MATERIALS AND METHODS

3.1. PLANT MATERIAL

The experimental plant of the present investigation was **CHIRATA [*Swertia chirayita* (Roxb. ex Fleming) Karsten]** of Family Gentianaceae. Chirata is an annual (Anon, 1982; Kirtikar and Basu, 1984), or a biennial or pluri-annual (Edwards, 1993) plant and dies after seed dispersal. Seed dispersal takes place once the plant has matured in late November or December (depending on the area). Matured chirata seeds were collected from different adjoining places of Darjeeling like Sepi (Rimbik), Khopi Dara (Maneybhanjang), Permaguri (Sukhia Pokhri), Lami Dara (Pussumbing) and Majidhura (Sukhia Pokhri) during the month of December-January. Plant seeds from Permaguri (Sukhia Pokhari) had the highest viability and germination percentage (Lama *et al*., 2012). Thus, the chirata seeds of this particular place *i.e.,* from Permaguri (Sukhia Pokhari) were collected locally and used as experimental material.

3.2. METHOD OF SEED COLLECTION

On maturity all semi-dried leaves were excised and from the mature inflorescence, the flower pods were manually separated and kept in dry paper bags. The collection exercise was generally done during foggy or humid day or in the early morning. The collected materials were sun dried for 48 hours allowing the flower pods to burst, releasing the seeds. The mature seed colour was dark brown and size of the seed was between 363-550 mm in length and 308-484 mm in width. The sieved and cleaned seed powder contained 50-60 seeds per milligram as determined microscopically.

The high value medicinal plant species, *Swertia chirayita*, is monocarpic in nature. The whole plant parts are used in different traditional and Indian system of medicine. This plant contains one of the bitterest compounds in nature which has great demand in medicinal and ethno botanical use. The chirata plant grows almost all over the Darjeeling Hills area at an altitude from 1900m onwards to 3000m above sea level. Important events during the life cycle of the chirata plant were determined from the average data of three planting seasons (Table1.6).

