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Research Article

IMPACT OF SALINITY ON ANTIOXIDATIVE ENZYMES AND METABOLITES RESPONSE IN TWO RICE CULTIVARS

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Abstract- Soil salinity, due to varying salt levels that occur in large terrestrial areas of the world severely affect crop yield. The present investigation was taken up to describe the potential biochemical and enzymatic responses in the leaves and roots of two rice cultivars CSR-23 (tolerant) and BPT- 5204 (sensitive) to different salt concentrations. Here, we observed significant changes in the biochemical and enzymatic responses between control and treatments as well as between the genotypes. The salt sensitive variety *i.e.,* BPT 5204 exhibited high reducing sugar and total phenol contents, compared to salt tolerant variety CSR 23. Catalase activity was significantly higher in leaves (46 %) and roots (5.5 %) of CSR 23 compared to BPT 5204. CSR 23 also showed an elevated Superoxide dismutase (SOD) activity in leaves (45.5 %) and roots (12.4 %) compared to BPT 5204. Similarly, Glutathione Reductase increased in the leaves (85 %) and roots (91.5 %) of CSR 23 as compared to BPT 5204. Highest nitrate reductase activity was observed in leaves of CSR 23(48 %) compared to BPT 5204, whereas there was no significant change in case of roots. BPT 5204 showed significantly higher NiR activity in leaves (18.3 %) and roots (17.5 %) as compared to CSR23. BPT 5204 showed significantly higher Total chlorophyll content in leaves and decreased in CSR 23 (25 %). The salt-tolerant cultivar CSR23 resisted salinity stress due to its ability to surmount oxidative stress via up-regulation of anti-oxidative enzymes and nitrogen assimilating enzymatic activities.

Keywords- Salinity, Anti-oxidative enzymes, Catalase, Superoxide Dismutase, Resistance

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Introduction

The productivity, growth and development of crop plants are determined by diverse interactions between their genomes and environmental factors such as biotic and abiotic stress [1]. One of the most vicious environmental factors that have affected 6 % of the world's total land area is salinity. 20 % of the irrigated land and 2 % of dry land agriculture area are affected by salinity directly or by secondary salinity [2]. Climate change also has influenced significantly for alarming increase in the salt affected area and it is assumed that 50% of the cultivable land will be salt affected by the middle of the twenty-first century [3]. Salt-induced osmotic stress, ionic toxicity, and a lower rate of photosynthesis increase the formation of reactive oxygen species (ROS), which disrupt the antioxidant defence system and consequently causes oxidative stress [4 and 5]. ROS are extremely reactive in nature because they can interact with a number of other molecules and metabolites such as DNA, pigments, proteins, lipids, and other essential cellular molecules which lead to a series of destructive processes [6 and 7]. Plants have adapted to acclimatize to different environmental conditions by evolving different strategies like complex signalling pathways- comprising of receptors, secondary messengers, phytohormones, and signal transducers to sense various stresses [8]. The immediate response of plants to high concentrations of salt is osmotic adjustment by reducing cell expansion, cell division, stomatal closure, and gradually reducing leaf area,[9].To circumvent effect of ROS, plants synthesize antioxidant molecules and various anti-oxidative enzymes [10,11and12]. This enzyme system includes superoxide dismutase (SOD), peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), and glutathione reductase (GR; EC 1.8.1.7). The primary scavenger in the detoxification of ROS in plants is SOD that converts superoxide anions to hydrogen peroxide (H_2O_2) and molecular oxygen [13]. A lot of studies have been done in plants for their anti-oxidative responses to various biotic and abiotic stresses including salt stress [14]. However, the knowledge about oxidative stress and ability of anti-oxidative response of local varieties is scanty. Considering the strategies discussed, our present study was conducted to investigate a comparative account of the impact of different levels of salinity-induced oxidative stress on growth and development in the salt-sensitive and a relatively salt-tolerant rice genotypes.

Materials and Methods

Plant material and experimental design

Two cultivars of Rice (*Oryza sativa* L.), CSR 23 (salt tolerant) and BPT5204 (salt sensitive) were chosen to study the biochemical response of antioxidant enzymes and nitrate assimilatory enzymes. Each cultivar was evaluated by pot culture experiments in polyhouse under controlled condition with different treatments of salinity T_1: Control 0 dS m^{-1},T_2: 8 dS m^{-1},T_3:12 dS m^{-1} and T_4: 16 dS m^{-1}) in a randomized complete block design with three replications .The salt solutions were prepared by dissolving appropriate amount of NaCl in distilled water for 8,12 and 16 dS m^{-1} and only distilled water for control. The pots were irrigated every two days with the above mentioned solutions.

Leaves and roots samples were collected from two genotypes of rice for biochemical analysis after 60 days of sowing. The samples were collected in an ice box and the tissue was processed immediately for superoxide dismutase

(SOD), catalase (CAT), glutathione reductase (GR), Nitrate (NR) and Nitrite reductase (NiR) enzyme extraction and assay. All the enzyme processing steps were carried out at 0˚C - 4˚C. The shade dried leaf samples were used for the preparation of alcoholic extracts used in phenol and reducing sugar estimation.

Enzyme extraction and determination of soluble protein content

Fresh leaf tissues after collection were processed immediately for enzyme extraction between (0˚C- 4˚C) and used for the assay. In order to measure the enzyme activities of SOD, CAT, GR, NR and NiR, 0.5g of leaf tissue, per treatment was taken, and ground into fine powder using liquid nitrogen and extracted in 2.0 mL of ice cold extraction buffer. Extraction buffer for CAT and SOD contained 0.05M sodium phosphate buffer of pH 7.0 and pH 7.8, respectively and for GR the extraction buffer included 0.1M Tris –HCl of pH 7.8 and 2mM dithiothreitol (DTT). Nitrate reductase (NR) and Nitrite reductase (NiR) enzyme extracts were prepared in 0.1 M phosphate buffer, pH 7.5, containing 10 mM cysteine. The extraction buffers of all the enzymes contained 1mM ethylenediaminetetraacetic acid (EDTA) and 1.5% w/v insoluble polyvinyl polypyrrolidone. Each enzyme homogenate was centrifuged at 14,000 rpm for 20 minutes at 4˚C and the supernatant maintained at 0˚C to 4˚C was used as enzyme source in the assay which was carried out immediately within 2-4 hours of leaf or root sample collection. An aliquot of supernatant was stored at -20˚C for protein analysis which was determined by Bradford method using bovine serum albumin as standard [15].

