Accumulation of Trehalose Mediates Salt Adaptation in Rice Seedlings

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Abstract: Trehalose is a non-reducing disaccharide that is present in diverse organisms, in which it serves as an energy source, osmolyte or protein/membrane protectant. In the plant kingdom, trehalose is biosynthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Over-expression of exogenous and endogenous gene encoding TPS is reported to be effective for improving abiotic stress tolerance in tobacco, potato, tomato, rice and Arabidopsis. Trehalose levels are generally low in plants because the presence of the enzyme trehalase which hydrolyze trehalose to glucose. Hence, it should be possible to direct increased trehalose accumulation by down regulating plant trehalase activity or by expressing the trehalose biosynthetic genes under stress-specific regulation. Validamycin A is a specific competitive inhibitor of trehalase and raises trehalose in plant tissue. The aim of the present work is to detect the change in the expression of TPS gene in rice plant (salt sensitive Sakha 103 and salt tolerance Agami M5) pre-soaked in Validamycin (30µM) and grown under saline condition (0, 50, 75, 100 mM NaCl) using Semi-quantitative RT- PCR. An additional point of interest was to study the possible roles of exogenous trehalase inhibitor validamycin A (30µM), on alleviating the harmful effects of salt stress on chlorophylls, carotenoids, total protein, free proline, total free amino acids, sugars and starch of rice plant (salt sensitive Sakha 103 and salt tolerance Agami M5). These results suggested that Validamycin A (30µM) has a regulatory role in increasing level of trehalose and improving salt tolerance in rice plant. Semi-quantitative RT-PCR indicated that the expression of this gene is upregulated in response to salt and validamycin treatments.

Key words: Rice · Salinity · Validamycin A · Trehalose · Organic solutes · Semi-quantitative RT-PCR · TPS

INTRODUCTION

Salinity is a major abiotic stress that reduces the yield of a wide variety of crops [1]. Salt stress exerts a range of adverse influence on plant growth and metabolism. Plant height, fresh and dry mass are all repressed by salt over a period of time in various species [2]. Photosynthesis is pronouncedly inhibited by salt stress, which is correlated with coordinately reduced stomatal conductance [3]. Therefore, investigators are aiming to understand the mechanisms by which plants respond and adapt to such stresses. Plant responses to salt stress have generally been conducted using anatomical, ecological, physiological and molecular approaches [4] in relation to regulatory mechanisms of ionic and osmotic homeostasis. In addition, salt adversely affects the metabolism of plants, resulting in substantial modifications in plant gene expression. These modifications may lead to the accumulation or depletion of certain metabolites, resulting in an imbalance in the levels of cellular proteins, which may increase, decrease, appear, or disappear after salt treatment [4]. Plant acclimation to salinity via the accumulation of compatible solutes, such as soluble sugars and part of free amino acids, is often regarded as a basic strategy for the protection and survival of plants under salt stress [5]. Trehalose is a non-reducing disaccharide that occurs in a large range of organisms and functions in the regulation of carbohydrate metabolism, as stress protection metabolite and storage carbohydrate [6, 7].

Recently, there is a focus of interest in the role of trehalose as it improves the performance of plants under drought, nutrition element or salinity [8]. Garg *et al.* [9] demonstrated that elevated Trehalose accumulation in rice plants also conferred high tolerance to salt stress: this was ascribed to its role in maintaining potassium in

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shoots and in reducing the sodium accumulation, so preserving the balance of Na and K [9]. The underlying mechanism by which trehalose improves plant response to salinity and other adverse environmental factors, is still unclear [10]. For example, it is suggested that trehalose is likely to function through its ability to scavenge reactive oxygen species, conferring protection to the machinery of protein synthesis [3]. In plants, trehalose is formed from UDP-glucose and glucose-6 phosphate and catalyzed by the enzyme trehalose- 6-phosphate synthase (TPS). Subsequently, this is dephosphorylated into trehalose by the enzyme trehalose- 6-phosphate phosphatase (TPP). Furthermore, trehalase, the key enzyme responsible for the hydrolysis of trehalose during degradation, is present in all organs of higher plants [11]. The latest reports show that exogenous validamycin A (trehalase inhibitor) treatment decreased the activity of trehalase which leads to the accumulation of trehalose in shoot and root of wheat plants. Raising trehalose level in the plant tissues was accompanied by increase in the sucrose content and starch content of the shoot [12]. Validamycin A also induced an increase in trehalose concentration in root nodules of *Medicago truncatula* by inhibiting trehalase activity, which then improved the response to salinity by increasing plant dry weight [13]. Finally, validamycin A increased cellulose and starch content and decreased total amino acid and nitrate content of mature tobacco plants [14]. Consequently, it should be possible to direct increased trehalose accumulation by down regulating plant trehalase activity or by expressing the trehalose biosynthetic genes under tissue- or stress specific regulation. Rice (*Oryza sativa* L.) is one of the most important food crops in the world and it is the staple food in most developing countries in Asia, South America and Africa [15]. Currently, 90% of the rice has been produced and consumed in Asian countries [16]. In areas such as Asia, Africa and Latin America where the demand for rice is a top priority, the population is expected to increase 1.5-fold by 2025 [17].

Most of the environmental constraints drastically decrease plant growth and development that lead to reduction in crop yield [18]. Consequently, discovery of stress-related functional genes and their regulatory elements have increased our knowledge about abiotic stress mechanisms [19]. Stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer. It is important to analyze the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation [20]. The aim of the present

work is to detect the change in the expression of TPS gene in rice plant pre-soaked in Validamycin A and grown under saline condition using Semi-quantitative RT- PCR. An additional point of interest was to study the possible roles of exogenous trehalase inhibitor validamycin A on alleviating the harmful effects of salt stress on chlorophylls, carotenoids, total protein, free proline, total free amino acids, sugars and starch of rice plant (salt sensitive Sakha103 and salt tolerance Agami M5).

MATERIALS AND METHODS

Plant and Chemical Materials: Two cultivars of rice (*Oryza sativa* L.) Sakha103 and AgamiM5 cultivar were obtained from Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt. Validamycin A was provided by Qianjiang Biochem. Co. Ltd., China and other chemicals were purchased from Sigma and Fisher group.

Methods: Seeds of both rice cultivars were surface sterilized with a 5% sodium hypochlorite solution for 5 min. After washing several times with distilled water, seeds were imbibed in Petri-dishes containing distilled water in a culture room at $24 \pm 2°C$ until the appearance of the white tip of the coleoptile. After imbibitions, the seeds of each cultivar were divided into two groups. Seeds of the 1st group were left in distilled water without any treatments (control). Seeds of the 2nd group were soaked in 30μM validamycin A [13] for 8 hours. Then seeds of both treatments were planted in plastic pots (diameter 30 cm and 40 cm depth); each pot contained 7 kg soil. Soil characteristics were: sandy loam in texture, sand 84.2%, silt 12.9%, clay 2.9%, pH 7.7, EC $0.5dSm^{-1}$ and organic matter 1.2%. Each pot contained ten plants; the seedlings were irrigated with tap water for 14 days under normal conditions.

