Activities of enzymes of fermentation pathways in the leaves and roots of contrasting cultivars of sorghum (*Sorghum Bicolor* L.) during flooding

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Abstract Flooding evoked a differential response in the activities of enzymes of fermentation pathway in leaves and roots of flood sensitive (S-308) and flood-tolerant (SSG-59-3) cultivars of sorghum. Activities of alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH) and alanine aminotransferase (AlaAT) enhanced in roots of SSG-59-3 during 72 h of flooding. In contrast, a transient increase in the activities was discerned in roots of S-308 up to 24 h flooding followed by a decline in activities of these enzymes. In leaves of SSG-59-3, the activities of ADH and LDH increased to about three fold during flooding stress as compared to that in the non-flooded control plants. Though elevation in activities of these enzymes was observed in leaves of S-308 up to 48 h of flooding, the magnitude of enhancement was much lower than that in SSG-59-3. Alanine aminotransferase activity depressed in leaves of both the cultivars but the level of decline was more pronounced in sensitive cultivar S-308 as compare to tolerant SSG-59-3. The amount of alcohol, lactic acid and alanine were higher in both roots and leaves of SSG-59-3 than that in S-308 during flooding stress. It is thus apparent that roots and leaves of flood tolerant variety tends to attain greater capacity to perform reactions of various fermentation pathways to sustain production of ATP under flooded conditions.

Keywords *Sorghum bicolor* · Alanine aminotransferase · Alcohol · Alcohol dehydrogenase · Flood-sensitive and tolerant cultivars · Lactate dehydrogenase · Lactic acid · Waterlogging

Abbreviations

ADH alcohol dehydrogenase
AlaAT alanine aminotransferase
GPT glutamate pyruvate transferase
LDH lactate dehydrogenase

Introduction

Higher plants have an absolute requirement for oxygen to sustain metabolism and growth. However, quite often they encounter waterlogged conditions of varying duration because of excessive rainfall, which results in depletion of soil oxygen and substantial yield losses (Setter and Waters 2003; Sairam et al. 2009). The biochemical consequence of limitations of oxygen on plant cell metabolism is the inhibition of respiratory ATP synthesis, which leads to a switch over to lower ATP synthesis (Kennedy et al. 1992). In order to meet energy demand of metabolic activities vital for survival, the affected plant parts might be switched over to fermentation mode of respiration thus ensuring sustained production of ATP, albeit at much lower rate (Jackson and Colmer 2005). The biochemical mechanisms for adaptation of plant to O₂ deficiency are not fully understood but the ability to maintain the active fermentative metabolism could be crucial for plant tolerance to anaerobiosis (Perata and Alpi 1993; Sachs et al. 1996; Perata et al. 1997; Boamfa et al. 2003; Ismail et al. 2009). Since three fermentative pathways are known to occur during waterlogging (Dennis et al. 2000), the present investigations were undertaken to ascertain

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whether the enzymes of these fermentative pathways respond differently upon flooding in a flood-sensitive (S-308) and flood-tolerant (SSG-59-3) cultivars of sorghum.

Materials and methods

Materials

Seeds of flood-tolerant (SSG-59-3) and sensitive (S-308) cultivars of sorghum (*Sorghum bicolor* L.) were obtained from the Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. All the biochemicals used were either from the *Sigma Chemical Co.* St. Louis, USA or from *Sisco Research Laboratory*, India.

Seed germination

Seeds of uniform size of both the varieties were surface sterilized and sown under natural condition in a screen house in 30×40 cm polyethylene bags filled with 10 kg washed river sand. The plants were supplied with modified Hoagland nutrient solution (Singla et al. 2004) at 7-d intervals. Twenty-day-old seedlings of each variety were divided in to two groups. One group of was subjected to flooding stress of different duration (0, 6, 12, 24, 48, 72 h) by irrigating them with excessive quantity of nutrient solution of 1/4th strength so that the level of water was 5 cm above the surface of sand. Nutrient solution was replenished daily to maintain this level of water in polyethylene bags throughout the course of the experiment. The other group was grown under normal non-flooded conditions and it served as corresponding control.