Antioxidant Enzyme Activities

Catalase Assay: CAT (EC 1.11.1.6) activity was spectrophotometrically determined by Beers and Sizers method [16]. The reaction mixture contained 2.98 mL of 16.65 mM hydrogen peroxide in 50mM phosphate buffer; pH 7.0 and 20 µL of enzyme extract was used to initiate the reaction. The decrease in absorbance at 240 nm was measured for 5 minutes using the substrate blank.. One unit of CAT is defined as the one µmole of H_2O_2 decomposed per minute at pH 7.0 at 25˚C and specific activity was expressed as µmole min $^{-1}$ mg^{-1} protein.

Superoxide dismutase: The activity of SOD, (EC 1.15.1.1) was assayed photochemically at 560 nm by the Beauchamp and Fridovich method. [17]. 3.0 mL of assay mixture contained 20 µL of enzyme extract, L-methionine (10 mM), p-nitrobluetetrazolium chloride (NBT) (33 µM), EDTA (0.66 µM) and riboflavin (3.3 µM) in a 50mM potassium phosphate buffer, pH 7.8. The assay was initiated by adding riboflavin and took place in a glass tube illuminated by a 15W fluorescent lamp at 25˚C for 20 minutes. The increase in absorbance of the blue formazan produced by NBT photo-reduction was measured at 560 nm. A blank was maintained with all the constituents but in the dark. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the NBT photo-reduction per minute and specific activity is expressed as IU per mg protein.

Glutathione reductase Assay: GR (EC 1.8.1.7) activity was determined spectrophotometrically by Mavis and Stellwagen method at 340nm [18]. The reaction mixture contained 100 µL of 30 mM oxidized glutathione, 1.5 mL of 100 mM potassium phosphate buffer with 3.4 mM EDTA, pH 7.6, 350 µL of 0.8 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and 950 µL of water. The decrease in absorbance at 340 nm on addition of 100 µL of enzyme to reaction mixture was recorded for 5 minutes. One GR unit is defined as the amount of enzyme that oxidizes 1.0 µmole of NADPH per minute at pH 7.6 at 25°C and specific activity is expressed as µmole min $^{-1}$ mg^{-1} protein.

Nitrogen Assimilatory Enzyme Activities

Nitrate reductase Assay: NR (EC 1.7.1.1) activity was spectrophotometrically determined at 540 nm by the method of Hageman and Reed [19]. A known weight (140 mg) of fresh tissue was cut into pieces and suspended in screw cap vials containing 3.5 ml. of incubation mixture (20 ml of 0.1M phosphate buffer, 20 ml of 5 per cent propanol and 10 ml of 0.2 per cent KNO3). The vials were sealed and kept in dark condition at 30°C for 2h. Nitrite released into the medium was determined by treating 1 ml. aliquot with 1 ml each of 1 % sulphanyl amide and 0.02 % N-1- napthyl ethylene diamine hydrochloride. After 20 min, solution is diluted to 5 ml with water and absorbance is measured at 540 nm. Reagent grade concentrations of nitrite(KNO2) solution was used to prepare standard curve. The nitrate reductase activity is expressed as nmoles of nitrite formed per hour per gram of fresh weight.

Nitrite reductase Assay: NiR (EC 1.7.7.1) activity was determined spectrophotometrically at 540nm by the method of Wray and Fido [20]. The 0.8 ml of reaction mixture contained 0.2 ml of 0.1M phosphate buffer, 0.1 ml of 5mM sodium nitrite, 0.1 ml of 1.5 mM methyl viologen, 50 µl of enzyme and distilled water. The reaction was started by adding 0.2 ml of the 2.5% sodium dithionite reagent and incubated for 10 minutes. The reaction was stopped by vigorously shaking the mixture until the dithionite was completely oxidized and the dye colour disappeared. For determination of nitrite consumed by enzyme 50 µl aliquot of above mixture was made to 2.0 ml using distilled water and 1.0 ml of 1 % sulphanilamide followed by 1 ml of 0.02 % NNED was added and incubated for 15 minutes. Blank was also processed in the similar way except for 50 µl of enzyme was added after the addition of sulphanilamide and NNED and read at 540 nm. The nitrite consumed by the action of enzyme was estimated from the nitrite standard curve. NiR activity is expressed as µmol of nitrite consumed /min and the specific activity as enzyme activity/mg protein.

Determination of total phenols and chlorophyll

Total Phenols: 5 gram of dried leaf tissue was extracted with 50 ml of 80% hot Ethyl alcohol [21]. The colorimetric method of Singleton and Rossi [22] was used for the determination of total phenols using the Folin–Ciocalteau reagent. The phenol content was expressed as mg per gram dry weight.

Total Chlorophyll: Chlorophyll content in leaf tissue was determined by Dimethyl sulphoxide (DMSO) method [23]. The values obtained were expressed as mg per gram fresh weight.

Statistical Analysis

The experimental data was analyzed statistically following the method described by Gomez and Gomez [24]. The results were expressed as mean and standard error of mean of three replicates of the biochemical parameter for each sample. $p \leq 0.05$ was used as significance level for "F" and "T" test. 2 factorial randomised Block design was used to calculate critical difference where ever "F" was found to be significant.

Results

Changes in activities of anti-oxidant enzymes in leaves and roots of rice cultivars due to salinity stress.