Salt Stress Treatments: Fourteen days old seedlings of the 1st and the 2nd groups from both varieties of rice plants were subjected to salt stress (0, 50, 75,100 mM NaCl) [21] for a period of 2 weeks. Preliminary experiments showed that NaCl treatment more than 100 mM caused damage too severe to investigate biological response. Phenotypic analysis was developed at 21 and 28 days plants old after stress conditions.

Plant Sampling for Analysis: Plant samples were taken at two growth stages (21 and 28 days after planting). The roots were discarded form the plant shoot system before backing in aluminum foil. Each packet of aluminum foil was then immediately frozen in liquid nitrogen and

stored in -80°C freezer until using for various biochemical analyses. Samples of shoot system were dried at 70°C for 24 h and dry weights estimated. Growth parameters like root length and shoot height were measured in 10 plants of fresh samples. Plants were weighed individually for their fresh weight and then kept for 72 h in oven at 70°C. Finally dry weight was determined by weighing the dried whole plants.

Physiological and Biochemical Analysis
Photosynthetic Pigments Extraction and Estimation:
Photosynthetic pigments (chlorophyll *a, b* and carotenoids) were measured in rice leaf tissues after extraction using (JENWAY 6305 UV/VIS) spectrophotometric apparatus and calculated using the formula of [22] given below:

Chlorophyll *a* mg /g FW= $(11.75 \times A_{663} - 2.35 \times A_{645}$ (×50/500

Chlorophyll *b* mg /g FW= $(18{:}61 \times A_{645} - 3.96 \times A_{663})$ ×50/500

Carotenoids mg /g FW= $(1000 \times A_{470})$-(2.27×Chl a)-(81.4×Chl b)/227×50/500.

Determination of Certain Minerals: The plant materials were digested by sulphuric acid-hydrogen peroxide procedure as described by Allen [23]. Phosphorous was determined according to Snell and [24] using ascorbic acid method. Potassium and sodium were determined photometrically using a flame photometer [25]. Calcium was determined photometrically by using the atomic absorption method described by Allen [23]. Results were expressed as mg/g dry wt. Total nitrogen content was determined using the micro-kjeldahl method [26].

Estimation of Carbohydrates: Sugars were extracted according to Homme *et al.* [27]. Total soluble sugars and sucrose was determined using modifications of the procedures of Yemm and Willis [28] and Handel [29], respectively. Starch contents were evaluated on the residual pellet left after ethanol extraction of soluble sugars by the method of Rose *et al.* [30]. Trehalose content was extracted according to the method described by Lynch *et al.* [31]. For trehalose quantitation, the anthrone reaction was used based on Umbreit *et al.* [32].

Estimation of Nitrogenous Constituents: The tissue extract was deproteinized using ethanol/acetone mixture and the free amino acids were then determined photometrically with-ninhydrin. Under appropriate test condition (buffer, solvent and boiling time), free amino acid can be determined directly in the extract by JENWAY 6305 UV/VIS at 580 nm [33]. Free proline content in the plant tissues was determined following the method of Bates *et al.* [34]. Total protein concentration of the supernatant was determined according to the method described by Bradford [35] with bovine serum albumin as a standard.

RNA Extraction and TPS Gene Expression Analysis:
The expression pattern of TPS was analyzed by semi quantitative RT-PCR using gene specific primer. For stress-induced expression assays, RNA was isolated from 21-day-old rice seedlings treated with 0, 50 and 75 mM NaCl alone and in combination with validamycin A. Samples were ground to a fine powder under liquid nitrogen in a mortar and pestle and total RNA was isolated using mini kit (Promega A3500, Madison, WI, USA) with an optional RNase-free DNase treatment. First strand cDNA was synthesized with 1 μg of total RNA and oligo (dT) 20 primers using Super Script III RNase H Reverse Transcriptase (Promega). The RT-PCR was carried out using the following TPS gene-specific primer:5´-CTGGCAACAGGCTCATCT-3`(forward) and 5´-CATCTTCACAACATCATCAGCCG-3` (reverse). Amplification of gene specific products from cDNA used the PCR cycle: initial denaturation 94°C for 2 minutes, denature (94°C) for 30 seconds, anneal (60°C) for 30 seconds and extend (72°C) for 2 minutes each for 30 cycles and final extension 72°C 10 minutes. The experiments were replicated at least three times. After RT-PCR, the PCR products from each sample were analyzed on 1% agarose gels.

Statistical Analysis: The obtained data were statistically analyzed using the one-way analysis of variance as described by Snedecor and Cochran [36]. Means were compared by LSD at 5% using SPSS program version16.

RESULTS AND DISCUSSION

The Change in Plant Growth Parameters in Rice Plants:
Studies on the growth and parameters of the salt tolerant rice cultivar AgamiM5 and its salt sensitive counterpart Sakha103 in response to salt stress showed significant differences. Agami M5 showed the highest value in all growth parameters (Fig. 1). Salt stress caused a significant reduction in all the growth parameters. The reduction was greater at higher NaCl concentrations (75 and 100 mM). The length of shoot, root and fresh, dry weight were
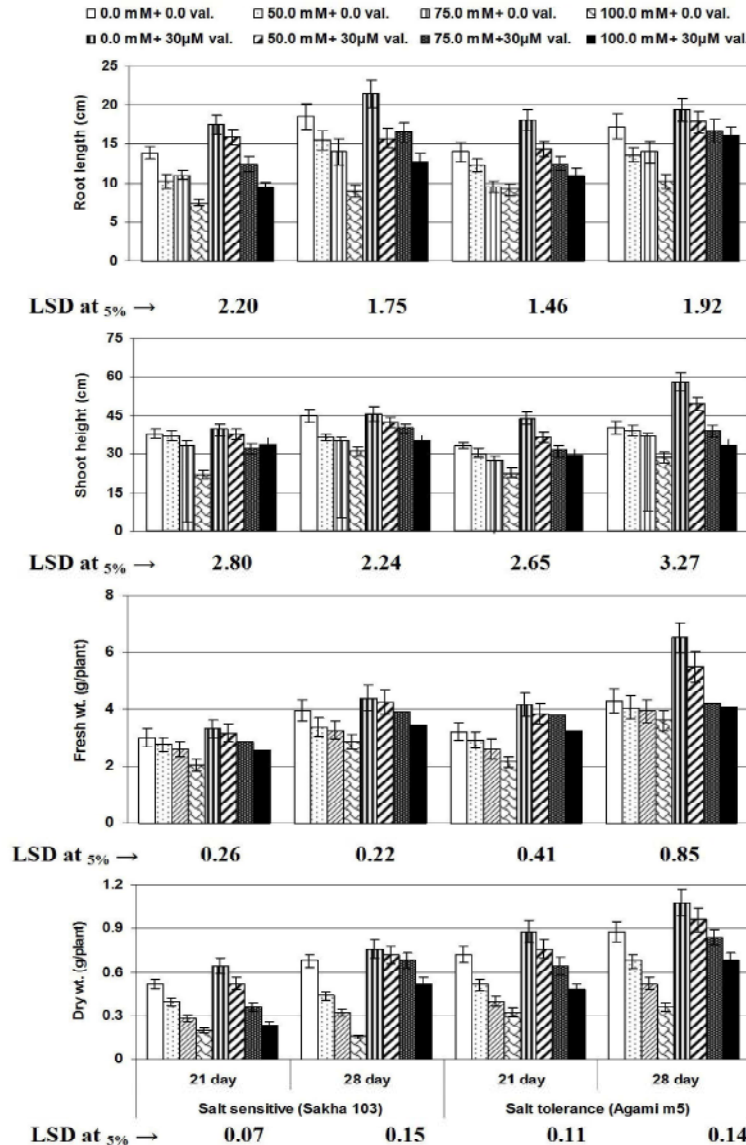
Fig. 1: Change in growth criteria of two rice cultivars shoots treated with 30 µM Validamycin A and grown under different conc. of NaCl.

