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Article

Proline and ascorbic acid content in leaves of NERICA mutant rice lines and their parents under rainfed condition

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Abstract: Proline and ascorbic acid are important osmolytes responsible for drought tolerance in rice. The present study was conducted to determine proline and ascorbic acid content in the leaves of NERICA mutant lines and their parents under rainfed condition. The experiment was conducted in the Biotechnology division of Bangladesh Institute of Nuclear Agriculture and in the laboratory of the department of Biochemistry and Molecular Biology, Bangladesh Agricultural University, Mymensingh. Proline and ascorbic acid contents were determinated by the method developed by Bates *et al*. 1973 and titrimetric method (Reo, 1954) respectively with three replication. As the experiment was conducted under rainfed condition and due to self-drought tolerance of NERICA lines, proline and ascorbic acid accumulate in significant amount. Free proline content in the leaves of mutant rice lines varied from 9.38 mg/100g to 25.46 mg/100g with a mean value of 17.59 mg/100g. Here maximum proline content was found in N_1/300/P-8-3-3 (25.46mg/100g) and minimum in N_4/350/P-2(1)-32-11 (9.38mg/100g). Ascorbic acid content in the leaves of rice plant varied from 1.12mg/100g to 1.98 mg/100g with a mean value of 1.59 mg/100g. Here maximum accumulation of ascorbic acid was found in N_1/250/P-7-2-1 (1.98mg/100g) and minimum in N_1/250/P-7-3-11 (1.12mg/100g).

Keywords: proline; ascorbic acid; NERICA mutant lines

1. Introduction

NERICA (New Rice for Africa) varieties of African origin has been introduced in Bangladesh to ensure the productivity of rice in drought condition and subsequently mutant lines are also developed for improving the performance further. proline is the most widely studied compatible solute synthesized from L-glutamic acid via pyrroline-5-carboxilate (P5C). Proline accumulates in many plant species in response to environmental stress such as drought, temperature and starvation starvation (Sairam *et al.*, 2002). High levels of proline enable a plant to maintain low water potentials. By lowering water potentials, the accumulation of compatible osmolytes involved in osmoregulation allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortages within the organism (Kumar *et al.* 2003). Ascorbic acid (AA) currently holds a significant position in plant physiology, mainly due to its possession of antioxidant and cellular reductant properties and its diverse roles in plant growth and development and the regulation of a broad spectrum of plant cellular mechanisms against environmental stresses.

2. Materials and Methods

2.1. Plant materials

The experiment was carried out with 31 rice germplasms in which promising 28 mutant lines from NERICA and 3 NERICA parental lines.

2.2. Mutant line production

The seeds of mother NERICA plants were treated with physical (250, 300 and 350 gamma-rays, unit is roentgen, R) mutagens and were grown as M_1 generation. Populations were grown with advanced mutants. Finally, 28 NERICA mutant lines were selected from M_4 and M_5 generations for this experiment.

2.3. Seedbed preparation

Seed bed was prepared by raising soil up to 5-10 cm from the field surfaces followed by puddling. Before puddling cow dung was applied @ 2kgm-2. The entire seed bed was than divided into three seed beds and small plots (50 cm X 50 cm) were prepared considering the 31 rice genotypes. Between the plots 10 cm distance was maintained. Drainage channels (30 cm) were prepared between seed beds to drain out excess water whenever needed.

2.4. Processing of seeds

After collection, admixtures were separated and the seeds were kept in an incubator oven for five days at 500C temperature for breaking of seed dormancy for better germination.

2.5. Sowing of pre-germinated seeds

The seeds were soaked into water for 24 hours and incubated in moist cloth sacks for 48 hours for quick germination. The pre-germinated seeds were sown in seedbed on 29th March, 2014.

2.6. Collection of leaf sample

Leaf samples were collected from young, vigorous leaves from 21 days old seedlings for biochemical studies. At first, the healthy portion of the youngest leaves of the tiller were cut apart with sterilized scissors and washed in ethanol (70%) and distilled water. The collected leaf samples were then kept in polythene bags with marking. The bags were placed in an ice box to carry it in the laboratory for avoiding any damage of the leaf tissues. Finally, the samples were stored in (-) 800 C freezer.

