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Research Article
Biochemical Traits at Reproductive Stage in Six Rice Genotypes under Drought Stress

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Abstract
Background and Objective: Drought is one of the main limitations of rice (*Oryza sativa* L.) productivity and is a severe problem in many regions of the world. In Bangladesh, drought affects approximately 11.54 million ha of the area under rice cultivation and usually occurs during the reproductive stage. Responses of six rice genotypes subjected to different drought levels (40% FC) and control (100% FC) were investigated in the laboratory to evaluate drought tolerance mechanism(s) based on osmolytes at the reproductive stage in rice genotypes. **Materials and Methods:** Twelve treatments (6 genotypes×2 irrigations) were arranged in CRD and the experiment was carried out at Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh, in two seasons. Drought was imposed at the reproductive stage where important biochemical traits of flag leaf at 75 days after transplanting were estimated. **Results:** During drought stress, proline and sugar accumulation increased and nitrate reductase activity reduced intolerant genotypes compared to sensitive ones. Proline and total sugar significantly increased under drought and nitrate reductase activity reduced during the drought which discriminated two genotypes consistently as drought tolerant. **Conclusion:** Due to increase osmolytes accumulation (proline and total soluble sugar) and decrease osmolyte like nitrate reductase activity of flag leaf at the reproductive stage, it may be concluded that the solute accumulation is one of the mechanisms for drought tolerance in rice genotypes.

Key words: Rice, drought stress, nitrate reductive activity, proline, total soluble sugar, reproductive stage

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Drought leading to water stress in plants is a major problem in reducing agricultural productivity especially in tropical, semi-arid and arid regions of the world. Drought is one of the major abiotic stresses that severely affect and reduce the yield and productivity of food crops worldwide up to 70%[1]. Under drought, the maintenance of leaf turgor may also be achieved by the way of osmotic adjustment in response to the accumulation of proline, sucrose, soluble carbohydrates, glycine betaine, nitrate reductase activity and other solutes in the cytoplasm which improve water uptake from drying soil. The process of accumulation of such solutes under drought stress is known as an osmotic adjustment which strongly depends on the rate of plant water stress[2]. Proline accumulation is a well-known metabolic response of plants to drought and other stresses[3]. Proline permits osmotic adjustment, stabilizes the structure of proteins and cell membranes, acts as a protective agent for enzymes and is a free radical scavenger and antioxidant[4-6]. The proline content increased as the drought stress progressed and reached a peak as recorded after 10 days stress and then decreased under severe water stress as observed after 15 days of stress[7]. Accumulation of proline under stress in many plant species has been correlated with stress tolerance[8]. In rice, the concentration of proline was remarkably increased during drought stress[9]. Abdula *et al.*[10] found that the increase of proline biosynthesis enhanced abiotic stress tolerance in rice varieties. The physiological response of the increase and accumulation of total soluble sugars in the leaves of plants is another important factor and is influenced by drought stress. The accumulation of soluble sugars in plants under drought stress is a result of a series of metabolism interactions. A complex essential role of soluble sugars in plant metabolism is well known as products of hydrolytic processes, substrates in biosynthesis processes, energy production but also in sugar sensing and signalling systems. Soluble sugars (sucrose, glucose and fructose) play an important role in maintaining the overall structure and growth of plants[11]. Lemoine *et al.*[12] suggested that soluble sugar regulation in plants was a very complex manner. Soluble sugar maintains the leaf water content and osmotic adjustment of plants facing the conditions of drought stress[13]. Recently it has been claimed that, under drought stress conditions, even sugar flux may be a signal for metabolic regulation[14]. Mostajean and Eichi[9] suggested that solute accumulation is one of the mechanisms for drought tolerance in rice. Measurement of nitrate reductase activity enzyme (NRA) becomes a key character in plant selection, especially Cempo Ireng black rice (*O. sativa*) results in predicting productivity because this enzyme is the first key in the pathway of synthesis of organic nitrogen compounds which have important aspects in the plant life cycle[15]. The results of research on several plants showed a positive correlation between NRA and yield. This underwater stress, leaf NR activity decreases[16-22]. The decrease in the quantity of NRA observed following the onset of water deprivation may have been caused by the decrease in foliar NO_3^-, [22-24]. Though so many research works regarding the effects of drought on biochemical traits in rice were carried out all over the world but little works on rice in these aspects in flag leaves at the reproductive stage were done especially in Bangladesh. So, considering the above lacking, the present research was carried out to evaluate drought tolerance mechanism(s) based on osmolytes behaviour at the reproductive stage in rice genotypes.