gradually decreased with increasing NaCl concentration. The reduction was more pronounced at 100 mM in both rice cultivars. According to the obtained results, pretreatment with validamycin A stimulated shoot and root length of rice seedlings of both Sakha103 and AgamiM5 cultivars under normal and salinity stress conditions. There was a significant decrease in shoot and root length of Sakha103 grown in 100 mM/l NaCl by 42% and 31% at 21 days old and 46% and 52% at 28 days old as compared with control. While, the pre-soaked with validamycin A (approximately 16%, 22% and 32%, 31%) length reduction compared to AgamiM5, which showed

(approximately 32%, 29% and 34%, 30%) length reduction at 21 and 28 days old treated plants, respectively, while the pre-soaked (approximately 11%, 18% and 21%, 7.5%) growth reduction at 100mM NaCl. Results for fresh and dry weights of the two rice cultivars grown in 0 to 100mM showed that fresh and dry weight of both cultivars were reduced significantly with increasing NaCl concentration. Soaking application of validamycin A enhanced fresh and dry weight of rice plants compared with plants treated with NaCl alone. AgamiM5 plants previously supplied with validamycin A showed the highest potential of growth compared with Sakha103.

Table 1:   Change in photosynthetic pigments (mg g^{-1}F.W.) in shoots of salt sensitive rice cultivar Sakha103 treated with 30μM Validamycin A and grown under different concentrations of NaCl

| | Days after salt stress treatments | | | | | | | | | |
| | Chlorophyll a | | Chlorophyll b | | Total Chlorophyll | | Carotenoids | | Total pigments | |
Treatments	21	28	21	28	21	28	21	28	21	28
T$_1$	51.48cd	60.78b	0.284b	0.281b	0.794b	0.788B	51.48cd	60.78b	52.27bc	61.57b
T$_2$	48.74d	42.85cd	0.211cd	0.214cd	0.655d	0.61D	48.74d	42.85cd	49.40bc	43.46C
T$_3$	46.21d	46.71c	0.188d	0.193d	0.551e	0.546De	46.21d	46.71c	46.76cd	47.26C
T$_4$	37.91e	37.22d	0.183d	0.162d	0.545e	0.495E	37.91e	37.22d	38.46d	37.72d
T$_5$	73.70a	87.37a	0.351a	0.420a	0.875a	0.944A	73.70a	87.37a	74.58a	88.32A
T$_6$	58.48b	59.19b	0.258bc	0.256bc	0.757bc	0.706C	58.48b	59.19b	59.24b	59.9B
T$_7$	55.78bc	46.91c	0.248bc	0.248bc	0.728c	0.708c	55.78bc	46.91c	56.51b	47.62C
T$_8$	50.81cd	46.49c	0.204cd	0.239c	0.594de	0.61d	50.81cd	46.49c	51.41b	47.1C
LSD 0.05	6.24	8.3	0.032	0.038	0.057	0.075	6.24	8.3	8.65	9.2

Means having the same letters in a column were not significantly different at p<0.05

where:

T$_1$=	0.0 mM+ 0.0 val.	T$_3$=	75.0 mM+ 0.0 val.	T$_5$=	0.0 mM+ 30μM val.	T$_7$=	75.0 mM+30μM val.
T$_2$=	50.0 mM+ 0.0 val.	T$_4$=	100.0 mM+ 0.0 val.	T$_6$=	50.0 mM+ 30μM val.	T$_8$=	100.0 mM+ 30μM val.

Studies on the growth parameters of salt tolerant rice cultivar AgamiM5 and its salt sensitive counterpart Sakha103 in response to salt stress showed significant differences. Growth of both Agami M5 and Sakha103 were affected by salinity. These results are similar to Dolatabadian *et al.* [37] and Sharma *et al.* [38], who found that salinity stress significantly decreased shoot and root length either fresh weight or dry weight in soybean and rice plants, respectively, in addition, total plant weight and leaves number were decreased due to salinity stress. Kumar *et al.* [39] quoted that salt tolerant rice cultivars generate larger biomass than sensitive ones irrigated with NaCl. Bandeoglu *et al.* [40] indicated that this retarded growth is due to inhibition of cell elongation due to higher concentration of Na$^+$ which causes membrane disorganization, inhibition of cell division and expansion [41]. Application of validamycin A caused trehalose accumulation and stimulated all growth parameters of rice seedlings of both (Sakha103 and Agami M5) under normal and salinity-induced stress conditions. Growth of both AgamiM5 and Sakha103 pre-soaked with validamycin A were least affected by salinity. Our results are in agreement with the findings of Chang *et al.* [3], who found that exogenous trehalose largely mitigated salt damage on growth of rice plants. Our results are also in agreement with those obtained by Gouffi *et al.* [42], who found that the increasing of biomass production with treatment of validamycin A may have been due to the role of trehalose as an osmoprotectant under adverse environmental conditions.

