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Evaluation of biochemical parameters in nitrate-hardened seeds of Indian mustard subjected to salt stress

Jai Bharti and CP Malik*

School of life Sciences, Jaipur National University, Jaipur, Rajasthan
**Corresponding author: cpm_malik@yahoo.com*

Abstract

Studies were conducted to evaluate the effects seed hardening (hydration-dehydration) treatments with KNO_3 on some physiological alterations (chlorophyll, carbohydrate, free amino acids and molecules concerned with peroxidation (H_2O_2, MDA), proline, antioxidative enzymes (SOD, CAT and APX)). The seedlings were subjected to salt stress (0.5% and 0.75%). Under salinity stress chlorophyll decreases over control whereas carbohydrates, free amino acids, MDA and proline contents increased over control. Nitrate hardening also increased activities of antioxidative enzymes (SOD, CAT, and APX) and lowered lipid peroxidation. Present investigations suggest that the hardening of seeds with KNO_3 for short duration induces the endurance capacity in Indian mustard seedlings to grow with salinity stressful conditions.

Key Words: *Malondialdehyde, superoxide dismutase, catalase, ascorbate peroxidase, proline*

Introduction

Indian mustard (*Brassica juncea*) is most widely grown among the rapeseed-mustard crops and is the second largest oil seed crop next to groundnut in terms of area and production in India. According to the United Nations Environmental Program approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flower and Yeo 1995). Growth and productivity of a plant is adversely affected due to abiotic stresses such as salinity as it triggers a biochemical changes in the life cycle of a plant. Salt stress constrained the growth and development of crop plants by limiting the crop yield and food production. This crop is mostly confined to arid and semi arid regions of the country. Average yield of this crop is 872 Kg/ha but in salt affected areas, the average yield is reduced to 580 Kg/ha. Among different *Brassica* species, Indian mustard shows higher tolerance to salinity stresses compared to other plain varieties. The sustained breeding efforts for the development of high yielding salt tolerant genotypes of Indian mustard have led to the development and release of a salt tolerant genotype CS 52. Salinity affects the metabolic pathways (Murumukar and Chavan, 1986) due to adverse effects of salinity through osmotic effects or ionic balance (Greenway and Munns, 1980). The salt stress lower the osmotic potential, increases ion toxicity and nutritional imbalance that lead to the formation of reactive oxygen species.

Seed priming helps in alleviating the adverse effects of abiotic stresses on germination and seedling growth. Generally Ca^{2+} and K^+ ions are decreased under saline condition (Kent and Lauchli 1985, Al-Harbi 1995, Lin and Kao, 1995). Priming might be affecting the concentration of these ions under salt-stressed conditions, thereby improving plant growth (Ashraf *et al*., 2003a, Kaur *et al*., 2003). Seed hardening (hydration-dehydration) treatments to seeds has been done using various chemicals like Ca (NO_3), GA_3, KNO_3, NaCl, KCl, KH_2PO_4, etc. (Kaur *et al*., 2008). In the present investigation the effects of various levels of salinity on the nitrate-hardened seeds has been evaluated physiologically in Indian mustard.

Materials and Methods

Seeds of *Brassica juncea* cultivars *viz.*, CS 52 and Shivalik were obtained from Central Soil Salinity Research Institute, Karnal, Haryana and Directorate of Rapeseed Mustard Research,

Bharatpur, Rajasthan, respectively. The CS 52 as salt tolerant whereas Shivalik non tolerant variety of *Brassica*. Seeds were hardened by presoaking in potassium nitrate for 6 hrs, then dried at 25ºC and brought to the original weight. These seeds are referred to as HS. Seeds were surface sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 minutes and (NHS and HS) were incubated in Petri plates lined with blotting paper. Salt solution of 0.5% and 0.75% concentration was added to the Petri plates. Seeds germinated in water alone were treated as controls. Biochemical and biophysical analyses carried out after 10 days of sowing and following parameters were determined. Each treatment was replicated thrice.

