BIOLOGIA PLANTARUM 47 (4): 555-560, 2003/4

# Activities of glycolytic enzymes in leaves and roots of contrasting cultivars of sorghum during flooding

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## Abstract

Activities of phosphofructokinase (PFK), fructose-1,6-bisphosphate aldolase (FBP aldolase) and pyruvate kinase (PK) increased progressively in the roots of flood-tolerant SSG-59-3 cultivar during flooding. In contrast, only a slight change in activities of PFK and FBP aldolase was discerned in the roots of flood-sensitive S-308 cultivar during initial stages of flooding followed by a decline in the activities of these enzymes. Although the activity of hexokinase (HK) was transiently elevated in roots of both the cultivars during flooding, the magnitude of increase was much more in SSG-59-3 than in the S-308. In leaves of SSG-59-3, HK activity increased during 12 h of flooding whereas only a minor change occurred in the case of S-308. Flooding resulted in depressed activities of PFK and PK in leaves of S-308 but that in SSG-59-3 rose following imposition of waterlogged conditions. Activity of FBP aldolase in leaves of tolerant cultivar also showed a steady enhancement during flooding. The total and reducing sugars content decreased in leaves and roots of the S-308 during flooding but in SSG-59-3 the amount was more or less comparable to that in corresponding non-flooded plants.

*Additional key words*: flood-sensitive and tolerant cultivars, fructose-1,6-bisphosphate aldolase, hexokinase, phosphofructokinase, pyruvate kinase, *Sorghum bicolor*.

## Introduction

Waterlogging causes depletion of soil oxygen and results in increased plant mortality and diminished yield of crop plants (Drew 1992). Deficiency of oxygen impedes respiration rate in the submerged parts of plants and production of ATP. In order to meet the energy demands of the metabolic activities vital for the survival, the affected plant parts might be expected to switch over to fermentative mode of respiration thus ensuring sustained production of ATP, albeit, at much lower rates. Though the precise biochemical mechanisms for adaptation of plants under oxygen insufficiency are not fully understood, it is believed that ability to maintain an active fermentative metabolism could be crucial for tolerance of plants to anaerobiosis (Perata and Alpi 1993, Sachs *et al.* 1996, Perata *et al.* 1997). Since fermentation requires an accelerated flux of sugars through glycolysis, the present investigations were undertaken to ascertain whether the enzymes of glycolytic pathway respond differently upon flooding in a flood-sensitive (cv. S-308) and a flood-tolerant (cv. SSG-59-3) cultivars of sorghum.

## Materials and methods

**Plant growth:** Seeds of tolerant (SSG-59-3) and non-tolerant (S-308) cultivars of *Sorghum bicolor* (L.) Moench were procured from Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. All the biochemicals were from either *Sisco Research Laboratories* (Bombay, India) or *Sigma Chemicals Co.* St. Louis, USA.

Seedlings of both sorghum cultivars were raised under natural conditions in a net house in 30 × 40 cm poly-ethylene bags filled with 10 kg of washed river sand.

*Received* 6 August 2002, *accepted* 24 February 2003.

*Abbreviations*: FBP aldolase - fructose-1,6-bisphosphate aldolase, HK - hexokinase, PEP - phosphoenolpyruvate, PFK - phospho-fructokinase, PK - pyruvate kinase.

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The plants were supplied with modified Hoagland nutrient solution (Dua 1987) at 7-d intervals. Twenty days old seedlings were divided into two groups. One group of the plant was then subjected to flooding stress of different durations (0, 6, 12, 24, 48 and 72 h) by irrigating them with excessive quantity of nutrient solution so that the level of water above the surface of sand was 5 cm. Nutrient solution was replenished to maintain this level of solution in the polyethylene bags throughout the course of the flooding treatment. The other group was grown under normal non-flooded conditions and it served as a corresponding control.

**Extraction and assay of enzymes:** Leaf and root extracts were prepared according to the procedure of Bouny and Saglio (1996) by macerating 1 g of the tissue in 5.0 $cm^3$ of 0.1 M cold Tris-HCl buffer (pH 8.0). The homogenate was gently squeezed through four layers of cheesecloth and centrifuged at 12 000 *g* for 30 min at 4 °C. The supernatant was carefully decanted and used for assaying the activities of various enzymes. Tissue extracts were prepared from three replicates of each treatment and enzyme activity in an individual extract was performed in duplicate. The presented data is, thus, representative of six determinations of each treatment.