The Change in Some Biochemical Compounds in Rice Plants

Photosynthetic Pigments: Chlorophyll (Chl. a, b), total chlorophyll, carotenoids and total pigments content of rice leaves were significantly decreased by salinity stress (50, 75, 100mM NaCl) as compared with control (Tables 1 and 2). Our results revealed that application of validamycin A was effective in reducing the inhibitory action of salt stress on photosynthetic pigments of rice plants. The total Chl. decreased by 31.4% and 37.2% in Sakha103 and 28.6% and 25.4% in Agami M5 cultivars, respectively, at 21 and 28 days after planting of 100mM NaCl salt application. While, the pre-soaked plants with validamycin A and treated with 100mM NaCl were decreased by 25.1% and 23% in total chlorophyll content of Sakha103 and 13.3% and 13.20% decrease in Agami M5 after 21 and 28 days after planting. Similarly, at 21 and 28 days after planting the plants treated with (100mM NaCl), decreased carotenoids content by 26.4% and 38.8%, respectively in Sakha103 and by 26.5% and 20.4% in Agami M5 compared to their respective controls. Upon validamycin A and salt stress treated plants, carotenoids contents increased significantly in presoaked plants of both the genotypes by 34% and 26% in Sakha103 and 0.5% and 2.5% in Agami M5. Carotenoids and total pigments follow the same trend throughout the experiment. Total pigments of salt stressed plants decreased significantly in both rice cultivars. However, validamycin A induced an increase in total pigment contents in both genotypes in control and salinized plants.

Table 2: Change in photosynthetic pigments (mg g^{-1}F.W.) in shoots of Salt tolerance rice cultivar Agami M5 treated with 30 μM Validamycin A and grown under different concentration of NaCl.

	Days after salt stress treatments									
	Chlorophyll a		Chlorophyll b		Total Chlorophyll		Carotenoids		Total pigments	
Treatments	21	28	21	28	21	28	21	28	21	28
T$_1$	0.917ab	0.883a	0.644ab	0.556B	1.561b	1.44a	107.27b	109.30a	108.56b	110.74a
T$_2$	0.811c	0.689c	0.463d	0.461C	1.274e	1.15e	79.20c	88.63b	80.48cd	89.78b
T$_3$	0.736d	0.631c	0.460d	0.450C	1.196f	1.08e	87.72c	88.20b	88.92c	89.28b
T$_4$	0.658e	0.626c	0.456d	0.447C	1.114g	1.07e	78.84c	87.04b	79.96d	88.11b
T$_5$	0.958a	0.877a	0.689a	0.637a	1.647a	1.51a	123.03a	113.18a	124.68a	114.70a
T$_6$	0.956a	0.843a	0.601b	0.613a	1.557b	1.46a	102.18b	112.9a	103.74b	114.36a
T$_7$	0.894bc	0.806b	0.520c	0.561b	1.414c	1.37c	86.16c	86.70b	87.58cd	88.07b
T$_8$	0.845c	0.690c	0.508cd	0.559b	1.353cd	1.25d	82.39c	89.13b	83.74cd	90.38b
LSD 0.05	0.058	0.095	0.06	0.068	0.068	0.062	10.18	12.52	7.98	10.25

Means having the same letters in a column were not significantly different at p<0.05

where:

T$_1$=	0.0 mM+ 0.0 val.	T$_3$=	75.0 mM+ 0.0 val.	T$_5$=	0.0 mM+ 30μM val.	T$_7$=	75.0 mM+30μM val.
T$_2$=	50.0 mM+ 0.0 val.	T$_4$=	100.0 mM+ 0.0 val.	T$_6$=	50.0 mM+ 30μM val.	T$_8$=	100.0 mM+ 30μM val.

The reduction in growth criteria by the influence of salinity could be attributed to the limiting effect of salinity-induced stress on the chlorophyll content and photosynthetic activity in maize plant [43]. Salt stress can reduce the leaf photosynthetic activity by affecting stomatal and non-stomatal factors. Lose turgor by osmotic effect can cause stomatal closure which lowers the supply of CO_2 to leaves. But salinity can also reduce photosynthetic activity by affecting the non stomatal attributes such as destruction of green pigments, lowering the leaf area or by decreasing the activity of photosynthetic enzymes in Calvin cycle [44]. Our results revealed that application of validamycin A greatly increased trehalose content which may reduce the inhibitory action of salt stress on photosynthetic pigments of both rice cultivar plants. These results are in agreement with that recorded by Jun *et al.* [45] who reported that trehalose maintains higher leaf chlorophyll contents in maize after sustained growth in the presence of NaCl. Zeid [43] suggested that trehalose may preserve the stability of the chloroplast envelope and maintained the osmotic potential of the chloroplast.

Sugars and Starch Contents: The behavior of the different carbohydrate fractions of two rice cultivars plants in response to salt application alone and /or with validamycin A are shown in Fig. 2. NaCl at the 50, 75 and 100 mM concentration significantly stimulated (TSS), sucrose and trehalose at 28 than 21days after planting. Fig. 2 illustrated that soaking of two rice cultivars plants with 30μm of validamycin A caused high significant increases in sugar fractions in the two genotypes as