2.7. Estimation of proline

The fresh leaf sample was collected at the seedling stage of rice. Proline content of leaves was determined according to the method developed by Bates *et al.* 1973. The overall procedure is given below:

2.7.1. Reagents

a. 3% aqueous sulfoselicylic acid
b. Acid ninhydrin: 1.25g of ninhydrin was dissolved in warm mixture of 30ml glacial acetic acid and 20ml of 6 M phosphoric acid with agitation, reagent stored at 4^0C for 24 hours.
c. Toluene

2.7.2. Working procedure

Fifty milligrams of fresh leaf sample was homogenized in a mortar with pestle using 10ml of 3% sulfo salicylic acid. The homogenate was centrifuged and then filtered through Whatman no. 1 filter paper. The extraction procedure was repeated and the two portions of the filtered were taken together. Two milliliter of the filtered was pipette into the test tube and 2ml acid ninhydrin and 2ml glacial acetic acid were added to it and the mixture was shaken well. The test tubes were incubated for one hour at 100^0C in a hot water bath. They were then transferred to an ice bath to terminate the reaction.

Four milliliter of toluene was added to each of the test tube, which was stirred vigorously for 15-20 seconds. The toluene the chromophore was separated from the aqueous phase and collected carefully. Absorbance of the collected toluene was measured at 520nm in a vis/uv spectrophotometer (Shimadzu, UV-1201) against reagent blank.

A standard curve was prepared with analytical grade proline and proline contents in sample were calculated by using the standard curve (Fig.1). Each analysis was done in duplicate from fresh leaf sample. Finally, the percentage of proline present in the leaves was expressed as mg/100g fresh leaves.

2.8. Estimation of ascorbic acid

Ascorbic acid was extracted with 6% Meta phosphoric acid from the leaf of rice was estimated by titrimetric method (Reo, 1954).

The following reagents were used for the estimation of L-ascorbic acid**.**

6% Meta phosphoric acid ($HP0_3$) solution: Sixty grams of Meta phosphoric acid was dissolved in about 500 milliliter distilled water. Eighty milliliters of glacial acetic acid was added and volume was made up to 1 liter with distilled water.

Dye solution (2, 6-dichlorophenol indophenol): Forty two milligrams of sodium bicarbonate and 52 mg of 2, 6-dichlorophenol indophenol was dissolved in small volume of water and make up to 200 mL with distilled water. The reagent was stored in a flask at 4°C and was used for 5 days.
Ascorbic acid standard solution: Ten milligram of pure ascorbic acid was dissolved in 100mL of 6% Meta phosphoric acid solution.

2.8.1. Extraction
Plant leaves were cut into pieces of 2-3 mm size. Portions of near about 1g of cut pieces were placed in the blender and 6% Meta phosphoric acid was added at the rate of 50 mL for 1g leaves. The plant tissues were crushed for 3 minutes with blender. The extract was filtrated through two layers of cloth. After filtration, the volume was made unto 50 mL with 6% Meta phosphoric acid. The filtrate was centrifuged for 10 minutes @ 2000 rpm and the supernatant was collected.

2.8.2. Procedure
Ten milliliter of extract was taken in a conical flask and titrated with the dye solution until the solution appeared pink and persisted for 10-12 seconds. The amount of dye needed for the titration was recorded. For the same sample the titration was repeated thrice. The result was calculated on the basis of dye required to titrate unit amount of ascorbic acid.

3. Results and Discussion
3.1. Proline estimation of leaves
A minimum of three replications from each of the genotypes were analyzed for proline content using the method developed by Bates *et al.* (1973) from leaf sample. The analyses of variance for proline content of selected rice genotypes are presented in Table 1. The values for proline content in the leaves of rice plant at seedling stage (21 days aged leaf) stage were presented in Table 2. Under rain fed condition the range of proline accumulation in mutant rice lines is 9.38mg/100g to 25.46 mg/100g with a mean value of 17.59 mg/100g. N_1/300/P-8-3-3 showed maximum amount of proline (25.46mg/100g), which is followed by N_{10}/300/P-3-7-3 (24.12mg/100g), N_{10}/300/P-2(1)-6-11 (22.78mg/100g) and N_{10}/300/P-2-3-5 (20.32mg/100g). N_4/350/P-2(1)-32-11 showed minimum accumulation of proline (9.38mg/100g) which is followed by N_1/300/P-9-9-13 (13.40mg/100g), N_1/350/P-2-3 (14.17mg/100g) and N_{10}/300/P-5-7-5 (14.31 mg/100g). On the otherhand, the parental NERICA lines N_1, N_4 and N_{10} contains 16.08, 25.46 and 14.74 mg/100g proline respectively. It is an established fact that water stress induces numerous metabolic alterations in plants. Free proline accumulation is one of the most dramatic stress characteristics. Investigation conducted by Goyal *et al.*1985, Sudhakar *et al.* 1989, Jha and Singh (1997) showed that drought tolerant rice varieties had positive correlation with the proline accumulation in the leaves in water stress condition. It was reported that proline content in plant during water stress condition could be increased mainly due to two reasons. Under water stress condition, increment in proline accumulation often did occur due to onset of adaptive process (Aspenall and Paleg, 1981). Many others reported that increase in proline accumulation might occur from cellular injury as well (Hanson and Nelson, 1978). Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Caballero *et al.*, 2005). Proline is a non-protein amino acid that forms in most tissues subjected to water stress and together with sugar, it is readily metabolized upon recovery from drought (Singh *et al.*, 2010). In addition to acting as an osmo-protectant, proline also serves as a sink for energy to regulate redox potentials, as a hydroxyl radical scavenger (Sharma *et al.*, 2000), as a solute that protects macromolecules against denaturation and as a means of reducing acidity in the cell (Kishor *et al.*, 2005). However, Vendruscolo *et al.* (2007) stated that proline might confer drought stress tolerance to wheat plants by increasing the antioxidant system rather than as an osmotic adjustment. Therefore; it seems that the accumulation rate was correlated with drought tolerance. During abiotic stress conditions, plants induce the synthesis of osmolytes such as soluble sugars and amino acids which contribute to turgor maintenance by osmotic adjustment (Arbona 2003 and Arbona 2008). Among amino acids, Proline is the main effector in this response (in addition to hexoses), contributing to around 50% of the osmotic adjustment in maize root tips (Nishizawa 2008). Indeed, increases in proline content have been reported in response to different abiotic stress conditions like salt stress (Yoshiba 1995, Arbona 2008), soil flooding, drought or extreme temperatures.

