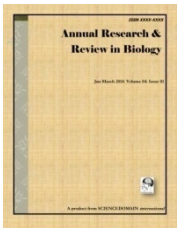

Annual Research & Review in Biology
4(21): 3274-3296, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Photosynthetic Differences in Mustard Genotypes under Salinity Stress: Significance of Proline Metabolism

Noushina Iqbal[1*], Shahid Umar[1*] and Nafees A. Khan[2]

[1]Department of Botany, Jamia Hamdard University, New Delhi 110062, India.
[2]Plant Physiology and Biochemistry Section, Department of Botany, Aligarh Muslim University, Aligarh 202 002, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author NI carried out the experimental work and searched literatures for the work on which this article is based and wrote the first draft of the manuscript. Authors SU and NAK supervised the work and helped in presentation of the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 31st March 2014
Accepted 2nd May 2014
Published 9th June 2014

ABSTRACT

Aim: To determine the importance of proline in protection of photosynthetic capacity under salt stress in mustard cultivars (*Brassica juncea* L. Czern and Coss.).
Design of the Study: Mustard plants were grown with or without 100mM NaCl treatments.
Place and Duration of Study: The experimental work was carried out in the herbal garden of the Department of Botany, Jamia Hamdard, New Delhi, India under natural day/night conditions, between November to December, 2013.
Methodology: Plants were sampled at 30 days after seed sowing to determine physiological, biochemical and growth parameters.
Results: Salinity stress significantly reduced the studied photosynthetic parameters in all the cultivars. However, Pusa Jai Kisan with higher proline content better protected its photosynthesis and showed higher tolerance to salinity stress. Higher proline content was a result of increased N assimilation and increased synthesis of proline synthesizing enzymes, pyrolline-5-carboxylate synthetase and γ-glutamyl kinase while decrease of proline oxidase, the proline degrading enzyme. Higher inherent and salinity induced proline content in Pusa Jai Kisan was responsible for higher water relations, photosynthetic pigments and growth under salt stress.

**Corresponding author: Email: naushina.iqbal@gmail.com, s_umar9@hotmail.com;*

Conclusion: Proline accumulation is an important strategy that plants operate to minimize the adverse effects of salinity stress on photosynthesis. Among all the cultivars proline content was maximum in Pusa Jai Kisan which led to comparatively higher osmotic potential and water potential, allowing the plants for higher intake of water and thereby resulting in increased stomatal opening and photosynthetic efficiency. Higher proline content resulted in lesser oxidative stress and consequently higher photosynthesis in Pusa Jai Kisan. The knowledge on the role of proline in protection of photosynthetic potential under salt stress could be exploited to develop tolerant line through intensive selection and breeding programs.

Keywords: Mustard; osmolytes; photosynthesis; proline; salinity.

ABBREVIATIONS

PNUE, Photosynthetic-Nitrogen Useefficiency; NR, Nitrate Reductase; N, Nitrogen; P5CS, pyrolline-5-carboxylate synthetase; GK, γ-glutamyl kinase; P_N, net photosynthetic rate; g_s, stomatal conductance; [$(CO_2)i$], intercellular CO_2 concentration, Chl, chlorophyll.

1. INTRODUCTION

Salinity is one of the most important abiotic stress that challenges sustainable agriculture globally and is shrinking the arable land at an alarming rate. About one-fifth of the world's irrigated land is affected by salinity [1] causing an adverse effect on plants photosynthesis and growth potential [2,3]. Although the plant's growth is controlled by a multitude of physiological, biochemical, and molecular processes, photosynthesis is a key phenomenon, which contributes substantially to the plant growth, development and productivity [4]. Decreased leaf turgor under salinity stress closes stomata which inturn reduces stomatal conductance and photosynthesis [5]. The salinity induced stomatal limitations for diffusion of gases ultimately alters photosynthesis and the mesophyll metabolism [5]. Kanwal et al. [6] reported that photosynthetic rate is adversely affected by salinity stress with maximum reduction in net carbon dioxide assimilation at higher level of salinity. Infact, photosynthetic attributes play crucial role in sustaining plant growth under saline stress [7].

Mustard is an economically important oilseed crop in India. Among the seven edible oilseeds cultivated in India, rapeseed-mustard contributes 28.6% to the total oilseeds production and ranks second after groundnut sharing 27.8% in the India's oilseed economy [8]. Among rapeseeds, *Brassica juncea* contributes 85% of the total rapeseed mustard production [9]. Its maximum distribution area is centered in the North-West agro-climatic zone, where the majority of ground water sources are highly saline and have medium to high sodicity problems [10]. Therefore, increasing mustard production under salinity stress is a growing concern.

In order to increase food production on salt stressed lands, understanding salt tolerance strategies is required. Plant species have demonstrated a wide degree of variation in their abilities to tolerate the toxic effects of salinity stress [11-13]. Different selection indicator could be used to assess inter-cultivar variation for salinity tolerance in different potential crops [14]. Ranganayakulu et al. [15] reported that osmolytes could be used as indicator for salt tolerance, as the variation in osmolytes accumulation is related to difference in salt tolerance. Accumulation of osmolytes is ubiquitous in plants [16] and helps plant to survive

extreme osmotic stress [17]. Among osmolytes, proline accumulation can be used as an indicator in selection for withstanding saline stress through the involvement in osmoregulation [18-19]. Proline is one of the most important osmolyte involved in osmotolerance in salinity stressed plants. It is a multifunctional amino acid and a major osmolyte that accumulates in plant cells in response to increased salinity [20-22]. The synthesis of proline in plants occurs mainly from glutamate, which is reduced to glutamate-semialdehyde (GSA) by the P5CS (pyrolline-5-carboxylate synthetase)enzyme, and converted to pyrroline-5-carboxylate (P5C). P5C intermediate is reduced to proline by P5C reductase (P5CR). Proline degradation occurs in mitochondria via the sequential action of proline dehydrogenase or proline oxidase (PDH or POX) producingP5C from proline, and P5C dehydrogenase (P5CDH), which regenerates glutamate from 5C. Another pathway of proline synthesis is via ornithine which is transaminated first byornithine-delta-aminotransferase (OAT) producing GSA and P5Cand then converted to proline.

Proline might act as a signaling molecule to modulate gene expression and influence cell proliferation [23]. Ashfaque et al. [24] reported that the application of H_2O_2 alleviated the salt inhibited photosynthesis probably through increase in proline content which acted as an antioxidant and protected photosynthetic machinery from salt-induced reactive oxygen species (ROS). Foliar applied proline has been reported to increase the photosynthetic attributes and yield characteristics in the cadmium-stressed *Cicer arietinum* plants [25]. Thus, there exists a relationship between proline accumulation, photosynthesis and salt tolerance.

The present study was conducted on four cultivars of mustard grown under salinity stress to study proline metabolism and associated changes in photosynthetic attributes. Such study would provide more insight into the development of salinity tolerant cultivars through intensive selection and breeding programs.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Mustard (*Brassica juncea* L.) cv. Pusa Jai Kisan, Basanti, Rohini and RH30 obtained from Pusa, New Delhi were surface sterilized with 0.1% HgCl2 followed by repeated washings with deionized water, and were sown in 15-cm-diameter earthen pots filled with acid-washed sand purified according to Hewitt [26] in the herbal garden of the Department of Botany, Jamia Hamdard, New Delhi, India under natural day/night conditions with a day and night temperature of 24/18±3ºC and relative humidity of 68±6%. Two plants per pot were maintained and were subjected to control (0mM NaCl) and treatment (100mM NaCl). Pots were saturated with 300mL of 100mM NaCl in the form of modified full strength Hoagland's nutrient solution every alternate day and 250ml of de-ionized water daily. The nutrient solution was replaced weekly. The control groups of plants were fed with 300ml nutrient solution every alternate day and 250ml of deionized water daily. The experiment followed a randomized complete block design and the number of replicates for each treatment was four (n=4). Measurements were done at 30 DAS and care was taken to select the leaves of same age for the determinations.

2.2 Estimation of Leaf Na^+ and Cl^- Content and Content of H_2O_2 and TBARS

The content of Na^+ and Cl^-in leaf was determined in the digested plant samples using Tri acid mixture (TAM), which is a mixture of nitric acid, sulfuric acid and perchloric acid in the ratio of 10:5:4. The content of Na+ was estimated using flame photometer (Khera-391: Khera Instruments, New Delhi), whereas Cl^- content was determined by titration against 0.02N silver nitrate solution using 5% K_2CrO_4 as indicator.

The content of H_2O_2 was determined following the method of Okuda et al. [27]. Leaf tissues (250mg) were ground in ice cold 200mM perchloric acid. After centrifugation at 1200×g for 10 min, perchloric acid of the supernatant was neutralized with 4M KOH. The insoluble potassium perchlorate was eliminated by centrifugation at 500×g for 3min. The reaction was started by the addition of peroxidase and the increase in absorbance was recorded at 590nm for 3 min.

The level of lipid peroxidation in leaves was determined by estimating the content of thiobarbituric acid reactive substances (TBARS) as described by Dhinsa et al. [28]. Leaf tissues (2.5g) were ground in 0.25% 2-thiobarbituric acid in 10% trichloroacetic acid using mortar and pestle. After heating at 95ºC for 30 min, the mixture was quickly cooled on ice bathand centrifuged at 10,000×g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of TBARS was calculated using the extinction coefficient (155mM^{-1} cm^{-1}).