Catalase (CAT) activity: CAT activity in the leaves of rice genotype differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-1] and [Fig-1a]. Among genotypes, CSR 23 showed significantly higher CAT activity (369.2 units/mg protein) in leaves compared to BPT 5204 (199.3 units/mg protein). Among interaction, CSR 23 showed lower CAT activity in control (182.1 units/mg protein) and increased (280.1, 419.7 and 595.2 units/mg protein) in salinity (8, 12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 showed higher CAT activity in control (349.8 units/mg protein) and the activity decreased (333.95 and 19.2 units/mg protein) in salinity(8, 12 and 12 dS m^{-1}) respectively. In case of root, catalase activity differed significantly under control and treatments in both the genotypes [Table-1] and [Fig-1b]. CAT activity was significantly low in roots compared to leaves under control (15.3 units/mg protein) and increased (19.8 and 30.5 units/mg protein) in salinity (8 and 16 dS m^{-1}) but it was low (18.7 units/mg protein) in medium salinity (12 dS m^{-1}). Among genotypes, CSR 23 showed significantly higher CAT activity (21.7 units/mg protein) in root compared to BPT 5204 (20.5 units/mg protein).

Superoxide dismutase (SOD): SOD activity in the leaves of rice genotype differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-2 and Fig-2a]. Among genotypes, CSR 23 showed significantly higher SOD

activity (4.9 units/mg protein) in leaves compared to BPT 5204 (2.6 units/mg protein).Among interaction CSR 23 showed lower SOD activity in control (2.27 units/mg protein) and increased (3.10, 6.80 and 7.43 units/mg protein) in salinity (8, 12 and 16 dS m^{-1})respectively. Genotype BPT 5204 also showed lower SOD activity in control (1.97 units/mg protein) and the activity increased (2.40, 2.80 and 3.50 units/mg protein) in salinity (8, 12 and 12 dS m^{-1}) respectively. In case of roots, SOD activity also differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-2] and [Fig-2b]. Among genotypes, CSR 23 showed significantly higher SOD activity (4.5 units/mg protein) in root compared to BPT 5204 (4.0 units/mg protein). Among interactions CSR 23 showed significantly lower SOD activity in control (2.3 units/mg protein) and increased (3.3, 5.5 and 7 units/mg protein) in salinity (8, 12 and 16 dS m^{1}). Genotype BPT 5204 also showed lower SOD activity in control (3.2 units/mg protein) and the activity increased (3.8, 4.1 and 5.1 units/mg protein) in salinity (8, 12 and 16 dS m^{-1}) respectively.

Table-1 *Assay of catalase activity in leaves and roots of rice*

Catalase (Units/mg protein)										
Treatment	Leaf					Root				
	CSR 23	% increase in treatment over control	BPT 5204	% decrease in treatment over control	Mean	CSR 23	% increase in treatment over control	BPT 5204	% increase or decrease in treatment over control	Mean
Control	182.1	-	349.8	-	**266.0**	16.5	-	14.0	-	**15.3**
8 dS m^{-1}	280.1	53.8	333.0	4.8	**306.5**	18.2	10.3	21.4	52.8	**19.8**
12 dS m^{-1}	419.7	130	95.0	72.8	**257.3**	26.3	37.2	31.2	122.8	**18.8**
16 dS m^{-1}	595.2	226.8	19.2	94.5	**307.2**	45.6	176.3	15.5	-10.7	**30.5**

G: Genotype T: Treatment

Table-2 *Assay of superoxide dismutase activity in the leaves and roots of rice*

SOD (Units/mg protein)										
Treatment	Leaf					Root				
	CSR 23	% increase in treatment over control	BPT 5204	% increase in treatment over control	Mean	CSR 23	% increase in treatment over control	BPT 5204	% increase in treatment over control	Mean
Control	2.27	-	1.97	-	**2.12**	2.33	-	3.20	-	**2.77**
8 dS m^{-1}	3.10	36.5	2.40	21.8	**2.75**	3.33	42.9	3.80	18.7	**3.57**
12 dS m^{-1}	6.80	199.5	2.80	42.1	**4.80**	5.53	137.3	4.13	29	**4.83**
16 dS m^{-1}	7.43	227.3	3.50	77.6	**5.47**	7.07	200.4	5.13	60.3	**6.10**

G: Genotype T: Treatment

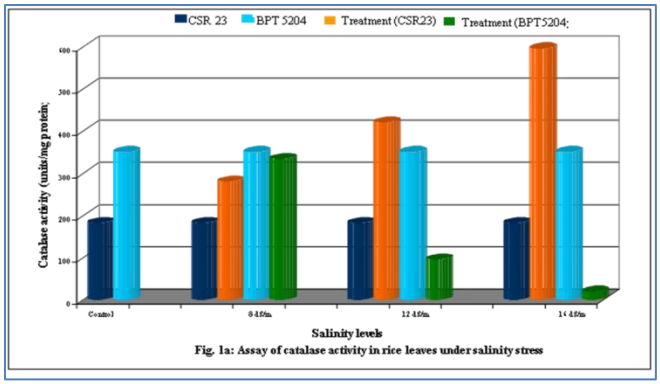

Fig-1a Assay of catalase activity in rice leaves under salinity stress

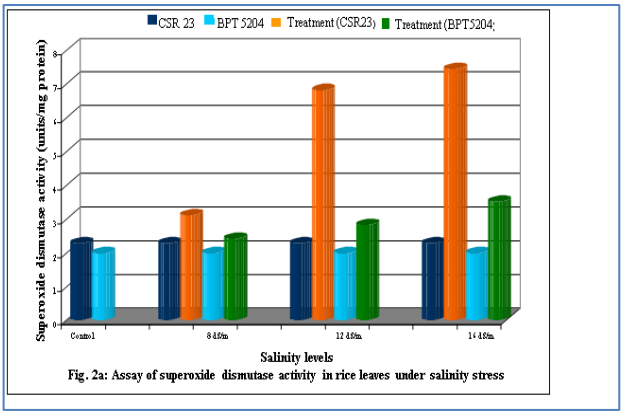

Fig-2a Assay of Superoxide dismutase activity in rice leaves under salinity stress.