Activity of hexokinase (HK; ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) was assayed spectrophotometrically (*LC-340B, Calbiochem-Behring Corp.*, USA) by coupling its reaction with glucose-6-phosphate dehydrogenase and then measuring the rate of reduction of $NADP^+$ (Tsai *et al.* 1970). The reaction mixture contained: 75 mM HEPES buffer (pH 7.5), 6.2 mM $MgSO_4$, 3.0 mM glucose, 0.625 mM $NADP^+$, 6.2 mM ATP, 2 units of glucose-6-phosphate dehydrogenase and 0.1 $cm^3$ of tissue extract.

Phosphofructokinase activity (PFK; ATP: fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) was measured by a coupled assay in presence of fructose-1,6-bisphosphate aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase using fructose-6-phosphate as the substrate (Ling *et al.* 1966). The assay mixture constituted of: 70 mM Tris-HCl buffer (pH 7.5), 4 mM fructose 6-phosphate, 10 mM $MgSO_4$, 4 mM ATP, 0.15 mM NADH and 2 units each of fructose-1,6-bisphosphate aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase, and 0.1 $cm^3$ of enzyme extract.

The coupled procedure for fructose-1,6-bisphosphate aldolase (FBP aldolase; D-fructose-1,6-bisphosphate-D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13), as described by Groves *et al.* (1966) and which is based on measuring rate of the oxidation of NADH in presence of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase during conversion of fructose 1,6-bis-phosphate to α-glycerophosphate was employed. The reaction mixture contained: 75 mM Tris-HCl buffer (pH 7.5), 2 mM fructose 1,6-bis phosphate, 0.04 mM $CaCl_2$, 0.06 mM cysteine-HCl, 15 mM KCl, 0.15 mM NADH and 2 units each of triosephosphate isomerase and α-glycerophosphate dehydrogenase and 0.1 $cm^3$ of the enzyme extract.

Pyruvate kinase (PK; ATP: pyruvate 2-O-phospho-transferase, EC 2.7.1.40) activity was determined according to the procedure of Dennis and Green (1975) by coupling the further reduction of pyruvate to lactate in the presence of lactate dehydrogenase (LDH) as the auxillary enzyme. The reaction mixture contained: 0.1 M Tris-HCl buffer (pH 7.6), 0.5 mM ADP, 1.0 mM PEP, 10 mM $MgCl_2$, 4.5 mM KCl, 0.15 mM NADH, 2 units LDH and 0.1 $cm^3$ of the enzyme extract:

The amount of soluble proteins in the tissue extract was estimated by the method of Lowry *et al.* (1951).

**Extraction and estimation of sugars:** Sugars were extracted from the freshly harvested samples by the method of Bouny and Saglio (1996). One g of tissue was thoroughly washed with water and then macerated in 5.0 $cm^3$ of 10 % (*v/v*) perchloric acid in a chilled pestle and mortar. After centrifuging the homogenate at 10 000 *g* for 30 min at 4 °C, the obtained supernatant was neutralized with $K_2CO_3$ and again centrifuged at 3 000 *g* for 15 min at 4 °C. Total sugars in the supernatant were estimated by anthrone method (Nelson 1944) and reducing sugars were determined using arsenomolybdate reagent (Nelson 1944, Somogyi 1945).

**Statistics:** The data was statistically analysed using a complete randomized design (CRD) where each observation was replicated thrice and, for each replication, the estimation was done in duplicate. The critical difference (CD) among variants was calculated at $P = 0.05$.

## Results and discussion

An almost constant activity of hexokinase (HK) was recorded in the roots of non-flooded (control) plants of both the cultivars during the course of experiment (Fig. 1*A*). However, after 6 h of flooding treatment, activity of this enzyme activity increased by about 5.5 and 2.3 times of that in respective controls of flood-tolerant and flood-sensitive cultivars. Thereafter, the activity began to decline at steady rate. Leaves of control plants of cultivar S-308 had significantly higher activity of hexokinase than in the leaves of cultivar SSG-59-3. Only a minor change in the activity of leaves of S-308 was noticeable during flooding whereas leaves of

SSG-59-3 possessed a 1.2 fold greater activity than in the control plants after 6 h of flooding (Fig. 1*B*). As in the case of roots, after this initial increase, the enzyme activity started to recede. Increase in activity of hexokinase in roots and shoots has been reported in *Echinochloa phyllopogon* and *E. crusparonis* plants upon subjecting to anoxia (Fox *et al.* 1998) whereas its activity was found to be lowered in roots of tomato (Germain *et al.* 1997).