2.3 Estimation of Proline Content and Assay of P5CS, GK Activity and Proline Oxidase Activity

Proline content was determined spectrophotometrically by adopting the ninhydrin method of Bates [29]. 1.5g of leaf tissue were homogenized in 3mL of 3% sulphosalicylic acid. The homogenate filtrate was reacted with 1mL each of acid ninhydrin and glacial acetic acid for 1h in a test tube placed in a water bath at 100ºC. The mixture was extracted with toluene and the absorbance was measured on a spectrophotometer (UV–vis L164, Elico, Hydrabad, India) at 520nm using L-proline as a standard.

To determine the activity of P5CS, GK and proline oxidase, enzyme extract was prepared by homogenizing 500mg leaf sample in 0.1M Tris-HCl buffer, pH 7.5 at 4ºC. The homogenate was centrifuged at 30,000*g* for 30 min. The supernatant was used as the crude extract enzyme preparation for P5CS activity and pellet was collected and used as extract for assay of GK and proline oxidase.

P5CS was assayed according to the method of Hayzer and Leisinger [30] with a slight modification the extract was kept in a freezer at -20ºC. The frozen sample was suspended in 10 mL of 0.1 M Tris-HCl buffer containing 1mM 1, 4- dithiothreitol (DTT) to rupture the cell and centrifuged at 30,000×g for 30min. The assay mixture contained 50 mM L-glutamate, 10mM ATP, 20mM $MgCl_2$, 100mM hydroxylamine HCl and 50mM Tris-HCl, pH 7.0 with 200µL of desalted extract in a final volume of 500 µL. The reaction was started by the addition of enzyme extract. After 30 min of incubation at 37°C, the reaction was stopped by the addition of 1.0mL $FeCl_3.3H_2O$ (2.5%, w/v) and trichloroacetic acid (6%, w/v) in 2.5M HCl. Protein was precipitated and removed by centrifugation at 12,000× g (4°C) and absorbance was recorded at 540nm against a blank identical to the above but lacking ATP. The amount

of - glutamyl hydroxamate was determined by the A540 by comparison with a standard curve of glutamyl hydroxamate [30]. Activity of GK was expressed in U mg^{-1} protein. One Unit (U) of enzyme activity is defined as µg of γ –glutamyl hydroxamate produced per min.

Proline oxidase activity was determined adopting the method of Huang and Cavalieri [31] with slight modification. The pellet was mixed with 1mL Tricine, KOH buffer (pH 7.5) containing 6M sucrose. This extract was used for the enzyme assay. The assay mixture contained 1.2mL of 50mM Tris-HCl buffer (pH 8.5), 1.2mL of 5mM $MgCl_2$, 0.1mL of 0.5mM NADP, 0.1mL of 1mM KCN, 0.1mL of 1mM phenazine methosulfate, 0.1mL of 0.06mM 2, 6 dichlorophenol indophenols (DCPIP) and 0.1mL of 0.1M proline in a final volume of 3mL. The increase in absorbance was recorded at 600nm at 25ºC using proline to initiate the reaction. Proline oxidase activity was expressed in U mg-1 protein. One Unit (U) of enzyme activity is defined as mM DCPIP reduced per min.

2.4 Activity of NR, N Content and PNUE

Activity of NR in leaf was measured by preparing enzyme extract using the method of Kuo et al. [32]. Activity of NR was assayed spectrophotometrically as the rate of nitrite production at 28ºC adopting the procedure of Nakagawa et al. [33]. The absorbance was read at 540nm after 10 min. The details of determination of activity of NR have been described earlier by Iqbal et al. [34].

Leaf N content was determined in acid-peroxide digested material using the method of Lindner [35]. PNUE was calculated by the ratio of photosynthesis rate to N contentper unit leaf area. Leaf area was measured using leaf area meter (LA 211, Systronic).

2.5 Determination of Leaf Water Potential and Osmotic Potential

Leaf water potential was measured on second leaf from top of the plant by using water potential system (Psypro, WESCOR). The leaf used for water potential measurement was frozen in liquid nitrogen in sealed polythene bags, which was thawed and cell sap was extracted with the help of a disposable syringe. The extracted sap was used for the determination of osmotic potential using a vapor pressure osmometer (5520,WESCOR, USA).

2.6 Determination of Photosynthetic Traits

Total Chl content was estimated using the method of Hiscox and Israelstam [36] by using dimethyl sulfoxide as an extraction medium and estimated and calculated by the method of Arnon [37]. The details of determination of Chl content have been described earlier by Iqbal and Khan [38]. All spectrophotometric analyses were conducted on a 171 UV-VIS spectrophotometer (Elico, Hyderabad, India).

Effective quantum yield of photosystem II (Φ PSII) was measured with the help of chlorophyll fluorometer (OS-30p, USA). Plants were dark-adapted for 30min, minimal fluorescence (Fo) was measured during the weak measuring pulses and a saturating pulse was used to obtain maximal fluorescence (Fm) and Φ PSII was calculated.

Leaf gas exchange parameters (net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration) were measured in fully expanded intact leaves in different

treatments and replications plants with the help of an Infra Red Gas Analyzer (CI-340 Photosynthesis system, CID Bio-Science, USA). The measurements were made between 10.00-11.30 a.m. at light saturating intensity on a sunny day. The atmospheric conditions at the time of measurements were: PAR, ~640μmol m−2s−1, air temperature, ~22ºC and relative humidity, ~70%.

Rubisco activity was determined spectrophotometrically by adopting the method of Usuda [39] by monitoring NADH oxidation at 30ºC at 340nm during the conversion of 3-phosphorglycerate to glycerol 3-phosphate after the addition of enzyme extract to the assay medium. The details of determination Rubisco activity have been described earlier by Iqbal et al. [34]. Protein was estimated according to Bradford [40] using bovine serum albumin as standard.

2.7 Tolerance Index

Tolerance index was calculated as the ratio of dry mass of 100mM NaCl treated plants to dry mass of control plants and expressed as in percentage.

2.8 Statistical Analysis

Data were statistically analyzed using analysis of variance (ANOVA) by SPSS statistics (ver. 17.0), and presented as treatment mean ± SE (n=4). Least significant difference (LSD) was calculated for the significant data at $p<0.05$. Bars showing the same letter are not significantly different by LSD test at $p<0.05$.

3. RESULTS

3.1 Effects of Salinity Stress on Leaf Na^+ and Cl^-Content and Oxidative Stress

Salt treatment resulted in an increase in Na^+ and Cl^- content in all the cultivars and maximally in RH30, whereas, Pusa Jai Kisan showed minimum accumulation of Na^+ and Cl^- content (Fig. 1). Pusa Jai Kisan accumulated 21.0% Na^+ and 16.7% Cl^- content whereas RH30 accumulated 42.1% Na^+ and 53.1% Cl^- content in comparison to control. All the cultivars experienced oxidative stress under salinity stress as evident from the increased content of H_2O_2 and TBARS.

Maximum oxidative stress was observed in RH30 followed by Rohini, Basanti and minimum oxidative stress was noted in Pusa Jai Kisan (Fig. 2). In RH30 oxidative stress in terms of both H_2O_2 and TBARS content was greater than all other cultivars. In comparison to Pusa Jai Kisan the H_2O_2 content was about 2.34 times and TBARS content was about 3 times higher when compared to their respective control.

3.2 Salinity Stress Decreases Nitrogen Content, Nitrate Reductase Activity, Photosynthetic-Nitrogen use Efficiency and Water and Osmotic Potential Differentially in Cultivars

In the presence of salt stress nitrate reductase (NR) activity and nitrogen (N) content decreased in all the cultivars. Pusa Jai Kisan exhibited maximum NR activity and a subsequent N content followed by Basanti, Rohini and RH30 under salt stress (Fig. 3).

Nitrogen content in RH30 decreased by 2.3 times and NR activity by 1.5 times approximately compared to that of Pusa Jai Kisan when compared with their respective control. Photosynthetic-nitrogen use efficiency (PNUE) in Pusa Jai Kisan was higher than all the cultivars and RH30 exhibited maximum decrease in the PNUE (Fig. 3).