Chlorophyll content

Total chlorophyll, chlorophyll-a and chlorophyll-b content were estimated spectrophotometrically (Coomb's *et al*., 1985) after extracting leaf discs (0.2 g) in 80% (v/v) acetone. The absorbance of the solution was recorded at 647and 664.

Determination of Carbohydrate and free amino acids content

The oven-dried leaf tissue was homogenized in hot ethanol and centrifuged at 2000 rpm for 10 min. Supernatant was clearly decanted off and 3 ml. of ethanol was added to the residue and recentrifuged. The extraction was repeated twice to ensure the complete recovery of sugars. The supernatant was evaporated in boiling water bath. The residue was eluted with 5 ml of 20% ethanol and subjected to analysis for total sugars (Yemn and Wills, 1954), reducing sugars (Sumner, 1935) and free amino acids (Lee and Takashi, 1966).

Estimation of lipid peroxidation

The level of lipid peroxidations in the membranes was measured in terms of MDA content (Heath and Packer, 1968). Total 0.2 g leaf tissue was extracted in 5 ml of 0.1% (w/v) TCA and centrifuged at 15000 rpm for 5 min. To 1 ml aliquot of the supernatant, 4 ml of 0.5% of TBA in 20% TCA was added. The mixture was heated at 95ºC for 30 min. and quickly cooled in ice bath. After centrifugation at 10000 rpm for 10 min. the absorbance of the supernatant was recorded at 532 nm.

Estimation of proline

Proline was estimated using the method of Bates *et al*. (1973). Leaf tissues were homogenized with 3% sulphosalicylic acid and the homogenate was centrifuged to 10 000 rpm for 10 min. The reaction mixture consisting of 2 ml each of supernatant, acid ninhydrin and glacial acetic acid was boiled at 100ºC for 1 hr. After termination of the reaction in ice bath, the reaction mixture was extracted with 4 ml toluene and the absorbance was read at 520 nm.

Determination of activities of antioxidant enzymes

Superoxide dismutase (EC 1.15.1.1)

The activity was assayed according to Dhindsa *et al*. (1981). The leaf tissue was homogenized in 50 mM phosphate buffer, 0.25% triton X-100 (m/v) and 1% polyvinyl pyrrolidone and centrifuged at 10 000 g for 10 min at 4ºC and supernatant treated as enzyme extract. The reaction mixture contained 13 mM methionine, 25 mM NBT, 0.1 mM EDTA, 50 mM sodium bicarbonate, 50 mM phosphate buffer (pH 7.8) and 0.1 ml of enzyme extract. The reaction was initiated by adding 2 mM riboflavin and exposing to 15 nm and the total SOD activity of the samples was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue-tetrazolium (NBT). 1 unit of SOD activity was defined as the amount of enzyme, which causes 50% inhibition of the photochemical reduction of NBT.

Ascorbate peroxidase (EC 1.11.1.11)

APX activity was determined by following oxidation of ascorbate as a decrease in absorbance at 290 nm (Nakano and Asda, 1981). Leaf tissue was homogenized in ice cold 50 mM phosphate buffer, centrifuged at 10 000 g at 4ºC. The assay was carried out at 20ºC in 3 ml reaction mixture containing 50 mM phosphate buffer pH 7.0, 0.1 mM EDTA, 0.5 mM ascorbic acid, and enzyme extract. The change in A_{290} was recorded at 30 s interval after addition of H_2O_2.

Catalase (EC 1.11.1.6)

Catalase activity was determined by the method of

Aebi (1983). The reaction mixture contain phosphate buffer (pH 7.0) 50 mM centrifuged at 10 000 g for 10 min. Reaction mixture contained supernatant and H_2O_2 in ratio 1:3 and absorbance recorded at 240 nm.