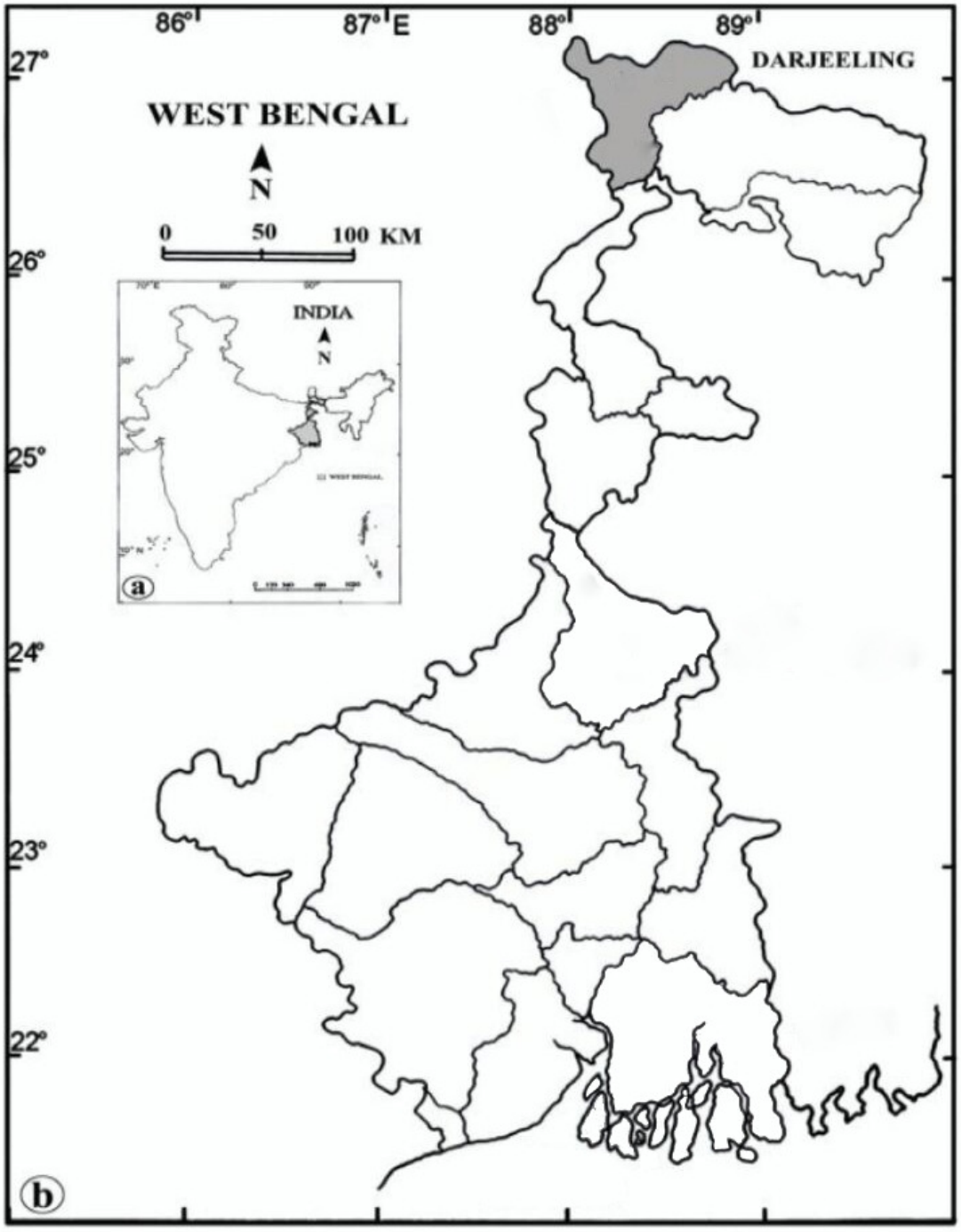

Fig. 3.1 Map of Darjeeling.

3.3. SOIL PREPARATION AND METHOD OF SEED SOWING AND TRANSPLANTING

All the field experiments of this investigation were performed in the experimental fields at Ramji Busty, latitude of 27°00.393'-N and longitude of 88°09.588'-E and an altitude of 6235 ft, Sukhia Pokhari, Darjeeling.

3.3.1. Nursery for plant materials

The land was ploughed 3-4 times to prepare for sowing. After adding adequate amounts of cow dung and organic compost, plots were prepared in terraced land. About 70% of the plot soil was field soil and the rest 30% of the composition was cow dung and compost manures. The plot was watered sufficiently and selected seed lots were broadcasted. Mature and healthy seeds were thoroughly mixed with semi dried cleaned sand and kept overnight. Next day, the mixture of seeds and sand were spread in the prepared plot manually. The whole plot was covered with natural coloured polythene and watered regularly. After seed germination, the following different developmental stages of the plant were collected and transplanted to plots for experimental purposes.

Selection of 3 different plant developmental stages for foliar treatment —

1] **Rosette stage [S1] -** The plant stayed at the first stationary phase of growth for a prolonged period of time when it reached 135-240 days. Active growth of the plant was stopped and it remained in 'rosette' form. The plant height was in between 5 to 8 cm.

2] **Sapling stage [S2]** - The plant with node, internode, branches and sub branches. The total height of the plant was from 45 to 85cm.

3] **Pre-flowering stage [S3]** - The mature plant with no apparent growth and bud formation.

For each and every stage and treatments, 25 numbers of uniformly grown plant seedlings were transplanted to each plot and treated accordingly. In each stage, plants were treated with three growth promoters, *viz.*, Gibberellins (GA_3), Indole Acetic Acid (IAA) and Kinetin (Kin) and three growth retardants, *viz.*, Maleic Hydrazide (MH), Alar (SADH) and Abscisic Acid (ABA). Two different concentrations of 100µg/ml and 200µg/ml of the Plant Growth Regulators were used; except for Abscisic Acid of which the concentration used was 10 µg/ml and 15µg/ml. The field was divided into subplots, each having an area of 4mx4m. In each of the subplots, twenty-five plants were transplanted for each treatment. The transplanted plants were irrigated at a regular interval.