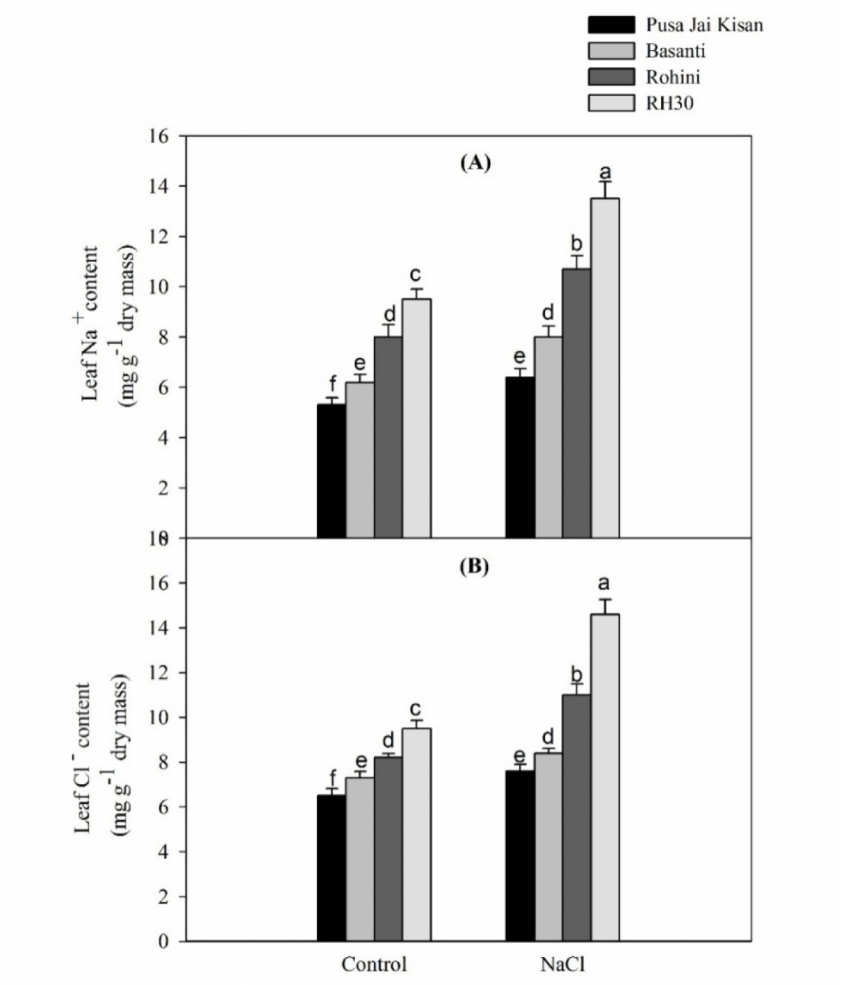

Fig. 1. Effects of NaCl (0 or 100mM) on Na^+ content (A) and Cl^- content (B) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

Salinity resulted in decreased leaf water potential and osmotic potential. Maximum decrease was noted in RH30 whereas Pusa Jai Kisan exhibited minimum decreased in water and osmotic potential in the presence of salt stress (Fig. 6).

3.3 Salinity Induces γ-glutamyl kinase and Inhibits Proline Oxidase for Proline Accumulation

Proline content increased with the increase in salt treatment. Both inherent and salinity induced proline content was higher in Pusa Jai Kisan and least in RH30. Pusa Jai Kisan with

maximum N content and NR activity exhibited maximum proline content followed by Basanti, Rohini and RH30. The increased proline content was in accordance with increased activity of γ-glutamyl kinase (GK) and pyrolline-5-carboxylate synthetase (P5CS) and decreased activity of proline oxidase. Maximum GK and P5CS activity was recorded in Pusa Jai Kisan and minimum in RH30. In contrast, Pusa Jai Kisan showed maximum decrease in proline oxidase activity whereas RH30 exhibited minimum decrease.

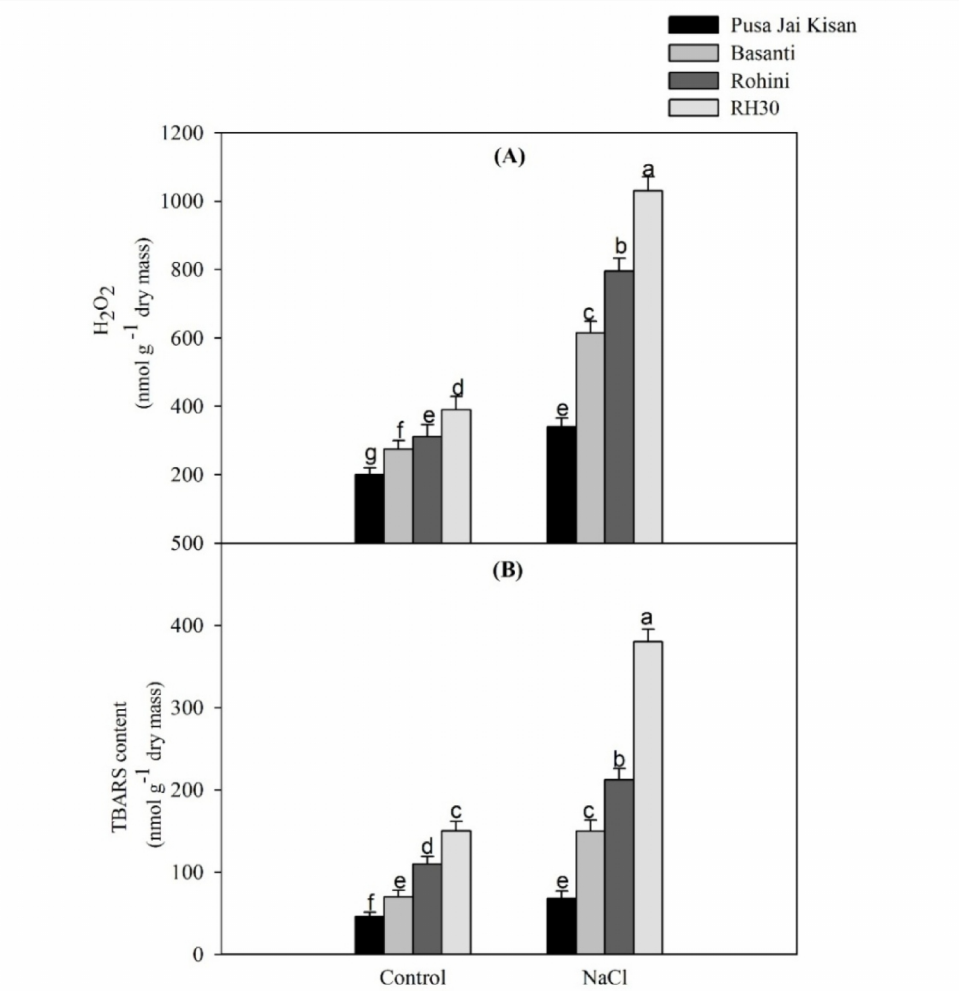

Fig. 2. Effects of NaCl (0 or 100mM) on H_2O_2 content (A) and TBARS content (B) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

Proline content increased by 112.3% in Pusa Jai Kisan, 78.1%, in Basanti, 64.1% in Rohini and 56.0% in RH30 upon salt treatment in comparison to their respective control. The GK activity in Pusa Jai Kisan was 2.8 times higher than that in RH30 compared to their control. In contrast, proline oxidase activity decreased by 38.3% in Pusa Jai Kisan, 18.5% in Basanti, 15% in Rohini, 13.1% in RH30 under salt stress in comparison to control (Fig. 4).

P5CS activity increased by 67.7% in Pusa Jai Kisan followed by 44.2%, 34.2%, and 25.0% in Basanti, Rohini and RH30 respectively in comparison to control (Fig. 5).

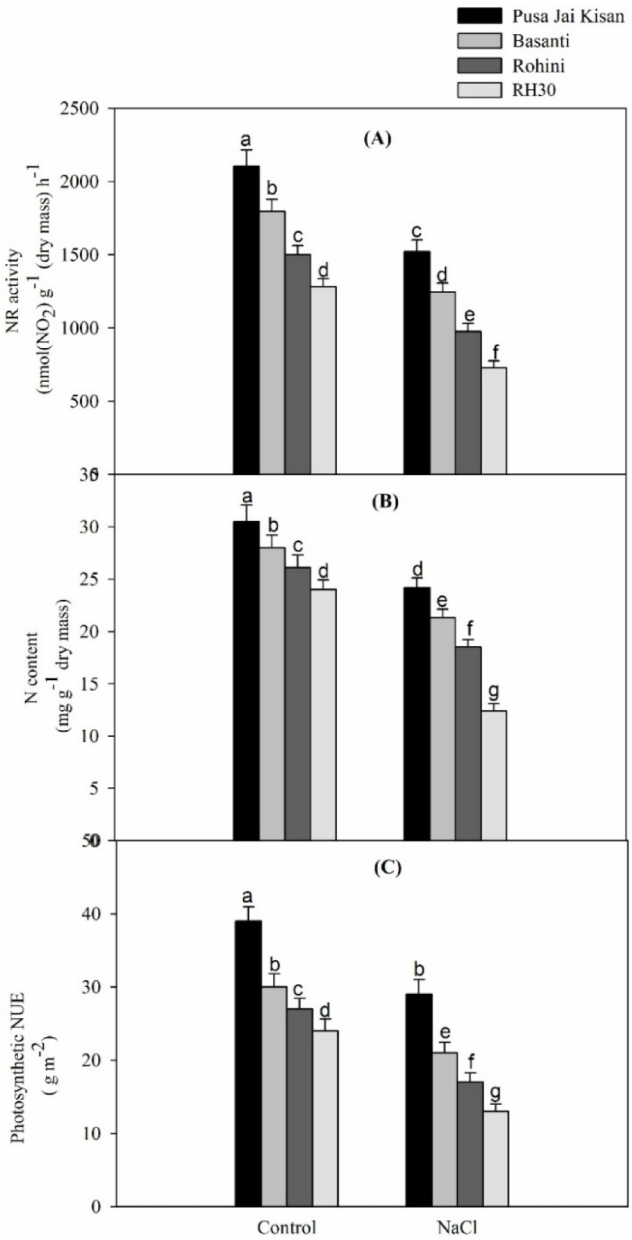

Fig. 3. Effects of NaCl (0 or 100mM) on leaf N content (A) NR activity (B) and PNUE (C) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