Results and Discussions

The effective concentration which reduced chlorophyll content significantly was 0.75%. Increased salt stress reduced the chlorophylls a, b and total chlorophyll content, whereas Chl a/b ratio also decreases (Tables 1 and 2).

Carbohydrates and free amino acids contents

Increased concentration of salt caused the increases of the total sugars, reducing sugars and free amino acid. Nitrate hardened seeds had enhanced germination and soluble sugars that facilitated an osmotic adjustment which is an adaptive response to stress. The enhancement of sugars in response to stress possibly acted as compatible solutes in the stabilization of biological structures (Tables 3 and 4).

Table 1 : Chlorophyll content (mg g^{-1} FW) as affected by different percentage of salt stress in tolerant variety *Brassica*

Chlorophyll contents	HS/NHS	Control	0.5	0.75
Chl a	HS	0.309	0.269	0.260
	NHS	0.231	0.233	0.177
Chl b	HS	0.230	0.216	0.210
	NHS	0.183	0.164	0.125
Total Chl	HS	0.539	0.485	0.470
	NHS	0.418	0.397	0.302
Ratio a/b	HS	1.34	1.24	1.23
	NHS	1.28	1.42	1.41

Table 2 : Chlorophyll content (mg g^{-1} FW) as affected by different percentage of salt stress in plain variety of *Brassica*

Chlorophyll contents	HS/NHS	Control	0.5	0.75
Chl a	HS	0.237	0.209	0.188
	NHS	0.227	0.208	0.167
Chl b	HS	0.182	0.163	0.143
	NHS	0.179	0.168	0.137
Total Chl	HS	0.419	0.372	0.338
	NHS	0.406	0.358	0.304
Ratio a/b	HS	1.30	1.28	1.27
	NHS	1.27	1.24	1.24

Lipid peroxidation

MDA, cytotoxic product of lipid peroxidation, increased under the salt stress in nitrate hardened seeds as compared to water control. Increase was higher in tolerant (CS 52) variety as compared to

Table 3: Carbohydrates and free amino acid contents (mg g^{-1}DM) as affected by different percentage of salt stress in tolerant variety of *Brassica*

Carbohydrate contents/ Free amino acids	HS/NHS	Control	0.5	0.75
Total sugars	HS	1.29	1.14	1.00
	NHS	1.23	1.09	0.95
Red. sugars	HS	0.74	0.42	0.29
	NHS	0.66	0.33	0.20
Free a.acids	HS	0.017	0.016	0.008
	NHS	0.015	0.013	0.006

Table 4: Carbohydrate and free amino acid content as affected by different percentage of salt stress in non tolerant variety of *Brassica*

Carbohydrate contents/ Free amino acids	HS/NHS	Control	0.5	0.75
Total sugars	HS	1.08	1.32	1.49
	NHS	0.91	1.41	1.30
Red. sugars	HS	0.36	0.56	0.60
	NHS	0.34	0.42	0.52
Free a.acids	HS	0.010	0.016	0.021
	NHS	0.007	0.011	0.017

plain variety of *Brassica juncea* (Tables 5 and 6). Salt stress induced lipid peroxidation is mostly attributed to increased production of ROS especially ·OH radicals in plant systems (Halliwell and Guteridge 1984). Possibly it affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methyl-CH_2-group that possess especially reactive hydrogen. Our results indicate that salt stress induced membrane disruption in plant seedlings.

