that it facilitated better study and photography.

For karyotypic study the drawings were made with camera lucida by using oil imersion. For determining the length of the chromosomes, 5 plates were studied and the average length of each individual chromosome was calculated from the data obtained. For the karyotype analysis the method of Levan et al. (1964) has been followed and idiogram of chromosomes was made. Slides were made permanant following the butyl alcohol and acetic acid series.

B) Method of Cultivation :

The plants required for experimentation were raised in the garden. They were propogated more or less in the same way, the farmers cultivate in the field.

The vigorous growing plant parts of Coleus forskohlii were chosen. The plants were raised in rows on soil beds prepared by previously mixing farmyard manure. 5 to 6 inch long stem cuttings with 3-4 pairs of leaves with a axillary bud are planted in rows keeping at a distance of 30 cm. The 60 cm. distance were maintained between the two rows. Cuttings are inserted in the soil deep leaving buds exposed. The plantation was raised in the early monsoon. After the mansoon is over they were irrigated once in a week. The usual dose of chemical fertilizer was given periodically. The experiment was carried out in a properly designed randomised blocks with three replication.

C) Determination of inorganic constituents :

Inorganic constituents of the leaf and the roots of Coleus forskohlii were determined in the digested sample, with the help of Atomic Absorption Spectrophotometer.

The matured leaf and the root samples were harvested at random from plants raised in the garden. They were washed thoroughly and blotted dry. The leaves and the roots were cut in the small pieces, weighed and kept in the oven for drying

at 80°C. After expelling the complete moisture, one gram leaf as well as the root samples were seperately acid digested in a mixture of perchloric acid and nitric acid. The acid digest was thus filtered through Whatman Filtre Paper No.1 and the volume was adjusted. The clean extract of the sample were then used to determine Mg, Mn, Co, Zn, Cu, Ca and Fe.

D) Estimation of Nitrogen :

To determine the nitrogen content of the leaf and root oven dried samples were taken. 0.5 gram leaf sample and the root sample were separately Kjeldahl digested in 10 ml of 50% sulphuric acid and a pinch of microsalt. The digested sample was made to volume. The nitrogen content was estimated spectrophotometrically using Nesseler's reagent by themethod of Hawk et al.(1948) at 560 nm on Shimmatzu Spectrophotometer.

E) Method of study of stomatal behaviour :

To know the behaviour of stomata on Coleus forskohlii both under natural condition as well as irradiated condition steady state Porometer LI 1600 USA was employed. The leaves were clamped in the midnoon and leaf temperature, gas exchange properly and transpiration rate were recorded by automatically by the Porometer and the values were computed for necessary data.

F) Mutation study :

To know the effect of induced mutation on forskolin content the vegetative portions were gamma irradiated by the curtsey of Bhabha Atomic Research Centre, Trombay, Bombay. To accomplish the same terminal cuttings of about 5 to 6 inch long portion with intact leaves were freshly harvested and quickly packed in a perforated polythene bags over a moist cotton wad. They were sent to BARC for irradiation. Following doses of radiation were given 500 R, 1000 R, 1500 R, 2000 R, 2500 R. For each of the dose 60 cuttings were exposed. No sooner did the irradiated cuttings reach the hands they were planted in a previously prepared garden beds on a ramdomized block design fashion and was watered.

Out of the 5 different doses exposed the cuttings which received 500 R only survived the rest had 100% mortality. From the 500 R M1 generation cuttings were raised for M2 in the same fashion as before. The effect of irradiation was studied on M2 generation.

G) Foliar application of Mg^{2+} and Mn^{2+} :

In order to study the effect of Mn^{2+} and Mg^{2+} on drug yield foliar spray method was adopted. This trial was undertaken in plants grown in garden. Prior to plantation soil beds were prepared in small plot. Initially while preparing the soil it was mixed with farmyard manure (FYM).

