MATERIALS AND METHODS

1. PROCUREMENT OF SEEDS AND MALEIC HYDRAZIDE

Seeds of the onion varieties N-53 and N-2-4-1 were purchased from Shatkari Beej Bhandar, Shahupuri, Kolhapur. Maleic hydrazide was obtained from s.d. fine-Chem Ltd., Bombay.

2. CHEMICAL TREATMENT

Healthy and uniform seeds of onion varieties viz. N-53 and N-2-4-1 were treated with 100, 500 and 1000 ppm MH in clean, dry petriplates for different durations as 1, 3, 6 and 12 hours. After the specific exposure period seeds were thoroughly washed with distilled water and used for further analysis.

3. METHODS

A. Cytological Studies

Cytological analysis in the root meristems and flower buds of *Allium cepa* was carried out to study the effect of Maleic hydrazide.

(i) *Mitotic Studies*

Cytotoxic effects of MH on somatic chromosomes of *Allium cepa* were studied by exposing seeds to various concentrations of MH for different durations. Five ml of solution of respective concentration of maleic hydrazide was used for treating 25 seeds in petriplates. After the chemical treatment seeds were washed for 24 h with distilled water for recovery and then allowed to germinate on moist

filter paper. Simultaneously untreated seeds were also germinated and used as control.

After the emergence of radicles the root tips were fixed in para dichlorobenzene (PDB) for 3 hrs. and then transferred in Carnoy's fluid (glacial aacetic acid and absolute alcohol in 1:3 proportion) for 24 hours. The fixed root tips were stored in 70 % alcohol and used for mitotic studies.

The pretreated root tips were washed thoroughly with distilled water and hydrolysed in 1 N HCl by gentle heating over a spirit lamp. Then squash of tip portion was prepared in 2 % acetocarmine and mounted in a drop of 45 % acetic acid. The squash preparation were observed under light microscope and data on mitotic index and chromosomal aberrations such as clumping/stikiness, gaps, rings, micronucleates, polyploidy, bridges and laggards were recorded at respective stages of mitosis. Percent mitotic index and aberrations were calculated by the following formulae

$$\text{Mitotic Index (\%)} = \frac{\text{Total No. of mitotic (dividing) cells in view}}{\text{Total No. of cells in that view}} \times 100$$

$$\text{Chromosomal Aberration (\%)} = \frac{\text{Number of mitotic cells showing abnormality}}{\text{Total No. of mitotic cells in that view}} \times 100$$

Ten counts of total mitotic cells and total abnormal cells were recorded for each treatment and each duration. Mean of these values was used to calculate the percentage.

(ii) ***Meiotic Studies***

The seeds of both the onion varieties were treated with MH using different concentrations and durations as described earlier. These treated seeds were washed with distilled water and sown in different field plots. The plants were allowed to grow untill mature and then bulbs were collected. These bulbs were stored for 30 days. Five bulb from each treatment were sown again in the field (removing upper 1/3 portion) to raise the plants.

After the emergence of flower head, young flower buds were fixed in freshly prepared Carnoy's fluid. Then they were transferred to 70 % alcohol and stored in a refrigerator at $5^{o}C$.

Slides were prepared by anther smear technique for the purpose of meiotic studies. Anthers from young flower buds of respective treatment were removed and hydrolysed for few seconds in 1 N HCl, washed, stained with 2 % acetocarmine and mounted in a drop of 45 % acetic acid.

Various chromosomal aberrations in pollen mother cells were recorded under a light microscope. The chromosomal abnormality was expressed as percentage of

specific abnormality out of total number of pollen mother cells. Chromosome abnormalities such as clumping/stickiness, bridges, laggards, rings, non-synchronous division, abnormal type of sporads were counted.

Mature anthers from flowers of onion were used to study pollen fertility. Pollen grains were stained by 2 % acetocarmine and mounted in a drop of 45 % acetic acid. The pollen sterility and fertility was expressed as percentage of sterile and fertile pollen grains out of the total number of pollen grains counted.

B. Physiology of Growth

The influence of maleic hydrazide on germination and the growth of the plants was examined in the two onion varieties

(i) *Seed Germination*

Seed treatment was carried out as described earlier. For each treatment 25 seeds were immersed in 5 ml of respective solution of MH. The treated seeds were washed thoroughly in distilled water and kept for germination in petriplates lined with filter papers. Sufficient amount of distilled water was added in each petriplate. All the petriplates were kept at room temperature in a dark room. After the emergence of radicles germination percentage was determined on the IIIrd, Vth and VIIth day.

(ii) *Growth Performance*

MH treated seeds of the two onion varieties were sown in field plots to raise the plants. Untreated seeds were also grown along with the treated ones. Various growth parameters like the number of roots, number of leaves, height of the plants, fresh weight, dry weight etc. were recorded after every month upto the maturity.

