Materials and Methods

B) METHODS

1) Moisture content and dry matter analysis

The leaf tissues from premature, mature, onset of senescence and senescent stage from various hormonal treatment and control were excised and brought to laboratory cleaned with distilled water and blotted to dry and fresh leaf tissue was weighed accurately and kept in the oven drying at 80^0C till constant dry weight was obtained. This gives fresh weight and dry weight content from the plant tissue in control and treated. The moisture percentage and dry matter percentage can be calculated by using the formula

$$\text{M (Moisture content) \%} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

$$\text{Dry matter \%} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

2) Photosynthetic Pigments

a. Chlorophylls

Chlorophylls were estimated following the method of Arnon (1949). Randomly sampled fresh leaves from premature, mature, onset of senescence and senescent stage on the plants were brought to laboratory, washed with distilled water and blotted to dry. Chlorophylls were extracted in 80% chilled acetone. 0.5g of fresh plant material was homogenized in cold mortar with pestle in dark. A pinch of $MgCO_3$ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Final volume of the filtrate was made to 100ml with 80% acetone. The filtrate was transferred into a conical flask wrapped with black paper to prevent photo-oxidation of the pigments. Absorbance was read at 663 nm and 645 nm on a UV-VIS double beam spectrophotometer (Shimadzu UV-190) using 80% acetone as a blank. Chlorophylls ($mg100^{-1}g$ fresh weight) were calculated using the following Formulae -

Chlorophyll 'a' = $12.7 \times A_{663} - 2.69 \times A_{645}$ ------- X

Chlorophyll 'b' = $22.9 \times A_{645} - 4.68 \times A_{663}$ --------Y

Total chlorophylls (a+b) = $(8.02 \times A_{663}) + (20.20 \times A_{645})$ -------- Z

Chl. a / Chl.b / total Chls. $= \frac{X/\ Y/Z \times \text{vol. of extract} \times 100}{1000 \times \text{weight of plant material (g)}}$

(mg 100 g^{-1} fresh weight)

b. Carotenoids

Carotenoids were extracted from the weighed amount of leaf material as per the procedure described for chlorophylls earlier. Carotenoids were estimated following the method described by Kirk and Allen, (1965). The absorbance was recorded at 480 nm on a UV-VIS double beam spectrophotometer (Shimadzu UV-190). The total carotenoids were calculated using the following formula –

$$\text{Total carotenoids (mg 100 g}^{-1}\text{ fresh weight)} = \frac{A_{480} \times \text{vol. of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}}$$

Where, 2500 = average extinction.

3) Total Polyphenols

The method of Folin and Denis (1915) was used for determination of the total polyphenol content in leaf tissue from premature, mature, onset of senescence and senescent stage (treated and control) of *Bougainville spectabilis.* Fresh leaf tissue (0.5 g) was crushed in 80 % acetone and filtered through Buchner's funnel. The residue was washed several times with 80 % acetone and the final volume was made 50 ml with 80% acetone. Two ml of plant extract along with a series of standard tannic acid (0.1 mg/ml) were taken in

separate Nessler's tubes and to each tube 10 ml of 20% Na_2CO_3 and 2 ml of Folin Denis reagent (100 g of sodium tungstate mixed with 20 g Phosphomolybdic acid in about 800 ml distilled water to this 200 ml 25% Phosphoric acid was added and the mixture was refluxed for 2-3 hours to room temperature and volume was made 1000 ml with distilled water). The final volume of reaction mixture was made 50 ml with distilled water. After 20 minutes absorbance was read at 660 nm with reagent blank. Total polyphenols were calculated with the help of std. curve of tannic acid and expressed as g 100 g^{-1} fresh weight.

4) Carbohydrate Status

a. Reducing sugars b. Total sugar and c. Starch

The sugars were estimated following the method described by Nelson (1944). Five hundred mg oven dried powder of leaf tissue from premature, mature, onset of senescence and senescent stage (each treatment and control) was extracted in 80% alcohol. The extract was filtered through Buchner's funnel using Whatmann No. 1 filter paper. The filtrate was condensed to 5 ml on hot water bath and to this 2 g lead acetate and potassium oxalate (1:1) were added for decolourization, 40 ml distilled water was added and aliquot was filtered through Buchner's funnel. The volume of filtrate (a) was measured and it served as an extract for determination of reducing sugars. From this 20 ml of filtrate was used for the determination of total sugars (b). For the estimation of

starch, the insoluble residue along with the filter paper was transferred to a 100ml capacity conical flask. To this conical flask 50ml distilled water and 5ml concentrated HCl were added and then these contents were hydrolysed at 15lbs pressure for half an hour. These conical flasks were cooled to room temperature, and the contents were neutralized by addition of anhydrous sodium carbonate (Na_2CO_3) and filtered through Buchner's funnel. The volume of filtrate (c) was measured and this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. The amount of glucose so formed is equivalent to the starch content in the residue.