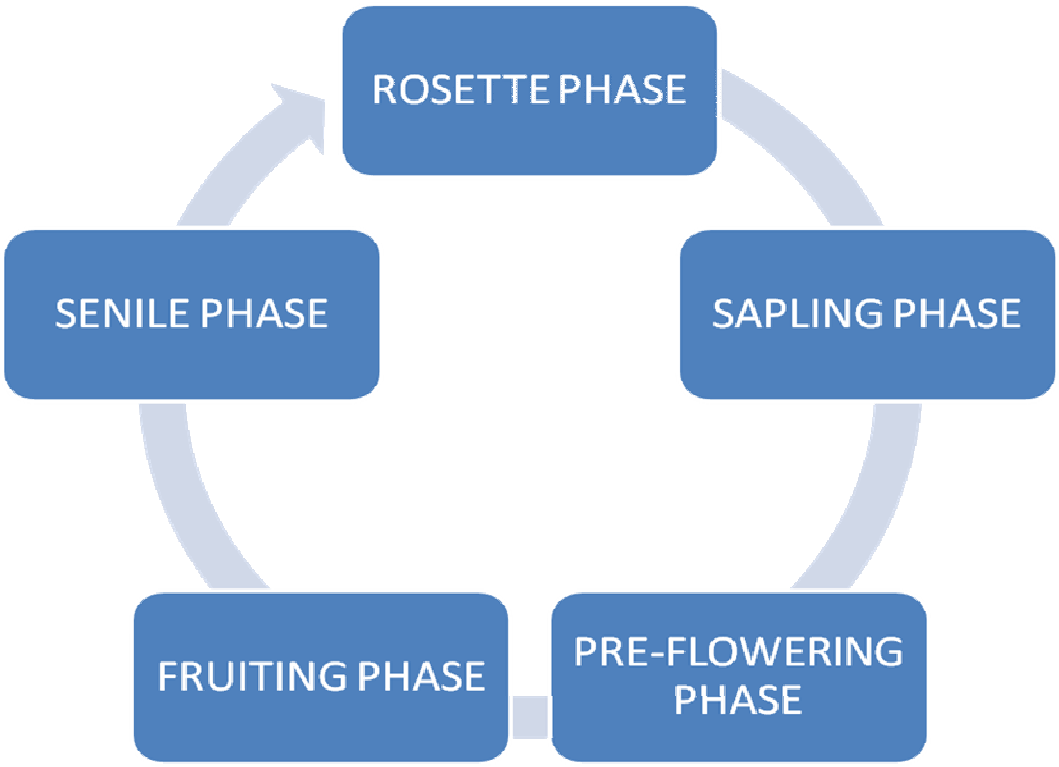

Fig. 3.2: Different phases in the life cycle of *Swertia chirayita*

Fig. 3.3: Life cycle of Swertia chirayita

Fig. 3.4: Different plant developmental stages for foliar treatment: A. Rosette stage (S1). B. Sapling stage (S2). C. Pre-flowering stage (S3).

Fig. 3.5: Experimental field showing different treatments

3.4. METEOROLOGICAL DATA

Meteorological data *viz.*, temperature, relative humidity and rainfall during the experimental years of 2009, 2010 and 2011 month-wise at Darjeeling were procured from the Principal Office of Agriculture, Government of West Bengal, Darjeeling and are tabulated in Tables 1.7, 1.8 and 1.9.

3.5. DESIGN OF EXPERIMENTS

In this investigation, experiments were designed under the following directions to analyze the effects of three growth promoters- GA_3, IAA and Kinetin and three growth retardants- MH, SADH and ABA on chirata plant (Ramadass *et al*., 1968):

- Analyses of the effects of growth promoters and growth retardants treated at rosette stage on growth, metabolism and yield.
- Analyses of the effects of growth promoters and growth retardants treated at sapling stage on growth, metabolism and yield.
- Analyses of the effects of growth promoters and growth retardants treated at pre-flowering stage on growth, metabolism and yield.

3.5.1. Analyses of the effects of growth promoters and growth retardants treated at rosette stage on growth, metabolism and yield.