compared with corresponding control. On the other hand, data appeared that treatment of seeds with validamycin A had a highest content of sugar fractions in Agami M5 than Sakha103-treated plants. Most striking results were seen in starch content where a sharp decrease was observed in Sakha103 plants with increasing salt stress upto100 mM NaCl compared with the control, whereas starch content was slight decreased significantly under the influence of salt stress in AgamiM5 plants compared to the respective control. Under the same conditions, starch markedly increased in both Sakha103 and Agami M5 at 21 and 28 days plant age in rice plants treated with NaCl and soaked with validamycin A as compared with untreated control. In the present study, trehalose accumulated in rice plants of the two cultivars in response to trehalase inhibition through validamycin A treatments. The increase in trehalose content was recorded under salinity stress in both validamycin A treated and untreated control and the magnitude of increase was in rice plants treated with validamycin A. Several researchers have used validamycin A to raise the level of trehalose in plants. Thus, Lo´pez *et al.* [13] reported that validamycin A was able to increase the level of trehalose in nodules of *Lotus japonicas* and *Medicago truncatula*. Trehalose significantly increased in rice seedlings by using validamycin A [46]. The obtained data revealed that pre-soaking seeds with validamycin A stimulated the accumulation of total soluble sugars and starch as compared with the corresponding control. Soluble sugar may play a key role in osmotic adjustment at the cellular level of plants under salt stress [47].
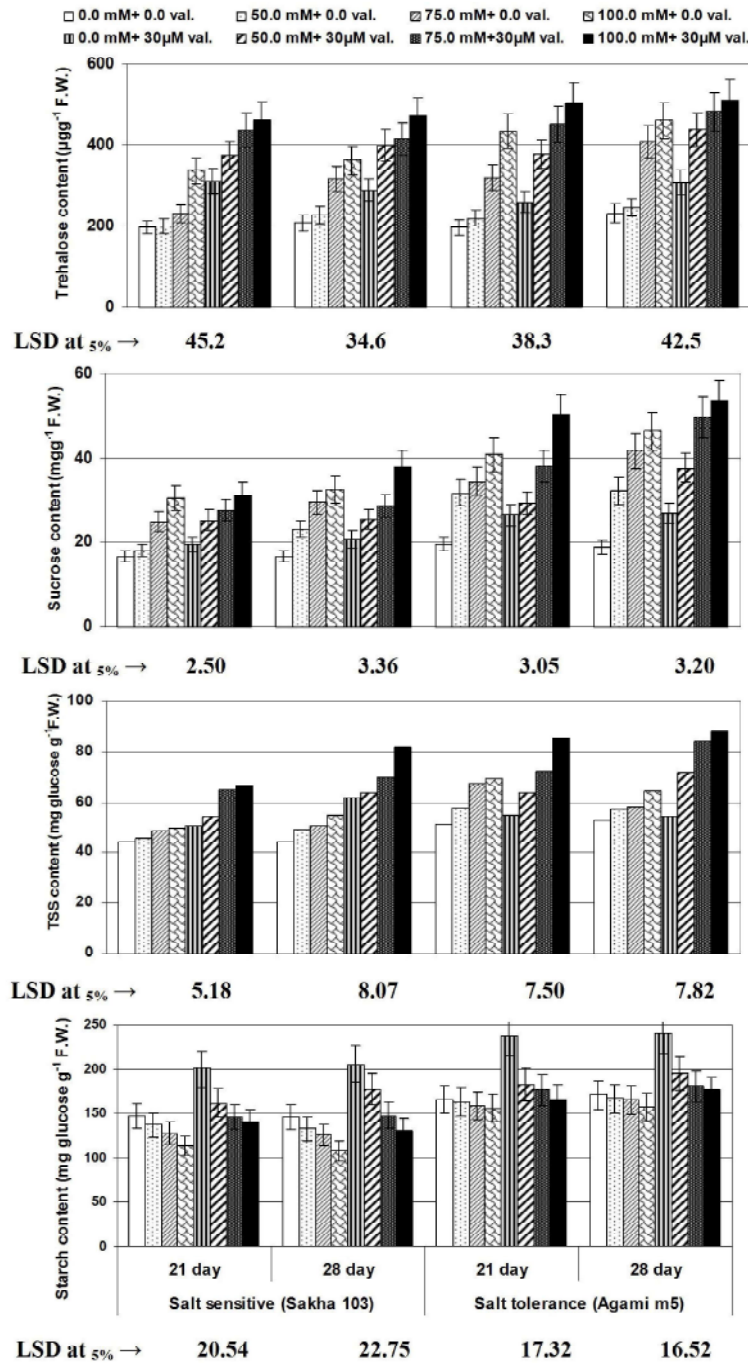
Fig. 2: Change in trehalose (µg trehalose g^{-1} F.W.), sucrose, total soluble sugars and starch (mg glucose g F.W.) contents in shoots of two rice cultivars treated with 30 µM Validamycin A and grown under different concentrations of NaCl.

In rice, soluble sugar content in salt-tolerant cultivar was significantly greater than in salt-sensitive one in plants exposed to salt stress [48]. The increasing of total soluble sugars in rice shoots may function as an osmotic adjustment to prevent water loss in the plant cells during salt stress [49]. Trehalose, which may play an important role in regulating carbohydrate allocation in plants during development, has often been proposed as acting as an osmoprotectant during periods of drought or water deficit- induced stresses [6]. It is clear from the present

Table 3: Change in Minerals content (mg g⁻¹dry weight) in shoots of two rice cultivars treated with 30 μM Validamycin A grown under different concentrations of NaCl

| | Sakha103 (Salt sensitive) | | | | | | Agami M5 (Salt tolerance) | | | | | |
| | 28 day | | | | | | 28 day | | | | | |
Treatments	K	Na	K/Na	N	P	Ca	K	Na	K/Na	N	P	Ca
0.0 mM+ 0.0 val.	145	37	3.90	45	15	35	168	36	4.7	100	16	31
50.0 mM+ 0.0 val.	118	52	2.26	40	12	35	131	48	2.7	92	14	37
75.0 mM+ 0.0 val.	95	55	1.70	38	10	34	119	52	2.3	87	11	41
100.0 mM+ 0.0 val.	67	76	0.90	33	8	36	100	62	1.6	70	7	45
0.0 mM+ 30μM val.	147	34	4.30	49	17	34	148	32	4.6	113	17	31
50.0 mM+ 30μM val.	142	38	3.70	44	14	33	145	41	3.5	107	17	37
75.0 mM+30μM val.	135	48	2.80	48	12	33	138	43	3.2	96	14	40
100.0 mM+ 30μM val.	110	56	2.00	41	11	36	122	51	2.4	83	10	43

study that the accumulation of trehalose in the rice plants is accompanied by increasing in the level of sucrose in both cultivars treated and untreated with NaCl⁺ validamycin A as compared to corresponding control. These results are in agreement with those reported by Garg *et al.* [46]. Trehalose may have an indirect effect on carbohydrate metabolism by interfering with photosynthetic capacity and the conversion and utilization of other sugars [50]. Meanwhile, plant tissues of the validamycin A-treated seeds showed a more accumulation of total soluble sugars in both stressed and unstressed rice cultivars. In Arabidopsis, exogenous application of trehalose induces accumulation of starch by increasing the activity of ADP-glucose pyrophosphorylase (AGPase), a major enzyme controlling starch synthesis [51].

In the present study, the starch content increased in both Sakha103 and AgamiM5 treated and untreated with NaCl in response to validamycin A than the corresponding control and consequence of trehalose accumulation. Our results are in agreement with those obtained by Bae *et al.* [52], who reported that starch was 3-fold greater in the trehalose treated samples than in the control of *Arabidopsis thaliana* seedlings. The increase in the level of total soluble sugar, sucrose and trehalose may be linked to the changes in starch content. Starch is an important component of plant tissues and accumulates in leaves as a temporary reserve form of carbon and is the principal component of dry mass accumulated in mature leaves, hence the accumulation of more starch in Agami M5 may be seen as the protective mechanism during stress conditions.

