MATERIALS AND METHODS

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Chemicals : Adenosine triphosphate, homologous series of diamines, gibberellic acid, guanidino acetic acid, guanidino butyric acid, kinetin, p-chloromercuri-benzoate, spermidine, spermine and triton x-100 were purchased from Sigma Chemical Co., U.S.A.; arginine, agmatine, iodoacetate, indole acetic acid, sodium deoxycholate and sucrose from British Drug House, England; 1-amino-2-napthol-4-sulfonic acid, from Fisher Scientific Company, U.S.A.; ammonium molybdate from May & Baker Ltd., Dagenham, England; sodium maleate, trichloro acetic acid and tris hydroxy methyl aminoethane from Loba Chemie Indo-Austranol Co. India; hydrogen peroxide, o-dianisidine and thiobarbituric acid from SD'S Lab-Chem Industry India; acetic acid, calcium chloride, magnesium sulfate, potassium chloride, sodium acetate, sodium bisulfate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium sulfate and sulfuric acid from Sarabhai M Chemicals, India and etherel from SISCO Research Laboratories, India. Dodine (Melprex Tech) was donated by Cyanamid of Great Britain, London; and guazatine was donated by Keno Gard AB, Sweden.

Plant Material : Certified Maize seeds (Zea mays L. Var. Ganga-2) were obtained from the local dealer of National Seed Corporation and stored at room temperature. Other seeds were obtained from local dealers.

Germination of seeds : Maize seeds were soaked in running tap water for 24 hr whereas pea, bengalgram and barley seeds were soaked for 8 hr and then kept in Petridishes on 2 layers of filter paper moistened with water and were allowed to germinate in dark at 22 ± 2°. The time when the seeds were kept for soaking was considered as zero time. In the studies where the maize scutella were incubated for a further period after excission, they were kept in Petridishes with 2 layers of moist filter paper for specified period at 22 ± 2°.

Preparation of slices and homogenate : The maize scutella were removed from the seeds and after removal of embryo they were cut transversly into thin slices with a razor blade. The slices were washed in cold distilled water until the wash water remained clear and were blotted on a filter paper and then used for enzyme assay. In case where homogenate rather than slices was used, a homogenate of the specified tissue (percentage given with assay system) was prepared by grinding with 0.95% KCl, pH 7, using a chilled pestle and mortar.

For treatment with amines and guanidines, the maize scutellum slices or homogenate from 4 or 8 day germinated seedlings were incubated for 1 hr at 37° in specified concentrations of these compounds. After incubation the slices were washed thoroughly with water and assayed for enzyme activity. In case of homogenates, the compounds were added to the homogenate and incubated at 37° for 1 hr before the other assay components were added. For experiments where amines, guanidines and sulfhydryl reagents were used to investigate their interaction, the scutellum slices were incubated at 37° for 1 hr with either amines, guanidines or sulfhydryl reagents first followed by the other compounds after washing with water.

Isolation of subcellular fractions : Maize scutella or other specified tissues (30g) from 4 day germinated seedlings were homogenized with 120 ml 0.25 M sucrose in 25 mM tris-maleate buffer pH 7.2. The homogenate was strained through 4 layers of cheese cloth and centrifuged at 1,000g for 10 min; 10,000g for 15 min; 40,000g for 30 min and 105,000g for 60 min. Pellets obtained from these fractions representing nuclear, mitochondrial, membrane and microsomal fractions were washed and suspended in the grinding medium. The fractions without any further purification were used for enzyme assay with or without preincubation with amines and guanidines for 1 hr at 37° as described earlier.

Solubilization of bound enzymes: Mitochondrial (10,000g) and membrane (40,000g) fractions were prepared from maize scutellum and were resuspended in the grinding medium. These fractions were later treated with calcium chloride (0.8 M), triton x-100 (1%) and sodium deoxycholate (1%) in the grinding medium for 1 hr. at 4° and then centrifuged to separate the solubilized enzyme in the supernatant and the residual enzyme. These concentrations were found to give optimal solubilization in preliminary trials. The residual enzyme fraction was resuspended in the grinding medium. Both the supernatant and the residual fractions were assayed for enzyme activity with or without preincubation with spermine or dodine.