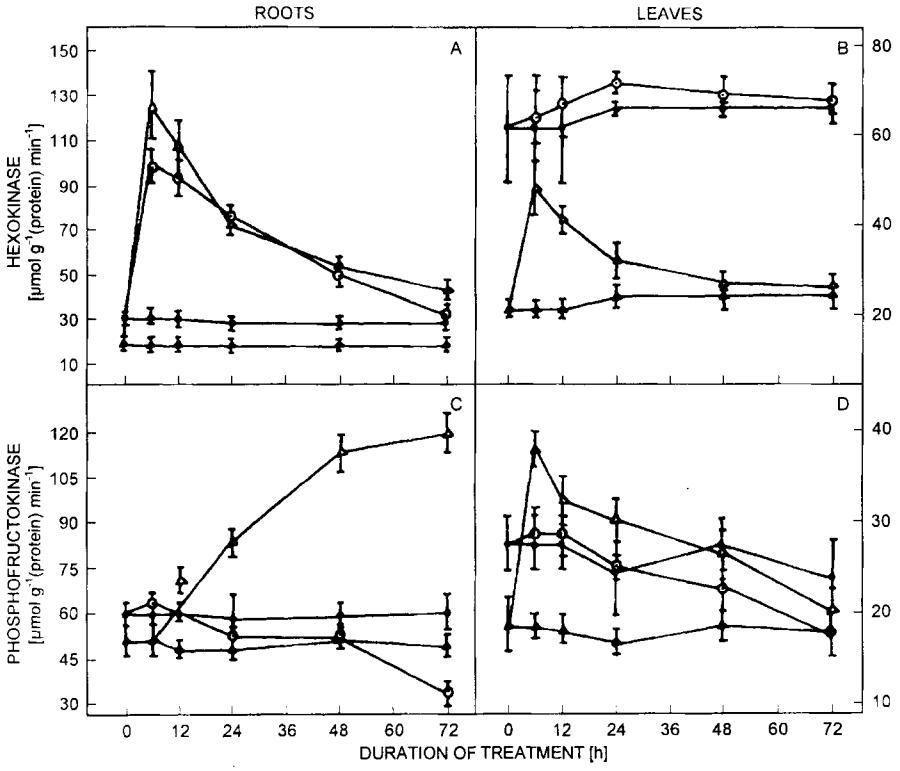


Fig. 1. Effect of flooding on activity of hexokinase (*A*, *B*) and phosphofructokinase (*C*, *D*) in roots (*A*, *C*) and leaves (*B*, *D*) of control (*closed circles*) and flooded (*open circles*) plants of the sensitive cultivar S-308 and in control (*closed triangles*) and flooded plants (*open triangles*) of tolerant cultivar SSG-59-3 of sorghum. The *bars* denote ± SE. [CD ($P$ = 0.05): HK roots 4.124 (cultivars), 7.14 (treatments), 10.10 (interactions); HK leaves 3.03 (cultivars), 5.24 (treatments), 7.41 (interactions); PFK roots 2.75 (cultivars), 4.76 (treatments), 6.63 (interactions); PFK leaves 1.81 (cultivars), 3.14 (treatments), 4.45 (interactions)].

A distinctly different response was observed in the activity of phosphofructokinase (PFK) in roots and leaves of cultivars SSG-59-3 and S-308. The roots of flooded and control plants of the sensitive cultivar had an identical activity of PFK up to 12 h of flooding treatment (Fig. 1*C*). However, after that the activity in roots of flooded plants was consistently lower than that in the non-flooded plants and after 72 h, roots of the former group of plants possessed only 55 % of the activity of that in their corresponding control. In contrast, after a lag of 12 h, activity of this enzyme in SSG-59-3 showed a steady enhancement in roots of flooded plants and after 72 h of flooding these had 2.4 times activity than in plants growing under normal conditions. PFK activity was markedly elevated in leaves of SSG-59-3 (Fig. 1*D*) particularly during early stages of flooding. Leaves of plants subjected to 6 h flooding showed two times activity than that in control plants after which the enzyme activity started to decrease. In contrast, no differences in the activity of PFK were discernible in leaves of S-308 between the flooded and non-flooded plants up to 24 h and thereafter leaves of former plants were found to have lower activity than their respective control. Increase in PFK activity under oxygen limitation has been earlier observed in anoxia-sensitive cultivar of soybean (Mohanty *et al.* 1993) and flood tolerant cultivar of rice (Gibbs *et al.* 2000). However, no difference in PFK activity was noticed in tissues of maize plants and *in vitro* cultured cells of rice (Bailey-Serres *et al.* 1988, Mohanty *et al.* 1993) kept under normal and anoxic conditions while a significant decrease was recorded in root tips of maize (Bouny and Saglio 1996).

