MATERIALS AND METHODS

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Chemicals : Putrescine, spermidine, spermine, cyclo-heximide, creatine and guanidino acetic acid were obtained from Sigma Chemical Co., U.S.A. Dodine (Melprex Tech.) was a gift from Cyanamid of Great Britain, London.

Potassium nitrate, potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, potassium iodide and glacial acetic acid were obtained from S.M. Chemical Co., Baroda. Sodium nitrate, hydroxylamine hydrochloride, manganese chloride and ethylenediamine-tetraacetic acid were obtained from BDH Laboratory, Bombay. N-1-napthylethylene diamine, adenosine-5-triphosphate, sodium dithionite, glutamine, casein, cysteine and sodium tungstate were obtained from Loba Chemie, Bombay.

Zinc acetate was obtained from Pfizer (India) Ltd., Bombay, sulfanilamide from IDPL, Hyderabad and methyl viologen from Koch Light Laboratory, England. All reagents used were of analytical grade.

Plant Material : Radish seeds (Raphanus sativus var. Suttons, Scarlet Globe) obtained from local dealer were germinated at 24° in continuous light or dark in Petri-dishes over two layers of filter paper moistened with the desired test compounds. The time when seeds were kept in

Petridishes was considered as 0 hr. The germination of seeds varied from 90-95%. At specified periods, cotyledons and embryonal axes were excised. Fresh weight of ten axes was recorded in duplicate for growth and the cotyledons were used for all the enzyme assays. All the data reported in the thesis are a mean of two independent sets of experiments. In each experiment 40-50 cotyledon pairs were pooled together for preparation of homogenate and each assay was carried out in duplicate.

Preparation of homogenates : A 10% (w/v) homogenate was used in all the enzyme assays. For nitrate and nitrite reductases, the homogenate was prepared in 10 mM PO_4 buffer, pH 7.5, containing 5 mM cysteine and 10 mM EDTA. For glutamine synthetase and alanine aminotransferase, 0.9% KCl was used. For protease, the homogenate was prepared in 10 mM acetate buffer, pH 5.5, containing 2 mM cysteine and for glutamate dehydrogenase the homogenate was prepared in 10 mM Tris-HCl buffer, pH 8, containing 2 mM cysteine. For glutamate synthase, the homogenate was prepared in 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM mercaptoethanol and 1 mM EDTA. The homogenate for glutamate dehydrogenase glutamate synthase, cytochrome c reductase and methyl viologen nitrate reductase was centrifuged at 8,000 x g for 15 min and the supernatant was used for assay. The above mentioned grinding media were found to show optimum activity in preliminary screening tests.

Enzyme assays : All the assays were carried out at 37°. One unit of enzyme is defined as the amount of enzyme required to utilize 1 μ mole of substrate/coenzyme h^{-1} or produce 1 μ mole of product hr^{-1}. The assay conditions described below for all the enzymes were found to give optimum activity in preliminary trials.

Nitrate reductase (NR) (E.C.1.6.6.1) was assayed according to the method of Hageman and Hucklesby (1971). The assay system consisted of PO_4 buffer, pH 7.5, 100 μmoles; KNO_3, 10 μmoles; NADH, 0.1 μmole and 0.2 ml of homogenate in a total volume of 4 ml. The reaction was terminated after 30 min by the addition of 0.5 ml of 1 M zinc acetate. In the control tubes, substrate was added after the termination of the reaction. Nitrite formed was estimated as described by Evans and Nason (1953). To 2 ml of aliquot were added 0.5 ml of sulfanilamide (1% w/v in 2 N HCl) and 0.5 ml of N-1-napthylethylene diamine (0.01% in 0.3 N HCl). The volume was made up to 4 ml with water. The tubes were allowed to stand for 15 min for colour development. The absorbance at 540 nm was read against a blank containing all reagents except nitrite.

Methyl viologen-NR activity was assayed using methyl viologen, 0.1 ml (25 mg/10 ml) reduced with sodium dithionite, 0.1 ml (17 mg/ml) as electron donor instead

of NADH. The reaction was terminated after 30 min and nitrite was estimated as described above.

Cytochrome c reductase activity was assayed by the method of Wray and Filner (1970). The assay system consisted of PO_4 buffer, pH 7.5, 100 µmoles; cytochrome c, 0.05 ml (2% w/v aqueous solution), NADH, 0.1 µmole and 0.2 ml of enzyme in a total volume of 3 ml. The reduction of cytochrome c was measured by following the rate of increase in absorbance at 550 nm for 3 min. NADH was omitted from the control tubes.

Nitrite reductase (NiR) (E.C.1.6.6.4) was assayed according to the method of Joy and Hageman (1966). The assay system consisted of PO_4 buffer, pH 7.5, 100 µmoles; $NaNO_2$, 1 µmole; methyl viologen, 0.1 ml (25 mg/10 ml); sodium dithionite, 0.1 ml (17 mg/ml) and 0.2 ml of enzyme in a total volume of 4 ml. The reaction was started by the addition of dithionite. In the control tubes, enzyme was added after the termination of the reaction. The reaction was terminated after 30 min by vigorous shaking of the tubes till the blue colour disappeared, followed by the addition of 0.5 ml of 1 M zinc acetate. The left over nitrite was estimated as mentioned above.

Glutamine synthetase (GS) (E.C.6.3.1.2) was measured as transferase activity according to the method of Tursh (1964).

The assay system consisted of acetate buffer, pH 5.5, 100 μmoles; glutamine, 10 μmoles; hydroxylamine HCl, 20 μmoles; $MnCl_2$, 10 μmoles; Na_2AsO_4, 10 μmoles; ATP, 2.5 μmoles and 0.5 ml of enzyme in a total volume of 4 ml. The reaction was terminated after 1 hr by the addition of .1 ml of $FeCl_3$ reagent, which was prepared by mixing equal volumes of 10% $FeCl_3$ in 0.2 N HCl, 24% TCA solution and 50% HCl. In the control tubes, glutamine was added after the termination of the reaction. The γ-glutamyl-hydroxamate formed was measured at 540 nm.