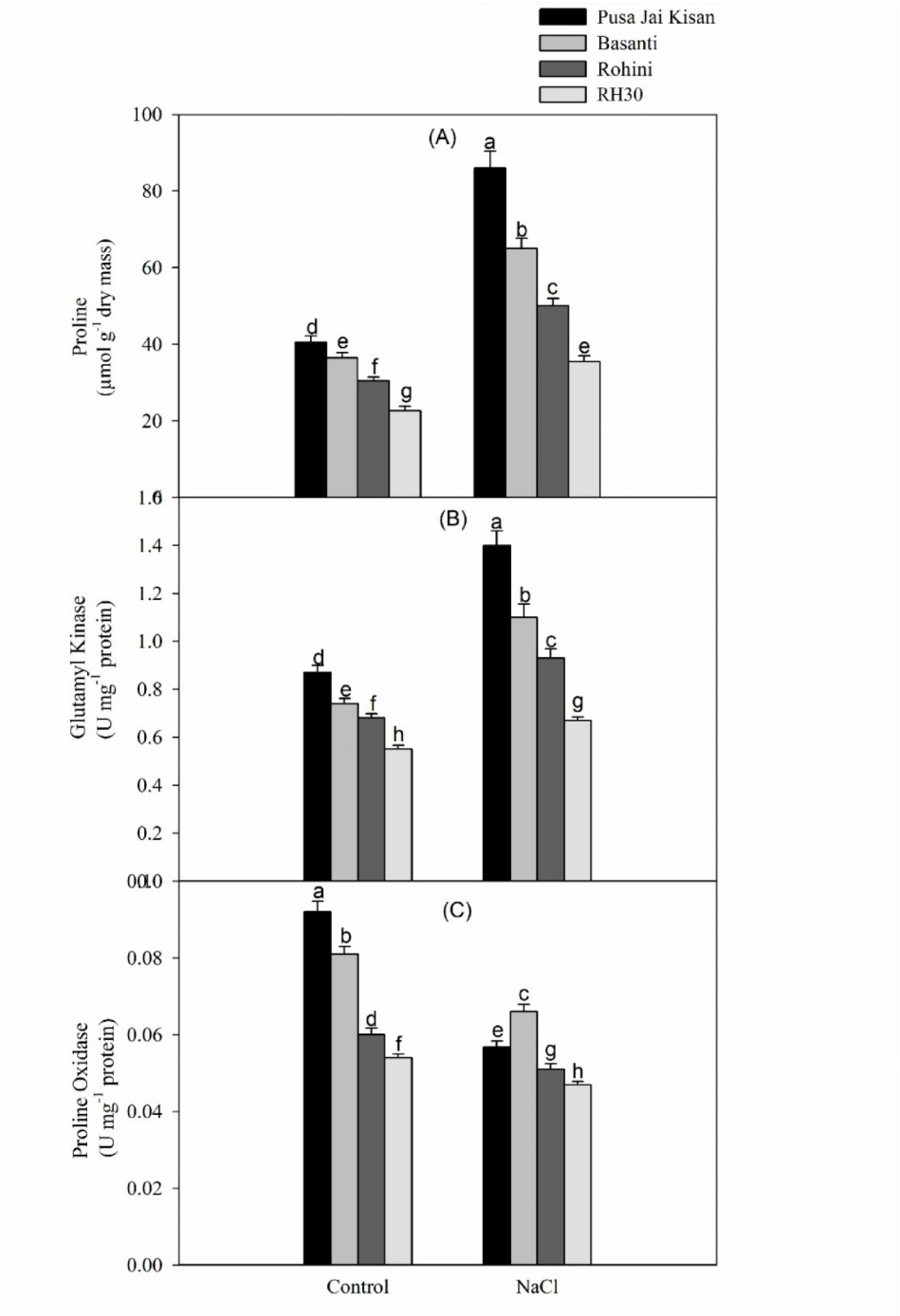

Fig. 4. Effects of NaCl (0 or 100mM) on proline content (A) GK activity (B) and proline oxidase activity (C) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

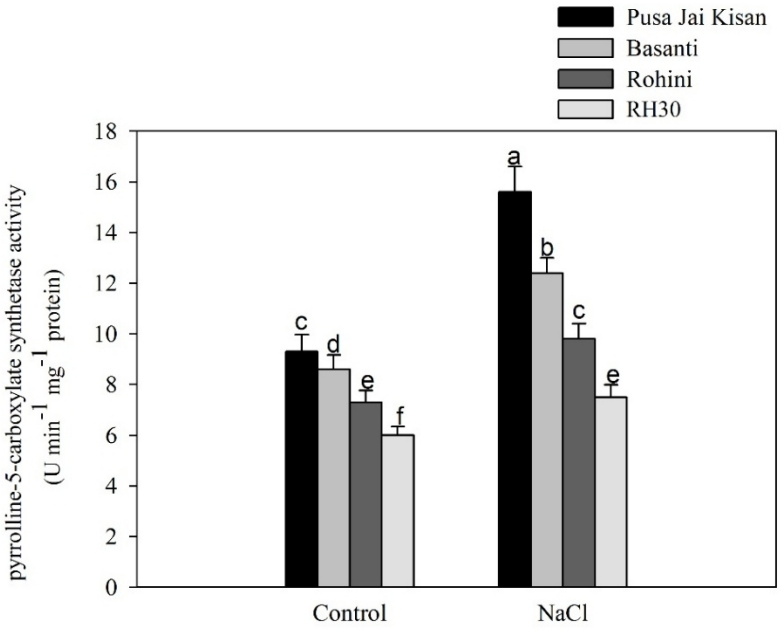

Fig. 5. Effects of NaCl (0 or 100mM) on P5CS activity of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

3.4 Variation in Photosynthetic Response of Cultivars to Salinity Stress and Their Tolerance Potential

Net photosynthetic rate (P_N), stomatal conductance (g_s,) and intercellular CO_2 concentration ($[CO_2]_i$) decreased substantially in the presence of 100mM NaCl. Maximum decrease was noted in RH30 whereas, Pusa Jai Kisan maximally protected its P_N, g_s, and ($[CO_2]_i$). Net photosynthesis decreased minimally by 13.3%, g_s by 27.5% and ($[CO_2]_i$) by 14.9% in Pusa Jai Kisan, whereas, maximum decrease of 47%, 55.7% and 36.5% in PN, g_s and ($[CO2]_i$) was observed in RH30 under salt stress in comparison to control (Fig. 7).

Chlorophyll (Chl) content decreased in all the cultivars with 100mM NaCl and maximally in RH30. Maximum Chl a, Chl b and total Chl content decreased in RH30 by 35.5%, 50.0% and 40.0% in the presence of NaCl whereas, maximum content of Chl a, Chl b and total Chl was observed in Pusa Jai Kisan. Pusa Jai Kisan exhibited minimum decrease in Chl a, Chl b and total Chl content of 21.4%, 24.6%, and 22.5% in the presence of NaCl compared to control (Fig. 8). Quantum yield of PSII decreased in the presence of salt with minimum decrease in Pusa Jai Kisan followed by Basanti, Rohini and RH30 (Fig. 9).

Rubisco activity was studied and it was observed that it decreased in the presence of salinity and maximum Rubisco activity was observed in Pusa Jai Kisan and minimum in RH30 under salinity stress (Fig. 9).

Pusa Jai Kisan with maximum proline content exhibited maximum protection of photosynthesis and had maximum tolerance index and RH with lowest proline content and

photosynthesis had minimum tolerance index. Basanti and Rohini lie in the middle of these two cultivars. Among them Basanti had higher tolerance index than Rohini (Fig. 10).

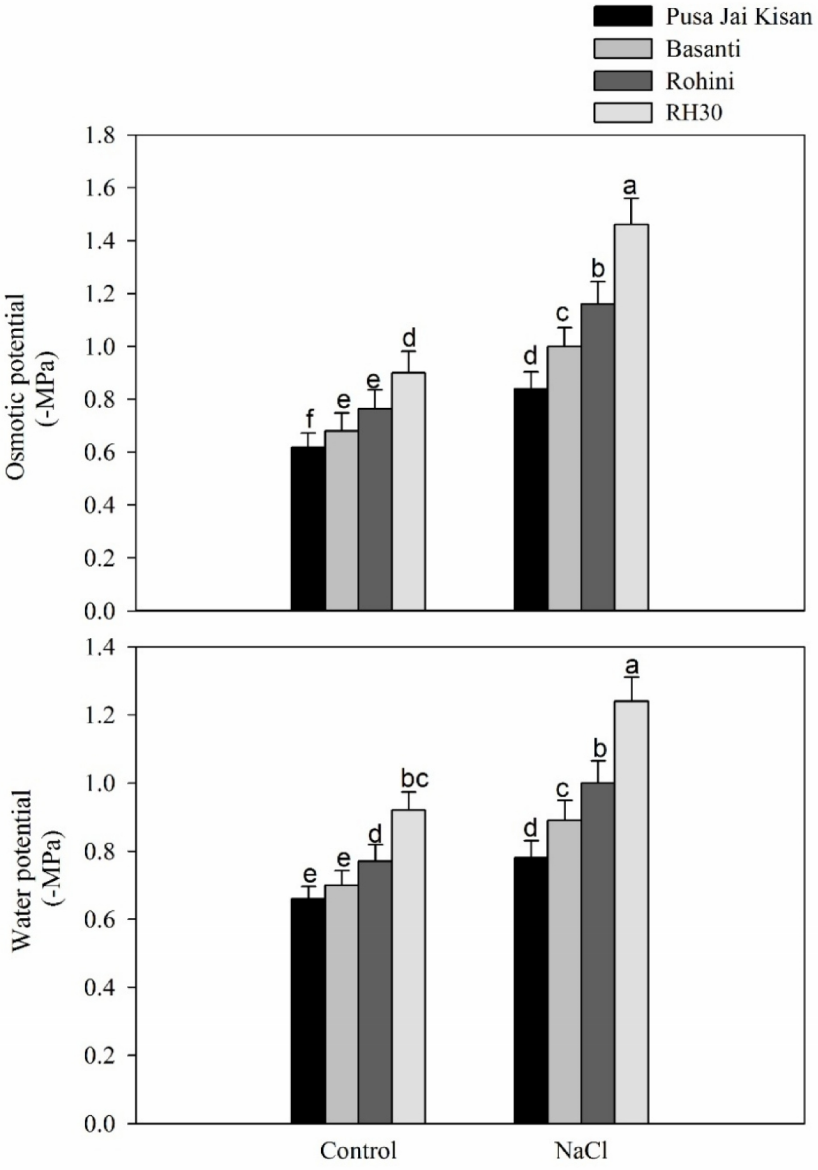

Fig. 6. Effects of NaCl (0 or 100mM) on osmotic potential (A) and water potential (B) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