Mineral Contents: When the plants were stressed with NaCl, the concentrations of most elements differed significantly from those in the control. Salinity stress caused significant decreases in nitrogen, phosphorous and potassium contents of two rice cultivars with the increases in salinity levels applications (Table 3). Pretreatment of rice seeds with validamycin A (30μM) induced significant accumulation of N and P under all salinity levels in both cultivars under both stress and non-stress conditions as compared with the corresponding salinity level. Regarding the effect of soaking seeds with validamycin A (30μM) of both rice cultivars on Ca⁺² contents the results recorded non significant variation between treatments as compared with the corresponding salinity level. Results in Table 3 showed the response of K⁺, Na⁺ and K⁺/Na⁺ ratio of both rice cultivars subjected to different salinity levels. The significant accumulation of sodium was increased with increasing salinity level in both cultivars. This accumulation of Na⁺ content was accompanied with significant gradual decrease in K⁺ content and K⁺/Na⁺ ratio in both cultivars. Sodium ion accumulation in the tolerant cultivar, Agami M5, was much lower than in the sensitive cultivar. In the meantime, Agami M5 cultivar showed higher significant values of K⁺/Na⁺ ratio as compared with Sakha103 cultivar. Soaking rice seeds in validamycin A (30μM) showed significant decrease in Na⁺ content in both cultivars at 50 and 75mM NaCl levels of salinity. Pretreatment of seeds with validamycin A (30μM) induced significant increases in K⁺ in both cultivars as compared with the corresponding salinity level especially at Agami M5 cultivar.

The N, P and K are primary nutrient elements which are very essential for plant growth and development [53]. Also most plants use K⁺ and Ca²⁺ rather than Na⁺ as an important component of osmotic adjustment, K⁺ and Ca²⁺ are essential macronutrients for all plants [54]. Consequently, crops growing in saline soils may suffer dual injury, Na⁺ toxicity and K⁺ or Ca²⁺ deficiency [55].
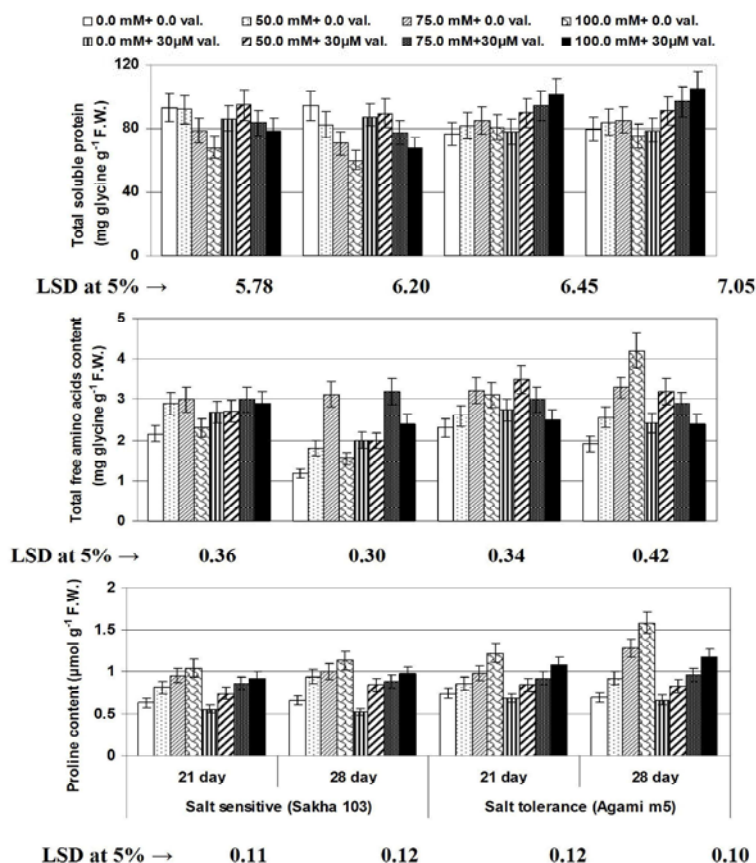
Fig. 3: Change in total soluble protein (mg g^{-1} F.W.), total free amino acid contents (mg glycine g^{-1} F.W.) and proline content (µmol g^{-1} F.W.) in shoots of two rice cultivars treated with 30µM Validamycin A grown under different concentrations of NaCl.

In the present study, our results indicated that accumulation of Na$^+$ in the leaves of both rice cultivars increased significantly under saline conditions. Exogenous application of validamycin A inhibited the accumulation of Na$^+$ in both cultivars of NaCl-stressed plants. This result was corroborated with Garg *et al.* [9], who showed that treatment with exogenous trehalose significantly reduced the salt induced accumulation of Na$^+$ in rice leaves. Accumulation of K$^+$, N and P in the leaves of both rice cultivars was significantly reduced due to salt stress. However, validamycin A through priming application enhanced the accumulation of K$^+$, N and P in both cultivars of the salt-stressed plants. According to Hajihashemi *et al.* [56], salt stress decreased the content of K$^+$ and P in the leaves and roots of salt-stressed wheat plants. The increased N and K concentrations with application of validamycin A have been a major factor for increasing dry weight production because both are components of many metabolically important compounds and play an important role in physiological function [57].

K$^+$/Na$^+$ ratio in both cultivars leaves indicated a great reduction with increasing salt concentration in the soil solution. Exogenous application of validamycin A increased K$^+$/Na$^+$ ratio in both cultivars of NaCl-stressed plants. Our results are in harmony with those reported by Zeid [43], who found that trehalose treatment increased K$^+$/Na$^+$ ratio in the leaves indicating alleviation of the adverse effects of Na$^+$ ions in the maize leaves.

Change in Nitrogenous Constituents in Shoots of Rice plants of Two Cultivars Pretreated with Validamycin A and Grown under Different Concentrations of NaCl: The changes in the different nitrogen fractions of rice plants soaked with validamycin A and subjected to salt stress with NaCl (50, 75 and 100 mM) are shown in Fig. 3.

Soluble Proteins: A continuous decrease in protein content with increasing salt stress was observed in both cultivars. Pretreatment of both rice seed cultivars with validamycin A (30µM) induced significant increases in

total protein content of rice plants under all salinity levels as compared with the corresponding salinity level. The observations also showed that Agami M5 could withstand salt stress in a better way than the other cultivar in terms of total protein content.

Total Amino Acids: The influence of salinity levels on free amino acids content of both rice cultivars is shown in Fig. 3. Total free amino acids content of both cv. increased in the two growth stages (21 and 28 days after planting) when salinity was increased but, in 100mM were lower than the other salt concentrations in Sakha103 cultivar. The maximum increase of total free amino acid contents increased by 1.7-fold in AgamiM5 treated with 100mM NaCl at 28 days plant age. In contrast, after the application of validamycin A (30μM) as seed soaking, the free fatty acids (FAA) contents of both cultivars decreased significantly than the corresponding salinity level.