The activity of fructose-1,6-bisphosphate aldolase (FBP aldolase) increased marginally by about 10 - 25 % in the roots of flooded seedlings of S-308 whereas the roots of flooded plants of SSG-59-3 had a much higher activity than their corresponding control throughout the duration of experiment (Fig. 2*A*). After 72 h of flooding

treatment, roots of these plants had two fold enzyme activity as compared to that in controls. Flooding caused a marked elevation in the activity of FBP aldolase in leaves of both the cultivars and 72 h flooding resulted in about 40 and 113 % increase in activity in leaves of S-308 and SSG-59-3, respectively (Fig. 2*B*). Raised activity of FBP aldolase in roots of maize seedlings on their transfer to anaerobic conditions has been documented by several investigators earlier (Kelley and Freeling 1984, Hake *et al.* 1985, Dennis *et al.* 1988).

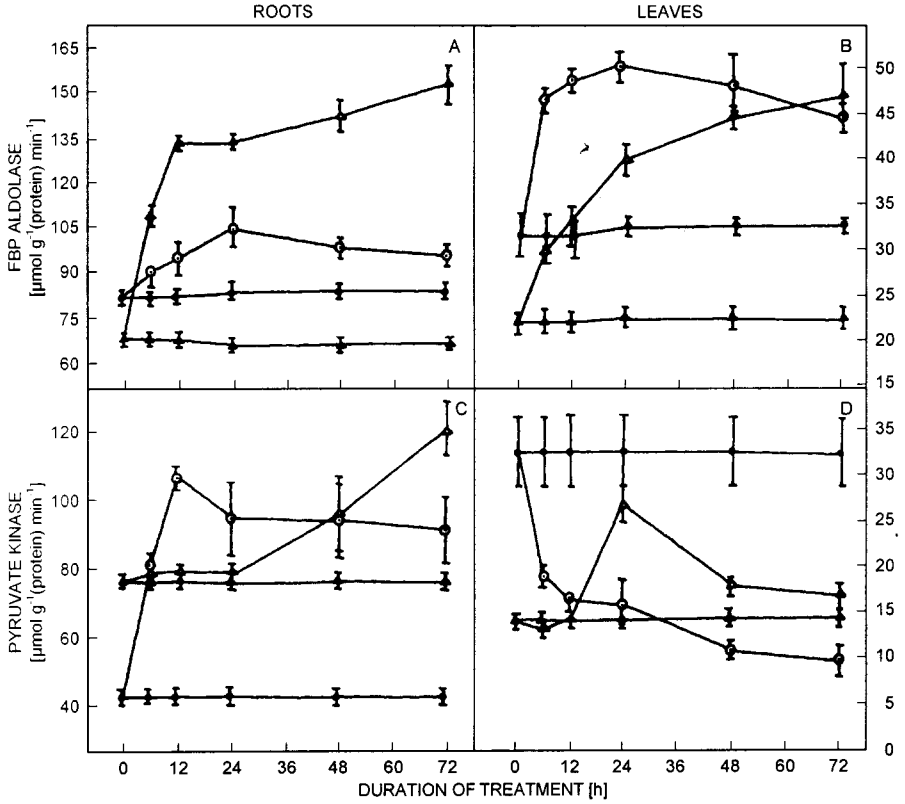


Fig. 2. Effect of flooding on activity of FBP aldolase (*A*, *B*) and pyruvate kinase (*C*, *D*) in roots (*A*, *C*) and leaves (*B*, *D*) of control (*closed circles*) and flooded (*open circles*) plants of the sensitive cultivar S-308 and in control (*closed triangles*) and flooded plants (*open triangles*) of tolerant cultivar SSG-59-3 of sorghum. The *bars* denote ± SE. [CD ($P = 0.05$): FBP aldolase roots 2.69 (cultivars), 4.66 (treatments), 6.60 (interactions); FBP aldolase leaves 1.79 (cultivars), 3.10 (treatments), 4.39 (interactions); PK roots 5.49 (cultivars), 9.50 (treatments), 13.43 (interactions); PK leaves non-significant (cultivars), 2.14 (treatments); 3.03 (interactions)].