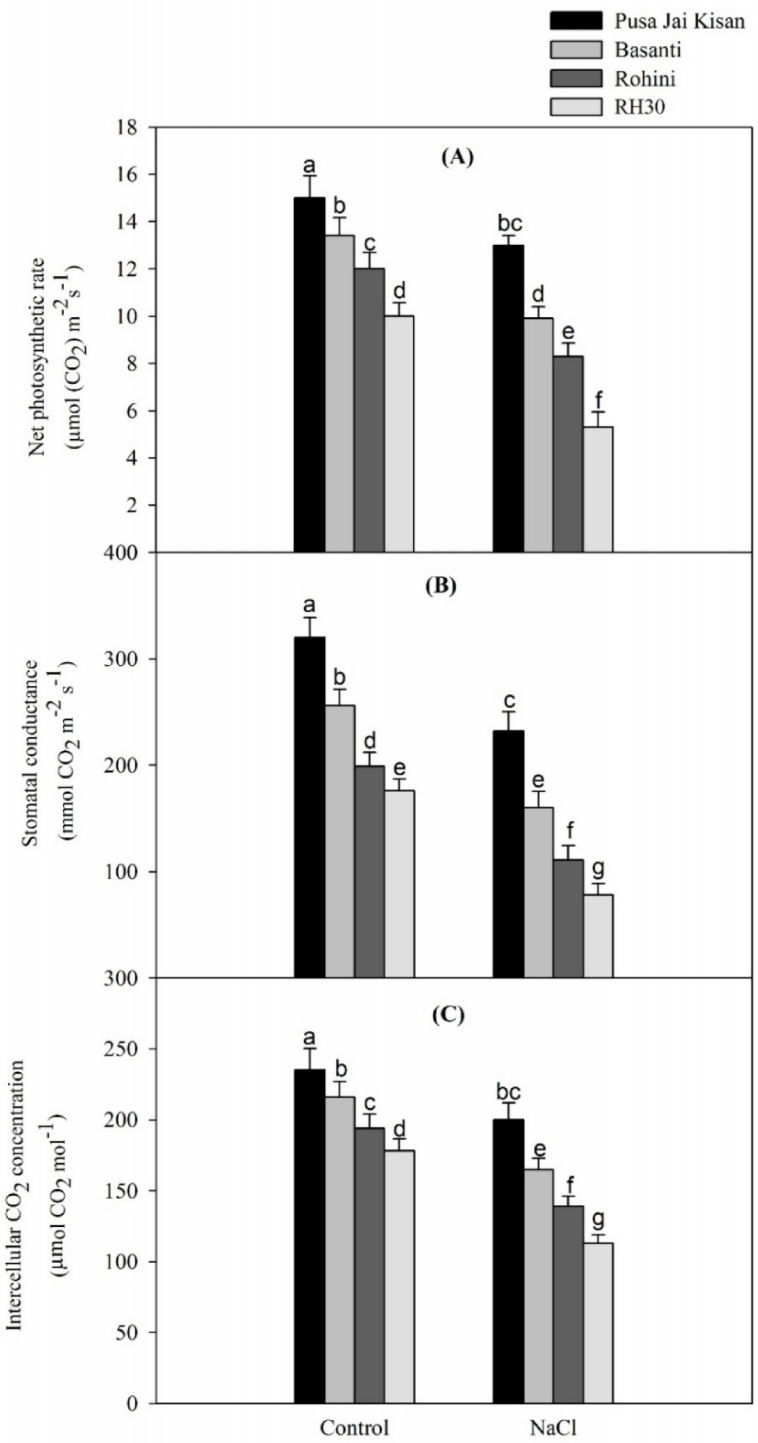

Fig. 7. Effects of NaCl (0 or 100mM) on P_N (A) g_s (B) and [$(CO_2)i$] (C) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

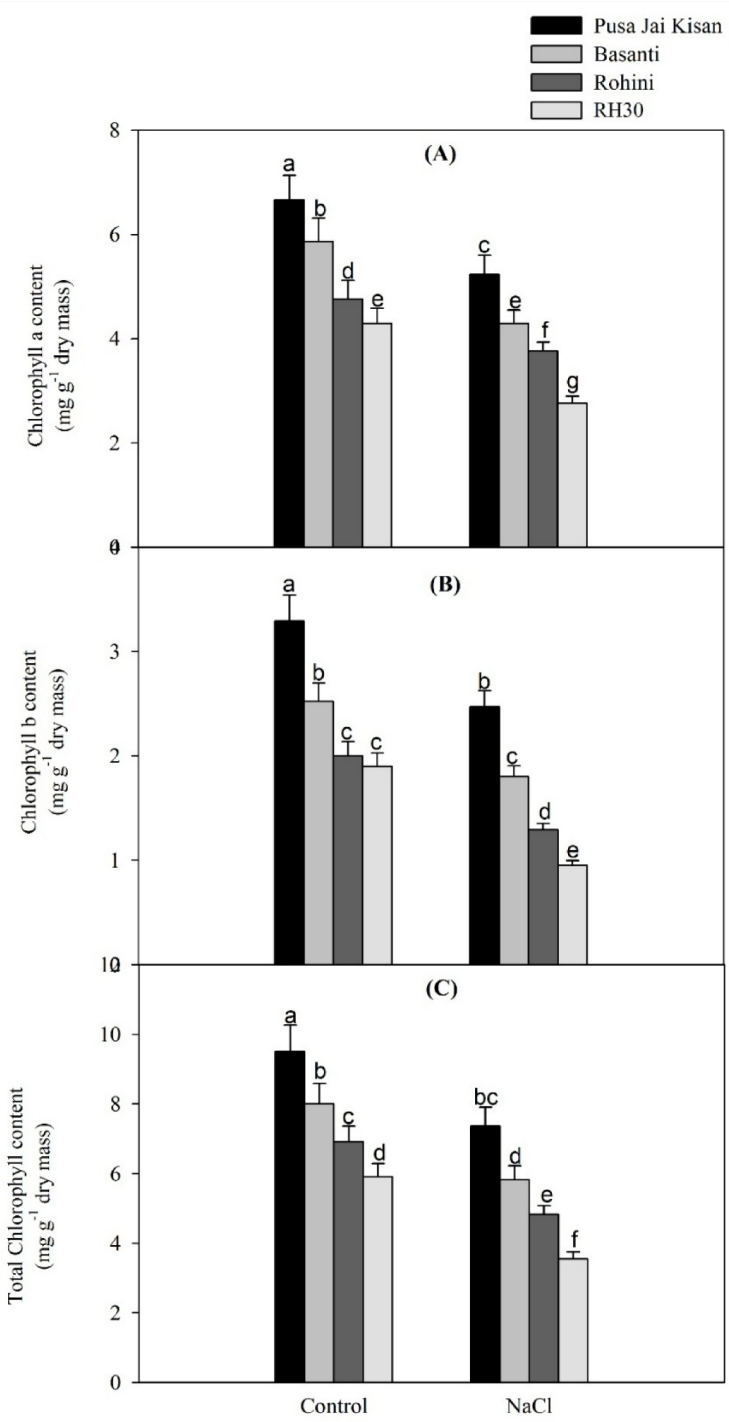

Fig. 8. Effects of NaCl (0 or 100mM) on Chl a (A) and Chl b (B) and total Chl content (C) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