Foliar application with aqueous solutions of GA_3 (100 and 200 µg/ml), IAA (100 and 200 µg/ml), Kinetin (100 and 200 µg/ml), MH (100 and 200 µg/ml), SADH (100 and 200 µg/ml) and ABA (10 and 15 µg/ml) containing Tween-20 (Surfactant) were given at the rosette stage of 135-240 days old plants for three consecutive days.

3.5.1.1. Growth analyses

For study of growth attributes of the plants, reading was done after ten days from the last date of foliar application of the PGRs and was repeated fortnightly. The reading was continued till the plants reached their senescence stage. Different physiological parameters like height of the plant, number of leaves, nodes and branches, stem circumference and internodal distance were recorded for each plant. Data were recorded from the mean values of five uniformly grown plants for each treatment.

3.5.1.2. Biochemical analyses

Biochemical analyses were carried out at five important life phases of chirata plant, *viz.*, at rosette, sapling, pre-flowering, fruiting and senile stages, taking samples from the leaves of treated plants.

3.5.1.2.1. Chlorophyll

To determine the chlorophyll level, leaf tissues (100 mg) were taken in 5ml methanol in test-tubes and kept in dark in laboratory conditions for 48 hours. The supernatant was decanted off and leaf samples were rinsed repeatedly with little amount of methanol. The supernatant and washing materials were pooled together to make the final volume to 10ml. Subsequently, the absorbance of the chlorophyll extracts was measured at 650nm, 663nm and 645nm for total chlorophyll, chlorophyll a and chlorophyll b respectively. The chlorophyll content was estimated following Arnon's principle (1949).

3.5.1.2.2. Carbohydrate

Carbohydrate levels (soluble and insoluble fractions) were determined following the method of McCready *et al.* (1950) with slight modifications. 100 mg sample from fresh and thoroughly cleaned leaves excluding midribs and veins were homogenized with boiling 80% ethanol and centrifuged at 6000g for 10 minutes. The supernatant was taken in a watch glass. This was repeated thrice, and the pooled supernatant was evaporated to dryness. Traces of chlorophyll, if any, adhering on the surface of the watch glass was carefully removed using solvent ether repeatedly. The remaining material in the watch glass was taken in a test-tube by washing it several times with 80% methanol and the volume was made up to 10ml. This was kept as a source of soluble carbohydrate. For the analysis of insoluble carbohydrate, the residue after centrifugation of the sample was digested with 5ml 25% H_2SO_4 at 80^oC in a water bath for 30 minutes. The extracted material after suitable dilution was taken as a source of insoluble carbohydrate.

For analyses and quantitative measurement of both the carbohydrate fractions, 1ml of the source sample from each was taken in a test-tube and 4ml of freshly prepared, pre-cooled, 0.2% anthrone reagent (200 mg anthrone in 10 ml analar sulphuric acid) was added to it. After 30 minutes the intensity of green colour was measured by

spectrophotometer at 620nm. Actual contents were determined from the standard curve prepared by using different concentrations of glucose solutions.

3.5.1.2.3. Protein

The chlorophyll-free leaf samples (100 mg wet tissue weight) were crushed with 80% ethanol and centrifuged at 6000g for 10 minutes. To make the pellet phenol-free, it was washed successively with 10% (w/v) cold trichloroacetic acid (twice), ethanol (once), ethanol: chloroform (3:1, v/v, twice), ethanol:ether (3:1, v/v, once) and finally with ether (once) following the method described by Kar and Mishra (1976). The pellet was evaporated to dryness. Protein was extracted from the pellet by digestion with 0.5M NaOH at 80^{o}C for 1 hour and estimated by Folin-phenol reagent measuring the O.D. value at 650nm in the spectrophotometer according to the method of Lowry *et al*., 1951. The quantitative determination was made by comparing the O.D. values of a standard curve previously prepared using bovine serum albumin (BSA Fraction-V-Powder, Sigma Chemical Company, USA).