The seedlings were raised in row plantation keeping 60 cm distance between the row and 30 cm between the seedlings. For raising the plantation terminal cuttings of 6 to 7 inch length were used. The treatments were applied to each plot separately. For foliar spray $MgCl_2.6H_2O$ and $MnCl_2.4H_2O$ is used as a source of Mg^{2+} and Mn^{2+} respectively. The solutions of desired concentrations are made in distilled water. They are sprayed in the following concentrations and combinations.

i) $MgCl_2$ 50 ppm

ii) $MgCl_2$ 100 ppm

iii) $MgCl_2$ 50 ppm

iv) $MgCl_2$ 100 ppm

v) $MgCl_2$ 50 ppm + $MnCl_2$ 50 ppm

The control was given spray of distilled water. The foliar spray was started when seedling were of 15 days old and continued periodically at the interval of a week. In all 5 sprays were given during their growth. The tubers were harvested after maturity and forskolin content of the tubers was determined by the TLC method.

H) <u>Effect of fertilizer on tuber yield and drug yield</u> :

The fertilizer trial was undertaken in departmental garden. The cuttings were raised in row plantation keeping 60 cm distance between the row and 30 cm between the plants.

For combination of NPK fertilizers, urea and diamonium phosphate was chosen as nitrogen source, murate of potash for potassium and diammonium phosphate is used as a source of phosphorus also. The fertilizers were tried in three different combinations such as 70:50:50, 50:70:50 and 50:50:70 of NPK. All these were split up in three doses. The experiment was designed maintaining three replication each of treatment and control. Three treatments were given at the intervals of 15 days after setting of cuttings. Water was adequately maintained avoiding waterlogging. After full maturity, the tubers were harvested and tuber yield per plant was worked out.

I) Assay of forskolin by TLC :

Forskolin content from root tubers of Coleus forskohlii is separated by TLC method as per Inamdar et al.(1984).

Preparation of sample :-

Root tubers of 5 month old plantations were harvested from the experimental plot. They were washed thoroughly and blotted dry. They were chopped into small slices and air dried in the laboratory for a period of a week. A kilogram dried root samples were crushed in the mortor with a pestle and powdered debris were seived out with a fine muslin cloth. From so collected fine powder from the root tubers 25 gram sample was extracted

first with 150 ml of benzene in a conical flask. Holding the container closed, it was extracted for 5 hours on a water bath at 45 to 50°C. It was then filtered. The residue was again extracted in 150 ml portion of benzene on a water bath at low temperature of 45 to 50°C. The process was repeated thrice. The benzene filtrate was collected together and concentrated in vacuo to 20 ml. This concentrated extract was then dried at room temperature and treated with 100 ml petroleum ether (B.P. 60-80°C). It was allowed to settle. Supernatent was then decanted. The process was repeated 2 to 3 times to remove all unnecessary compounds such as proteins, organic acids, sugar, colouring matters etc. The residue so remained is dried at room temperature in dark to avoid photochemical reaction. This residue was then treated with 50 ml portion of methanol and process was repeated twice. The methanolic solution is then separated and concentrated in vacuo. This concentrate which look as a brown solid mass is dissolved in 5 ml portion of chloroform and was then passed through column of charcoal for purification and solidified. 30 ul portion of the semipurified extract and the standard (1 mg dissolved in 0.5 ml chloroform) was loaded on the bed of Kisselgur on a TLC plate and run.

J) <u>TLC Separation</u> :

The TLC plate was prepared as per the method of Stahl's (1958). The Kisselgur (Silica gel G) of Emark previously mixed

with binder was used for the purpose. The powder was mixed with distilled water and slurry was prepared. This slurry was spread over the TLC glass plate and spread uniformly with the help of applicator. After setting of the film on the plate, the plate was activated by heating at 120°C in an oven. The known quantity of standard as well as the sample, loaded side by side and was run in a glass chamber using the solvent benzene + ethyl acetate mixed in a proportion of 85 : 15. The chamber was made airtight. After completely running solvent as well as the solute the plate was removed and dried. To detect the forskolin spots Forskolin Stahl's Reagent No.11 was sprayed. The forskolin spots developed pink colour. The fronts of the spots of standards and the sample were compared. The intensity of the colour as detected by the eye was considered for quantitative difference in different plant samples.

Preparation of Stahl's Reagent No.11 :

The spray reagent was prepared as per method of Inamdar et al.(1984). It was prepared by mixing 50 ml of glacial acetic acid, 0.5 ml of anisaldehyde and 1 ml of conc. sulphuric acid in a conical flask. The mixture was thoroughly shaken to facilitate better mixing.