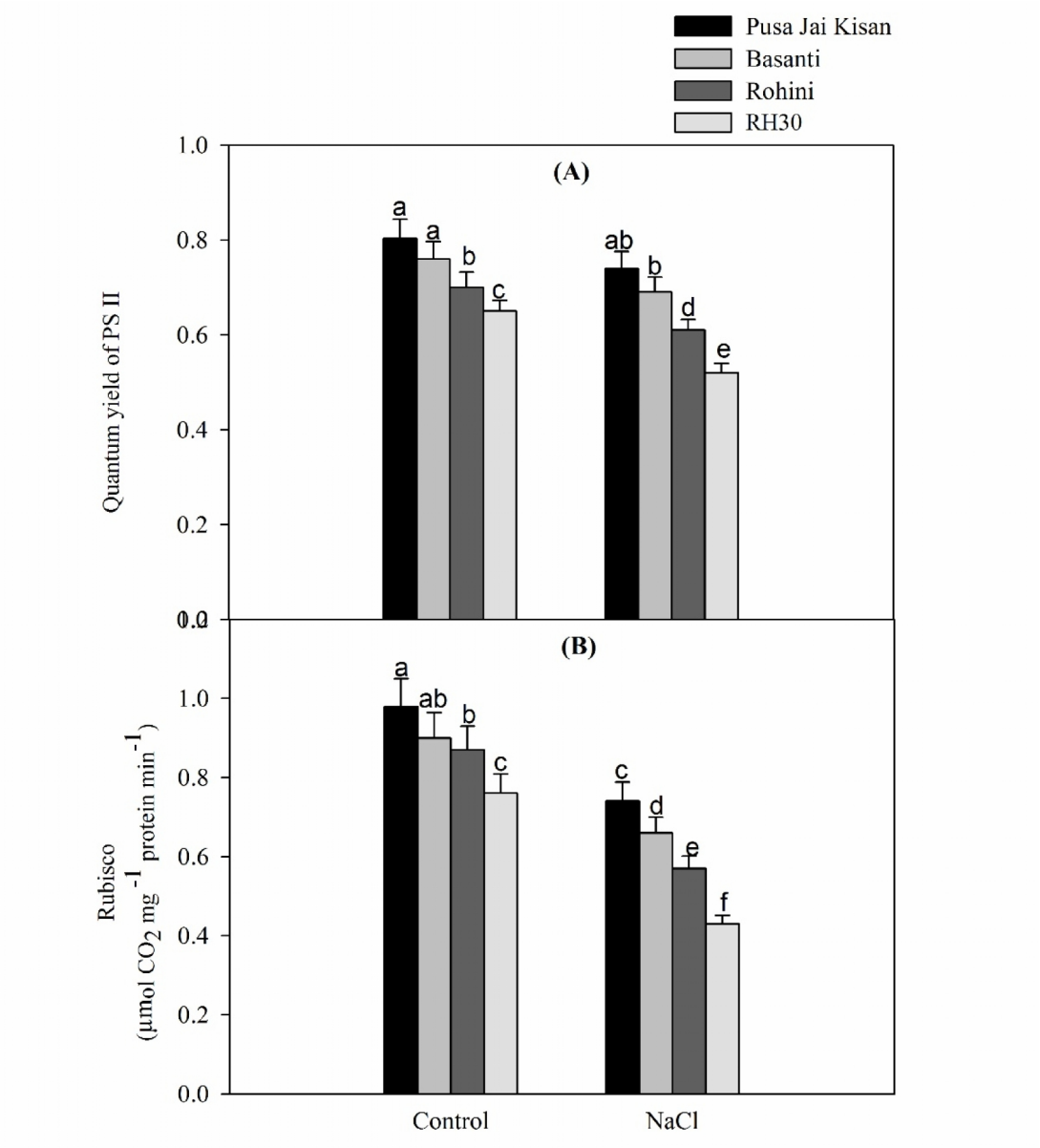

Fig. 9. Effects of NaCl (0 or 100mM) on Chl fluorescence (A) and Rubisco activity (B) of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

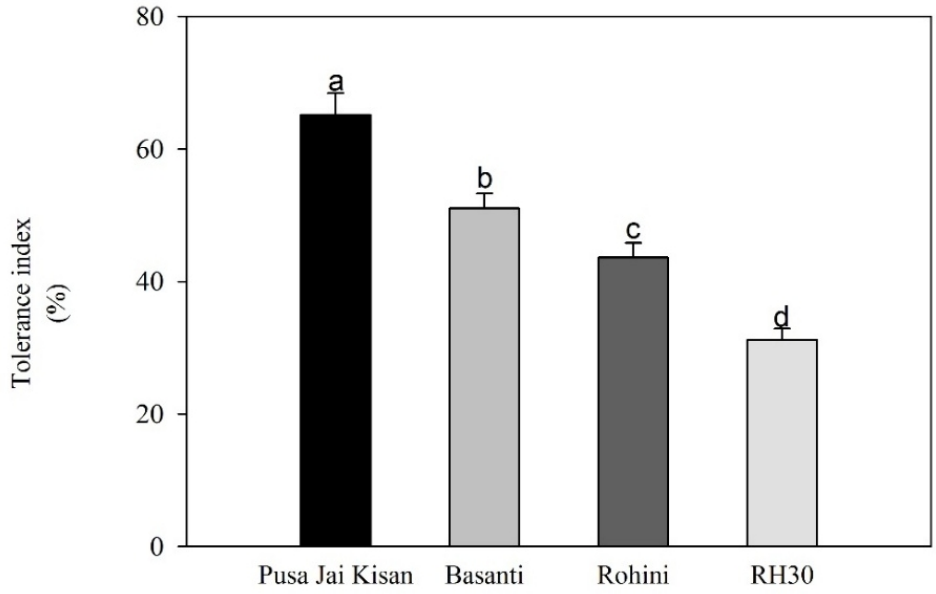

Fig. 10. Effects of NaCl (0 or 100 mM) on tolerance index of mustard (*Brassica juncea* L.) at 30 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

4. DISCUSSION

Photosynthesis is the key factor that determines plants growth and productivity. The decline in productivity in many plant species under salinity stress is often associated with reduction in its photosynthetic ability [41]. Photosynthetic reduction under salinity stress could be associated with salinity-induced ionic and osmotic stress. In the present study it was observed that salinity reduced the plants water and osmotic potential in all the cultivars and maximally in RH30. Lower osmotic potential due to salt stress affect water use efficiency in plants [42] and the lower water potential leads to decrease in photosynthesis through inducing stomatal closure, decreasing stomatal conductance, altered Chl fluorescence and decrease in activity and content of Rubisco enzymes [43-44]. Galle et al [45] reported that stomatal closure has been shown to act as the initial and most prominent limitation to CO_2 assimilation, as diffusion of CO_2 from the atmosphere to the sites of carboxylation in the chloroplast is impaired.

The main physiological processes that are adversely affected by salinity includes osmotic stress, ionic toxicity, oxidative stress and nutrient deficiency [46,47]. Oxidative stress leads to the formation of reactive oxygen species [3,11] that causes lipid peroxidation, damages DNA, reduces photosynthesis and disturbs the mineral nutrient status [3,48]. Salinity adversely effects photosynthesis through changes in photosynthesizing tissue, disturbance in water balances and homoeostasis of Na^+ and Cl^- ions [11,46] and may cause disturbance in the uptake of nutrients such as nitrogen (N), phosphorus, potassium and calcium [2]. In order to reduce salinity-induced photosynthetic inhibition plants accumulate osmolytes among which proline has been recognized as a major contributor to salinity tolerance. In the present study, minimum accumulation of Na^+ and Cl^- content and oxidative stress (H_2O_2 and TBARS content) and higher photosynthesis in Pusa Jai Kisan compared to other cultivars,

under salt stress, is attributed to maximum accumulation of proline content. Proline accumulation under stress is correlated with osmotic adjustment and improves plant salinity tolerance [49]. Proline accumulates in cells and balance the osmotic difference between the cell's surroundings and the cytosol [50]. It also plays roles in scavenging free radicals, stabilizing subcellular structures, and buffering cellular redox potential under stresses. The salinity stress responsive genes, whose promoters contain proline responsive elements (PRE, ACTCAT), are also known to be induced by proline [51]. Pusa Jai Kisan with comparatively higher proline content helped in maintaining osmotic balance and exhibited increased leaf water and osmotic potential and had higher stomatal conductance, CO_2 assimilation, ΦPSII, Rubisco activity and photosynthesis. The lesser oxidative stress in Pusa Jai Kisan compared to Basanti, Rohini and RH30 led to lesser damage to the photosynthetic apparatus.

Proline has been reported to function as a scavenger of ROS [23,24]. Goudarzi and Pakniyat [52] reported that salinity induced proline content in wheat could be used to screen tolerant and susceptible genotypes. Ahmad et al. [53] reported that salt tolerant ecotypes of *Agrostis stolonifera* accumulated more proline in response to salinity compared to salt sensitive ecotypes. Petrusa and Winicov [54] found that salt tolerant alfalfa plants rapidly doubled their proline content in the roots, whereas in salt sensitive plants the increase was slow. In the present study also Pusa Jai Kisan accumulated maximum proline content and RH30 showed minimum accumulation of proline exhibiting the higher tolerance potential of Pusa Jai Kisan and thus higher photosynthesis protection.

The increase in proline content under salinity stress was due to increase in proline biosynthesizing enzymes, P5CS and GK whereas decrease in the proline degrading enzyme, proline oxidase. Pusa Jai Kisan with highest proline accumulation had higher P5CS activity and GK activity and RH30 had least activity of both. In contrast, Pusa Jai Kisan exhibited maximum decrease in the activity of proline oxidase whereas, RH30 had minimum decrease. Basanti and Rohini were found to lie in the intermediate range between the two extremes. It has been reported that proline biosynthetic enzymes, pyrroline-5-carboxylate reductase and ornithine aminotransferase increased to a larger extent in tolerant lines of *B. juncea* compared to non-tolerant lines. In contrast, the activity of proline degrading enzyme, proline oxidase decreased under salt stress in the leaf tissues of all the lines of *B. juncea* [55]. Claussen [56] reported that proline accumulation under stress conditions may be caused by induction of proline biosynthesis enzymes, reduction in the rate of proline oxidation conversion to glutamate, decrease utilization of proline in proteins synthesis and enhancing proteins turnover. The higher proline accumulation in Pusa Jai Kisan was responsible for higher photosynthetic performance of this cultivar. The trend of photosynthetic performance was in accordance with the amount of proline accumulated in the cultivars. Silva-Ortega et al. [57] reported that proline accumulation plays a critical role in protecting photosynthetic activity in *Opuntia streptacantha* plants under salinity stress.