However, whether Proline can counteract and protect against abiotic stress or not is still a question of debate. The biosynthesis of proline is activated under dehydration whereas rehydration induces the opposite path way (Figure 5.1); the target enzyme is a pyrroline-5-caboxylate synthetase (P5CS) located mainly in cytoplasm (Arbona, 2008). Recently, it has been suggested that proline over accumulation could increase ROS and MDA production probably via pyrroline-5-carboxylate and by inhibition of ABA and ethylene biosynthesis resulting in a decrease in stress tolerance.

3.2. Estimation of ascorbic acid

A minimum of three replications from each of the genotypes were analyzed for ascorbic acid content by titrimetric method (Reo, 1954) from leaf sample. The analyses of variance for ascorbic acid content of mutant rice genotypes are presented in Table 2. Ascorbic acid plays an important role in the metabolism of plants. The values for ascorbic acid content in the leaves of NERICA mutant rice lines at seedling stage (21 days aged leaf) were presented in Table 3. The highest ascorbic acid was recorded in N_1/250/P-7-2-1 (1.98 mg/100g) and the lowest value was recorded in N_1/250/P-7-3-11 (1.12mg/100g). Yarosh (1959) observed increase in ascorbic acid activity in cotton plant growing in water stress condition. As the experiment was conducted under rain fed condition without any artificial irrigation and inbuilt drought tolerance of NERICA rice significant amount of ascorbic acid was accumulated. At the initial stage the amount of ascorbic acid was higher but with the time span it was decreased as plant might use up a certain amount of ascorbic acid to scavenge ROS produced due to drought stress. Ascorbic acid content has also been reported to decrease with age and water stress condition of rice, Sorghum and Maize crops (Anwar *et al*., 1986).

Table 1. Analysis of variance (ANOVA) for proline content.

Source of variation	Degrees of freedom	Sum of square	Mean square	F-value	Level of significance
Genotypes	30	1224.69	40.823	96.89	**
Replication	2	3.39	1.699	4.03	
Error	60	25.27	0.421		
Total	92	1253.36			

** indicates significant at 0.01 probability

Table 2. Analysis of variance (ANOVA) for ascorbic acid content.

Source of variation	Degrees of freedom	Sum of square	Mean square	F-value	Level of significance
Genotypes	30	6.437	0.215	51.601	**
Replication	2	0.018	0.009	2.200	
Error	60	0.250	0.004		
Total	92	6.70			

** indicates significant at 0.01 probability

Table 3. Mean performance of 31 rice genotypes for proline and ascorbic acid content.

Genotypes	Proline (mg/100g)	Ascorbic acid (mg/100g)
N_4/350/P-2(1)-32-11	9.38 l	1.82 bcdef
N_1/300/P-9-9-13	13.40 k	1.78 cdefg
N_{10}/300/P-2(1)-11-(1)	14.74 j	1.28 j
N_1/350/P-2-2-4	15.20 ij	1.92 ab
N_1/250/P-7-2-1	14.74 j	1.98 a
N_{10}/300/P-2(1)-6-11	22.78 c	1.82 bcdef
N_4/250/P-2(5)-11-13	20.10 d	1.23 jk
N_{10}/300/P-3-7-1	16.24 hi	1.41 i
N_{10}/300/P-3-7-3	24.12 b	1.79 cdefg
N_{10}/300/P-5-7-5	14.31 jk	1.82 bcdef
N_4/250/P-2(6)-26(1,3,4)	17.76 fg	1.74 efgh
N_4/300/P-3(4)-10-9	18.76 ef	1.47 i
N_1/250/P-7-3-12	16.08 hi	1.49 i