Extraction and assay of enzymes and metabolites

Preparation of enzyme extracts Enzyme extracts of roots and leaves were prepared by macerating 1.0 g of the tissue in 5.0 cm³ of 0.1 M cold Tris-HCl buffer (pH 8.0) as described by Bouny and Saglio (1996). Homogenate was gently squeezed through four layers of cheesecloth and centrifuged at 10 000 g for 30 min at 4 °C. The supernatant was used for assaying the various enzymes. Tissue extracts were prepared in three replicates of each treatment and the enzyme assay in an individual extract was performed in duplicate. The data is thus, representative of six determinations of each treatment.

Enzyme assays Alcohol dehydrogenase (ADH; EC 1.1.1.1) activity was determined spectrophotometrically (*LC-340B*, *Calbiochem Behring Corp.*, USA) by measuring the rate of reduction of NAD⁺ (Hanson and Jacobsen 1986). The reaction mixture contained 65 mM Tris-HCl buffer (pH 9.5), 30 mM ethanol, 1 mM NAD⁺ and 0.1 cm³ of enzyme extract. Increase in absorbance due to production of NADH was

recorded at 30 °C. Lactate dehydrogenase (LDH; EC 1.1.1.27) was determined following the rate of reduction of NAD⁺ using lactate as substrate (Hanson and Jacobsen 1986). The assay mixture constituted of 70 mM Tris-HCl buffer (pH 8.5), 40 mM lactate, 1.2 mM NAD⁺ and 0.1 cm³ of tissue extract. Alanine aminotransferase (AlaAT; EC 2.6.1.2) activity was measured by coupling its reaction with lactate dehydrogenase using alanine and 2-oxoglutarate as substrates (Good and Muench 1992). The assay mixture contained 100 mM Tris-HCl buffer (pH 8.0), 25 mM alanine, 5 mM 2-oxoglutarate, 0.15 mM NADH, 2 units of LDH and 0.1 cm³ of tissue extract. Decrease in absorption was recorded spectrophotometrically at 340 nm at 30 °C.

Estimation of soluble protein The amount of soluble protein in the tissue extract was estimated by the method of Lowry et al. 1951.

Extraction and estimation of alcohol, lactic acid and alanine Alcohol, lactic acid and alanine were extracted from freshly harvested samples by the method of Bouny and Saglio (1996). One g of tissue thoroughly washed with cold distilled water was macerated in 5 ml of 10% (v/v) perchloric acid in a chilled pestle and mortar. The homogenate was centrifuged at 10,000×g for 30 min at 4 °C and the supernatant obtained was neutralized with KHCO₃ and again centrifuged at 4,000×g for 15 min at 4 °C.

Amount of alcohol in the supernatant was estimated following its oxidation by alcohol dehydrogenase using NAD⁺ as electron acceptor. The reaction mixture contained 100 mM Tris-HCl buffer (pH 9.0), 15 mM NAD⁺, 2 units of ADH and 0.1 cm³ of tissue extract (Bernt and Gutmann 1974). Increase in absorption due to production of NADH was measured. Lactic acid was determined following its oxidation by lactate dehydrogenase using NAD⁺ as electron acceptor. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 1.5 mM NAD⁺, 2 units of LDH and 0.1 cm³ of tissue extract. The NADH production was measured (Guttmann and Wahlefeld 1974). L-alanine was estimated by converting it to pyruvate using GPT. The pyruvate formed was determined with the aid of LDH using NADH as reductant (Grassl 1974). The reaction mixture consisted of 92 mM Tris-HCl buffer (pH 7.6), 0.6 mM NADH, 7.5 mM α-ketoglutarate, 2 units of LDH and 0.05 cm³ of supernatant.

Statistical analysis

The data was statistically analyzed using complete randomized design (CRD) where each observation was taken in triplicate and, for each replication, the estimation was done in duplicate. The critical difference among variants was calculated at $P \geq 0.05$.