Pospisilova et al. [58] through their study on transgenic tobacco plants (M51-1) constitutively over-expressing a modified gene for the proline biosynthetic enzyme P5CS F129A, studied that the transgenic plants with higher proline content under optimum conditions, had lower transpiration rate and stomatal conductance probably to conserve water and had higher contents of chlorophyll and xanthophyll cycle pigments compared to their wild type plants.

Studies have been conducted with transgenic plants over-expressing proline biosynthetic enzyme confirming the role of proline in influencing plants photosynthetic ability. Molinari et al. [59] reported that Chl content and the variable to maximum Chl fluorescence ratio

(Fv/Fm) was higher in transgenic sugarcane plants compared to the wild type plants under water stress conditions. During simultaneous drought and heat stress, dissociation of the oxygen-evolving complex was bypassed by proline feeding electrons into photosystem II, maintaining the acceptable NADPH level in transgenic soybean plants [60]. Under drought stress, the stomatal conductance was slightly higher in transgenic chickpea plants compared to wild type under both control and stress conditions [61]. Enhanced biomass production was observed in transgenic indica rice overexpressing P5CSF129A gene for proline synthesis under salt stress [62].

Pusa Jai Kisan with higher proline content was found to have higher N-assimilation. Increased N led to enhanced proline production. Being a constituent of proline, N availability is directly linked with the regulation of proline metabolism. Tarighaleslami et al. [63] reported that leaf proline content increased with the application of N fertilizer. Rais et al. [64] have shown that both individual and combined application of N and sulfur resulted in increased NRA, N content, proline accumulation and alleviated salt stress effects on photosynthetic efficiency and growth of *Brassica juncea.*

Both NR activity and N content and PNUE were highest in Pusa Jai Kisan under salinity stress and least in RH30. N content and metabolism decreases under salinity stress [3,65]. N is an essential element that affects photosynthesis because it is a major constituent of Chl, thylakoid protein and many enzymes of photosynthetic carbon reduction cycle [66,67]. An increase in N availability results in higher leaf N content resulting in strong positive correlation between photosynthesis and leaf N content [68,69]. With larger N supply and increased PNUE, the increased allocation of N to Rubisco may increase photosynthesis. N application increases the photosynthetic electron transport rate of PS II reaction center significantly, and promotes the photosynthetic electron flow towards photochemistry, making more photosynthetic electron take part in Rubisco carboxylation and leading to the significant increase of Pn [70]. N content decreased in all the cultivars under salinity stress, however, in comparison to all other cultivars, Pusa Jai Kisan had higher N-assimilation for accumulating more proline under salinity stress. Higher PNUE in Pusa Jai Kisan was responsible for higher allocation of N to the photosynthetic machinery and higher Rubisco activity. The lesser decrease in N content and higher proline accumulation was responsible was higher Chl content and quantum yield of PSII and higher protection of photosynthesis under salinity stress. Chl content and quantum yield of PSII decreased in all the cultivars exposed to salinity stress but maximally in RH30 whereas, Pusa Jai Kisan with highest proline accumulation maximally protected its Chl content.

Thus, mustard genotypes differentially responded to physiological processes under salinity stress. The higher photosynthetic traits in Pusa Jai Kisan could be related to higher accumulation of proline in this cultivar which is known to be involved in salt tolerance. It was further verified that Pusa Jai Kisan with highest proline content was also having higher tolerance index followed by Basanti, Rohini and RH30. The same order of increase was observed for proline content.

5. CONCLUSIONS AND FUTURE PROSPECTS

Photosynthesis is the determining factor for plant growth and productivity and is adversely effected by salinity. The response of plants photosynthetic traits to salinity depends on various factors and proline accumulation is one among them. The accumulation of proline is a strategy to tolerate salt-induced adverse effects and reduce photosynthetic inhibition. Proline helps to maintain osmotic balance and regulate other physiological processes

favorably. It can be used as a screening criterion for salt tolerance because of its involvement in osmoregulation and protection of photosynthetic functions.

To develop salt tolerant cultivars it is important that sufficient genetic variation exist such that exploitation of these resources make selection and breeding of trait possible. Further, knowledge on the degree of salt tolerance through involvement of proline could provide an approach to manipulate the key regulatory points of proline metabolism to target up-regulation of proline biosynthesis for the development of salt tolerant cultivars. Focus on enhancing proline production under salinity stress and increasing PNUE would provide a novel strategy to increase photosynthesis and subsequently plant growth under salinity stress. Non tolerant cultivars could be made tolerant via enhancing their proline production potential.

ACKNOWLEDGEMENTS

First author is thankful to the University Grants Commission, New Delhi for DS Kothari Post-Doctoral Fellowship (File No.-No.F.4-2/2006 (BSR)/13-848/2013(BSR)) and to the Head, Department of Botany, Jamia Hamdard University, New Delhi for providing research facilities.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Finkel E. With phenomics, plant scientists hope to shift breeding into overdrive. Science. 2009;325:380-381.
2. Khan NA, Nazar R, Anjum NA. Growth, photosynthesis and antioxidant metabolism in mustard (*Brassica juncea* L.) cultivars differing in ATP- sulfurylase activity under salinity stress. Sci Hort. 2009;122:455-460.
3. Nazar R, Iqbal N, Syeed S, Khan NA. Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in two mungbean cultivars. J Plant Physiol. 2011;168:807-815.
4. Dubey RS, Photosynthesis in Plants under Stressful Conditions. In: A handbook of photosynthesis. Taylor and Francis Group, LLC; 2005.
5. Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. Ann Bot. 2009;103:551-560.
6. Kanwal H, Ashraf M, Shahbaz M. Assessment of salt tolerance of some newly developed and candidate wheat (*Triticum aestivum* L.) cultivars using gas exchange and chlorophyll fluorescence attributes. Pak .J Bot. 2011;43:2693-2699.
7. Siddiqi, Ashraf EHM, Hussain M, Jamil A. Assessment of inter variety variation for salt in safflower using gas exchange character as selection criteria. Pak J Bot. 2009;41:2251-2259.
8. Shekhawat K, Rathore SS, Premi OP, Kandpal BK, Chauhan JS. Advances in agronomic management of Indian mustard (*Brassica juncea* (L.) Czernj. Cosson): An overview.Int. J Agron; 2012. Available: http://dx.doi.org/10.1155/2012/408284.

9. Yadava DK, Vasudev S, Singh N, Mohapatra T, Prabhu KV. Breeding major oil crops: Present status and future research needs. In: Gupta S K (Ed.) Technological Innovations in Major World Oil Crops. 2012;17-51.
10. Sharma P, Sardana V, Banga SS. Salt tolerance of Indian mustard (*Brassica juncea*) at germination and early seedling growth. Environ ExpBiol 2013;11:39–46.
11. Khan MIR, Iqbal N, Masood A, Khan NA. Variation in salt tolerance of wheat cultivars: Role of glycinebetaine and ethylene, pedosphere. 2012;22:746–754.
12. Hichem H, Mounir D, Naceurc EA. Differential responses of two maize (*Zea mays* L.) varieties to salt stress: Changes on polyphenols composition of foliage and oxidative damages. Industrial Crops and Products. 2013;30:144-151.
13. Munir S, Siddiqi EH, Bhatti KH, Nawaz K, Hussain K, Rashid R, Hussain I. Assessment of inter-cultivar variations for salinity tolerance in winter radish (*Raphanus sativus* L.) using photosynthetic attributes as effective selection criteria. World App Sci J. 2013;21:384-388.
14. Ulfat M, Athar HR, Ashraf M, Akram NA, Jamil A. Appraisal of physiological and biochemical selection criteria for evaluation of salt tolerance in canola (*Brassica napus* L.)",Pak J Bot. 2007;39:1593-1608.
15. Ranganayakulu GS, Veeranagamallaiah G, Sudhakar C. Effect of salt stress on osmolyte accumulation in two groundnut cultivars (*Arachis hypogaea* L.) with contrasting salt tolerance. African J Plant Sci.2013;7:586-592.
16. Bohnert HJ, Nelson DE, Jensen RG. Adaptations to environmental stresses. The Plant Cell. 1995;7:1099-1111.
17. Lang F. Mechanisms and significance of cell volume regulation. J Am Coll of Nutr. 2007;26:613S–623S.
18. Haroun SA. Fenugreek growth and metabolism in response to gibberellic acid and sea water. Assuit Univ J Bot.2002;31:11-12.
19. Ueda A, Yamamoto-Yamane Y, Takabe T. Salt stress enhances proline utilization in the apical region of barley roots. Biochem Biophy Res Comm. 2007;355:61-66.
20. Wang ZQ, Yuan YZ, Qu JQ, Lin GH, Zhang CF. Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. J Plant Physiol. 2007;164:695–701.
21. Mattioli R, Costantino P, Trovato M. Proline accumulation in plants. Plant Signal. 2009;4:1016–1018.
22. Iqbal N, Umar S, Khan NA, Kham MIR. A new perspective of phytohormones in salinity tolerance: Regulation of proline metabolism. Environ Exp Bot. 2014;100:34–42.
23. Szabados L, Savouré A. Proline: A multifunctional amino acid. Trends Plant Sci. 2010;15:89–97.
24. Ashfaque F, Khan MIR, Khan NA. Exogenously applied H_2O_2 promotes proline accumulation, water relations, photosynthetic efficiency and growth of wheat (*Triticum aestivum* L.) under salt stress. Ann Res Rev Biol. 2014;4:105-120.
25. Hayat S, Hayat Q, Alyemeni MA, Ahmad A. Proline enhances antioxidative enzyme activity, photosynthesis and yield of *Cicer arietinum* L. exposed to cadmium stress. Acta Botanica Croatica. 2014;71:323-335.
26. Hewitt EJ. Sand and water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureaux, Farnham Royal. 1966;547.