(iii) *Initiation of Flowering*

Bulbs harvested from the mature plants were used to raise the plants to observe floral initiation.

C. Biochemical Analysis of Onion Leaves

A few organic and inorganic constituents in the leaves of *Allium cepa* growing under the influence of MH were analysed after I^{st} and the III^{rd} month.

(i) ***Photosynthetic Pigments***

(a) *Total Chlorophylls*

Chlorophylls were estimated following the method by Arnon (1949). Fresh plant material (0.5 g) was crushed in 80 % acetone containing 4 ml ammonia per liter. A pinch of $MgCO_3$ was added to enhance the extraction. The extract was filtered through Buchner's funnel using Whatman No. 1 filter paper. The volume of filtrate was adjusted to 50 ml with 80% acetone and transferred to a conical flask wrapped with a black paper to prevent photooxidation of the pigments. The

absorbance was measured at 663 nm and 645 nm on Shimadzu spectrophotometer.

Chlorophylls (mg/100 g of fresh tissue) were calculated using formulae -

$$\text{Chl.a, } X = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{Chl.b, } Y = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{Chl.(a+b), } Z = 8.02 \times A_{663} + 20.2 \times A_{645}$$

$$\text{Chl.a/Chl.b/ Chl.(a+b) (mg)} = \frac{\text{X or Y or Z X Volume of extract}}{\text{1000 X weight of plant material (g)}}$$

(b) *Carotenoids*

The same acetone extract was used to determine carotenoid content in the onion leaves. The method is given by Kirk and Allen (1965). Absorbnance was red at 480 nm. The following formula was used to calculate the amount.

$$\text{Carotenoids (mg/100g)} = \frac{A_{480} \times \text{Volume of extract} \times 10 \times 100}{2500 \times \text{Weight of plant material (g)}}$$

Where, 2500 = Average extinction.

(ii) *Total Polyphenols*

Polyphenols were estimated following the method suggested by Folin and Denis (1915). Fresh plant material (0.5 g) was crushed in 80% acetone and filtered through Buchner's funnel using Whatman filter paper. The residue on the filter paper was washed several times with 80% acetone and final volume of the extract was adjusted to 50 ml.

Two ml extract was treated with 10 ml of 20 % Na_2CO_3 in Nessler's tube. The volume was adjusted to 35 ml with distilled water followed by 2 ml of Folin-Dennis reagent. Final volume of reaction was adjusted to 50 ml with distilled water. Absorbance was measured after half an hour at 660 nm using a reaction blank. A standered curve of polyphenols, obtained by using standered tannic acid (0.1 mg/ml) was used to calculate the amount of polyphenols.

(iii) *Carbohydrates*

Total sugars and starch were estimated using the method of Nelson (1944). Extract was prepared by grinding 1g fresh leaves in 80% alcohol. The extract was filtered through Buchner's funnel using Whatman No. 1 filterpaper and condensed on a water bath to about 2-3 ml. A mixture (about 2g) of lead acetate and potassium oxalate (1:1) was added with constant stirring. The contents were mixed with sufficient amount of water (20 ml) and filtered through Buchner's funnel using Whatman No. 1 filterpaper. The volume of the filtrate was adjusted to 25 ml with distilled water.

A known amount of filtrate was transferred to a conical flask containing 2 ml conc. HCl. The flask was closed with a cotton plug and autoclaved for 30 minutes under 15 lbs pressure. After cooling to room temperarture the contents were neutralized with unhydrous Na_2CO_3 and

filtered again. The volume of the filtrate was recorded. This filtrate was used for the estimation of total sugars.

The residue on the filter paper during alcoholic extraction was transferred along with the filter paper to a conical flask containing 5 ml conc. HCl and 15 ml distilled water. It was hydrolysed at 15 lbs pressure for half an hour and then cooled to room temperature. The contents were neutralized with unhydrous Na_2CO_3 and filtered. The volume of the filtrate was measured. This filtrate was used for the estimation of starch.

Estimation of sugars was carried out colorimetrically using Arsenomolybdate reagent to determine cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent. The sample (0.1 ml) was heated with 1 ml alkaline copper tartarate on a boiling water bath for 10 minutes. One ml of Arsenomolybdate reagent was added to each tube after cooling. The volume was adjusted to 10 ml with distilled water. Absorbance was read after 10 min. at 560 nm on Shimadzu spectrophotometer. Simultaneously a set of standard glucose (0.1 mg/ml) was prepared in the same manner along with a blank and used to obtain the standard curve. The amount of total sugars and starch was calcultaed using the calibration curve.