Glutamate dehydrogenase (GDH) (E.C.1.4.1.2) was assayed according to the method of Bulen (1956). The assay system consisted of Tris-HCl buffer, pH 8, 250 μmoles; $(NH_4)_2SO_4$, 400 μmoles; 2-oxo-glutarate, 10 μmoles; NADH, 0.1 μmole and 0.1 ml of enzyme in a total volume of 3 ml. The reaction was started by the addition of NADH and the decrease in absorbance at 340 nm was measured for 3 min. $(NH_4)_2SO_4$ was omitted from the control tubes.

Glutamate synthase (GOGAT) (E.C.1.4.1.13) The assay for this enzyme was attempted according to the method of Beevers and Storey (1976). The assay system consisted of Tris-HCl buffer, pH 7.5, 250 μmoles; Glutamine, 10 μmoles; 2-oxo-glutarate, 20 μmoles; NADH, 0.1 μmole and 0.1 ml of enzyme. The reaction was started with the addition of

NADH and decrease in absorbance at 340 nm was measured. But no measurable activity could be obtained. Then a number of modifications were also tried but since none of the modifications could give any measurable activity, this enzyme was not studied further.

Alanine aminotransferase (AAT) (E.C.2.6.1.2) was assayed according to the method of Tonhazy et al. (1950). The assay system consisted of PO_4 buffer, pH 7.5, 100 µmoles; alanine, 10 µmoles; 2-oxo-glutarate, 10 µmoles; pyridoxal phosphate, 0.1 µmole and 0.5 ml of enzyme in a total volume of 2.5 ml. The reaction was terminated after 30 min by the addition of 0.5 ml of 10% TCA. In the control tubes, enzyme was added after the termination of the reaction. 1 ml of 2,4-dinitrophenyl hydrazine solution (0.1 g in a mixture of 20 ml concentrated HCl and 80 ml water) was added to each assay tube. After incubation at 37° for 5 min, 2 ml of toluene was added. After vigorous mixing the tubes were allowed to stand for 5 min and 0.5 ml aliquot of toluene layer was taken. To this was added 5 ml of alcoholic KOH (2.5 g in 100 ml of 95% ethanol) and colour was allowed to develop for 5 min. The absorbance was read at 540 nm.

Protease (E.C.3.4.4) was assayed according to Tappel (1968). The assay system consisted of acetate buffer, pH 5.5, 200

µmoles; casein, 50 mg and 0.5 ml of enzyme in a total volume of 4 ml. The reaction was terminated after 1 hr by the addition of 1 ml of 10% TCA. In the control tubes, TCA was added prior to the enzyme. The absorbance change at 280 nm was measured using tyrosine as the standard.

Estimation of nitrate : A 10% (w/v) homogenate was prepared in 0.9% KCl and the proteins were precipitated by the addition of 1 M zinc acetate. The tubes were centrifuged and the nitrate in the supernatant was estimated according to the method of Sloan and Sublett (1966) involving reduction of nitrate by metallic cadmium and the subsequent measurement of nitrite. Cadmium was prepared by placing zinc rods in 20% cadmium sulfate. The mossy growth of cadmium on the rods was scraped off after 4 hr, washed in distilled water and then with 2 N HCl. It was then rinsed with distilled water and stored under 0.4 M NH_3-NH_4Cl buffer, pH 9.6. To 0.2 ml of sample was added 2 ml of 0.4 M NH_3-NH_4Cl buffer, pH 9.6, 2 g of cadmium and the volume made upto 4 ml with 1 M $MgCl_2$. The tubes were stoppered and shaken manually for 3 min. The contents were then filtered and the nitrite formed was measured as follows : to 1 ml of filtrate were added 0.5 ml each of iodine reagent (0.36% I_2 in 0.95% KI solution), $NaAsO_2$ (0.8% w/v in water), sulfanilamide (1% w/v in 2 N HCl) and N-1-napthyle-

thylene diamine (0.01% in 0.3 N HCl). The tubes were allowed to stand for 15 min and the absorbance read at 540 nm.

In vitro induction of nitrate reductase : Seeds were grown in the absence of a nitrogen source in continuous light or dark condition for the specified periods. Cotyledons were excised and pretreated with test compounds for 30 min. NR was induced with 10 mM KNO_3 over a period of 3 hr unless otherwise mentioned. At the end of the induction period, cotyledons were washed with distilled water and homogenized. NR activity was assayed as described earlier.

Metabolic pool of nitrate : was assayed according to the method of Ferrari et al. (1973) using excised cotyledons from seeds grown in the absence of nitrate. NR was induced by nitrate (10 mM) in dark in the absence and presence of spermine (1 mM) over a period of 3 hr. The metabolic pool was assessed by incubating the cotyledons under anaerobic condition in darkness at 37° in 100 mM phosphate buffer for 1 hr. The nitrite released in the medium was estimated as mentioned earlier.

Subcellular fractionation : A 10% (w/v) homogenate was prepared by grinding the tissue in 0.9% KCl containing 0.5 M sucrose. The homogenate was centrifuged at 100 x g for 15 min. The supernatant was centrifuged at 1,600 x g

for 30 min to separate the chloroplasts. The resulting supernatant was then centrifuged at 10,000 x g for 15 min to separate the mitochondrial fraction. The chloroplast and mitochondrial fractions after washing were suspended in the grinding medium. GS and AAT activity were assayed in the chloroplast, mitochondrial and supernatant fractions.