An enhancement in pyruvate kinase (PK) activity was recorded in the roots of S-308 and SSG-59-3 cultivars due to flooding (Fig. 2*C*). After 72 h, roots of these plants exhibited about 2.15 and 1.60 times activity than that in their corresponding controls. A different response of PK in leaves of flooded plants of S-308 was observed and activity of this enzyme diminished substantially under waterlogging (Fig. 2*D*). Flooding treatment for 6 and 72 h depressed PK activity in leaves of this cultivar by 50 and 75 %, respectively. In contrast, the enzyme activity in leaves of SSG-59-3 increased by about 80 % after 24 h of the treatment and was maintained more or less at this level during rest of the period. Anoxia tolerant cells of rice were found to contain higher PK activity (Mohanty *et al.* 1993) which is at variance to no change in activity of this enzyme in tolerant and intolerant cultivars of rice (Umeda and Uchimiya 1994).

The amount of total sugars in roots of the treated seedlings of SSG-59-3 remained more or less comparable to that in the controls upto 48 h of flooding (Fig. 3*A*). However, in the case of S-308 a perceptible depletion was observed particularly during the later stages and after 72 h of flooding these contained 32 % less total sugars than the control (Fig. 3*A*). A similar effect of flooding on total sugar content in leaves of these cultivars was obtained and after 72 h the leaves of S-308 had 45 % less total sugars than that in non-flooded control (Fig. 3*B*). A decrease of 33 % and 13 % occurred in the content of reducing sugars in roots of S-308 and SSG-59-3 plants, respectively, following 72-h flooding (Fig. 3*C*). Thus, on the basis of per cent reduction, the observed decline in total sugar content (32 %) in roots of S-308 was largely due to lower amount of reducing sugars (33 %). However, in terms of absolute amount of sugars the decrease in total sugar content (8.3 mg) was largely due to decrease in non-reducing sugars (7.5 mg). Quali-

tatively, similar pattern was obtained for leaves of flooded plants of these cultivars and after 72 h of the treatment, the leaves of S-308 and SSG-59-3 had 37 and 15 % lower content of reducing sugars, respectively, than the corresponding non-flooded plants (Fig. 3*D*).