MATERIALS AND METHODS

Study area: The study was conducted at the Crop Physiology and Biochemical Laboratory of the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh from September, 2015 to October, 2016.

Sample collection: The pot experiment was conducted with six rice genotypes viz. Binadhan-13, BRRIdhan34, Ukunimadhu, RM-100-16, Kalizira and NERICA mutant. The soil was collected from the field of BINA Farm. The topsoil was non-calcareous dark grey floodplain with loamy texture belonging to the AEZ Old Brahmaputra Floodplain. The collected soil was pulverized. Inert materials, visible insect pests and plant propagules were removed. The soil was dried in the sun, crushed carefully and thoroughly mixed.

Methodology: The experiment was set in a two factorial CRD with three replications in two seasons. The first factor was rice genotypes and the second factor was irrigations: Control (100% FC) and drought (40% FC) stresses treatments. Drought (40% FC) was imposed at anthesis till maturity. Biochemical parameters like proline and total sugar were studied from the flag leaf samples 75 days after sowing.

Determination of proline: Proline accumulation in fresh flag leaves was determined according to the method[25]. Free proline was extracted from the leaves of plants using aqueous sulfosalicylic acid. The filtrate (1 mL) was mixed with equal volumes of glacial acetic acid and ninhydrin reagent (Dalynn Biologicals-Catalogue No. RN70) (1.25 g ninhydrin, 30 mL of glacial acetic acid and 20 mL 6 M phosphoric acid) and incubated for 1 hr at 100°C. The reaction was stopped by placing the test tubes in cold water. The samples were vigorously mixed with 3 mL toluene. The light absorption of the toluene phase was estimated at 520 nm using Pharmacia LKB- Novaspec II model spectrophotometer (Sweden). The proline concentration was finally determined using a standard curve. Free proline content was expressed as μmol g^{-1} of plant parts.

Determination of total sugar: Total sugar content was determined according to the procedure outlined by Hu *et al.*[26]. Total sugar was extracted by boiling the fresh flag leaf tissue (0.1 g) in 5 mL of 80% ethanol contained in a test tube for 10-15 min in a water bath. The extraction was repeated at least twice with an additional 5 mL of 80% ethanol. To remove the pigment chloroplast, the leaf extract solution was made up to 20 mL volume with distilled water. Then 0.5 mL of leaf extract was taken in a test tube and 0.5 mL of 5% phenol solution was added. Thereafter, 3 mL concentrated H_2SO_4 was added to the mixture and it became hot and appeared orange in colour. Total sugar content was determined according to the procedure outlined by Hu *et al.*[26]. After cooling the supernatant at room temperature, the optical density was measured at 490 ηm wavelengths using the spectrophotometer (Systronics UV-VIS 118-India) and the result was expressed in mg g^{-1} fw.

Determination of nitrate reductase (NRase): Nitrate reductase (NRase) in the flag leaf at the reproductive stage was determined following the method of Ahmad *et al.*[27]. Buffer solution (assay solution) was prepared with 0.1 M potassium buffer solution at pH 7.5. This buffer solution was prepared by mixing the solution of K_2HPO_4 and KH_2PO_4, 1.5% potassium nitrate (w/v), 1.5% propanol (v/v) and 0.05% Nutritionix or triton x-100. 1% Sulphanilic acid was prepared by dissolving in 3 M H_2SO_4 and 0.2% NEDD (N-1-Napthylethylenediamine dihydrochloric-acid) in 100 mL distilled water. About 0.05 g chopped fresh leaf sample was poured in 5 mL assay solution in 20 mL vial. The solution was infiltrated into the sample by shaking in a vacuum desiccator's (Terra Universal, USA) for 15 min and was incubated in a water bath at 28-30°C for 1/2 hrs. About 1 mL sulphanilic acid was taken in a 10 mL test tube, 1 mL incubated extracted solution was pipetted in the test tube containing sulphanilic acid then 1 mL of 0.02% NEDD solution was added and allowed 20-25 min for colour development. Finally, reading was taken at 540 ηm wavelengths by a spectrophotometer (Systronics UV-VIS, 118-India). The NRA was expressed in μmol NO_2^- g^{-1} fw h^{-1}. The percent reduction of each parameter under drought stress compared to control was calculated for each genotype using the formula:

$$\text{Relative increase or decrease (\%)} = \frac{\text{Data for control treatment-Data for drought treatment}}{\text{Data for control treatment}} \times 100$$

Mathematically, the (-) ve value indicate an increase and (+) ve one decrease.

Statistical analysis: The collected data were analyzed statistically following Completely Randomized Design by R software computer package programme developed[28-30]. Duncan's Multiple Range Test (DMRT) adjudged the treatment means[31].