27. Okuda T, Matsuda Y, Yamanaka A, Sagisaka S. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. Plant Physiol.1991;97:1265-1267.
28. Dhindsa RH, Plumb-Dhindsa P, Thorpe TA. Leaf senescence correlated with increased level of membrane permeability, lipid peroxidation and decreased level of SOD and CAT. J Exp Bot. 1981;32:93–101.
29. Bates LE, Waldren RP, Teare ID. Rapid determination of free proline for water stress studies. Plant Soil. 1973;39:205-207.
30. Hayzer DJ, Leisinger TH. The gene-enzyme relationships of proline biosynthesis in *Escherichia coli*. J Gen Microbiol.1980;118:287-293.
31. Huang AHC, Cavalieri AJ. Proline oxidase and water stress induced proline accumulation in spinach leaves. Plant Physiol.1979;63:531-535.
32. Kuo TM, Warner RL, Kleinhofs A. In vitro stability of nitrate reductase from barley leaves. Phytochemistry. 1982;21:531-533.
33. Nakagawa H, Poulle M, Oaks A. Characterization of nitrate reductase from corn leaves (*Zea mays* cv W64A x W182E): two molecular forms of the enzyme. Plant Physiol. 1984;75:285-289.
34. Iqbal N, Khan NA, Nazar R, Teixeira da Silva JA. Ethylene-stimulated photosynthesis results from increased nitrogen and sulfur assimilation in mustard types that differ in photosynthetic capacity. Environ Exp Bot. 2012;78:84– 90.
35. Lindner RC. Rapid analytical methods for some of the more common inorganic constituents of plant tissues. Plant Physiol.1944;19:76-89.
36. 36 Hiscox JD, Israelstam GF. A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot. 1979;57:1332-1334.
37. Arnon DI. Copper enzymes in isolated chloroplasts of polyphenoloxidase in *Beta vulgaris.* Plant Physiol.1949;24:1-5.
38. Iqbal N, Khan NA. Variation in growth, photosynthesis function and yield of five mustard (*Brassica juncea* L.) cultivars under high cadmium stress. Plant Stress (special issue I). 2010;4:87-93.
39. Usuda H. The activation state of ribulose 1,5-bisphosphate carboxylase inmaize leaves in dark and light. Plant Cell Physiol.1985;26:1455-1463.
40. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein tilizing the principle of protein-dye binding. Anal Biochem.1976;72:248-254.
41. Ziska LH, Seemann JR, De Jong TM. Salinity induced limitations on photosynthesis in *Prunus salicina*, a deciduous tree species. Plant Physiol.1990;93:864–870.
42. Cha-um S, Kirdmanee C. Effect of salt stress on proline accumulation, photosynthetic ability and growth of two maize cultivars. Pak J Bot. 2009;41:87-98.
43. Plaut Z. Photosynthesis in plant/crops under water and salt stress. In: Pessarakli M (Ed.), Handbook of Plant and Crop Physiology, New York, Marcel Dekker. 2003;587-603.
44. Flexas J, Medrano M. Drought inhibition of photosynthesis in C_3 plants: Stomatal and non-stomatal limitations revisited. Ann Bot. 2002;89:183-189.
45. Galle A, Florez-Sarasa I, Tomas M, Pou A, Medrano H,Ribas-Carbo M, Flexas J.The role of mesophyll conductance during water stress and recovery in tobacco (*Nicotiana sylvestris*): Acclimation or limitation? J Exp Bot. 2009;63:3499-3509.

46. Munns R, Tester M. Mechanisms of salinity tolerance. Ann Rev Plant Biol. 2008;59:651-681.
47. Daneshmand F, Mohammad JA, Khosrow MK. Effect of acetyl salicylic acid (Aspirin) on salt and osmotic stress tolerance in *Solanum bulbocastanum In vitro*: Enzymatic antioxidants. Am-Eurasian J Agricul Environ Sci. 2009;6:92-99.
48. Turan S, Tripathy BC. Salt and genotype impact on antioxidative enzymes and lipid peroxidation in two rice cultivars during de-etiolation. Protoplasma 2012;250:209-222.
49. Vinocur B, Altman A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opi Biotechnol. 2005;16:123–132.
50. Wani SH, Singh NB, Haribhushan A, Mir JI. Compatible solute engineering in plants for abiotic stress tolerance -role of glycine betaine. Current Genomics. 2013;14:157-165.
51. Chinnusamy V, Jagendorf A, Zhu JK. Understanding and improving salt tolerance in plants. Crop Sci. 2005;45:437–448.
52. Goudarzi M, Pakniyat H. Salinity causes increase in proline and protein contents and peroxidase activity in wheat cultivars. J App Sci. 2009;9:348-353.
53. Ahmad I, Wainwright SJ. Stewart GR. The solute and water relation to *Agostis stolonifera* ecotypes differing in their salt tolerance. New Phytol.1981;87:615-629.
54. Petrusa LM, Winicov I. Proline status in salt tolerant and salt sensitive alfalfa cell lines and plant in response to NaCl. Plant Physiol Biochem.1997;35:303-310.
55. Madan S, Nainawatee HS, Jain Rk, Chowdhury JB. Proline and proline metabolizing enzymes in In vitro selected NaCl tolerant (*Brassica juncea* L.) under salt stress. Ann Bot. 1995;76:51-57.
56. Claussen W. Proline as measure of stress in tomato plants. Plant Sci. 2005;168:241-248.
57. Silva-Ortega CO, Ochoa-Alfaro, AE, Reyes-Agüero JA, Aguado-Santacruz GA, Jiménez-Bremont JF. Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. Plant Physiol Biochem. 2008;46:82-92.
58. Pospisilova J, Haisel D, Vankova R. Responses of transgenic tobacco plants with increased proline content to drought and/or heat stress. Am J Plant Sci. 2011;2:318-324.
59. Molinari HBC, Marur CJ, Daros E, Freitas de Campos MK, Portela de Carvalho JFR, Filho JCB, Pereira FLP, Vieira ELJ. Evaluation of the stress - inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiol Plant. 2007;130:218-229.
60. De Ronde JA, Cress WA, Kruger GHJ, Stre-asser RJ, Van Staden J. Photosynthetic Response of Trans-genic Soybean Plants, Containing an Arabidopsis P5CR Gene, during Heat and Drought Stress. J Plant Physiol. 2009;161:1211-1224.
61. Bhatnagar-Mathur P, Vadez V, Devi MJ, Lavanya M, Vani G, Sharma KK. Genetic Engineering of Chickpea (*Cicer arietinum* L.) with the P5CSF129A Gene for Osmoregulation with Implications on Drought Tolerance. Mol Breeding. 2009;23:591-606.
62. Kumar V, Shriram V, KaviKishor PB, Jawali N, Shitole MG. Enhanced Proline Accumulation and Salt Stress Tolerance of Transgenic Indica Rice by over-Ex-pressing P5CSF129A Gene. Plant Biotechnol Reports. 2010;4:37-48.
63. Tarighaleslami M, Zarghami R, Boojar MMA, Oveysi M. Effects of drought stress and different nitrogen levels on morphological traits of proline in leaf and protein of corn seed (*Zea mays* L.). Am-Eurasian JAgric Environ Sci. 2012;12:49-56.

64. Rais L, Masood A, Inam A, Khan NA. Sulfur and nitrogen co-ordinately improve photosynthetic efficiency, growth and proline accumulation in two cultivars of mustard under salt stress.J Plant Biochem Physiol. 2013;1:1.
65. Khan N, Syeed S, Masood A, Nazar R, Iqbal N. Application of salicylic acid increases contents of nutrients and antioxidative metabolism in mungbean and alleviates adverse effects of salinity stress. International J Plant Biol; 2010. doi:10.4081/pb.2010. e1.
66. Marschner H. Mineral nutrition of higher plants. New York: Academic Press.1995;229–299.
67. Rathke GW, Behrens T, Diepenbrock W. Integrated nitrogen Management strategies to improve seed yield, oil content and nitrogen efficiency of winter oilseed rape (*Brassica napus* L.): A review. Agric Ecosys Environ. 2009;117:80-108.
68. Huber SC, Sugiyama T, Alberte RS. Photosynthetic determinants of growth in maize plants: effects of nitrogen nutrition on growth, carbon fixation and photochemical features. Plant Cell Physiol. 1989;30:1063–1072.
69. Connor DJ, Hall AJ, Sadras VO. Effect of nitrogen content on the photosynthetic characteristics of sunflower leaves. Austr J Plant Physiol.1993;20:251–263.
70. Zhang XC, Yu XF, Ma YF. Effects of nitrogen application and elevated atmospheric CO_2 on electron transport and energy partitioning in flag leaf photosynthesis of wheat. Ying Yong Sheng Tai Xue Bao. 2011;22:673-680.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=552&id=32&aid=4862