For estimation of reducing sugars and total sugars, 0.4 ml (a and b respectively) and 0.1 ml (c) filtrates were taken in a set of separate test tubes respectively for the estimation of starch. Different concentrations of glucose (0.1 mg ml^{-1}) were taken in separate test tubes. In each test tube requisite amount of distilled water was added to make final volume 1 ml. In case of blank 1 ml distilled water was taken instead of filtrate or standard glucose. To this 1 ml Somogyi's alkaline copper tartarate reagent (4g $CuSO_4$, $5H_2O$, 24 g anhydrous Na_2CO_3, 16 g Na-K-tartarate and 180g anhydrous Na_2SO_4 dissolved in 1 liter distilled water) was added and then the tubes were kept in boiling water bath for 10 minutes. After cooling to room temperature, 1 ml Nelson's Arsenomolybdate reagent (25g Ammonium molybdate dissolved in 450

ml distilled water, 3 g sodium arsenate dissolved in 25 ml distilled water, 21 ml concentrated HCl. These ingredients were mixed well and digested for 48 hours at 37°C) and carefully added. The reaction mixtures were further diluted to 10 ml with distilled water. The absorbance readings were recorded on a UV-VIS double beam spectrophotometer (Schimadzu UV-190) at 660 nm. The amount of reducing sugars, total sugars and starch were estimated with the help of calibration curve of standard glucose (0.1 mg ml^{-1}) and the values were expressed as g $100g^{-1}$ dry tissue.

5) Inorganic constituents

a. Preparation of acid digestion

Acid digestion method of Toth *et al.,* (1948) has been employed for the analysis of inorganic constituents. Plant material was carefully washed in water and blotted to dryness. The leaves from premature, mature, onset of senescence and senescent stage of *Bougainvillea spectabilis* were separated and subjected to drying at 60^0C for 10 days till dried plant material had constant weight. The oven dried plant material was powdered. Five hundred mg of oven dried powdered leaf material was transferred to 150 ml capacity beaker to which 20 ml concentrated HNO_3 was added. The beakers were covered with watch glass and kept till the primary reactions were completed. Then these beakers were heated slowly to dissolve solid particles. After cooling to room temperature,

10ml of perchloric acid (60%) were added to it and mixed thoroughly. Then these beakers were heated strongly until a clear and colourless solution (about 2-3 ml) was obtained. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day these extracts were filtered through dry Whatmann No.44 (Ash less) filter paper. Filtrates so obtained were used for estimation of different inorganic constituents.

i) Sodium, Potassium, Calcium, Magnesium and Iron

Sodium, Potassium, Calcium, Magnesium and Iron were estimated using Atomic Absorption Spectrophotometer (AAS). For standardization of various concentrations of sodium (20-100 ppm) and potassium (20-100 ppm) and calcium (20-100 ppm) from NaCl, KCl, $CaCl_2$, $MgCl_2$and Fe_2O_3 respectively were prepare using standard solutions, standard curve for these elements were prepared. The acid digests were analyzed in the similar manner. Wherever necessary the appropriate dilutions were applied for the analysis.

ii) Phosphorus

The method of Sekine *et al.,* (1965) was employed for estimation of Phosphorus. Phosphorus reacts with 'molybdate vanadate' reagent to give yellow coloured complex. By estimating calorimetrically the intensity of colour developed and by comparing it with the colour intensity of known standards, Phosphorus content was estimated.

4 ml of acid digest were taken in test tube and to two ml of 2 N HNO_3 and 1 ml of 'molybdate vanadate' reagent (A-25 g ammonium molybdate in 500 ml of distilled water, B- 1.25 g ammonium vanadate in 500 ml 1 N HNO_3, A and B were mixed at the time of experiment) were added. Then final volume of each test tube was adjusted to 10 ml with distilled water. After 20 minutes, color intensity was measured at 420 nm using a reaction blank without acid digest or standard phosphorus.

Calibration curve of standard phosphorus was prepared from standard phosphorus solution containing mg per ml (0.110 g KH_2PO_4 per liter = 0.025 mg P^{5+} ml $^{-1}$) with the help of standard curve the amount of phosphorus in the plant material was calculated and it was expressed on dry weight basis.

6) Enzyme studies

a. Enzyme Nitrate reductase: (EC 1.6.6.1)

Activity of enzyme nitrate reductase was determined following the *in vivo* method described by Jaworski (1971).