Proline: Rapid accumulation of free proline is a typical response to salt stress. The rate of salt stress-induced proline accumulation was considerably higher in Agami M5 than its counterpart, Sakha103. In control plants the proline content was almost similar in both cultivars, however, as the magnitude of salinity stress increased, the rate of proline accumulation was observed much higher in AgamiM5 (64.9-129% of control at the 21and 28 days plant age, respectively) as compared to Sakha103 (65.1- 73.9% of control at the 21 and 28 plant age, respectively) at 100 mM NaCl stress (Fig. 3). In contrast, after the application of validamycin A, the proline contents of both cultivars decreased significantly as compared with their respective control and corresponding salinity level. Osmotic adjustment is an important mechanism of salinity tolerance and occurs through compatible solutes accumulation in stressed plants [58]. Many functions have been postulated for proline accumulation in plant tissues, proline and free amino acids could be involved in the osmotic adjustment of plants [59] and could also be a protective agent of enzymes and membranes [60]. Proline may not only act as an osmoregulator but also plays an important role in the protection of enzymes and the structure of macromolecules and as a reservoir of energy and nitrogen for utilization upon exposure to salinity [61]. Interestingly, the priming of rice seeds with validamycin A decreased the concentration of this osmoticum when compared with control. Similar results were obtained by Nounjana *et al.* [62], who reported that supplements of rice with trehalose

negatively affected proline amounts in both unstressed and salt-stressed conditions resulting in a significant reduction in proline. Furthermore, exogenous trehalose also reduced proline accumulation in two maize cultivars under drought stress while increasing biomass production, improving plant water relations and some key photosynthetic attributes [63]. It appears that the priming of rice seeds with validamycin A in response to NaCl treatments or sowing in saline soil has a significant role in high alleviation of salinity stress. It may be presumed that osmoprotective effects of the accumulated trehalose reduced the need for plants to accumulate proline.

A gradual increase in amino acid at high salinity level could be due to increased degradation of protein. Amino acid accumulation occurs not only under salinity but also under water stress in higher plants [64]. The changes in soluble protein showed a reverse trend to that of free amino acids implying that the increase in protein content may be at the expense of amino acids and that the salinity influenced the inter conversion of these compounds. Protein content in the tissues of many plants declined under drought or salinity stress, because of proteolysis and decreased protein synthesis [65]. However, the priming of rice seeds with validamycin A increased the concentration of soluble protein; it decreased the content of free amino acids in both cultivars when compared with the corresponding salinity level. Similar results were obtained by Best *et al.* [14], who reported that exogenous validamycin A (trehalase inhibitor) treatment decreased total amino acid of mature tobacco plants.

TPS Gene Expression Analysis Induced by Validamycin A and Salt Stress: Rice *TPS* gene expression has been determined in both salt sensitive and salt tolerance cultivars treated and/or untreated with validamycin A under salt stress at 50 and 75mM NaCl using semi-quantitative RT-PCR. A 230-bp *TPS* fragment was amplified by RT-PCR (primer pair p1/p2). Fig 4 shows that *TPS* is constitutively expressed (30 cycles RT-PCR) in leaves of both rice cultivars under stressed and non stressed conditions treated and untreated with validamycin A, but there were higher transcript amounts in salt-stressed leaves treated with validamycin A (30 cycles RT-PCR (Fig. 4). In addition, increased *TPS* transcript amounts were observed in Agami M5 (Fig.4b) tissues compared to Sakha103 (Fig.4a) in all treatments. Trehalose expression has two-step pathway involving two enzymes: trehalose-6-phosphate synthase *(TPS)* and trehalose-6-phosphate phosphatase *(TPP)*. Constructs with both *TPS* and *TPP* genes are often used in plant

(a) Expression analysis of *TPS* gene in salt sensitive rice cultivar (Sakha 103)

(b) Expression analysis of *TPS* gene in salt tolerance rice cultivar (Agami M5)

1-Control; 2-50 mM NaCl; 3- 75 mM NaCl; 4- validamycin A only; 5-50 mM NaCl+ validamycin A; 6- 75 mM NaCl+validamycin A.
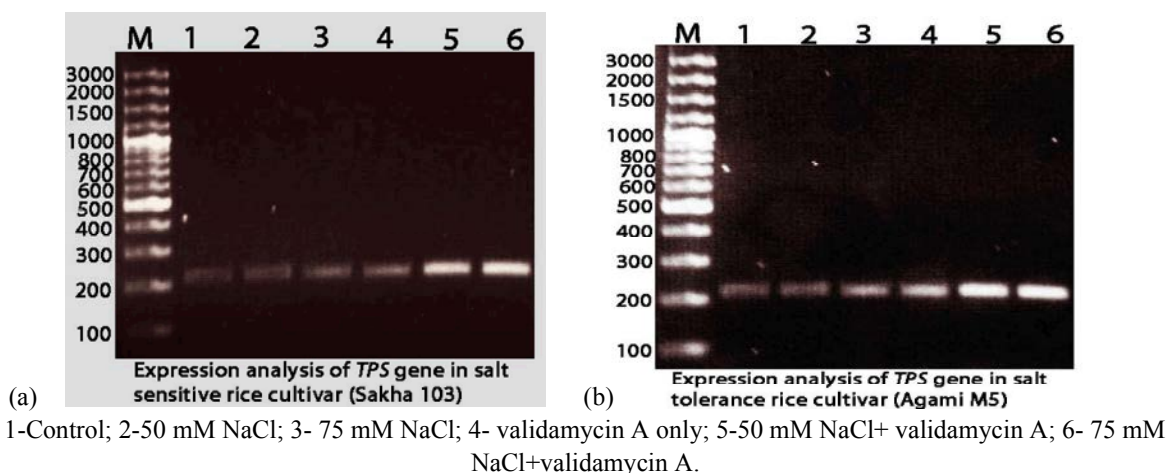
Fig. 4: a, b: Expression analysis of *TPS* gene in salt sensetive(Sakha 103)and salt tolerance (AgamiM5) rice cultivars in response to treatment with different concentrations of NaCl alone or in combination with 30μM validamycin A.

transformation to increase trehalose accumulation. From the literature, it is clear that trehalose and T6P accumulate in plants treated with the potent trehalase inhibitor "validamycin A". When the cytoplasmic trehalose level increases, its feedback inhibition of trehalose phosphate phosphatase *(TPP)* activity enhanced the level of T6P [12]. Our results are in accordance with such theory and seem to indicate that, when aiming to increase stress tolerance, the use of the validamycin A would be sufficient to increase the expression level of trehalose-6-phosphate synthase gene.