Results and discussion

An almost constant level of alcohol dehydrogenase (ADH) was recorded in the roots of non-flooded (control) plants of both cultivars during the course of experiment (Fig. 1). After 24 h of flooding treatment, the activity of this enzyme increased to 2.1 times to that of respective control plants of flood-tolerant and flood-sensitive cultivars of sorghum. Thereafter, the activity began to decline at steady rate in flood-sensitive cultivar S-308 and at 72 h stage, the increase was non-significant. Contrarily, the roots of flood-tolerant *cv.* SSG-59-3 exhibited a constant increase in ADH activity over a period of 72 h of waterlogging stress and had 2.3 times activity than that in the non-flooded control. Leaves of control plants of S-308 had significantly higher activity than that of SSG-59-3. Flooding for a period of 6 to 48 h resulted in increase in activity of ADH from 28–99% in the sensitive variety S-308 which declined upon extending the period of stress to 72 h. At this stage, ADH was only 17% higher than that in control. In the leaves of resistant cultivar SSG-59-3, ADH activity increased steadily over the course of 72 h to 2.3 times than that in non-flooded control (Fig. 1b). Induction of ADH during anaerobiosis has been observed in many plant species such as maize (Good and Muench 1993; Subbaiah and Sachs 2003), mungbean (Sairam et al. 2009), wheat and barley (Perata et al. 1997) soybean (Mohanty et al. 1993), pigeonpea (Kumutha et al. 2008), rice (Perata et al. 1997; Gibbs et al. 2000) and grasses (Kato-Noguchi 2000). ADH activity has been reported to increase under anoxia in all parts of plants such as roots (Drew 1997), shoots (Muench et al. 1993; Kato-Noguchi 2000), seedlings (Setter et al. 1994) and coleoptile (Perata et al. 1997). Roots of control plants of flood-tolerant SSG-59-3 had significantly higher activity of ADH than that in the sensitive variety S-308. Concomitant with our findings, higher ADH activity has been observed in roots of resistant cultivars of pigeonpea (Kumutha et al. 2008), mungbean (Sairam et al. 2009) and chrysanthemum (Yin et al. 2009).

Flooding stress resulted in increase in the activity of LDH in roots and leaves of both the tolerant (SSG-59-3) and sensitive (S-308) varieties. In roots of S-308, the LDH activity enhanced over a period of 24 h of flooding to 2.5 fold as compared to that of control. Upon extending the period of stress from 24 to 72 h LDH activity declined, however, it was still higher than that in the control. In roots of SSG-59-3, LDH activity increased constantly over a period of 72 h to 3.3 times than that of non-flooded control plants (Fig. 1c). Data presented in Fig 1d. show that LDH activity in leaves of S-308 (control) was almost double than that in the non-flooded control of SSG-59-3. In S-308, an increase in activity from 13 to 40% was observed upon flooding the plants over a period of 6 to 48 h. Subsequent stress

to 72 h resulted in drastic decrease in activity to 80% of that in control plants. However, in leaves of SSG-59-3, activity increased during 6 to 72 h of flooding stress from 30 to 200% as compared to that in control plants. Induction in LDH under waterlogging conditions has been reported in barley, wheat and rice (Hoffman et al. 1986; Couldwell et al. 2009).

From the results presented in Fig. 1e, it is evident that AlaAT activity increased to 2.7 fold in roots of seedlings of S-308 flooded for 24 h and decreased thereafter. At 72 h of stress, activity was nearly the same as that in the control. However, the roots of flooded plants of SSG-59-3 showed an enhancement in activity throughout the duration of experiment. After 72 h of flooding stress, roots of these plants had 2.1 fold more enzyme activity as compare to that in the controls. In contrast, AlaAT declined during flooding stress in the leaves of both the cultivars (Fig. 1f). In SSG-59-3, AlaAT remained almost constant up to 12 h of flooding and impeded thereafter to 85 and 77 per cent respectively at 48 h and 72 h of waterlogging. However, in S-308, AlaAT activity was 22% less at 48 h stress and further decreased to 56% after 72 h of flooding treatment. Good and Crosby (1989b) reported elevation of AlaAT activity in roots of several cereals but activity decreased in leaf tissue in these species. Enhanced activity of AlaAT in seedlings of *Medicago truncatula* under hypoxia (Ricoult et al. 2006) has been reported to be due to up regulation of AlaAT gene (Limami et al. 2008).

The influence of flooding on alcohol concentration in the roots and leaves of these varieties was also monitored (Fig. 2a, b). The amount of alcohol in the roots of treated seedlings of S-308 remained unaffected upon flooding up to 12 h and increased thereafter by 1.4 times over control. Further, flooding up to 72 h resulted in lowering of alcohol content to the level comparable in the control. In SSG-59-3, the flooding for 6 h did not affect alcohol content of roots but thereafter, a marked increase in amount of alcohol occurred and about 6 times more alcohol content accumulated in roots after 72 h of flooding (Fig. 2a). A similar effect of flooding on alcohol content of leaves of these varieties was obtained and after 72 h, SSG-59-3 and S-308 had 87 and 19 per cent higher alcohol content than their respective controls (Fig. 2b). The increase in the amount of ethanol under waterlogging conditions (Kolb and Joly 2009) with higher rate of ethanol synthesis in flood-tolerant cultivar (Gibbs et al. 2000) may help the plant to generate ATP under low oxygen conditions (Ismail et al. 2009).