Proline

During stress nitrogen compounds *e.g*. proline increased and alterations in its contents reflected the extent of stress and endurance of the system. Under normal conditions proline is produced from ornithine but under stress it is made directly from glutamate. Accumulation of proline is an adaptive mechanism for reducing the level of accumulated NADH and the acidity ($2NADH + 2H^+$) is used for synthesizing each molecule of proline from glutamic acid. Proline (osmolyte) protecting the enzymes, stabilization of machinery of protein synthesis, regulation of cytosolic acidity and also acts as an effective singlet oxygen quencher. Increase in both proline and MDA content in tolerant variety (CS 52) with increasing salt stress are indicative of a correlation between free radical generation and proline accumulation. Proline also helps in osmotolerance (Kishoe *et al*., 1995, Bajji *et al*., 2000). Of particular significance in the present

Table 5 : MDA (µM g^{-1} FW) and Proline contents (µM g^{-1} FW) as affected by different percentage of salt stress in tolerant variety of *Brassica*

MDA/Proline	HS/NHS	Control	0.5	0.75
MDA	HS	8.06	10.0	12.5
	NHS	10.3	14.1	18.0
Proline	HS	2.97	3.49	4.67
	NHS	1.72	2.51	3.75

Table 6 : H_2O_2, MDA (µg g^{-1} FW) and Proline content (µM g^{-1} FW) as affected by different percentage of salt stress in plain variety of *Brassica*

MDA/Proline	HS/NHS	Control	0.5	0.75
MDA	HS	3.87	9.03	15.16
	NHS	2.90	6.77	11.29
Proline	HS	1.63	2.28	2.58
	NHS	1.52	1.95	2.06

studies that appeared to be an indicator of salt injury to the seedlings was increase the elevation of proline and MDA more in tolerant variety (CS 52) as compared to plain variety of *Brassica juncea*. (Tables 5 and 6).

Antioxidants

Under the stress condition there is formation of ROS and superoxide radicals. The productive mechanism adopted by plants to scavenge harmful ROS and peroxides include several antioxidative enzymes, such as SOD, CAT and APX. These antioxidative enzymes are important components in protecting plant cells from oxidative stress as they increased in plant when exposed to stress conditions. These radicals not only react with most cellular components, but also spontaneously combine with peroxides H_2O_2 to form singlet oxygen and hydroxyl radical which is disruptively reactive.

Superoxide dismutase (SOD)

SOD converts the superoxide H_2O_2 into water and oxygen. Superoxide is charged molecule and cannot cross biological membranes. H_2O_2 which is formed as a result of SOD action is capable of diffusing across membranes and is thought to fulfill a signaling function in defense responses. SOD enzymes increased under the stress of salt. In hardened seeds SOD increased as compared to non-hardened seeds. Increased level of this free radical scavenging enzymes due to the oxidative stress during priming could also protect the cell against membrane damage due to lipid peroxidation occurring naturally.

Catalase (CAT)

CAT increased under the stress. CAT is a common enzyme found in nearly all living organism which are exposed to oxygen, where it function as to catalyze the decomposition of H_2O_2. Catalase is critical for maintaining the redox balance during oxidative stress. It functions as a cellular sink for H_2O_2.

Ascorbate peroxidase (APX)

APX increased under stress condition. APX play an important role in reducing oxidative stress by catalyzing the reduction of H_2O_2 using ascorbate as a substrate. They catalyze the transfer of electrons from ascorbate to peroxide producing dehydroascorbate and water as products. Thus, the increase in activities of APX by salt stress suggested increased production of H_2O_2. The role of this enzyme has been proposed to be important in regulating the levels of reduced and oxidized glutathione and NADPH.

Table 7: Antioxidant enzymes content as affected by different percentage of salt stress in tolerant variety of *Brassica*

Enzymes	HS/NHS	Control	0.5	0.75
SOD	HS	1.02	1.34	1.39
(IU)	NHS	0.60	1.15	1.28
CAT	HS	0.64	0.86	1.218
(µM $ml^{-1}g^{-1}$ FW)	NHS	0.43	0.75	0.96
APX	HS	1.33	1.67	2.14
(µM $ml^{-1}g^{-1}$ FW)	NHS	0.80	1.07	1.33

Table 8: Antioxidant enzymes content as affected by different percentage of salt stress in plain variety of *Brassica*