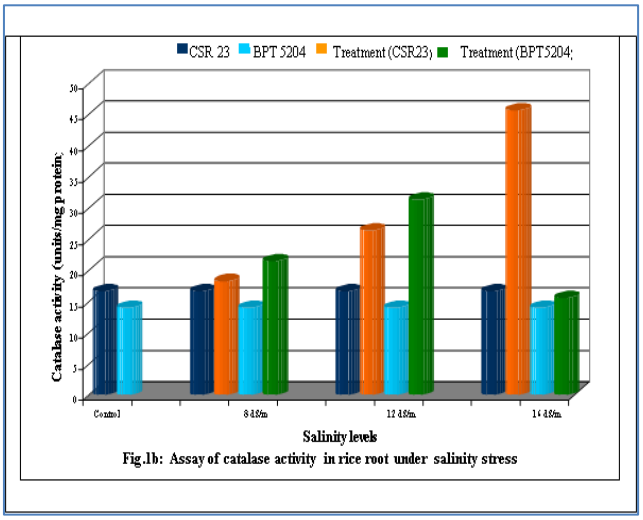

Fig-1b Assay of catalase activity in rice roots under salinity stress

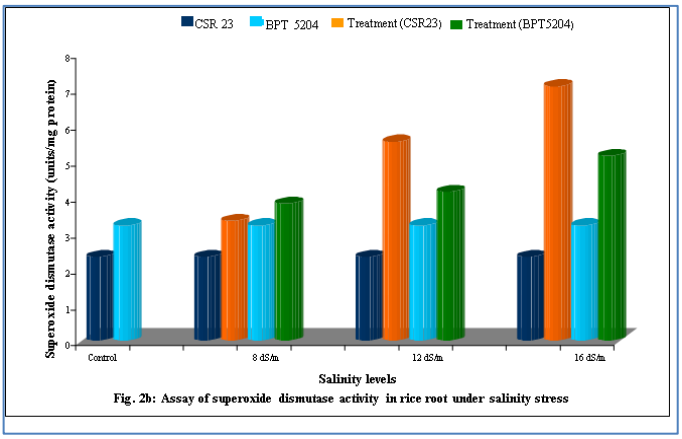

Fig-2b Assay of Superoxide dismutase activity in rice roots under salinity stress.

Glutathione reductase (GR): The GR activity in the leaves of rice genotype differed significantly($p \leq 0.05$) under control and treatments in both the genotypes [Table-3] and [Fig-3a] Among genotypes, CSR 23 showed significantly higher GR activity (23.4 units/mg protein) in leaves compared to BPT 5204 (3.3 units/mg protein).Among interaction CSR 23 showed lower GR activity in control (13.8 units/mg protein) and increased (20.8, 26.9 and 32.4 units/mg protein) in salinity (8,12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 showed very lower GR activity in control (0.83 units/mg protein) and the activity increased (1.5, 5.05 and 5.8 units/mg protein) in salinity (8, 12 and 16 dS m^{-1}) respectively. In case of roots, GR activity differed significantly($p \leq 0.05$) under control and treatments in both the genotypes [Table-3] and [Fig-3b] Among genotypes, CSR 23 showed significantly higher GR activity (13.2 units/mg protein) in root compared to BPT 5204 (1.12 units/mg protein) Among interaction CSR 23 showed higher GR activity in control (9.1 units/mg protein) and increased (10.8, 15.3 and 17.9 units/mg protein) in salinity (8, 12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 also showed lower GR activity in control (0.83 units/mg protein) and the activity increased (0.9, 1.3 and 1.4 units/mg protein) in salinity (8, 12 and 16 dS m^{-1}) respectively.

Table-3 *Assay of glutathione reductase activity in leaves and roots of rice*

Glutathione reductase (Units/mg protein)										
Treatment	Leaf					Root				
	CSR 23	% increase in treatment over control	BPT 5204	% increase treatment over control	Mean	CSR 23	% increase in treatment over Control	BPT 5204	% increase in treatment over control	Mean
Control	13.87	-	0.83	-	**7.35**	9.17	-	0.83	-	**5.00**
8 dS m^{-1}	20.80	49.9	1.53	84.3	**11.17**	10.80	17.7	0.91	9.6	**5.86**
12 dS m^{-1}	26.90	93.9	5.05	508.4	**15.98**	15.30	66.8	1.31	57.8	**8.30**
16 dS m^{-1}	32.40	133.5	5.80	598.7	**19.10**	17.90	95.2	1.44	73.4	**9.67**

G: Genotype T: Treatment

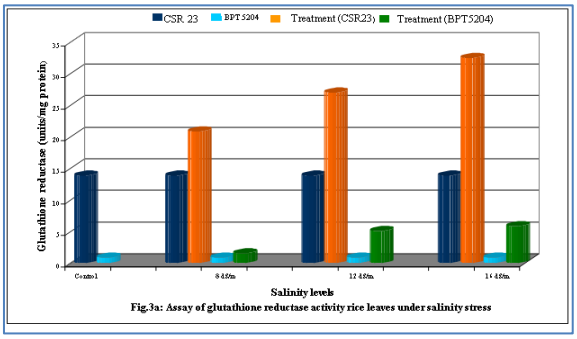

Fig-3a Assay of Glutathione reductase activity in rice leaves under salinity stress.

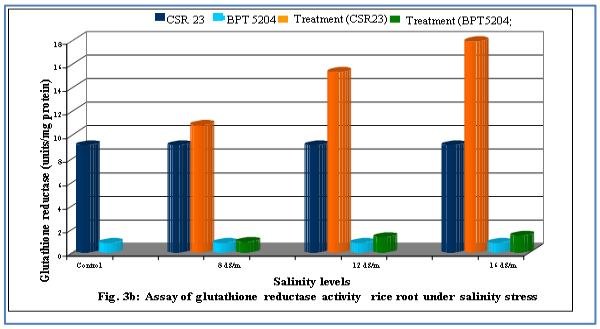

Fig-3b Assay of Glutathione reductase activity in rice roots under salinity stress.

Changes in activities of Nitrogen assimilatory enzymes in leaves and roots of rice cultivars due to salinity stress.

