MATERIALS AND METHODS

Seeds of paddy (Oryza sativa L. var. Bhura Rata) were obtained from State Department of Agriculture, Gujarat. Selected seeds of uniform size were surface sterilized with 0.1% mercuric chloride for three minutes and were washed thoroughly with distilled water and blotted. These seeds were then transferred to sterilized Petri dishes containing Whatman No. 1 filter paper moistened with distilled water or salt solution (NaCl 0.05, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0%). Each Petri dish contained 50 seeds and each treatment was replicated 4 times. Experiments were repeated thrice. The seeds were set for germination at 30±1°C under dark conditions. The rate of germination was noted at 24 hrs interval for a period of 120 hrs. The length of root and shoot system of 5-day-old seedlings were also recorded. Salt at a concentration of 2.0% brought about total inhibition of seed germination (Table 1) and hence attempts were made to induce germination in seeds under the toxic level of salt by treating (pre-sowing soaking for a period of 4 hours - optimum duration of soaking observed - Table 3) the seeds with various chemicals such as :

Gibberellic acid (GA_3) (5, 10, 25 mg/l),

Succinic acid (SA) (5, 10, 20 mg/l),

Proline (5 , 10, 25 mg/l),

Thiourea (0.2, 0.5, 1.0%),

Cycocel (CCC)	(500, 1000, 1500 mg/l)
2-Chloroethanol	(0.02, 0.04, 0.06, 0.08, 0.1, 0.5%) and
Indol Acetic Acid (IAA)	(1, 2, 4 mg/l)

were tried. The seeds were then tried for their germination under the influence of 2% salt as described earlier. Among different chemicals tried only GA_3 (10 mg/l) could induce germination of seeds significantly (Table 16) and hence further studies were carried out with 10 mg/l GA_3.

Determination of dry weight of endosperm

Samples of 50 seeds were collected during different stages of germination, 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 using mortar and pestle.

Extraction of soluble and reducing sugars

Aliquots of 100 mg powdered material were taken in centrifuge tubes, few drops of cold 80% ethanol and 0.5 ml distilled water were added to each tube to prevent clumping

by stirring well. 5 ml of boiling 80% ethanol was added to each tube and stirred well to remove soluble and reducing sugars (Mc Cready et al., 1950). The tubes were allowed to cool and they were spinned down at 10,000 r.p.m. for 15 minutes. The supernatant was collected and the residue was repeatedly (4 times) extracted with hot ethanol and all the supernatants were pooled together.

Extraction and estimation of starch

The residue (after the removal of soluble sugars) obtained after the ethanolic extraction was used for the extraction of starch with 52% perchloric acid as described by Mc Cready et al. (1950). The residue was suspended in 0.5 ml of distilled water, 6.5 ml of 52% perchloric acid was added and stirred continuously for 5 min. It was stirred frequently for 20 min. 10 ml of distilled water was added to it and the residue was spinned down at 6,000 r.p.m. for 15 min. The residue was extracted again with perchloric acid and the volume of the pooled supernatant was made upto 100 ml. The supernatant was dry filtered through Whatman No. 1 filter paper and the residue was discarded. 10 ml of the perchloric acid extract was hydrolysed with 10 ml of 1N HCl for 3 hrs in boiling waterbath. It was then neutralized with 2N

sodium carbonate and the volume was made upto 25 ml. An aliquot of this extract was used for the estimation of its reducing sugar content by following the method of Folin and Malmros (Umbreit et al., 1959). The estimated glucose content was then converted into starch equivalent by multiplying the glucose value with 0.9.

Estimation of total soluble sugars and reducing sugars

The pooled ethanol supernatant was evaporated to dryness at 40°C and the residue was dissolved in little warm water and the total volume was made upto 50 ml. The total soluble sugars of the filtrate was estimated using Anthrone reagent (Yemm and Willis, 1954) and reducing sugars by the method of Folin and Malmros (Umbreit et al., 1959).

Estimation of total nitrogen

50 mg dried powder of the endosperms was digested with 2N H_2SO_4 containing copper selinate (20 mg/100 ml) in a microkjeldahl'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 0.3 N KOH and was incubated for 18 hours at 37°C and the residue was sedimented at 10,000 r.p.m. for 15 minutes. The supernatant was collected into a volumetric flask. The residue was repeatedly washed till no more protein was left out in the residue. The volume was made upto desired amount. 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 (Sigma).

