CHAPTER II

MATERIAL AND METHODS
Study on the betacyanin biosynthesis was carried out with the seedlings of Amaranthus caudatus and Amaranthus polygamus and the seedlings of Raphanus sativus (var: Crimson French Breakfast) were used in the studies on anthocyanin biosynthesis. Seeds of Amaranthus caudatus and Raphanus sativus (var: Crimson French Breakfast) were obtained from Suttons and Son's Ltd., Calcutta, India. Seeds of Amaranthus polygamus were obtained from the Ahmedabad co-operative fruit and vegetable growers Association, Ahmedabad, India.

Seeds of average and uniform size were sprinkled over a moistened filter paper placed in 11 cm petridishes and incubated in dark at 25 ± 1°C. Amaranthus caudatus seeds required about 30 h for germination. Seeds of Amaranthus polygamus germinated within 24 h after soaking. Seeds of Radish also germinated in about 24 h. Germinated seeds were transferred to petridishes with filter paper containing 5 or 6 ml of distilled water or test solution and exposed to light or incubated in dark for the desired period. All manipulations were carried out in dark under a green safety lamp. Cotyledons, hypocotyls and roots were isolated in dark under a green
safety lamp immediately before or after the light exposure. Unless otherwise stated the intact seedlings and the isolated parts were subjected to light treatment of intensity 500 Lux using fluorescent tubes. The intensity was measured using Lange's Lux meter.

Red, far-red lights:

Red light was obtained by using the red plastic filter (640-660 nm) of the Imperial laboratories, England. Two tungsten filament bulbs of 100 watts each served as the light source. The two bulbs were fitted inside a lamp fixed to a table with a platform 2 ft below the lamp. Filters of size 6" x 12" were inserted at the base of the lamp through a shutter. Far-red light was obtained by combining the blue and red filters.

Preparation of solutions:

Cytokinins: 6-furfurylaminopurine of Fluka, Switzerland was used in the present investigation, 6-r-r-dimethyl allylaminopurine and Zeatin (mixed isomers, 6-4-dihydroxy-3-methylbut-2-enylaminopurine) were from Sigma chemicals, USA and 6-Benzylaminopurine was from Koch Light Laboratories, England. Required amount of cytokinins were dissolved directly in hot distilled water to make the solution. Stock solution of 20 μg/ml was prepared for all the cytokinins.
Gibberellic acid:

Gibberellic acid (GA3) of 90% purity from Sigma chemicals, USA has been used. Solutions were made by dissolving the substance in hot distilled water. Stock solutions were either 100 μg/ml or 10 μg/ml strength.

Indoleacetic acid:

Indoleacetic acid used was from E. Merck, Germany and was of 99% purity. A stock solution of 100 μg/ml was prepared by first dissolving the required amount of the substance in few drops of absolute alcohol and later making up the volume with distilled water.

Abscisic acid:

A stock solution of 20 μg/ml abscisic acid prepared by dissolving the substance in hot distilled water was used.

L-Tyrosine and L-Dihydroxyphenylalanine:

L-tyrosine of Nutritional Biochemical Corporation, Ohio and L-Dihydroxyphenylalanine of Sigma Chemicals have been used. Stock solutions of both the precursors were made to $10^{-3} M$ or $10^{-3} \times 2 M$ concentrations. Required amount of precursor was dissolved in hot distilled water to make the solution. L-Dihydroxyphenylalanine underwent auto-oxidation when exposed to
light and a black paper was wrapped around the flask immediately after making the solution to prevent auto-oxidation.

L-Phenylalanine and Trans-cinnamic acid:

Stock solutions of either $10^{-3} \times 2\text{M}$ or $10^{-3} \text{M}$ of L-phenylalanine and trans-cinnamic acid were prepared by dissolving the required amount of the substance in hot distilled water. The same precaution used for L-dihydroxyphenylalanine was also used for trans-cinnamic acid solution to prevent auto-oxidation. L-phenylalanine used was from Nutritional Biochemical Corporation, Ohio and trans-cinnamic acid was from Sigma Chemicals, USA.

Sucrose and Sodium acetate:

Sucrose and sodium acetate of analar grade from BDH Laboratories, India have been used. Stock solutions of $10^{-2} \times 2\text{M}$ or $10^{-2} \text{M}$ were made by dissolving the substance in required amount in distilled water. Sodium acetate solution used was buffered with the same concentration of glacial acetic acid prepared by direct dilution with distilled water and pH adjusted to 6.3.

All the stock solutions were stored in refrigerator. The lower concentrations were prepared
by further dilutions of the stock solutions using distilled water.

Hypocotyl length was measured by placing the seedlings directly over the mm graph paper and reading to the nearest 0.5 mm. Growth readings were taken for 20 or 30 seedlings per set. Each experiment was carried out in triplicates and repeated thrice. The mean calculated and given in the table along with the standard deviation is the mean of three sets in triplicates.

Estimation of anthocyanin and betacyanin:

Anthocyanin content was estimated following the method of Thimann and Edmondson (1949). Extraction was carried out in cold with 0.3N hydrochloric acid. 10-20 seedlings were used at a time and the pigment was extracted in 10 ml of cold 0.3N hydrochloric acid at 5°C. No maceration was required for the tender seedlings. The time taken for complete extraction was 30 to 36 h. Extracted pigment was measured as optical density at 540 nm in spectronic-20 (Bausch and Lomb).

Betacyanin was estimated in the intact seedlings or the isolated parts after the method of Malaviya and Laloraya (1966) - based on the method of Thimann and Edmondson (1949) for anthocyanin extraction. 25 seedlings or isolated parts were taken in 5 ml of 0.3 N
hydrochloric acid at a time. Extraction time was shorter than it took for anthocyanin extraction and the pigment could be completely extracted in 24 h in cold. Betacyanin extracted was measured in Klett Summerson colorimeter equipped with a green filter 540 nm.

Both anthocyanin and betacyanin were estimated as units/seedling or units/isolated organ using the following formula:

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\text{Anthocyanin or betacyanin unit/Seedling or isolated organ} = \frac{\text{Klett reading or OD} \times \text{ml of Extract}}{\text{No. of seedlings or isolated organs}}.
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Estimations were undertaken in triplicates and repeated thrice. The mean of the three sets in triplicates along with standard deviation is given in the tables.