Nitrate reductase (NR): NR activity in the leaves of rice genotype differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-4] and [Fig-4a] Among genotypes, CSR 23 showed significantly higher NR activity (129.4 nmoles of NO_2/g/h fresh weight) in leaves compared to BPT 5204 (67.2 nmoles of NO_2/g/h fresh weight). Among interaction CSR 23 showed higher NR activity in control (217.6 nmoles of NO_2/g/h fresh weight) and decreased (128.4, 105.8 and 65.7 nmoles of NO_2/g/h fresh weight) in salinity (8,12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 showed higher NR activity in control (120.8 nmoles of NO_2/g/h fresh weight) and the activity decreased (80.5, 37.1 and 30.2 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively. In case of roots, NR activity differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-4] and [Fig-4b]. Among genotypes, BPT 5204 showed higher NR activity (36.4 nmoles of NO_2/g/h fresh weight) in roots compared to CSR 23 (35.2 nmoles of NO_2/g/h fresh weight). Among interaction CSR 23 showed higher NR activity in control (48.1 nmoles of NO_2/g/h fresh weight) and decreased (35.1, 29.2 and 28.3 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 also showed higher NR activity in control (47.0 nmoles of NO_2/g/h fresh weight) and the activity decreased (40.3, 34.8 and 23.4 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively.

Table-4 *Assay of nitrate reductase activity in the leaves and roots of rice*

Nitrate reductase (nmoles of NO_2/g/h)										
Treatment	Leaf					Root				
	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean
Control	217.6	-	120.9	-	**169.2**	48.17	-	47.03	-	**47.6**
8 dS m^{-1}	128.5	40.9	80.5	33.4	**104.5**	35.13	27	40.40	14	**37.7**
12 dS m^{-1}	105.9	51.3	37.2	69.2	**71.5**	29.28	39.2	34.87	25.8	**32**
16 dS m^{-1}	65.7	69.8	30.2	75.0	**47.95**	28.32	41.2	23.48	50	**25.9**
Grand mean	**129.4**		**67.2**		**98.3**	**35.23**		**36.4**		**35.8**

G: Genotype T: Treatment

Nitrite reductase (NiR): NiR activity in the leaves of rice genotype differed significantly ($p \leq 0.05$) under control and treatments in both the genotypes [Table-5] and [Fig-5a]. Among genotypes, BPT 5204 showed significantly higher NiR activity (23.4 nmoles of NO_2/g/h fresh weight) in leaves compare CSR 23 (19.1 nmoles of NO_2/g/h fresh weight). Among interaction CSR 23 showed lower NiR activity in control (27.6 nmoles of NO_2/g/h fresh weight) and decreased (21.9, 16.1 and 11.4 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}). Genotype BPT 5204 showed higher NiR activity in control (35.3 nmoles of NO_2/g/h fresh weight) and the activity decreased (29.9, 17.1 and 11.3 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}), respectively. In case of root, NiR

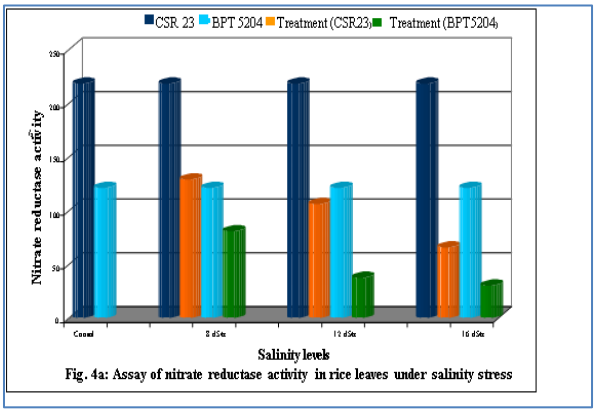

Fig. 4a: Assay of nitrate reductase activity in rice leaves under salinity stress

Fig-4a Assay of Nitrate reductase activity in rice leaves under salinity stress.

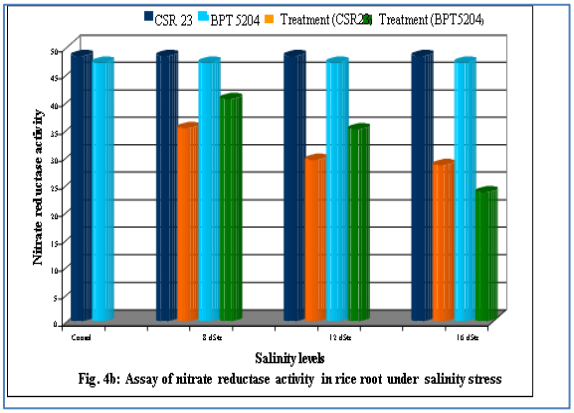

Fig. 4b: Assay of nitrate reductase activity in rice root under salinity stress

Fig-4b Assay of Nitrate reductase activity in rice roots under salinity stress.

activity differed significantly ($p \leq 0.05$) [Table-5] and [Fig-5b] under control and treatments in both the genotypes. NiR activity was significantly high in root under control (99.8 nmoles of NO_2/g/h fresh weight) and decreased (58.9, 49.8 and 47.3 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively. Among genotypes, BPT 5204 recorded significantly higher NiR (70.3 nmoles of NO_2/g/h fresh weight) in root compared to CSR 23 (57.6 nmoles of NO_2/g/h fresh weight) under control and treatments in both the genotypes *i.e.* NiR activity in root represented in [Table-7]. NiR activity was significantly high in root under control (99.8 nmoles of NO_2/g/h fresh weight) and decreased (58.9, 49.8 and 47.3 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively. Among genotypes, BPT 5204 recorded significantly higher NiR activity (70.3 nmoles of NO_2/g/h fresh weight) in root compared to CSR 23 (57.6 nmoles of NO_2/g/h fresh weight) Among interaction CSR 23 showed higher NiR activity in control (95.5 nmoles of NO_2/g/h fresh weight) and decreased (46.3, 44.7 and 44.0 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively. Genotype BPT 5204 also showed higher NiR activity in control (104.1 nmoles of NO_2/g/h fresh weight) and the activity decreased (71.5, 55 and 50.6 nmoles of NO_2/g/h fresh weight) in salinity (8, 12 and 16 dS m^{-1}).

Changes in activities of phenols and chlorophyll pigments in leaves of rice cultivars due to salinity stress.

Total phenols: Phenol content in the leaves of rice genotypes differed significantly ($p \leq 0.05$) [Table-6 and Fig-6] under control and treatments. Among treatments phenol content was significantly lower (1.4 g/100 g dry weight) in control and increased *i.e.* (1.8, 2.2 and 2.7 g/100 g dry weight) during salinity stress (8, 12 and 16 dS m^{-1} respectively). Among genotypes, salt sensitive genotype BPT 5204 showed significantly high phenol content (2.34 g/100 g dry weight) compared to salt tolerance CSR 23 (1.77 g/ 100 g dry weight).