RESULTS

The interaction effect of drought (40% FC) and control (100% FC) irrigations with genotypes on proline, total sugar accumulation and nitrate reductase activity was significant at $p \leq 0.05$ (Table 1-2).

Table 1 showed that 40% FC exhibited more or less inhibitory effects compared to control in both the years. The genotypic variation among the genotypes based on proline accumulation (μ mol g^{-1} fw), sugar accumulation (mg g^{-1} fw) and NRA (μmol NO_2 g^{-1} fwt h^{-1}). In both seasons, all the genotypes showed high proline accumulation in 40% FC compared to control. Proline accumulation of those genotypes ranged from 0.157-2.788 and 0.102-1.961 (μ mol g^{-1} fw) in two consecutive years, respectively. From the result of two years, it was observed that genotypes: NERICA mutant and BINA dhan-13 had higher in season 1 and 2. The total sugar at the reproductive stage of those genotypes ranged from 1.113-1.730 and 1.023-1.568 (mg g^{-1} fw) in two consecutive years, respectively. From the result of two years it was observed that genotypes: NERICA mutant, BINA dhan-13 and RM100-16 had higher in season 1 and 2, lower in Kalizira, Ukunimodhu and BRRI dhan-34 in both seasons. Nitrate reductase activity varied from 0.186-0.246 and 0.121-0.166 (μmol NO_2 g^{-1} fwt h^{-1}) in both seasons, respectively. One genotype NERICA mutant had lower (0.221 and 0.166 μmol NO_2 g^{-1} fwt h^{-1}) NR activity.

In Table 2, In season 1, a relative increase of proline accumulation (μ mol g^{-1} fw) was higher in the genotypes Binadhan-13 (-127.27) and NERICA mutant (-36.21) (average of 81.74%) compared to others (average of 18.79%). In season 2, it was also higher in the above two genotypes (average of 74.43%) compared to the rest (average of 7.81%), except genotype RM-100-16 which genotype did not show any

Table 1: Effect of drought (40% FC) on nitrate reductase activity (NRA), proline and total sugar during grain filling period of six rice genotypes in two seasons

Treatments	Proline (μmol g^{-1})	Total sugar (mg g^{-1} f wt)	NRA (μmolNO$_2$ g^{-1} fwt h^{-1})
Season-1			
Drought			
Control	1.375^{b}	1.637^{a}	0.254^{a}
40% FC	1.670^{a}	1.346^{b}	0.187^{b}
Genotypes			
Binadhan-13	2.788^{a}	1.510^{ac}	0.242^{a}
Kalizira	0.157^{b}	1.458^{bc}	0.246^{a}
RM-100-16	0.198^{b}	1.443^{c}	0.186^{c}
Ukunimodhu	2.74^{b}	1.113^{d}	0.20^{bc}
BRRIdhan34	2.747^{a}	1.695^{ab}	0.228^{ab}
NERICA mutant	2.297^{a}	1.730^{a}	0.221^{a-c}
Season-2			
Drought			
Control	0.948^{b}	1.236^{b}	0.181^{a}
40% FC	1.095^{a}	1.428^{a}	0.113^{b}
Genotypes			
Binadhan-13	1.847^{b}	1.392^{abc}	0.140^{ab}
Kalizira	0.102^{c}	1.358^{bc}	0.146^{ab}
RM-100-16	0.187^{c}	1.429^{ab}	0.121^{b}
Ukunimodhu	0.194^{c}	1.023^{d}	0.143^{ab}
BRRIdhan34	1.838^{b}	1.221^{c}	0.165^{a}
NERICA mutant	1.961^{a}	1.568^{a}	0.166^{a}

Values under each factor having a common letter(s) in a column do not differ significantly at $p \leq 0.05$ as per DMRT

Table 2: Combined effect of genotype and drought (40% FC) with control (100% FC) irrigations on proline and total sugar in six aromatic rice genotypes in two seasons