3.5.1.2.4. Free amino acids

100 mg of chlorophyll free leaf samples were taken in a clean and pre chilled mortar with pestle. Then the tissue was homogenized with 5ml 80% ethanol and the disorganized tissue was centrifuged at 5000rpm for 10 minutes. The supernatant was then taken as the source of free amino acid content (Dwivedi *et al.,* 1979). From the stock solution, amino acid was estimated following the method of Moore and Stein (1948). A test tube containing 1ml of 0.3% ninhydrin solution (in 80% ethanol) was kept in a water bath for 15 minutes, with glass marble at the top of the test tubes. When the reaction turned to violet colour, the test tubes were taken out, cooled and the volume was made up to 5ml with 80% ethanol. The absorption of the solution was measured at 570 nm in a spectrophotometer. The quantitative estimation was made by comparing the O.D. values of the standard curve prepared by glycine as the reference amino acids.

3.5.1.2.5. Nucleic acids

Extraction of nucleic acids (DNA and RNA) was made from 100 mg fresh (wet tissue weight) leaves following the method described by Cherry (1962). The levels of

DNA and RNA were estimated from a common stock employing the method of Markham (1955), modified by Choudhuri and Chatterjee (1950).

3.5.1.2.5.1. RNA: For the estimation of RNA, 3ml of diluted nucleic acid extract in a test-tube was treated with an equal volume of freshly prepared orcinol reagent (1 gram AR grade orcinol dissolved in 100ml of concentrated HCl containing 100mg 0.1% $FeCl_2$.6H2O), and boiled in a water bath for 20 minutes with a glass marble at the top of the test-tube. The mixture was then cooled, and the intensity of the blue-green colour was measured at 700nm in the spectrophotometer. The blank used contained a mixture of 3ml distilled water and 3ml orcinol reagent which were treated in an identical manner. RNA level was calculated from the O.D. values from a standard curve prepared with yeast RNA.

3.5.1.2.5.2. DNA: For the estimation of DNA, 1ml of the nucleic acid extract in a test-tube was mixed with 5ml freshly prepared diphenylamine reagent (100ml glacial acetic acid BDH.AR + 2.7ml H_2SO_4 + 1gram AR grade diphenylamine). The mixture was boiled in a water bath for 30 minutes with a glass marble at the top. After cooling, the intensity of the bluish colour was measured at 610nm in the spectrophotometer. DNA content was quantified from the O.D. values of a standard curve prepared with herring sperm DNA.

3.5.1.2.6. Lipids

Leaf tissues (2grams) of each treatment were homogenized with a little neutral sand with 10ml of a mixture of chloroform and methanol. The homogenate was centrifuged at 4000g for 10 minutes (Cox and Pearson, 1962). The supernatant was taken in a separating funnel. A pinch of Sudan III was added and the mixture was shaken thoroughly. 10-15ml of 0.05 N KCl was added and shaken well. It was kept for 30 minutes for layer separation. The lower chloroform layer containing fat was taken in a pre-weighed conical flask and the chloroform was evaporated over a heater. When the chloroform was totally evaporated, the weight of the flask with fat sample was taken.

3.5.1.2.7. Total phenols

Leaf tissues (50 mg) were homogenized with 5 ml of 80% boiled ethanol at 60-65^0 for 5 minutes. The supernatant was taken in a test-tube, to which 4ml of distilled water was added and then kept in a hot water bath till the alcohol is completely evaporated

following the method of Mallick and Singh (1980). The water content was taken in a separating funnel to which 4 ml of solvent ether was added and shaken vigorously for 5 minutes. It was kept upright for 15 minutes with a clamp and a stand. The upper layer was taken in a beaker to which 3 ml of distilled water was added. The beaker was kept in open air for complete dryness. 1 ml of water extract was taken in a test-tube, along with 3 ml of 5% Na_2CO_3 and 1 ml of Folin. It was then kept in boiling water bath for 1 hour. The reading was taken at 650nm.

3.5.1.2.8. Catalase activity

Leaf tissues (200mg) of each treatment were homogenized with 8ml of chilled 0.1M phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH=6.8). The homogenate was centrifuged in the cold at 3000g for 15minutes followed by 10,000g for 20minutes. The volume of the supernatant was made up to 10ml with the same buffer, and this was assayed following the method of Snell and Snell (1971) modified by Biswas and Choudhuri (1978). The reaction mixture for catalase consisted of 1ml of the above extract and 1ml of H_2O_2 (0.0025M), incubated together at 37^oC for 2minutes. The reaction was stopped by adding 2ml 0.1% titanium sulphate in 25% H_2SO_4 (v/v), and the mixture was centrifuged at 6000g for 15 minutes. The intensity of the yellow colour was measured at 420nm. The blank was prepared by inactivating the enzyme with the addition of titanium sulphate prior to H_2O_2 addition.