Table-5 *Assay of nitrite reductase activity in leaves and roots of rice*

Treatment	Nitrite reductase (nmoles of NO_2/g/h)									
	Leaf					Root				
	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean
Control	27.69	-	35.34	-	**31.6**	95.59	-	104.1	-	**99.8**
8 dS m^{-1}	21.19	23.4	29.97	15.1	**25.5**	46.35	51.5	71.59	31.2	**58.9**
12 dS m^{-1}	16.12	42.7	17.19	51.3	**16.6**	44.74	53.1	55.09	47	**49.8**
16 dS m^{-1}	11.46	58.6	11.38	67.7	**11.4**	44.05	53.9	50.69	51.3	**47.3**

G: Genotype T: Treatment

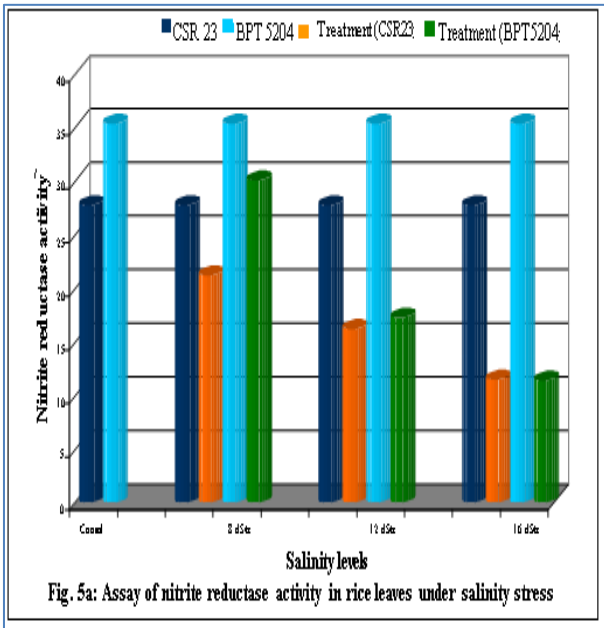

Fig. 5a: Assay of nitrite reductase activity in rice leaves under salinity stress

Fig-5a Assay of nitrite reductase activity in rice leaves under salinity stress

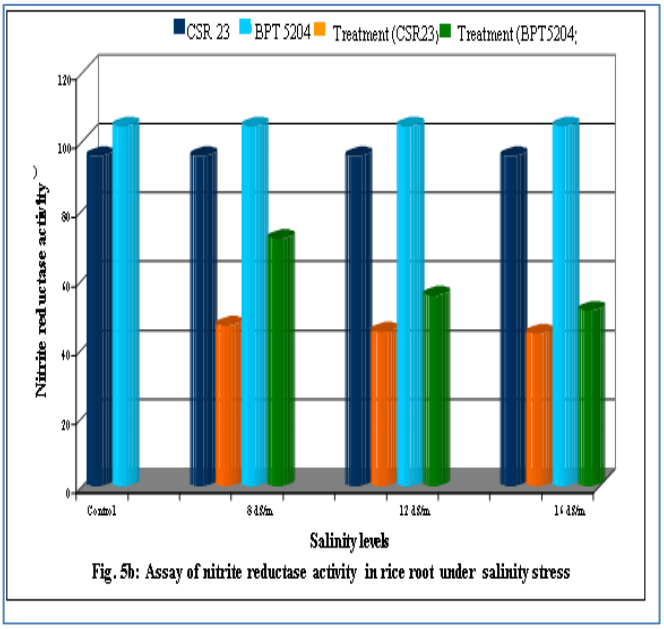

Fig. 5b: Assay of nitrite reductase activity in rice root under salinity stress

Fig-5b Assay of nitrite reductase activity in rice roots under salinity stress

Table-6 *Estimation of total phenol in rice leaves*

Treatment	Total phenol (g % dry weight)				
	Leaf				Mean
	CSR 23	% increase in treatment over control	BPT5204	% increase in treatment over control	
Control	1.13	-	1.80	-	**1.47**
8 dS m^{-1}	1.58	39.8	2.01	11.6	**1.80**
12 dS m^{-1}	1.92	69.9	2.30	27.7	**2.25**
16 dS m^{-1}	2.47	118.5	2.50	38.8	**2.73**

G: Genotype T: Treatment

Chlorophyll 'a', Chlorophyll 'b' and Total chlorophyll: Chlorophyll 'a', Chlorophyll 'b' and Total chlorophyll in the leaves of rice genotypes differed significantly (p≤ 0.05) [Table-7] and [Fig-7a] under control and treatments, *i.e.* represented in [Table-7]. Among treatments chlorophyll 'a' in the leaves of rice genotype was significantly higher (0.233 mg/100 g fresh weight) in control and decreased (0.167, 0.122 and 0.081 mg/100 g fresh weight) during increased salinity (8, 12 and 16 dS m^{-1}) respectively. Among genotypes, CSR 23 recorded significantly high chlorophyll 'a 'content (0.191 mg/100 g fresh weight) compared to BPT 5204 (0.11 mg/100 g fresh weight). Among interaction both the genotypes showed significantly high chlorophyll 'a' content in controls (0.30 mg/100 g fresh weight in CSR 23 and 0.16 mg/100 g fresh weight in BPT 5204), decreased (0.19, 0.15 and 0.11 mg/100 g fresh weight in CSR 23 and 0.14, 0.08 and 0.04 mg/100 g fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively.

Chlorophyll 'b' in the leaves of rice genotype was significantly (p≤ 0.05) [Table-7] and [Fig-7b] higher (0.552 mg/100 g fresh weight) in control and decreased (0.347, 0.293 and 0.239 mg/100 g fresh weight) in increased salinity (8, 12 and 16 dS m^{-1}) respectively. Among genotypes, BPT 5204 recorded significantly higher chlorophyll 'b' content (0.385 mg/100 g fresh weight) compared to CSR 23 (0.33 mg/100 g fresh weight). Among interaction both the genotypes showed higher chlorophyll 'b' content in controls (0.41 mg/100 g fresh weight in CSR 23 and 0.68 mg/100 g fresh weight in BPT 5204), decreased (0.37, 0.31 and 0.21mg/100 g fresh weight in CSR 23 and 0.32, 0.27 and 0.25 21mg/100 g fresh weight in BPT 5204) in salinity (8, 12 and 16 dS m^{-1} respectively).