(iv) *Total Soluble Proteins*

Extraction - Fresh plant material (1 g) was homogenized in 0.14 M cold saline (NaCl) solution. The

extract was filtered and centrifuged for 15 minutes. The supernatant was used as a source of proteins.

Estimation - For estimation of proteins Biuret method described by Gornall *et al.* (1949) was used. When proteins are treated with an alkaline solution of copper sulphate the peptide linkages are broken down giving a characteristic violet colour to the solution. This reaction is termed as 'Biuret' reaction and was first demonstrated on Biuret which is the product of pyrogenic decomposition of urea. One ml plant extract was mixed with eight ml Biuret reagent and it was incubated at 37^{o}C for 30 minutes. The absorbance of violet colour developed, was measured at 540 nm. Simultaneously a set of reaction mixtures containing different concentrations of standard Casein (20 mg/ml) was prepared to obtain a standard curve of proteins. This curve was used to determine the amount of proteins.

(v) *Ribonucleic Acid Content*

RNA content was determined following the method of Schneider (1957). The furfural derivative formed by ribose, released during RNA hydrolysis, reacts with orcinol in presence of ferric chloride to produce a green colour which can be measured spectrophotometrically at 665 nm.

Extraction - Fresh plant material (1 g) was extracted in 10 % cold TCA and then centrifuged for 10 minutes at 1000 g. The pellet was washed thrice with cold

TCA and then extracted again with 5 % perchloric acid at 85°C for about half an hour on a water bath. After cooling to R.T. it was centrifuged at 5000 g for 15 minutes. The supernatant used as a source of RNA.

Estimation - One ml plant extract was mixed with 4 ml perchloric acid and 3 ml orcinol reagent (Freshly prepared) and boiled on a water bath for an hour. The absorbance was measured at 665 nm after cooling the reaction mixture. Standard RNA solution (1 mg/ml) was used to obtain a calibration curve for calculating the amount of RNA.

(vi) ***Ascorbic Acid***

A titrimetric method given by Sadasivam and Manickam (1992) was used to determine the ascorbic acid content. Ascorbic acid is extracted in metaphosphoric or oxalic acid to lower down the pH and to stabilize its content by preventing catalytic oxidation. Fresh plant material (0.5 g) was extracted in 4 % oxalic acid. The extract was centrifuged for 15 minutes and the supernatant was used as the source of ascorbic acid.

Estimation - Ascorbic acid is oxidised to dehydroxyascorbic acid by reducing 2,6 -dichlorophenolindo-phenol (a blue dye) to a pink colourd solution. Oxalic acid is used as a titrant. The capacity of plant extract to reduce the dye (V_2 ml) is directly proportional to ascorbic acid content. Standard ascorbic acid (100 µg/ml) is

titrated against the dye till the appearance of persistent pink colour. The amount of the dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid taken for titration. The amount of ascorbic acid is calculated as follows :

$$\text{Ascorbic acid (mg/100g)} = \frac{0.5 \text{ mg}}{V_1 \text{ ml}} \times \frac{V_2 \text{ ml}}{5} \times \frac{100}{\text{Wt of the sample}} \times 100$$

(vii) ***Elemental Analysis***

(a) *Nitrogen*

Nitrogen was estimated following the method of Hawk et al. (1948). Oven dried plant material (0.5g) was transferred to Kjeldahl flask containing 10 ml of 1:1 H_2SO_4, a pinch of microsalt and a few glass bids (to avoid bumping). The flask was heated on a low flame till a colourless solution was obtained. After cooling the contents were diluted with distilled water to adjust 100 ml volume. It was then filtered through Whatman No.1 filter paper and used for nitrogen estimation.

The filtrate (2 ml) was taken in a Nessler's tube along with a drop of 8% $KHSO_4$ and the volume was made to 35 ml with distilled water. Then freshly prepared Nessler's reagent (15 ml) was added and mixed thoroughly. The absorbance was recorded at 520 nm after 10-15 minutes. A blank solution containing all the ingradients except nitrogen source was used to adjust zero absorbance. Standard

curve was obtained by using different concentrations of ammonium sulphate (0.05 mg/ml) in place of the filtrate.

(b) *Preparation of extract for other elements*

Acid digest was prepared using the method of Toth et al. (1948) oven dried plant material (0.5 g) was treated with 20 ml concentrated HNO_3 in a beaker covered with a watch glass. When the initial reaction was subsided the beaker was heated slowly on a hot plate to dissolve the plant material. After cooling to R.T., 10 ml of perchloric acid were added into the beaker and it was heated again to obtain a clear solution, reduced to 2 to 3 ml. It was then cooled to R.T., diluted to 100 ml with distilled water and kept over- night. Next day it was filtered through Whatman No.1 filter paper. This acid digest was used to estimate inorganic elements such as Phosphorus, Potassium, Calcium, Magnesium, Copper and Zinc.