A weighed amount of the leaf discs from premature, mature, onset of senescence and senescent stage from each treatment and control of *Bougainvillea spectabilis* were separately incubated in the medium containing 1ml 0.1M KNO_3, 2 ml 5% n-propanol, 5 ml 0.2 M phosphate buffer (pH 7.5) and 2 ml 0.5%Triton X-100 for 1 hour in dark under anaerobic conditions. After 1h, 1ml of reaction mixture was taken out and mixed with 1ml 1% sulfanilamide (1% in 2N HCL) and 1 ml 0.02% NEEDA [N-1-(naphthyl)-Ethylenediamine dihydrochloride], while mixture of 1ml incubation medium, without plant tissue 1ml of sulfanilamide and 1ml NEEDA served as a blank. The absorbance was read at 540 nm on UV-VIS double beam spectrophotometer (Shimadzu UV-190). The standard curve was prepared with the help of different

concentrations of KNO_2 and enzyme activity is expressed as µg of NO_2 liberated h^{-1} g^{-1} of fresh tissue.

b. Enzyme Peroxidase (EC 1.11.1.7)

To study peroxidase activity the method of Maehly (1951) was followed. Randomly sampled 0.5g of fresh leaves from premature, mature, onset of senescence and senescent stage of *Bougainvillea spectabilis* were homogenized in 15 ml ice-cold (1/15 M) phosphate buffer (pH-6.8) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 5 ml of 1/15 M Acetate buffer (pH-5), 0.5 ml of 0.1% guaiacol, 1 ml enzyme extract , 2 ml distilled water, 0.5 ml 0.08% H_2O_2. The reaction mixture was incubated at 30°C. After 15 minutes of incubation, 1 ml 1 N H_2SO_4 was added to stop the reaction, followed by measurement of absorbance at 470 nm. The soluble proteins in the enzyme extract were determined according to the method of Lowry *et al.,* (1951).The enzyme activity was expressed as unit h^{-1} mg^{-1} protein.

c. Enzyme Polyphenol oxidase (EC 1.10.3.2)

Activity of enzyme polyphenol oxidase from leaf tissue of premature, mature, onset of senescence and senescent stage of *Bougainvillea spectabilis* was studied according to the method of Mahadevan and Sridhar (1982). Five hundred milligram of plant material of leaf tissue (from each treatment and control) was homogenized in 15 ml cold 0.1 M phosphate buffer (pH-6.8). The homogenate was filtered through 4 layered muslin cloth. The filtrate so obtained was centrifuged at 10,000 rpm on cooling centrifuge for 20 minutes. The supernatant served as enzyme source. The assay mixture contained 4 ml 0.1 M phosphate buffer (pH-6.1), 1 ml 0.01 M catechol prepared in 0.1 M phosphate buffer (pH-6.1) and 0.5 ml enzyme and it was mixed well. The increase in absorbance at 30 seconds interval up to 180 seconds at 495 nm was recorded. The soluble proteins in the enzyme extract were determined according to the method of Lowry *et al.,* (1951). The enzyme activity was expressed as ΔOD min^{-1} mg^{-1} protein.

d. Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase was determined following the method described by Giannopolitis and Ries (1977), with slight modifications.

Enzyme was extracted by homogenizing 0.5 g fresh leaf tissue from premature, mature, onset of senescence and senescent stage of *Bougainvillea spectabilis* in 10 ml, 150 mM cold potassium phosphate buffer (pH-7.8) containing 1% PVP, to protect enzyme from the action of polyphenols. Then it was filtered through 4- layered muslin cloth and the filtrate so obtained was centrifuged at 10,000 x g for 20 min at 0 - 4°C. The supernatant was used as an enzyme source.

An enzyme assay mixture contained 2 ml potassium phosphate buffer pH-7.8, 0.2 ml methionine (13 mM), 0.1 ml Nitroblue tetrazolium (75μM), 0.5 ml EDTA (0.1 mM), 0.1 ml enzyme and 0.1 ml riboflavin (2M) was added lastly and immediately the absorbance was measured at 560 nm on UV-VIS double beam spectrophotometer (Shimadzu UV-190). Then the assay mixture was exposed to full sunlight for 30 min and again the absorbance was read at 560 nm. The enzyme activity is expressed as Δ OD $h^{-1}mg^{-1}$ of protein.

Soluble Protein

The soluble proteins in the enzyme extract were determined following the method described by Lowry *et al.,* (1951). In test tube, 0.1 ml enzyme extract was taken and diluted to 1 ml with distilled water. To

this 5 ml of 'Reagent – C' solution (50 ml of 'A' containing 2% sodium carbonate in 0.1 N aqueous NaOH was mixed with 1 ml of 'B' containing 0.5% copper sulfate in 1%, Na-K tartarate), was added, mixed well and allowed to stand for 15 minutes at room temperature. After 15 minutes 0.5 ml Folin-Ciocalteau's phenol reagent was added with immediate mixing. This was allowed to stand for 30 minutes in dark and intensity of developed blue colour was measured at 660 nm on UV-VIS double beam spectrophotometer (Shimadzu UV-190).