MATERIALS AND METHODS

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Paddy seeds (Oryza sativa L. var. GR-3) were obtained from the State Department of Agriculture, Gujarat State. Selected seeds of uniform size were surface sterilized with 0.1% mercuric chloride for 3 minutes and were washed thoroughly with distilled water and blotted. These seeds were then transferred to sterilized Petri dishes (9.0 cm diameter) containing Whatman filter paper No. 1 moistened with distilled water or salt solutions (NaCl, 0.05, 0.075, 0.1, 0.15 and 0.2 M). Each Petri dish contained 25 seeds and each treatment was replicated 5 times. The seeds were set for germination at 30±1°C under dark conditions. The rate of germination was noted at an interval of 24 hours for a period of 120 hours. The length of root and shoot systems of 5-day-old seedlings was also recorded. The germination of seeds was reduced to 22% by 0.2 M NaCl as compared to the cent per cent observed in the control. Hence, attempts were made to enhance the germination percentage at 0.2 M NaCl by treating (pre-sowing soaking for a period of 4 hours - optimum duration of soaking observed) the seeds with various chemicals such as :

Calcium chloride ($CaCl_2$)	(0.1, 0.2, 0.3 M);
Succinic acid (SA)	(5, 10, 20, 25 mg/l);
Proline	(0.01,0.02,0.03,0.04%);

Polyethylene glycol (PEG,MW 6,000)	(Soaking for 4, 12, 24, 48 hrs);
Kinetin	(2.5, 5,10,15,20 mg/l);
Gibberellic acid (GA_3)	(5,10,15,20 mg/l).

Among different chemicals tried, GA_3 (10 mg/l) could increase the germination significantly (88%) and hence further studies were carried out with 10 mg/l GA_3.

Determination of dry weight of endosperm

Samples were collected at periodic intervals, the husk and axis were removed and the endosperms were collected. These endosperms were dried at 80°C till they attained a constant weight.

The dried endosperms thus obtained were ground to a fine powder.

Extraction and estimation of reducing sugars, total soluble sugars and starch

The method used for extraction of starch and sugars is essentially the one described by McCready et al. (1950).

Aliquots of 200 mg dry powder of endosperms were taken in centrifuge tubes. Few drops of cold 80% ethanol and 1 ml

of distilled water were added to each tube to avoid clumping by constant stirring. 10 ml of boiling 80% ethanol was added with constant stirring. The tubes were allowed to cool and were spinned at 6,000 r.p.m. for 10 minutes. The supernatant was collected and the residue was repeatedly (4 times) extracted with hot ethanol and all the supernatants were pooled together. The supernatant was evaporated to dryness at 40°C. The residue was dissolved in a little warm water and the total volume was made upto 50 ml. The contents of total soluble sugars and reducing sugars present in this sample were estimated according to the procedure of Yemm and Willis (1954) and Nelson and Somogyi (Hawk et al., 1954) respectively.

To the residue remained after ethanolic extraction 2.5 ml of distilled water was added. The tubes were placed in ice water and 3.25 ml of diluted perchloric acid (52%) was added to each tube with constant stirring. 10 ml of water was added after 15 minutes and the residue was spinned down at 6,000 r.p.m. The supernatant was collected into a 100 ml volumetric flask. The residue was extracted again with perchloric acid and the supernatant was pooled together. The final volume of the supernatant was dry filtered through Whatman filter paper No. 1 and the residue was discarded. An aliquot of this extract was used for the estimation of its glucose content by the procedure mentioned above (Yemm

and Willis, 1954). The estimated value of glucose was multiplied by 0.9 to convert it to starch.

Estimation of total nitrogen

50 mg dry powder of endosperms was digested with 2N H_2SO_4 containing copper selenite (20 mg/100 ml) in a micro-Kjeldahl's flask till the solution became colourless. The volume of the colourless solution was made upto 25 ml. The nitrogen content in an aliquot of digested sample was estimated using double iodide reagent (Umbreit et al., 1959).

Extraction and estimation of total protein

100 mg of dry powder of endosperms was suspended in 5 ml of 0.3 N KOH and was incubated for 18 hours at 37°C and the residue was sedimented at 6,000 r.p.m. for 15 minutes. The supernatant was collected into a volumetric flask. The residue was repeatedly (4 times) washed till no more protein was left out in the residue. The volume was made upto 50 ml with water. An aliquot was used for the estimation of protein following the procedure of Lowry et al. (1951) and compared with a standard established with bovine albumin.