The amount of lactic acid in the roots of both the cultivars showed a perceptible enhancement when these were subjected to flooding for different periods of time. The maximum increase was observed after 6 h of flooding in both the cultivars with higher level (53%) of enhancement in SSG-59-3 as compared to that in S-308 (Fig. 2c). Further

Fig. 1 Effect of flooding on activity of alcohol dehydrogenase (a, b), lactate dehydrogenase (c, d) and alanine aminotransferase (AlaAT, e, f) in roots (a, c, e) and leaves (b, d, f) of control (□, ○) and flooded (■, ●) plants of sensitive cultivar S-308 and tolerant cultivar SSG-59-3 respectively of sorghum. The bar denotes $\pm$ SE [CD] ($P=0.05$): ADH roots 5.32 (cultivars), 7.98 (treatments), 9.62 (interactions);

ADH leaves 3.78 (cultivars), 5.42 (treatments), 6.39 (interactions); LDH roots 8.91 (cultivars), 9.74 (treatments), 10.53 (interactions); LDH leaves 2.89 (cultivars), 4.07 (treatments), 5.66 (interactions); AlaAT roots 4.31 (cultivars), 6.94 (treatments), 7.39 (interactions); AlaAT leaves 3.55 (cultivars), 5.21 (treatments), 6.79 (interactions)]

increase in the period of stress to 72 h caused progressive decline in the amount of lactic acid to the level of control. A similar effect of flooding on lactic acid content in the leaves of both the varieties was obtained with maximum accumulation after 6 h of flooding stress (Fig. 2d). The initial burst of lactic acid production during flooding stress reported in the present investigation is known to occur when plant tissue are transferred from aerobic to anaerobic conditions (Greenway and Gibbs 2003; Felle 2005).

In the roots of S-308, alanine content increased upon flooding up to 24 h to 1.67 times than that in the control. Further flooding up to 72 h resulted in decline in alanine content to the level of the control. However, in the roots of SSG-59-3, alanine content increased steadily with the increase in period of stress to 72 h and at this stage the roots possessed about 3.6 times the alanine content than that in the control (Fig. 2e). Similar effect of waterlogging was discerned in the leaves of both the varieties, with maximum alanine contents at

Fig. 2 Effect of flooding on activity of alcohol (a, b), lactate (c, d) and alanine (e, f) in roots (a, c, e) and leaves (b, d, f) of control ($\square$ -, $\circ$ -) and flooded ($\blacksquare$ -, $\bullet$ -) plants of the sensitive cultivar S-308 and tolerant cultivar SSG-59-3 respectively of sorghum. The bars denote $\pm$ SE [CD ($P=0.05$): alcohol roots 2.93 (cultivars), 5.58 (treatments), 6.11 (interactions); alcohol leaves 3.46 (cultivars), 4.82 (treatments),

5.99 (interactions); lactate roots 1.91 (cultivars), 3.15 (treatments), 5.04 (interactions); lactate leaves 2.42 (cultivars), 4.25 (treatments), 4.87 (interactions); alanine roots 2.71 (cultivars), 4.24 (treatments), 5.30 (interactions); alanine leaves 3.02 (cultivars), 4.75 (treatments), 7.08 (interactions)]

24 h in S 308 ($102.3 \mu\text{mol g}^{-1}$ fresh weight) and a progressive increase up to 72 h of flooding stress in the resistant variety SSG-59-3 ($465.7 \mu\text{mol g}^{-1}$ fresh weight) (Fig. 2f). Alanine content has been reported to enhance under anaerobic conditions (Good and Muench 1993; Ricoult et al. 2006).