Enzymes	HS/NHS	Control	0.5	0.75
SOD	HS	0.09	0.10	0.12
(IU)	NHS	0.04	0.06	0.09
CAT	HS	0.52	0.74	0.94
($\mu M\ ml^{-1}g^{-1}$ FW)	NHS	0.38	0.59	0.78
APX	HS	0.96	1.13	1.29
($\mu M\ ml^{-1}g^{-1}$ FW)	NHS	0.78	0.91	1.10

Catalase, superoxide dismutase, ascorbate peroxidase and another key enzyme quenching free radicals also increases during priming. Increased levels of these free radical scavenging enzymes due to the oxidative stress during priming could also protect the cell against membrane damage due to lipid peroxidation. (Tables 7 and 8).

Discussion

Salt stress causes hyper osmotic stress and ion disequilibrium by causing a water potential imbalance between the apoplast and symplast by this there is decrease in turgar. The Cellular response to turgar reduction is osmotic adjustment which is done by compartmentalization of compatible osmolytes and osmoprotectants (Bohnert, 1995; Bohnert and Jensen, 1996). Chlorophyll content was reported to decrease in salt resistant crops like mustard (Singh *et al*., 1990).

There is accumulation of soluble carbohydrates in plants under the salt stress (Murakeozy *et al*., 2003). Carbohydrates such as sugars (glucose, fructose, sucrose, fructans) and starch accumulate under salt stress (Parida *et al.*, 2002). Salt stress accumulates amino acids and amides (Mansour, 2000). Total free amino acids in the leaves have been reported to be higher in salt tolerant than in salt sensitive lines of sunflower (Ashraf and Tufail, 1995)

Osmotic adjustment by the plant is through accumulation of some kind of compatible solutes such as proline, betaine and polyols to protect membranes and proteins (Delauney and Verma, 1993). There is also report that proline stabilize cellular proteins and membranes in presence of high concentrations of osmoticum (Yancey, 1994 and Errabii *et al*. 2006). Beside salt stress increasing amount of proline (Poustini *et al*., 2007), was also reported in several other stress conditions such as cold (Charest and Phan, 1990) and U.V (Tian and Lei, 2007) in wheat.

Under the salt stress there is formation of reactive oxygen species. Antioxdants and antioxidative enzymes (SOD, APX, CAT) eliminate the ROS by quenching the free radicals and ultimately alleviates the oxidative stress. ROS such as super oxide redical, hydroxyl radical causes the peroxidation of lipid in the membrane as a result the membrane get damaged. Salt stress resulted in accumulation of H_2O_2 in leaves due to activity of SOD. Higher lipid peroxidation indicates the extent of oxidative injury to cell membranes in terms of MDA. Priming repair the membrane system and initiate the reactivation of membrane bound enzymes and enhance the germination (Burgass and Powell 1984, Rao *et al.*, 1987).

Antioxidative enzymes play a vital role in conferring tolerance to abiotic stress. Free-radical scavenging enzyme such as catalase and superoxide dismutase increases during priming of seeds.

Salt stress inhibited chlorophyll synthesis by impairing uptake of essential elements such as Mg and Fe by the seedlings and hence affects the photo system. Nitrate hardened seeds had enhanced germination and soluble sugars. Soluble sugars facilitated osmotic adjustments which is an adaptive response to stress. Increase in both hydrogen peroxide and MDA content is indicative of a correlation between free radical generation and membrane damage. During stress nitrogen compounds e.g. proline increased and alterations in

its contents reflected the extent of stress and endurance of the system. Increased activity of free radical scavenging enzymes (catalase. superoxide dismutase, ascorbate peroxidase) in HS decreased oxidative stress protecting the cell against damage due to lipid peroxidation. Antioxidative enzymes quench the free radicals generated by lipid peroxidation and provide protection to the cell. Present investigations suggest that the hardening of seeds with nitrate salts could overcome deleterious effects of salt stress by accentuating nitrogen containing compounds e.g free amino acids, proline etc.

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