(c) *Phosphorus*

Phosphorus was estimated following the method suggested by Sekine (1965). Acid digest (2 ml) was reacted in a test tube with 2N HNO_3 (2 ml) followed by Molybdate Vanadate reagent (1 ml). The mixture was shaken properly after making a volume of 10 ml with distilled water. It was then allowed to stand for 20 minutes and absorbance was read at 420 nm against a blank. Phosphorus content was calculated by using a standard curve of phosphorus.

(d) *Potassium, Calcium, Magnesium, Copper and Zinc*

These elements were analysed using the acid digest on atomic absorption spectrophotometer. (Perkin-Elmer 3030 model). The readings were available in ppm concentrations which were further converted into grams per 100 g of dry weight.

D. Biochemical Analysis of Bulbs

(i) *Organic and Inorganic Constituents*

A few organic and inorganic constituents from onion bulbs were determined after a growth period of four months. Outer scales (1-2) were used for this analysis. Carbohydrates, Ascorbic acid and different elements were determined following various methods described earlier in the biochemical analysis of leaves.

(ii) *Anthocyanins*

Anthocyanins were estimated by the method described by Mancinelli *et al.* (1975). Fresh plant material (0.5g) was extracted in methanol (20 ml) containing 1% HCl. The extract was filtered and then centrifuged for 5 minutes at 1000 g. Supernatant was transferred to another centrifuge tube and it was again centrifuged for 10 to 15 minutes at full speed. The clear supernatant obtained during the second centrifugation was used to measure the absorbance at 530 and 657 nm against a solvent blank. The amount of

anthocyanins was expressed in terms of absorbance units calculated as follows :

Anthocyanins (absorbance units) = $A_{530} - (0.33 \times A_{657})$

4. PREPARATION OF STAIN AND REAGENTS

(A) Acetocarmine : (Mitotic and meiotic studies)

2 g acetocarmine powder was dissolved in 100 ml of 45 % glacial acetic acid. It was boiled for 10 to 15 minutes on hot plate and filtered through Whatman No. 1 filter paper.

(B) Folin-Denis Reagent : (Total polyphenols)

100 g sodium tungstate and 20 g phosphomolybdic acid were dissolved in 200 ml distilled water. 50 ml 25 % phosphoric acid were added and it was refluxed for 2-3 hours using water condenser. The volume was adjusted to one litre with distilled water.

(C) Somogyi's Alkaline Copper Tartarate Reagent : (Carbohydrates)

4 g $CuSO_4$, $5H_2O$; 24 g unhydrous Na_2CO_3; 16 g Na-K tartarate and 180 g unhydrous Na_2SO_4 were dissolved in one litre distilled water.

(D) Arsenomolybdate Reagent : (Carbohydrates)

25 g of ammonium molybdate were dissolved in 450 ml distilled water to which 21 ml concentrated H_2SO_4 were

added 3 g of sodium arsenate (Na_2HAsO_4, $7H_2O$) were dissolved in 25 ml distilled water. The solutions were mixed well and kept in an incubator at 37 ^oC for 48 hours before use.

(E) Biuret Reagent : (Soluble proteins)

0.5 g $CuSO_4$, $5H_2O$ and 6 g Na-K tartarate were dissolved in 500 ml of distilled water. 300 ml of 10 % NaOH were added to this solution with swirling. The volume was adjusted to 1000 ml with distilled water.

(F) Orcinol Reagent : (Ribonucleic acid content)

This reagent consisted of 3 solution a, b and c.

a) 1% orcinol in distilled water - 1 gm orcinol powder was dissolved in 100 ml distilled water.

b) Concentrated HCl.

c) Ferric Chloride - 10 gm $FeCl_3$, $6H_2O$ were dissolved in 100 ml distilled water

10 ml a + 40 ml b +1 ml c were mixed just before use.

(G) DCPIP Solution : (Ascorbic acid)

45 mg of sodium bicarbonate were dissolved into a small volume of distilled water. 52 mg 2,6,-dichlorphenolindophenol were dissolved in it and the volume was adjusted to 200 ml with distilled water.

(H) Nessler's Reagent : (Total Nitrogen)

Nessler's A : 7 g of KI and 10 g of HgI_2 were dissolved in 40 ml distilled water.

Nessler's B : 10 g of NaOH were dissolved in 50 ml of distilled water.

The reagent was prepared fresh by mixing A and B (4:5) at the time of estimation.

(I) Molybdate Vanadate Reagent : (Phosphorus)

Solution A : 1.25 g of ammonium vanadate was dissolved in 500 ml of 1N HNO_3.

Solution B : 25 g of ammonium molybdate were dissolved in 500 ml of distilled water.

The reagent was prepared fresh by mixing A and B in equal proportions at the time of estimation.