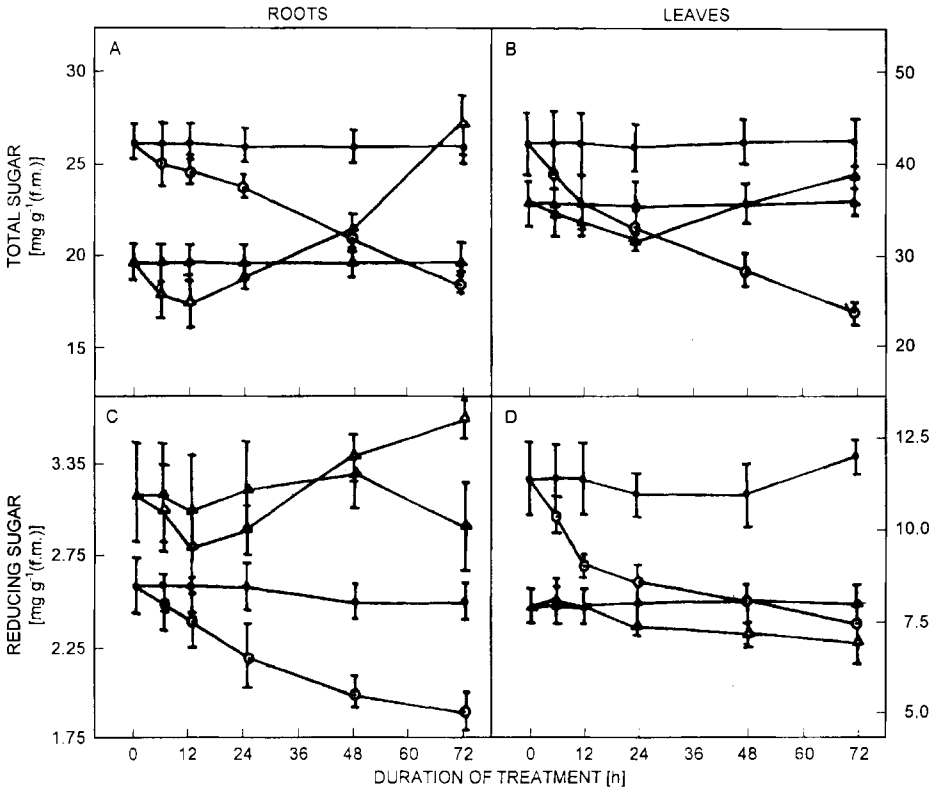


Fig. 3. Effect of flooding on total sugars (*A*, *B*) and reducing sugars contents (*C*, *D*) in roots (*A*, *C*) and leaves (*B*, *D*) of control (*closed circles*) and flooded (*open circles*) plants of the sensitive cultivar S-308 and in control (*closed triangles*) and flooded plants (*open triangles*) of tolerant cultivar SSG-59-3 of sorghum. The *bars* denote ± SE. [CD ($P$ = 0.05): total sugar roots 2.19 (cultivars), 3.32 (treatments), 4.86 (interactions); leaves 3.81 (cultivars), 4.35 (treatments), 7.68 (interactions); reducing sugars roots 0.12 (cultivars), non-significant (treatments), 0.29 (interactions); leaves 0.39 (cultivars), 0.68 (treatments), C. 0.96 (interactions)].

Sugar phosphates, which are the principal substrates for glycolytic pathway, are produced by phosphorylation of hexoses by HK. A sharp elevation in activity of this enzyme was observed in the roots of both S-308 (flood-sensitive) and SSG-59-3 (flood-tolerant) cultivars of sorghum, particularly, during the initial stages of flooding. However, as compared to the corresponding non-flooded plants, the magnitude of increase of HK activity in SSG-59-3 was considerably greater than that in S-308. It is also evident that flooding elicited a differential effect on activities of PFK (Fig. 1*C*) and FBP aldolase (Fig. 2*A*) in roots of these two cultivars of sorghum. Whereas a progressive enhancement in activities of these enzymes occurred in roots of SSG-59-3 during flooding, only a small change was detectable up to about 48 h of the treatment in S-308 followed by a decline. These two cultivars also differed in response of PK activity. There was no enhancement of PK activity in roots after 12 h of flooding of S-308 but in the case of SSG-59-3, the enzyme activity further increased after 24 h.

The results presented also reveal differences in activities of the examined enzymes in leaves of these two cutivars. Thus, unlike in leaves of S-308 where only a minor change in activity of HK was detected, that in SSG-59-3 was elevated to about 2 times during 12 h of flooding of that in the corresponding plants. Similarly, activities of PFK and PK tended to decline in the leaves of flooded plants of S-308 (Fig. 1*D*, 2*D*). In contrast that in SSG-59-3 (Fig. 1*D*, 2*D*) rose on imposition of water-logged conditions. FBP aldolase activity in SSG-59-3 leaves also showed a continuous and steady enhancement during the experiment while that in S-308 remained unaltered following an initial increase. Flooded plants of SSG-59-3 were found to possess 2.13 times FBP aldolase activity than in the non-flooded plants as compared to the corresponding value of 1.37 times in leaves of S-308. Even though leaves of flooded plants were not submerged and remained exposed to air, their metabolism also was evidently perturbed upon flooding. It has previously been demonstrated that flooding causes closure of stomata (Newsome *et al.* 1981), which by hindering free exchange of gases between the leaves and atmosphere,

would create partial anaerobiosis within these organs.

Evidently, with the exception of HK, the tolerant cultivar (SSG-59-3) maintained higher activities of the other glycolytic enzymes. The likely limitation for carrying out fermentative respiration due to restricted supply of hexose phosphate because of failure to maintain high activity of HK could be circumvented by the action of sucrose phosphate synthase. This enzyme catalyzes reversible reaction involving transformation of sucrose to UDP-glucose and fructose-1-phosphate. Similarly, further phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate might be mediated by PP-dependent PFK. Even though during the present studies the influence of flooding on the activities of these two enzymes was not examined, sucrose phosphate synthase and PP-dependent PFK have been implicated in performing these important roles under anoxic conditions (Germain *et al.* 1997, Gibbs *et al.* 2000). Nonetheless from our investigations it appears that the flood-tolerant cultivar SSG-59-3 is endowed with greater metabolic adaptability to sustain fermentative respiration than the sensitive cultivar (S-308) under flooding and this could be one of the important contributory factors for its flood tolerance character.