Reconstitution of peroxidase solubilized from membrane fraction: Membrane (40,000g) fraction from maize scutellum was prepared as described earlier, resuspended in the grinding medium and was then treated with 0.8 M calcium chloride for 1 hr. at 4°. It was then centrifuged at 40,000g to separate the residual and supernatant enzymes. The solubilized enzyme (supernatant fraction) was dialysed against 50 fold diluted grinding medium for 48 hr. at 4° with two changes of the buffer to remove Ca^{++}. The enzyme solubilized from the homogenate after dialysis or the cytosolic enzyme were then mixed with the residue fraction of the membrane in a dialysis bag and further dialysed

against 50 fold diluted grinding medium at 4° for 48 hr. After dialysis the fraction was centrifuged at 105,000g to separate the left over enzyme in the soluble fraction from the reconstituted residual enzyme. The reconstituted fraction was then incubated with spermine or dodine and assayed for enzyme activity. In another study the residue fraction from the membrane was treated with spermine or dodine before carrying out the reconstitution.

Phytochrome mediation of the enzyme: Maize scutella along with the embryo were excised from the seedlings germinated for different days and were exposed to red light for 5 min (red light source consisted of 100 W incandescent light passed through a klett 660 filter giving 300 ergs/Cm^2/sec at seedling height) or far-red light for 15 min. (far-red light consisted of a 60 W incandescent light passed through two layers of blue one layer each of red and yellow cellophane sheets and 1 Cm water layer giving 1450 ergs/Cm^2/sec at seedlings height) or red followed by far-red. After the light exposures the scutella were transferred to dark and allowed to incubate at 22 $\pm$ 2°. At specified period, the embryo was removed and the scutellum slices were prepared for enzyme assay under green safe light (green light consisted of a 40 W fluorescent lamp covered by 4 layers of green cellophane giving less than 15 ergs/Cm^2/sec at seedling height).

For the studies on the interaction of phytochrome with polyamines and guanidines, the scutella along with the embryo were excised from 3 day old seedlings and were kept in Petridishes containing 2 layers of filter paper moistened with 2 mM test solutions and exposed to lights immediately or they were first exposed to lights, transferred to dark and then 2 hr after exposure they were transferred to Petridishes containing compounds. In another set the scutella were first treated with the compounds in dark and 2 hr after this they were exposed to lights and again kept in dark for incubation.

Enzyme assays

Peroxidase : Peroxidase activity was assayed essentially according to the method of Shanon et al. (1966). The assay system consisted of acetate buffer pH 5.5, 250 µmoles; 0.1 ml of 0.25% o-dianisidine; 0.1 ml of 3% H_2O_2 and enzyme (0.1 ml of 1% homogenate or 40 mg of slices) in a total volume of 4 ml. After incubation for 30 sec. at room temperature, the reaction was terminated with 0.5 ml of 5N H_2SO_4 and the absorbance of the coloured complex was measured at 400 nm. Enzyme unit is defined as the amount of enzyme giving 1.0 absorbance unit change per min under the assay conditions.

ATPase : ATPase activity was assayed essentially according to the method of Wheeler et al. (1979). The assay system consisted of acetate buffer, pH 5.5, 50 μmoles; $MgSO_4$, 10 μmoles; ATP, 10 μmoles and enzyme (0.1 ml of 5% homogenate or 200 mg slices) in a total volume of 3 ml. After incubation at 37° for 1 hr the reaction was terminated by 0.5 ml 10% TCA and the liberated phosphorus was estimated in the centrifuged supernatant according to the method of Fiske and Subbarow (1925). Enzyme unit is defined as μmoles of phosphate released per hr under the assay conditions.

Polyamine oxidase: Polyamine oxidase activity was assayed essentially according to the method of Naik et al. (1979). The assay system consisted of phosphate buffer, pH 6.6, 50 μmoles; spermidine, 10 μmoles; pyridoxal phosphate, 0.1 μmole and enzyme (0.5 ml, 10% homogenate) in a total volume of 3.5 ml. After incubation at 37° for 60 min, the reaction was terminated by adding 0.5 ml of 10% TCA. Δ^1-pyrroline formed during the reaction was estimated from the centrifuged supernatant. Enzyme unit is defined as the μmoles of Δ^1-pyrroline formed per hr under the assay conditions.

Lipid peroxidation : The level of lipid peroxidation in maize scutellum tissue was measured as malondialdehyde (MDA). The content of MDA was determined according to the method of

Dhindsa et al. (1981). 250 mg of tissue was homogenized in 5 ml of 0.1% TCA and the homogenate was centrifuged for 5 min. To 1 ml of the supernatant aliquot 4 ml of TCA-TBA reagent (20g of TCA dissolved in 100 ml of water to which 500 mg thiobarbituric acid (TBA) was added and heated to assist in the dissolution of TBA) was added. The tubes were kept in water bath at 95° for 30 min and then quickly cooled in ice bath, centrifuged for 10 min and the absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was substracted. Concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} Cm^{-1} (Heath and Packer, 1968).