Genotypes	Irrigation	Proline (μ mol g^{-1} fw)	Total sugar (mg g^{-1} fw)	NRA (μmol NO$_2$ g^{-1} fw h^{-1})
Season 1				
Binadhan-13	100% FC	0.12^{d}	1.19^{de}	0.266^{a+}
	40% FC	0.28^{d} (-127.27)	1.69^{abc} (-42.6)	0.22^{ab} (18.05)$^{++}$
Kalizira	100% FC	0.15^{d}	1.45^{cd}	0.26^{a}
	40% FC	0.17^{d} (-16.55)	1.47^{cd} (-1.17)	0.23^{ab} (11.83)
RM-100-16	100% FC	2.57^{bc}	1.85^{ab}	0.21^{abc}
	40% FC	2.92^{ab} (-13.49)	1.54^{bcd} (16.91)	0.16^{cd} (25.70)
Ukunimodhu	100% FC	2.71^{bc}	1.02^{ee}	0.25^{a}
	40% FC	3.23^{a} (-19.06)	1.20^{de} (-17.38)	0.15^{d} (42.52)
BRRIdhan34	100% FC	2.47^{c}	1.39^{cd}	0.27^{a}
	40% FC	3.11^{a} (-26.06)	1.63^{abc} (-17.33)	0.19^{bcd} (31.11)
NERICA mutant	100% FC	0.232^{d}	1.49^{cd}	0.26^{a}
	40% FC	0.32^{d} (-36.21)	1.97^{a} (-32.68)	0.19^{bcd} (27.63)
Season 2				
Binadhan-13	100% FC	0.116^{cde}	1.02^{d}	0.15^{cd}
	40% FC	0.27^{c} (-135.34)	1.43^{b} (-40.12)	0.13^{cde} (14.29)
Kalizira	100% FC	0.09^{e}	1.27^{bc}	0.16^{bc}
	40% FC	0.10^{de} (-5.05)	1.45^{b} (-13.85)	0.13^{cde} (19.13)
RM-100-16	100% FC	0.27^{cd}	1.44^{b}	0.16^{c}
	40% FC	0.11^{de} (59.02)	1.41^{b} (2.21)	0.12^{c-e} (27.60)
Ukunimodhu	100% FC	1.88^{ab}	0.97^{d}	0.22^{ab}
	40% FC	2.04^{a} (-8.28)	1.08^{cd} (-11.69)	0.11^{cde} (47.71)
BRRIdhan34	100% FC	1.75^{b}	1.35^{b}	0.16^{c}
	40% FC	1.93^{a} (-10.11)	1.43^{b} (-6.30)	0.09^{e} (45.22)
NERICA mutant	100% FC	1.73^{b}	1.37^{b}	0.23^{a}
	40% FC	1.96^{a} (-13.52)	1.76^{a} (-28.41)	0.09^{de} (58.79)

$^{+}$Data were separately analyzed for both seasons, in a year in each column, figures having a common letter(s) do not differ significantly at $p \leq 0.05$ as per DMRT, $^{++}$Figures within parenthesis indicates (%) decrease (+) or increase (-) at 40% FC compared to control

increase (59.02) (Table 2). These results pointed out that the magnitude of proline accumulation varied between the seasons and genotypes. However, in both the years, Binadhan-13 and NERICA mutant had consistently shown higher relative increase under drought compared to control (100% FC). In season 1, the relative increase of sugar accumulation (mg g^{-1} fw) was higher in genotypes Binadhan-13 (-42.6) and NERICA mutant (-32.68) (average of 37.65%) compared to all remainders (average of 11.96%). In season-2, it was also higher in genotypes Binadhan-13 (-40.12) and NERICA mutant (-28.41) (average of 34.27%) compared to other genotypes (average of 10.61%) (Table 2). These results pointed out that the magnitude of sugar accumulation enhancement varied between the seasons and genotypes. However, in both years, it was consistently higher in Binadhan-13 and NERICA mutant. In season-1, the magnitude of NRA relative reduction was lower in genotypes Binadhan-13 (18.05) and Kalizira (11.83) (average of 14.94%) compared to others (average of 31.74%). In season-2, once again it was lower in genotypes Binadhan-13 (14.29) and Kaizira (19.13) (average of 16.71%) compared to the rest (average of 44.83%) (Table 2).

These results pointed out that the magnitude of NRA relative reduction varied between genotypes and seasons but the pattern was almost similar in the genotypes. The above results indicated that the genotypes Binadhan-13 and NERICA mutant had lower NRA reduction, higher proline and sugar accumulation indicating irrespective of genes greater tolerance to drought and that these biochemical traits, particularly proline and soluble sugar could be better drought selection index.