## References


Bailey-Serres, J., Kloeckener-Gruissem, B., Freeling, M.: Genetic and molecular approaches to the study of the anaerobic response and tissue specific gene expression in maize. - Plant Cell Environ. **11**: 351-357, 1988.

Bouny, J.M., Saglio, P.H.: Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. - Plant Physiol. **111**: 187-194, 1996.

Dennis, D.T., Green, T.R.: Soluble and particulate glycolysis in developing casterbean endosperm. - Biochem. biophys. Res. Commun. **64**: 970-975, 1975.

Dennis, E.S., Gerlach, W.L., Walker, J.C., Lavin, M., Peacock, W.J.: Anaerobically regulated aldolase gene of maize, A chimeric origin? - J. biol. Chem. **202**: 759-767, 1988.

Drew, M.C.: Soil aeration and plant root metabolism. - Soil Sci. **154**: 259-268, 1992.

Dua, A.: Effect of NADH on activity of nitrate reductase in rice leaves. - M.Sc. Thesis, CCS Haryana Agricultural University, Hisar 1987.

Fox, T.C., Green, B.J., Kennedy, R.A., Rumpho, M.E.: Changes in hexokinase activity in *Echinochloa phyllopogon* and *Echinochloa cruspavonis* in response to abiotic stresses. - Plant Physiol. **118**: 1403-1409, 1998.

Germain, V., Ricard, B., Raymond, P., Saglio, P.: The role of sugars, hexokinase and sucrose synthase in the determination of hypoxically induced tolerance to anoxia in tomato roots. - Plant Physiol. **114**: 167-175, 1997.

Gibbs, J., Morrell, S., Valdez, A., Setter, T.L., Greenway, H.: Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. - J. exp. Bot. **51**: 785-796, 2000.

Groves, W.E., Calder, J., Rutter, W.J.: Fructose-1,6-diphosphate aldolase from *Clostridium perfringens*. - In: Wood, W.A. (ed.): Methods in Enzymology. Vol. 9. Pp. 486-491. Academic Press, New York 1966.

Hake, S., Kelley, P.M., Taylor, W.C., Freeling, M.: Coordinate induction of alcohol dehydrogenase-1, aldolase and other anaerobic RNAs in maize. - J. biol. Chem. **260**: 5050-5054, 1985.

Kelley, P.M., Freeling, M.: Anaerobic expression of maize fructose-1,6 diphosphate aldolase. - J. biol. Chem. **259**: 673-677, 1984.

Ling, K.H., Paetkau, V., Marcus, F., Lardy, H.A.: Phosphofructokinase from skeletal muscles. - In: Wood, W.A. (ed.): Methods in Enzymology. Vol. 9. Pp. 425-429. Academic Press, New York 1966.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275,1951.

Mohanty, B., Wilson, P.M., apRees, T.: Effect of anoxia on the growth and carbohydrate metabolism in suspension cultures of soybean and rice. - Phytochemistry **34**: 75-82, 1993.

Nelson, N.: A photometric adaptation of Somogyi's method for the determination of glucose. - J. biol. Chem. **153**: 375-380, 1944.

Newsome, R.D., Kozlowski, T.T., Tang, Z.C.: Responses of *Ulmus americana* seedling to flooding of soils. - Can. J. Bot. **60**: 1688-1695, 1981.

Perata, P., Alpi, A.: Plant responses to anaerobiosis. - Plant Sci. **93**: 1-17, 1993.

Perata, P., Gugliemmetti, L., Alpi, A.: Mobilization of endosperm reserves in cereal seeds under anoxia. - Ann. Bot. **79**: 49-56, 1997.

Sachs, M.M., Subbaiah, C.C., Saab, I.N.: Anaerobic gene expression and flooding tolerance in maize. - J. exp. Bot. **47**: 1-15, 1996.

Somogyi, M.: A new reagent for determination of sugars. - J. biol. Chem. **160**: 61-69, 1945.

Tsai, C.V., Salamini, F., Nelson, O.E.: Enzymes of carbohydrate metabolism in developing endosperm of maize. - Plant Physiol. **46**: 299-306, 1970.

Umeda, M., Uchimiya, H.: Differential transcript levels of genes associated with glycolysis and alcoholic fermentation in rice plants (*Oryza sativa* L.) under submergence stress. - Plant Physiol. **106**: 1015-1022, 1994.