Genotypes	Proline (mg/100g)	Ascorbic acid (mg/100g)
N_{10}/300/P-2-3-5	20.32 d	1.63 h
N_1/300/P-9-5-12	15.32 ij	1.18 jk
N_{10}/300/P-2(1)-8	16.92 gh	1.75 defg
N_4/250/P-2(5)-11-10	17.42 g	1.70 fgh
N_4/250/P-9-5-3	17.42 g	1.15 k
N_1/250/P-7-3-11	20.10 d	1.12 k
N_1/250/P-7-13-15	19.31 de	1.87 abcd
N_1/250/P-7-13-12	15.26 ij	1.85 bcde
N_1/350/P-2-3	14.17 jk	1.63 h
N_1/300/P-9-5	18.87 ef	1.89 abc
N_{10}/300/P-3-7-6	16.08 hi	1.73 efgh
N_1/300/P-8-3-3	25.46 a	1.42 i
N_1/250/P-6-2-8	16.08 hi	1.73 efgh
N_1/250/P-7-3	22.78 c	1.67 gh
N_1/300/P-9-5-6	16.08 hi	1.21 jk
N_1 Parent	16.08 hi	1.13 k
N_4 Parent	25.46 a	1.78 cdefg
N_{10} Parent	14.74 j	1.45 i
CV (%)	3.69	4.04
Maximum	25.46	1.98
Minimum	9.38	1.12
Mean	17.59	1.59
$LSD_{(0.05)}$	1.06	0.103
Level of significance	**	**

Genotypes with the different letter (s) are significantly different.

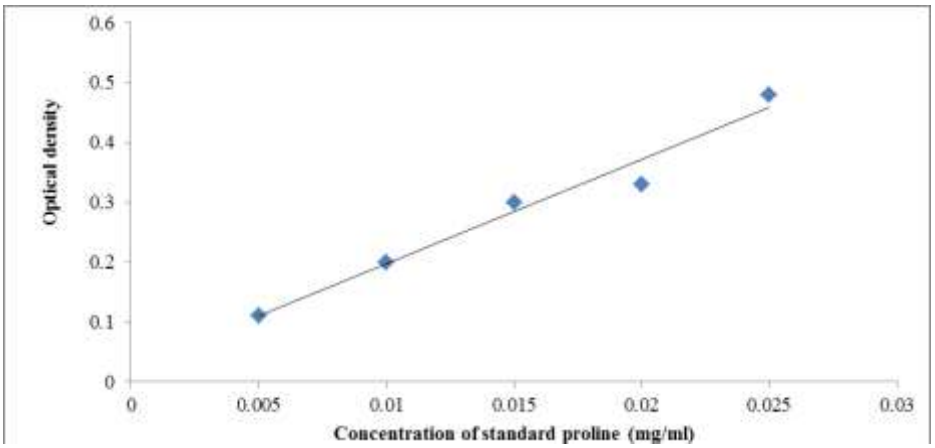

Figure 1. Standard curve for proline.

4. Conclusions

NERICA mutant lines are established as drought tolerant due to higher accumulation of proline under stress condition. Proline content in plant in water stress condition could be increased mainly due to two reasons. Under water stress condition, increment in proline accumulation often did occur due to onset of adaptive process and that was a genetic response as well as inhibition of ROS over proline and ultimately mechanical damage of plant tissue. N_1 parent showed maximum accumulation of proline (25.46mg/100g) as the experiment conducted under rain fed condition without any artificial irrigation. N_4/350/P-2(1)-32-11 showed minimum accumulation of proline (9.38mg/100g). Proline increase makes a relation with drought resistance observed by many researchers. Ascorbic acid content in plant in water stress condition could be increased but due to prolong drought condition ascorbic acid content decreased as plant utilize for scavenging reactive oxygen species (ROS). The highest ascorbic acid was recorded in N_1/250/P-7-2-1 (1.98mg/100g) and the lowest value was recorded in plant N_1/250/P-7-3-11 (1.12mg/100g). Under water deficit condition the ascorbic acid was decreased. Ascorbic acid decrease makes a relation with proline increment observed by many researchers. So after biochemical

evaluation of rice genotypes, it was revealed that, N_1/300/P-8-3-3 is best suited in drought prone areas as ascorbic acid decreases with increased accumulation of proline under rain fed condition.

Conflict of interest

None to declare.

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