DISCUSSION

In this 2 years study, we analyzed the effects of drought stresses and subsequent recovery on the accumulation and degradation of proline, soluble sugar and nitrate reductase activity content in different rice genotypes at the reproductive stage. Proline is one of the most common compatible osmolytes in water-stressed plants. The proline accumulated in plants under water stress can protect the cell by balancing the osmotic potential of cytosol with that of vacuole and external environment[32]. In rice leaves, the concentration of proline accumulation tended to be increased under drought stress. In both the years, at 40% FC the degree of the relative increase in leaf proline accumulation was more than 5 times higher in Binadhan-13 and NERICA mutant than remainder genotypes. This suggests that proline accumulation in the flag leaf was less affected by drought in the former than in the latter group of rice genotypes. It could be argued that the 5 times higher proline accumulation might have helped osmotic adjustment thereby water potential, leading to stable cell growth, stomatal diffusions and photosynthesis[33]. The results expressed that proline was highly accumulated in leaves of rice plants under drought stress compared to control conditions and that more severe drought stress resulted in more proline accumulation. These results agreed with other previous studies, which mentioned that proline is highly accumulated under drought stress conditions[3,9,32]. The role of proline in the adaptation and survival of plants under drought was also observed by Watanabe *et al.*[33] and Saruhan *et al.*[34] in cereals. Accumulation of proline content under drought stress indicates accumulated proline might act as a compatible solute regulating and reducing water loss from the plant cell during water deficit[30] and play important role in osmotic balance[31]. Proline accumulates under stress also supplies energy for survival and growth and thereby helps the plants to tolerate stress condition[34]. Studies have indicated that soluble sugars are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source organs to sink organs[11]. Several studies have shown that the soluble sugar content increases under drought stress. The physiological response of the increase and accumulation of total soluble sugars in the leaves of plants is another important factor and is influenced by drought stress. In both the years, at 40% FC the degree of increase of total sugar accumulation in flag leaf was more than three-fold in Binadhan-13 and NERICA mutant than in the other three genotypes. It suggests that total soluble sugar accumulation in the flag leaf was less affected by drought in tolerant genotypes. This suggests that the drought-sensitive genotypes had a lower amount of assimilating to support the developing grains as compared to the drought-tolerant genotypes that had a higher amount of assimilating to feed the grains. The accumulation of sugars in response to drought stress is also quite well documented[11,12]. Recently it has been claimed that, under drought stress conditions, even sugar flux may be a signal for metabolic regulation[9,15]. Previously[9] suggested that solute accumulation is one of the mechanisms for drought tolerance in rice[35]. This study also assayed nitrate reductase activity in the flag leaf under drought stress during grain filling. Increased drought stress will reduce NRA due to a decrease in the gradient of water potential between the environment and plant tissue, which plants respond to by closing the stomata mechanism on the leaves to maintain the plant's water potential. Closure of the stomata will cut the supply of carbon dioxide to mesophyll cells as a result, the rate of photosynthesis in these cells is reduced by the level of water shortage. According to Sing *et al.*[32], the smaller the efficiency of photosynthesis will affect the amount of reducing power (NADPH2/NADH2) produced so that electrons are not available in normal quantities. The resulting NADPH2 is not enough to support the activity of the nitrate reductase enzyme, so increasing drought stress can reduce nitrate reductase activity[15], activity of the enzyme in plants gives the best estimate of the nitrogen status of the plant and is very often correlated with growth[33,34]. In situations of water deprivation, maximal foliar extractable NRA was decreased[17,33,34,36,37] which is similar to the current result. The decrease in the quantity of NRA observed following the onset of water deprivation may have been caused by the decrease in foliar NO_3^-[24,36,37]. Metabolism of nitrate reductase activity in rice was affected by both drought stress conditions and characteristics of genotypes.

CONCLUSION

Proline and soluble sugar starch contents in leaves of rice genotypes were significantly increased under drought stress with some exceptions in comparison with susceptible genotypes. Nitrate reductase activity content in leaves of rice genotypes was significantly decreased. In comparison with susceptible genotypes, the Nitrate reductase activity content of tolerant varieties was less negatively affected by drought stresses. The smallest decrease in reductase activity and increase in value, especially in the parameters of proline and total soluble sugar show the ability of plants to defend themselves against drought stress. Nitrogen reductase activity (NRA), proline and total sugar accumulation showed interaction effects between genotype and irrigation treatments and two genotypes had shown, respectively lower, higher and higher values under drought compared to control irrigation (100% FC). Therefore, osmoprotectants like increased proline and total sugar contents and decreased nitrate reductase activity in flag leaf at reproductive stage may also be considered another important index of drought-tolerant mechanism in rice genotypes. Thus, characters may be considered during the development of drought tolerance genotypes.

SIGNIFICANCE STATEMENT

This study discovers biochemical traits like increased proline and total sugar contents which may be considered an important index of drought-tolerant mechanism in aromatic rice genotypes. This study will help the researcher to uncover the critical areas of yield attributes of rice genotypes under drought stress particularly at the reproductive stage that can be beneficial for drought-prone areas of Bangladesh, that many researchers were not able to explore. Thus a new theory on the development of drought-tolerant rice genotypes may be arrived at.

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