Extraction and estimation of phytin phosphorus

The method employed for the extraction of phytic acid

phosphorus is essentially the one followed by Ergle and Guinn (1959). 100 mg dry powder of endosperms was taken in a beaker and 50 ml of 0.5 N HCl was added to it. The mixture was stirred for 2 hours. The mixture was filtered and the filtrate was neutralized with 2% NaOH using phenolphthalein as an indicator and it was rendered slightly acidic with HCl and made upto 100 ml. Two aliquots of 20 ml each were taken in centrifuge tubes and were treated each with 4 ml of 0.05% $FeCl_3$ solution. The tubes were heated by keeping them in a boiling waterbath for 15 minutes to floculate the precipitate of ferric phytate. The tubes were then centrifuged at 6,000 x g for 15 minutes and the pellets were washed with 5 ml of N/6 N HCl in order to remove the inorganic phosphorus present. The pellets were suspended in 2 ml of distilled water and heated in a boiling waterbath for 2 minutes. 2 ml of 2% NaOH was added to each tube and heating was continued for another 15 minutes. The tubes were centrifuged and the supernatants were transferred into two Kjeldahl's flasks. To each flask containing sodium phytate, 1 ml of H_2SO_4 and 1 ml of 60% $HClO_4$ were added. The samples in the flasks were heated till they became colourless. The flasks were cooled and 40 ml of water was added to each tube. The content of the flask was neutralized using 40% NaOH and phenolphthalein as an indicator. The final volume of the

neutralized sample was made upto 100 ml with distilled water. The inorganic phosphorus content of the sample thus obtained was estimated using the colorimetric method of Fiske and Subbarow (1925).

Extraction and estimation of inorganic phosphorus

The method employed for extraction of inorganic phosphorus is essentially that of Holden (1951). 500 mg dry powder of endosperms was chilled and 5 ml of cold distilled water and 5 ml of 0.4 N $HClO_4$ were added to it. The mixture was stirred and was allowed to stand for 15 minutes. At the end of 15 minutes, the mixture was centrifuged at 6,000 x g for 15 minutes and the supernatant was collected. The residue was repeatedly extracted (4 times) and all the supernatants were pooled together. The inorganic phosphorus content of the supernatant was estimated colorimetrically as mentioned earlier.

Extraction of amylases

1 g of fresh endosperm tissue was ground at 0-4°C with a pinch of neutral glass powder for 5 minutes using chilled mortar and pestle and 5 ml of chilled distilled water was added to get a homogenate. The homogenate was spinned at 6,000 r.p.m. for 15 minutes at 2-4°C and the supernatant was used as the source of amylases.

Assay of total amylolytic activity

The assay system contained 0.5 ml 0.1 M. acetate buffer (pH 4.6), 0.5 ml 1% soluble starch and 1 ml homogenate. The reaction was carried out at 30°C for 30 minutes and an aliquot of 1 ml was estimated for the amount of maltose released using 3,5-dinitrosalysilic acid (DNSA) (Bernfeld, 1955). The protein content of the homogenate was estimated by the method of Lowry et al. (1951). The total amylolytic activity was expressed as micromoles of maltose formed per mg protein per 30 minutes.

Assay of α-amylase

The homogenate used for assay of total amylolytic activity was heated at 70°C for 45 minutes to inactivate the β-amylase; it was then cooled and assayed for its α-amylase activity as mentioned earlier. The difference between the total amylolytic activity and α-amylase activity gives the value of β-amylase activity.

Isozyme separation of amylases

Disc gel electrophoresis was carried out according to the method described by Davis (1964). The crude extracts used for assay of α- amylase and total amylolytic activity were loaded on polyacrylamide gels containing 0.1%

soluble starch. The electrophoresis was carried out by using Tris-glycine buffer (pH 8.3) for about 3 hours with a current of 1 mA per tube (5 x 110 mm). After electrophoresis, the gels were incubated in 0.1 M acetate buffer (α-amylase, pH 4.6; β-amylase, pH 3.4) for 1 hour. The gels were then stained with 1 mM I_2- 2 mM KI reagent.

Extraction of maltase

500 mg fresh endosperms were ground for 5 minutes with 0.01 M Tris-HCl buffer (pH 7.5) in a chilled mortar and pestle. It was then centrifuged at 6,000 r.p.m. for 15 minutes at 4°C and the supernatant was used as the enzyme source.

Assay of maltase

The assay system contained 0.9 ml 0.2 M sodium phosphate buffer (pH 6.5), 0.3 ml maltose (25 mM) and 0.3 ml crude extract. The reaction was carried out at 30°C for 30 minutes. The reaction was terminated by immersing the tubes in boiling waterbath for 5 minutes. The glucose content of the reaction mixture was determined as mentioned earlier. The enzyme activity was expressed as mg glucose formed per mg protein per 30 minutes.

Extraction of Invertase

The homogenate used for the assay of amylases was used for the assay of invertase.

Assay of Invertase

The assay system contained 1 ml 0.1 M acetate buffer (pH 4.6), 0.5 ml 2% sucrose and 0.5 ml homogenate. The reaction was carried out at 30°C for 30 minutes and the reaction was terminated by keeping the tubes in a boiling waterbath for 10 minutes. An aliquot from the reaction mixture was used for the estimation of glucose according to the procedure of Nelson and Somogyi. The enzyme activity was expressed as µg of glucose formed per mg protein per 30 minutes.