3.5.1.2. 9. Peroxidase activity

For assay of peroxidase, 100mg leaf tissues were homogenized in cold 0.05M sodium phosphate buffer (pH= 6.5). The homogenate was centrifuged at 10,000g for 10 minutes. The pigments were removed from the supernatant by adsorbing with activated charcoal and filtered. The filtrate was used as enzyme source.

The activity of this enzyme was assayed following the method of Kar and Mishra (1976) with slight modifications. 5ml of the assay mixture containing 300µM sodium phosphate buffer (pH=6.8), 50µM H_2O_2, 50µM catechol and 1ml of crude enzyme extract were incubated at 25°C for 5minutes; the reaction was stopped with the addition of 1ml of 10% H_2SO_4. The colour was read at 430nm in the spectrophotometer.

3.5.1.2.10. RNase activity

100 mg fresh leaves were homogenized with 5ml of 0.1M sodium phosphate buffer (pH=6.4) at 0^oC and centrifuged at 10,000g for 20 minutes. The supernatant was made up to 10ml with the same buffer solution and this was used as crude enzyme source. Assay was made as per the method described by Biswas and Choudhuri (1978).

The reaction mixture for RNase consisted of 1ml of the enzyme extract and 1ml of yeast RNA (1mg/ml) dissolved in 0.1M sodium phosphate buffer (pH=5.7). The mixture was then incubated for 30 minutes at 37^oC, and the reaction was stopped by adding 0.2ml perchloric acid (70%). After centrifugation at 6000g, the supernatant was mixed with 5ml of BSA (0.5µg/ml) dissolved in 0.1M sodium acetate buffer (pH=4.0). After 5minutes the turbidity developed was stabilized with 2ml of 0.1% gelatin and measured at 420nm. The activity of this enzyme was expressed following the principle of Flick and Qualset (1975).

3.5.1.2.11. Alpha amylase activity

100mg of leaf tissue were taken in a pre-chilled mortar with pestle and homogenized with 8 ml of 0.1% Sodium Acetate buffer (pH 5) and centrifuged at 6000 rpm for 15 minutes in cold condition then, the supernatant was used as the source of enzyme amylase. From the supernatant, 2ml enzyme solution was mixed with equal amount of 0.2% starch solution & incubated at 37^0C for 30 minutes. The reaction was stopped with 2ml Iodine- HCl solution (60 mg KI and 6 mg Iodine dissolved in 100 ml 0.1 N HCl). The density was then measured at 610 nm in spectrophotometer (Biswas and Choudhuri (1978).

3.5.1.2.12. IAA-oxidase activity

Extraction of this enzyme was made from 100mg leaf tissue with 12ml of cold 0.2M sodium phosphate buffer (pH=6.1). The activity of IAA-oxidase was assayed following the method of Gordon and Weber (1951) and modified by Ramadas *et al.,* 1968. The reaction mixture contained 1ml of 1mM 2, 4-dichlorophenol, 1ml of 1mM $MnCl_2$, 6ml of 0.03M sodium citrate buffer (pH4.5) and 1ml of enzyme extract. This was incubated for 50 minutes at room temperature and then the reaction was stopped by adding 1ml of 20% $HClO_4$ to the mixture. 1ml of the assay mixture was reacted with 3ml of

Salkowski reagent (50ml of $HClO_4$ + 1ml of 0.5N $FeCl_3$), and the reading was taken at 525 nm in the spectrophotometer.

In case of enzyme assay, a zero time control was taken as blank and the activity of each enzyme was expressed as [{ΔA X V} / {t X v}], where ΔA is the change in absorbency at zero time control, V is the total volume of the filtrate taken for the assay, t is the time in minutes of incubation with substrate and v is the volume of the filtrate taken for the assay (Flick & Qualset, 1975). The activity of each enzyme was expressed as unit/gram fresh weight/hour.