Total chlorophyll in the leaves of rice genotype was significantly (p≤ 0.05) [Table-7] and [Fig-7c] higher (0.44 mg/100 g fresh weight) in control and decreased (0.24, 0.18 and 0.14 mg/100 g fresh weight) in increased salinity (8, 12 and 16 dS m^{-1}) respectively. Among genotypes, BPT 5204 recorded significantly higher total chlorophyll content (0.287 mg/100 g fresh weight) compared to CSR 23 (0.215 mg/100 g fresh weight). Among interaction both the genotypes showed high total chlorophyll content in controls (0.314 mg/100 g fresh weight in CSR 23 and 0.566 mg/100 g fresh weight in BPT 5204), decreased (0.25, 0.16 and 0.12 mg/100 g fresh weight in CSR 23 and 0.22, 0.20 and 0.16 mg/100 g fresh weight) in salinity (8, 12 and 16 dS m^{-1}) respectively.

Table-7 *Estimation of chlorophyll 'a', chlorophyll 'b' and total chlorophyll in rice leaves*

Treatment	Chlorophyll (mg/100 g fresh weight)														
	Chlorophyll 'a'					Chlorophyll 'b'					Total chlorophyll				
	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean	CSR 23	% decrease in treatment over control	BPT 5204	% decrease in treatment over control	Mean
Control	0.303	-	0.163	-	**0.233**	0.415	-	0.689	-	**0.552**	0.315	-	0.566	-	**0.440**
8 dS m^{-1}	0.190	36.6	0.143	12.5	**0.167**	0.374	9.7	0.322	52.9	**0.348**	0.260	16.1	0.224	60.7	**0.242**
12 dS m^{-1}	0.157	50	0.087	50	**0.122**	0.313	24.3	0.275	60.2	**0.294**	0.166	48.3	0.202	64.2	**0.184**
16 dS m^{-1}	0.113	63.3	0.048	68.7	**0.081**	0.219	48.7	0.259	63.2	**0.239**	0.123	61.2	0.161	71.4	**0.142**

G: Genotype T: Treatment

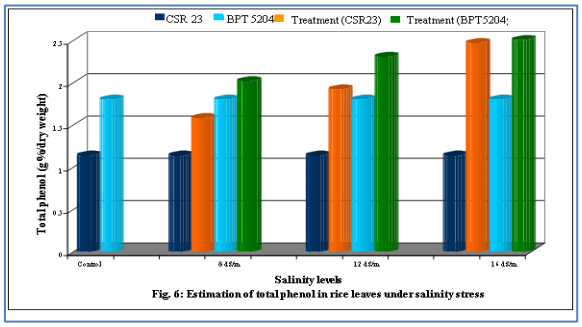

Fig-6 Estimation of total phenols in rice leaves under salinity stress

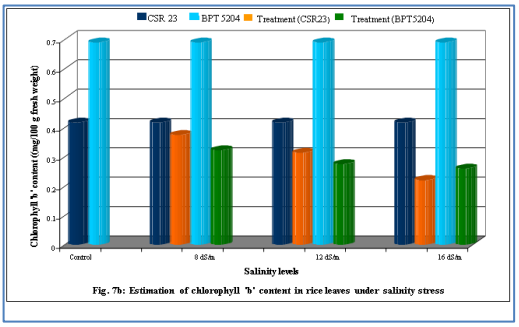

Fig-7b Estimation of chlorophyll 'b' content in rice leaves under salinity stress

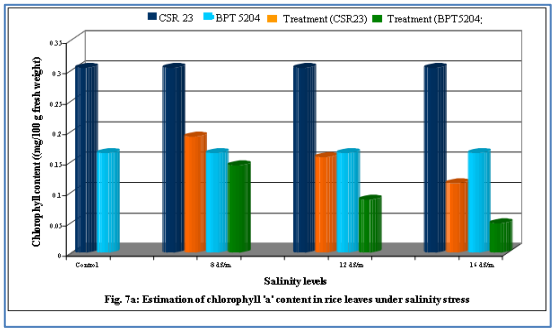

Fig-7a Estimation of chlorophyll 'a' content in rice leaves under salinity stress

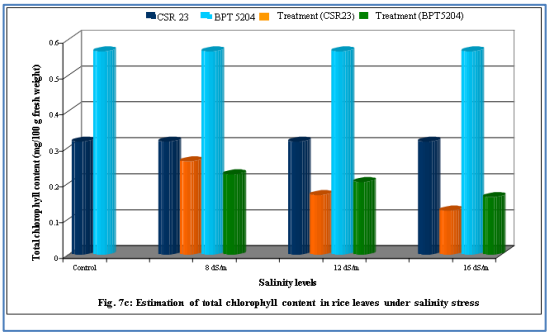

Fig-7c Estimation of Total chlorophyll content in rice leaves under salinity stress

Discussion

Changes in activities of defence enzymes in leaves and root of Rice cultivars salinity stress.

Catalase

Generally, the Catalase (CAT) has a defensive role against the ROS. Up to a certain level ROS production under any stress may work as a signal for triggering acclamatory /defence responses via transduction pathways, which have H_2O_2 as a secondary messenger [24,25]. According to the results obtained [Table-1] among the genotypes, CSR 23 recorded highest CAT activity in both leaves and roots. Salt treatments induced an increase in catalase activity in leaves and roots of both genotypes, implying an efficient detoxification of H_2O_2 and thereby contribute to the ROS tolerance of the species. The increased induced CAT activity in CSR 23 may be related to increased tolerance of the genotype to oxidative stress [26] thus exhibiting a more efficient tolerance mechanism during salinity, by maintaining H_2O_2 homeostasis.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) constitutes the first line of defense against reactive oxygen species (ROS) with in a cell [27]. SOD catalyzes the conversion of two superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2^-) and alleviates oxidative stress. In the present investigation [Table-2] CSR 23 exhibited higher SOD activity compared to BPT 5204 in both leaves and roots under different levels of salinity. Similar results were reported by [28] who studied antioxidant responses of rice seedlings to salinity stress. Hence it could be concluded that the salt-tolerant varieties exhibit better protection mechanism against increased free radical production by maintaining the specific activity of antioxidant enzymes and are less vulnerable to oxidative stress.