Extraction of protease

500 mg fresh endosperms were ground for 3 minutes in 5 ml medium consisting of 50 mM Tris-HCl (pH 7.6), 1 mM EDTA and 0.5 mM mercaptoethanol along with equal amount of neutral glass powder using a chilled mortar and pestle. The homogenate was centrifuged at 6,000 r.p.m. for 15 minutes at 0-2°C and the supernatant was used as the enzyme source.

Assay of protease

The proteolytic activity was measured by the capacity of extract to liberate α-amino group from casein. The assay system contained 1 ml 0.2 M Tris-maleate buffer (pH 5.0), 1 ml 2% heat denatured casein and 1 ml of crude enzyme. The tubes were incubated at 40°C for one hour. The reaction was terminated by the addition of 1 ml of 20% TCA to the incubation medium which was then allowed to stand for 30 minutes at room temperature. The precipitated protein was removed by filtration through Whatman filter paper No. 44. The solution (filtrate) was used for the determination of its α-amino groups using the method described by Spices (1957), and the results were corrected with a blank to which TCA was added before the addition of enzyme extract. The enzyme activity is expressed as µg of tyrosine liberated per mg protein per hour.

Extraction of phytase

1 g fresh endosperms were ground for 5 minutes, with a pinch of neutral glass powder in 0.01 M Tris-HCl buffer (pH 7.0). The extract was filtered through cheese cloth and then was centrifuged at 6,000 r.p.m. for 15 minutes at 2-4°C. The supernatant was used as enzyme source.

Assay of phytase

The method employed for the assay of phytase is the one followed by Guardiola and Sutcliffe (1971). The assay system contained 2 ml 0.2 M acetate buffer (pH 3.8), 1 ml 10 mM sodium phytate and 1 ml crude enzyme extract. The tubes were incubated at 35°C for 30 minutes. The reaction was terminated by the addition of 1 ml of 20% TCA. and after allowing it to stand for 30 minutes at room temperature, the precipitated protein was removed by centrifugation at 5,000 x g for 15 minutes. The amount of inorganic phosphorus present in the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as µg of inorganic phosphorus liberated per mg protein per 2 hours.

Extraction of ATPase

The crude enzyme extract used for the assay of phytase was used as a source of enzyme for assay of ATPase.

Assay of ATPase

The assay system contained 0.25 ml 0.2 M Tris-HCl buffer (pH 7.5), 0.25 ml 0.02 M ATP (Na salt), 0.1 ml 0.05M $MgCl_2$ and 0.4 ml crude enzyme extract. The tubes were

incubated at 28°C for 15 minutes. The reaction was terminated by adding 1 ml of 20% TCA. The tubes were centrifuged at 5,000 x g for 15 minutes and the inorganic phosphorus content of the supernatant was estimated as mentioned earlier. The enzyme activity is expressed as µg of inorganic phosphorus liberated per mg protein per 15 minutes.

Extraction of dehydrogenases

2 g fresh endosperms were ground for 3 minutes, with a pinch of neutral glass powder in 10 ml medium consisting of 0.1 M Tris-HCl (pH 8.0), 0.01 M EDTA, 1 M KCl and 0.4% mercaptoethanol. It was squeezed through 2 layers of cheese cloth and filtrate was centrifuged at 1,000 r.p.m. for 15 minutes. The supernatant obtained was again centrifuged at 14,000 r.p.m. for 60 minutes. All the operations were carried out at 0-4°C. The supernatant was used as a source of dehydrogenases.

Assay of succinic, pyruvic, and α-ketoglutaric dehydrogenases

The assay system contained 0.4 ml 0.1 M phosphate buffer (pH 7.4), 0.5 ml 0.2 M substrate (sodium salt of succinic acid or pyruvic acid or α-ketoglutaric acid), 1 ml 0.1% tetrazolium chloride (TTC), 0.1 ml 0.1% PMS (phenazine

methosulfate), 0.5 ml crude enzyme and 0.5 ml H_2O. The reaction was started by adding the crude enzyme. A blank was prepared omitting substrate. The tubes were incubated at 37°C for 30 minutes. The reaction was terminated by adding 7 ml of acetone. The tubes were shaken well and were centrifuged for 5 minutes. The colour developed was read at 420 mμ. The activity was expressed as increase in O.D. per mg protein per 30 minutes.

Assay of glucose-6-phosphate dehydrogenase

The assay system contained 1 ml 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml 0.003 M NADP (TPN), 0.1 ml 0.05 M $MgCl_2$ 0.2 ml enzyme, 0.1 ml 0.02 M glucose-6-phosphate and 1.5 ml distilled water. The reaction was started by adding glucose-6-phosphate. The increase in O.D. was read at 340 nm. Blank was prepared omitting glucose-6-phosphate. The activity of enzyme is expressed as increase in O.D. per mg protein per minute.

Measurement of O_2 uptake

The rate of O_2 uptake by seeds/seedlings was measured manometrically (Umbreit et al., 1959).

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