One of the important consequences of flooding is the blockade of transfer of air and oxygen between the soil and

the atmosphere, thereby lowering the partial pressure of oxygen in the root zone. The stomata of plants growing under flooding conditions also undergo closure (Sena-Gomes and Kozłowski 1980; Newsome et al. 1981) which prevents free exchange of gases between the leaves and atmosphere. The low oxygen conditions within the plant tissues evidently impairs the oxidation of NAD(P)H and

generation of ATP through mitochondrial electron transport chain, conceivably survival of plants under oxygen limiting condition might be related to their capacity to carry anaerobic mode of fermentative respiration so that plant can meet the requirement for ATP despite the much lower efficiency of ATP production (Sairam et al. 2009). In order to fulfill the energy demand of cells, higher rate of fermentation would be required. This could be accomplished by elevation in the activities of the enzymes of fermentation pathways viz. pyruvate decarboxylase, ADH, LDH and AlaAT which catalyze the reactions leading to the production of ethanol, lactic acid and alanine. In the present investigations, changes in activities of these enzymes in the flood-tolerant and flood-sensitive cultivars during flooding were examined. Alcoholic fermentation has been recognized as the principal catalytic pathway for recycling NAD^+ to maintain glycolysis and substrate level phosphorylation in the absence of oxygen (Kennedy et al. 1992; Good and Muench 1993). An increase in the activity of ADH was observed in the roots and leaves of both S-308 (flood-sensitive) and SSG-59-3 (flood-tolerant) cultivars of sorghum. However, as compared to the corresponding non-flooded plants, the magnitude of increase in ADH was greater and up to 72 h of flooding in SSG-59-3 while in S-308, the increase was only upto 24 h and 48 h of treatment in roots and in leaves respectively (Fig. 1a, b). Similar effect of flooding stress was observed on the activity of LDH in roots and leaves of these two cultivars (Fig. 1c, d). It is also evident that flooding elicited a differential response on activity of AlaAT (Fig. 1e, f) in these cultivars of sorghum. Whereas a progressive enhancement in activity of this enzyme occurred in roots of SSG-59-3 after 12 h flooding till the duration of the experiment (72 h), only a small change was detectable up to 24 h of treatment in S-308 followed by a decline. Like ADH and LDH, the amount of ethanol and lactic acid increased in roots of both the varieties. However, increase was more and for longer duration in both the tissues of treated seedlings of SSG-59-3 as compared to that of S-308 (Fig. 2a–d). Alanine content also showed the similar trend (Fig. 2e, f) though there was decline in the activity of AlaAT in S-308.

The results presented also reveal prominent differences in behavior of the examined enzymes in leaves of these cultivars of sorghum. Thus, unlike the leaves of S-308, where a change in the activity of ADH was detected up to 48 h, that in SSG-59-3, was elevated to about 2.3 folds during 72 h of flooding of that in the control plants. Similarly, activities of LDH also increased in the leaves of both cultivars but tended to decline after 48 h of treatment in S-308 whereas in leaves of SSG-59-3, enhancement was observed during the course of investigation. In contrast, AlaAT declined

in leaves of S-308 but enhanced transiently up to 6 h of flooding in that of SSG-59-3 (Fig. 2f). Even though leaves of flooded plants were not submerged and remained exposed to air, their metabolism was also evidently perturbed upon flooding. It may be because of closure of stomata by flooding (Newsome et al. 1981) which by prohibiting free exchange of gases between the leaves and atmosphere would create partial anaerobiosis within these organs.

Results obtained during the present investigation indicate that activities of all the examined enzymes were elevated and maintained at consistently higher levels throughout the duration of experiment in roots of flood-tolerant cultivar (SSG-59-3) than in flood-sensitive S-308 variety. More or less similar differences were also perceived in response of enzymes in the leaves of these cultivars of sorghum. The only exception was AlaAT that declined after initial burst. In addition whereas the amount of alcohol, lactic acid and alanine in roots of sensitive cultivar remained almost at par with that in the non-flooded control, the level of these metabolites in the tolerant cultivar was higher than that in the control.

Accumulation of significant amount of alcohol and alanine under waterlogging conditions shows the operation of ethanol and alanine fermentation pathways flooding. Higher levels of these molecules in leaves and roots of tolerant cultivar (SSG-59-3) than that in S-308 indicate higher rate of fermentation thus endowing the better survival capacity to SSG-59-3 under anaerobic conditions than S-308. Transient burst of lactic acid production seems to acidify the cytoplasm (Couldwell et al. 2009), thereby, triggering ethanol fermentation (Fox et al. 1995). Induction in the activity of enzymes of fermentation pathways in both the species of sorghum under waterlogged conditions may function as a metabolic to rescue when respiration is arrested. From our investigations, it appears that the flood tolerant variety SSG-59-3 is endowed with greater metabolic adaptability to sustain fermentative respiration than the sensitive variety, S-308 under flooded conditions and this could be one of the contributory factors for its flood tolerance.

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