Extraction and estimation of phytin

The method employed for the extraction of phytic acid phosphorus is essentially the one followed by Ergle and Guinn (1959) 100 mg powder was taken in a beaker and 50 ml of 0.5 N HCl was added to it. The mixture was stirred for 2 hours on a magnetic stirrer. The mixture was filtered and the filtrate was neutralized with 2 per cent NaOH using phenolphthalein as 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 6000 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 per cent 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 per cent $HClO_4$ were added. The samples in the flask 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 per cent NaOH and phenolphthalein as indicator. The final volume of the neutralized sample was made upto 100 ml with distilled water.The inorganic phosphorus content of the samples thus obtained was estimated using the colorimetric method of Fiske and Subbarow (1925).

Extraction of amylase

500 mg of fresh endosperm tissue was ground with a pinch of neutral glass powder for 10 minutes using chilled

mortar and pestle and 5 ml of 0.1 M acetate buffer (pH 5.5) was added to get a homogenate. The homogenate was spinned at 10,000 r.p.m. for 15 minutes at 0° to 2°C and the supernatant was used as the source of amylase.

Assay of total amylolytic activity

The assay system contained 0.5 ml 0.1 M citrate buffer (pH 5.0), 0.5 ml 1% soluble starch and 1 ml enzyme. 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 (Bernfeld, 1951).

Assay of α-amylase

The enzyme was heated at 70°C for 15 minutes to inactivate the β-amylase and the heated enzyme after cooling was estimated for its α-amylase activity as mentioned above. The difference between the total activity and α-amylase activity was designated as β-amylase activity. The enzyme activity is expressed as μmoles of maltose formed per mg protein per thirty minutes.

Extraction of invertase

About 500 mg tissue was ground for ten minutes at

0-2°C using a pinch of neutral glass powder. The enzyme was extracted with 5 ml of 0.1 M acetate buffer pH 4.6. The residue was spinned down at 6,000 r.p.m. for 15 minutes. The supernatant was used as enzyme source.

Assay of invertase

The reaction mixture contained 1 ml of 0.1 M acetate buffer pH 4.6, 0.5 ml 2% sucrose and 0.5 ml enzyme extract. The reaction mixture was incubated at 30°C for 30 minutes and the reaction was terminated by keeping it 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 Folin and Malmros (Umbreit et al., 1959). The enzyme activity is expressed as µgm of glucose formed per mg protein per hour.

Extraction of maltase

250 mg of the endosperm tissue was ground to a fine paste 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 a period of 15 minutes at 4°C and the supernatant was used as the enzyme source.

Assay of maltase

The assay system contained 0.3 ml maltose (25 mM), 0.9 ml 0.2 M sodium phosphate buffer (pH 6.5) and 0.3 ml enzyme. The assay was carried out at 38°C for 30 minutes. The enzyme activity was terminated by immersing the tubes in boiling waterbath for 5 minutes. The glucose content of the reaction mixture was determined using Folin and Malmros method (Umbreit et al., 1959). The enzyme activity was expressed as µgms of glucose released per mg protein per 30 minutes.

Extraction of protease

About 250 mgs of fresh endosperms immediately after harvesting were ground in a 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

Proteolytic activity was measured by the capacity of extract to liberate α-amino group from casein. In routine

assay 1 ml extract was incubated with 1 ml of 2% heat-denatured casein and 1 ml of 0.2 M Tris-maleate buffer at pH 5.0 for 2 hours at 40°C. The reaction was terminated by the addition of 1 ml of 20 per cent 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 No. 44 filter paper. The solution 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. The protein content of the dialysed extract was estimated as mentioned previously. The enzyme activity is expressed as μg of tyrosine liberated per mg protein per 2 hours.

Extraction of phytase

Freshly harvested endosperms (1 gm) were ground with a pinch of neutral glass powder and extracted with 0.01 M Tris-HCl buffer (pH 7.0) and filtered through cheese cloth. It was then centrifuged at 6,000 r.p.m. for 10 minutes and was used as the enzyme source. All operations were carried out at temperature range 0-4°C.

Assay of phytase

The method employed for the assay of phytase is the

one followed by Guardiola and Sutcliffe (1971). 1 ml enzyme extract was incubated with 1 ml of 10 mM sodium phytate and 2 ml of 0.2 M acetate buffer at pH 5.0 for 30 minutes at 35°C. The reaction was terminated by the addition of 1 ml of 20 per cent 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 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 120 minutes. The protein content of the extract was estimated according to the procedure of Lowry et al. (1951).

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