3.5.1.2.13. Bitter principle content

Chirata plant was dried and cleaned. The plant was separated in three groups - (i) stem and branches, (ii) roots and (iii) leaves and inflorescence sticks. These parts were ground in mechanical grinder and made powder using No. 60 sieve. 20 grams of plant powder was mixed with boiling water containing 0.5 grams of calcium carbonate and extracted with boiling water till the last portion of the extract was devoid of bitterness. It was then concentrated in vacuum and the residue was dissolved in hot alcohol. It was filtered while hot and the residue was washed thrice on the filter with 10 ml portions of hot alcohol; the alcohol was removed from the filtrate and the residue was taken up repeatedly with 25, 15, 15, 15 and 15 ml of hot water. The aqueous extract was shaken repeatedly with 25, 20, 15, 15 and 10 ml of ethyl alcohol. The ethyl acetate extracts were collected, evaporated, dried and weighed. The percentage of bitter principle was then calculated (Mukharjee, 2005).

3.5.1.3. Yield analyses

After senescence, yield data were analyzed in terms of total water weight and dry weight of the whole plant. Yield attributes included number of flowers, length of the whole plant including the roots (in centimetre) and total weight of the whole plant (in grams). The wet weight of the whole plant was taken immediately after uprooting and the decrease in water content was recorded at a regular interval till the weight became constant to determine the dry weight of the plants.

Each experiment was carried out three times and mean values are given in the tables. Statistical evaluation of the results has been done at treatment and replication

levels. LSD (least significant difference) values at 5% level are included in the tables (Clarke, 1969).

3.5.2. Analyses of the effects of growth promoters and growth retardants treated at sapling stage on growth, metabolism and yield.

Foliar application with aqueous solutions of GA_3 (100 and 200 μg/ml), IAA (100 and 200 μg/ml), Kinetin (100 and 200 μg/ml), MH (100 and 200 μg/ml), SADH (100 and 200 μg/ml) and ABA (10 and 15 μg/ml) containing Tween-20 (Surfactant) were given at the sapling stage of 205-235 days old plants for three consecutive days.

3.5.2.1. Growth analyses

After ten days from the last date of foliar application with the growth promoters and retardants, physiological data, measured in terms of the parameters mentioned earlier in case of rosette treatment, were recorded fortnightly. Like rosette treatment, data were analyzed from the mean values of five uniformly growing plants. The reading was continued till the plants reached their senescence stage.

3.5.2.2. Biochemical analyses

Biochemical parameters analyzed were also the same as done in rosette treatment. Methods of extraction and estimation of the components are described earlier. Data were recorded at sapling, pre-flowering, fruiting and senile stages of plant development.

3.5.2.3. Yield analyses

The yield data recorded include the number of flowers, total length of the whole plant, including the root, as well as wet weight and dry weight of the plants as done earlier.

3.5.3. Analyses of the effects of growth promoters and growth retardants treated at pre-flowering stage on growth, metabolism and yield.

Foliar application with aqueous solutions of GA_3 (100 and 200 μg/ml), IAA (100 and 200 μg/ml), Kinetin (100 and 200 μg/ml), MH (100 and 200 μg/ml), SADH (100 and

200 µg/ml) and ABA (10 and 15 µg/ml) containing Tween-20 (Surfactant) were given at the pre-flowering stage of 325-350 days old plants for three consecutive days.

3.5.3.1. Growth analyses

Physiological data recorded in this experiment were the same as done in case of rosette and sapling treatments. The data were recorded after ten days from the last date of foliar application and was repeated fortnightly. Here also, the reading was continued till the plants reached their senescence stage.

3.5.3.2. Biochemical analyses

Biochemical data analyzed were also the same as done in rosette and sapling treatments. Extraction and estimation methods of the components were described earlier. Data were recorded at pre-flowering, fruiting and senile stages of plant development.

3.5.3.3. Yield analyses

In this experiment, yield data analyzed include number of flowers, total length of the whole plant and the wet weight and dry weight of the whole plant as done earlier.