Glutathione reductase (GR)

Ascorbate/glutathione cycle is another important pathway involved in the control of ROS level in the plant tissue, in which Glutathione reductase (GR) plays a major role.GR is a flavo-protein, an oxido-reductase which catalyses the reduction of glutathione disulphide (GSSG) to the sulphydryl form GSH. This enzyme employs NADPH as a reductant. [29]. In this study it is observed that CSR23 showed increased GR activity in both leaves and roots as compared to BPT 5204 [Table-3]. Similar result was reported by [30] which revealed that GR activity increased with time in control and salt-stressed plants of BR5033 and BR5011 of maize genotypes. Thus, increase in catalase, superoxide dismutase and glutathione reductase could be taken as support for the notion that the components of the ascorbate-glutathione cycle are co-regulated [31].

Changes in activities of nitrogen assimilating enzymes, phenols and chlorophyll pigments in leaves of rice cultivars due to salinity stress.

Nitrate reductase (NR)

Nitrate reductase catalyses the first step of nitrate assimilation in plants and algae, which appears to be rate limiting step in the acquisition of nitrogen in most cases [32]. The primary role of NR in plants is NAD(P)H-dependent reduction of nitrate to nitrite, which is subsequently reduced to ammonium by the nitrite reductase. [33]. In the present study [Table-4] the NR showed decreased activity in both leaf and roots in of both cultivars as compared to respective controls. Among genotypes CSR 23 maintained higher NR activity compared to susceptible BPT 5204. [34] Found similar negative impact of NaCl on NR activity which was more pronounced for salt sensitive bean cultivar. Hence the result indicates that BPT 5204 was more sensitive to the osmotic effect; and inhibition of NR could be explained by toxicity exerted by Na^+ and Cl^-.

Nitrite reductase (NiR)

The primary role of Nitrite reductase in plants is reduction of nitrite to ammonia. The nitrite reduction takes place by enzymatic mechanisms that include the reaction catalyzed by nitrite reductase (NiR) in the cytosol [35] and [36]. In the present investigation [Table-5] the susceptible variety BPT 5204 was able to retain higher activity of the NiR compared to the resistant variety CSR23. This could be attributed to the fact that Non-enzymatic nitrite reduction occurs spontaneously in the apoplast due to the acidic conditions or to the presence of ascorbic acid or phenols [37].

Total phenols

As per the result obtained, among the genotypes, salt sensitive genotype BPT 5204 recorded high total phenol content compared to salt tolerant genotype *i.e.* CSR 23, salt treatment induced increase in total phenol content in leaves of both genotypes [Table-6]. The increase in phenol content may be attributed to the positive regulation of phenol (secondary metabolites) synthesis under stress due to salt stress. Phenols play an important role in cyclic reduction of reactive oxygen species (ROS) such as superoxide anion and hydroxide radicals, H_2O_2 and singlet oxygen, which in turn activate a cascade of reactions leading to the activation of defensive enzymes [39]. The decrease in phenol levels may be due to their consumption in reduction of ROS as cited in the above reference along with simultaneous suppression of phenol synthesis machinery in response to biotic stress.

Chlorophyll 'a', Chlorophyll 'b' and Total chlorophyll

Changes in the chlorophyll content of leaf tissues is an important indicator of disturbed chloroplast development and impaired photosynthetic capacity in plants exposed to a broad spectrum of biotic and abiotic stress. [40,41]. As per the results obtained in this study Chlorophyll 'a', Chlorophyll 'b' and Total chlorophyll in the leaves of both rice genotypes decreased due to increased salinity. Among genotypes CSR 23 recorded higher Chlorophyll 'a', Chlorophyll 'b' and Total chlorophyll compared to BPT 5204. Similar result was reported by [42]. Increased salinity levels reduced chlorophyll a, chlorophyll b contents and total chlorophyll. This could be attributed to the destruction of chlorophyll pigments and the instability of the pigment protein complex [43]. It is also attributed to the interference of salt ions with the de-novo synthesis of proteins, the structural component of chlorophyll, rather than the breakdown of chlorophyll [44]

Conclusion

The current study was aimed at identifying a local rice genotype for breeding program and for popularization of the variety among the local farmers. The result of the study has indicated convincingly that CSR 23 has better tolerance mechanism to salinity stress compared to BPT 5204.

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Application of research: The resistant cultivar CSR 23 will be used for breeding further breeding program to come up with a better resistant varieity. The information obtained from the present investigation will be utilized to design and conduct molecular experiments involving marker assisted selection , transcriptome analysis and stress response protein identification .

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Author Contributions:

Shashi Kumar: The graduate student has carried out the research work as a part of her M.Sc. Programme.

Kiran Kamalakar Mirajkar: Principle Investigator, who has conceptualized, designed the research programme and arranged for all the infrastructural facilities required for the research programme.

Aravind Kumar – professor of Agronomy has guided and helped us in conducting pot culture studies in poly house**.**

Supreeth kulkarni – Graduate Assistant , management of poly house and handling instruments and conducting analysis and drafting**.**

Renuka Sudarshan Patil: Research Associate, contributed to designing and

calibration of biochemical investigations.

Abbreviations:
PAL: Phenylalanine ammonia Lyase
POX: Peroxidase
PR proteins: Pathogenesis related proteins**.**
CAT: Catalase
SOD: Supreroxide Dismutase
GR: Glutathione Reductase
NR: Nitrate Reductase
NiR: Nitrite Reductase
NBT: p-nitrobluetetrazolium chloride
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
NADPH: ß-nicotinamide adenine dinucleotide phosphate (reduced form)
NNED: N-1- napthyl ethylene diamine hydrochloride
DMSO: Dimethyl sulphoxide

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest: None declared

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