The re-upregulation of both rice cultivars *TPS* appears to lead to an accumulation of trehalose and to maintain the expression of genes that produce osmoprotectants because the content of trehalose is too low to serve as a protectant [66]. Earlier reports indicated that *Arabidopsis* and many other higher plants, accumulate trehalose at only trace levels [67]. This is probably due to the low-level activity of synthesis enzymes and relatively high level of trehalase activity (hydrolytic enzyme) [68]. The accumulation of trehalose was increased dramatically in soybean, cowpea, tobacco and *Arabidopsis* after trehalase activity was inhibited by validamycin A [69]. The high level of *TPS* mRNA in Agami M5 was found to be present in the plant treated with validamycin A alone or in combination with NaCl. This expression pattern is consistent with the increase in *TPS* activity. These results suggested that rice *TPS* participates in the response to salt stress in both cultivars. This confirms the important role of TPS in sugar metabolism and within the plant, which could explain its role in plant stress tolerance [70]. Jiang *et al.* [71] suggested that the major role of trehalose in higher plants is not osmotic protection, but signal transduction. Finally, Fernandez *et al.* [72] reviewed that, exogenous application of trehalose in rice significantly reduces damage caused by salt stress. Its action results in: (i) preservation of root integrity; (ii) reduction of both Na^+ accumulation and chlorophyll loss in leaf blades; (iii) growth inhibition and (iv) moderation of the expression of the osmotically responsive salt gene.

REFERENCES

1. Ashraf, M. and M.A. Foolad, 2007. Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. Environ. Exp. Bot., 59: 206-216.

2. Chen, Z., T.A. Cuin, M. Zhou, A. Twomey, B.P. Naidu and S. Shabala, 2007. Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. J. Exp. Bot., 58: 4245-4255.

3. Chang B., L. Yang, W. Cong, Y. Zu and Z. Tang, 2014b. The improved resistance to high salinity induced by trehalose is associated with ionic regulation and osmotic adjustment in *Catharanthus roseus*. Plant Physiology and Biochemistry, 77: 140-148.

4. Lee, M.H., E.J. Cho, S.G. Wi, H. Bae, J.E. Kim, J.Y. Cho, S. Lee, J.-H. Kim and B.Y. Chung, 2013. Divergences in morphological changes and antioxidant responses in salt-tolerant and salt-sensitive rice seedlings after salt stress. Plant Physiology and Biochemistry, 70: 325-335.

5. Poor, P., K. Gemes, F. Horvath, A. Szepesi, M.L. Simon and I. Tari, 2011. Salicylic acid treatment via the rooting medium interferes with stomatal response, CO_2 fixation rate and carbohydrate metabolism in tomato and decreases harmful effects of subsequent salt stress. Plant Biol. (Stuttg), 13: 105-114.

6. Eastmond, P.J. and I.A. Graham, 2003. Trehalose metabolism: a regulatory role for trehalose-6-phosphate?. Curr. Opin. Plant Biol., 6: 231-235.

7. Paul, M., 2007. Trehalose 6-phosphate. Curr. Opin. Plant Biol., 10: 303-309.

8. Chang, B.W., W.W. Cong, Q. Chen, Y.G. Zu and Z.H. Tang, 2014a. The influence of different forms and concentrations of potassium nutrition on growth and alkaloid metabolism in *Catharanthus roseus* seedlings. J. Plant Interact., (9): 370-377.

9. Garg, A.K., J.K. Kim, T.G. Owens, A.P. Ranwala, Y.D. Choi, L.V. Kochian and R.J. Wu, 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Appl. Biol. Sci., 99: 15898-15903.

10. Paul, M.J., L.F. Primavesi, D. Jhurreea and Y.H. Zhang, 2008. Trehalose metabolism and signaling. Annu. Rev. Plant Biol., 59: 417-441.

11. Schluepmann, H. and M. Paul, 2009. Trehalose Metabolites in Arabidopsis-Elusive, Active and Central. American Society of Plant Biologists, the Arabidopsis Book, pp: 1- 17.

12. Ahmed, H.E., A.Y. Elhusseiny, A.K. Maimona and E.A. Qaid, 2013. Trehalose Accumulation in Wheat Plant Promotes Sucrose and Starch Biosynthesis. Jordan J. of Biol. Sci., 6: 143-150.

13. Lo´pez, M., N.A. Tejera and C. Lluch, 2009. Validamycin A improves the response of *Medicago truncatula* plants to salt stress by inducing trehalose accumulation in the root nodules. Journal of Plant Physiology, 166: 1218-1222.

14. Best, M., K. Koenig, K. McDonald, M. Schueller, A. Rogers and R.A. Ferrieri, 2011. Inhibition of trehalose breakdown increases new carbon partitioning into cellulosic biomass in *Nicotiana tabacum*. Carbohydrate Research, 346: 595-601.

15. Mustafa, R.E.M., 2005. Physiological and Molecular Responses to Abiotic Stress in Rice (*Oryza sativa*) and Characterization of an Up-Regulated Gene Family. Univ. of Arkansas.

16. Paranthaman, R., K. Alagusundaram and J. Indhumathi, 2009. Production of Protease from Rice Mill Wastes by *Aspergillus niger* in Solid State Fermentation. World Journal of Agricultural Sciences, 5: 308-312.

17. Sasaki, T., 2002. Rice genomics to understand rice plant as an assembly of genetic codes. Current Sci., 83: 834-839.

18. Shahbaz, M. and M. Ashraf, 2013. Improving salinity tolerance in cereals. Crit. Rev. Plant Sci., 32: 237-249.

19. Thomashow, M.F., 2001. So what's new in the field of plant cold acclimation? Lots! Plant Physiol., 125: 89-93.

20. Seki, M., A. Kamei, K. Yamaguchi-Shinozaki and K. Shinozaki, 2003. Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr Opin Biotech., 14: 194-199.

21. Sawada, H., I.S. Shim and K. Usui, 2006. Induction of benzoic acid 2-hydroxylase and salicylic acid biosynthesis - Modulation by salt stress in rice seedlings. Plant Sci., 171: 263-270.

22. Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol., 148: 350-82.

23. Allen, S.E., 1989. Chemical Analysis of Ecological Materials. 2nd Edition. Blackwell Scientific Publications, Melbourne.

24. Snell, F.D. and C.T. Snell, 1967. The Colorimetric Methods of Analysis Including Photometer Method. D. Van. Nostrand Company Inc. Princeton, New Jersey, Toronto, London, 4 (217): 3rd pp: 437-438.

25. Jackson, M.L., 1973. Soil Chemical Analysis. Prentice Hall of India Private Limited, New York, pp: 61-73.

26. Jacobe, M.B., 1958. The Chemical Analysis of Food and Food Products. Van Nostrand Co. Inc., New York.

27. Homme, P.M., B. Gonzalez and J. Billard, 1992. Carbohydrate content, frutane and sucrose enzyme activities in roots, stubble and leaves of rye grass (*Lolium perenne* L.) as affected by source/link modification after cutting. J. Plant Physiol., 140: 282-291.

28. Yemm, E.W. and A.J. Willis, 1954. The respiration of barley plants IX. The metabolism of roots during assimilation of nitrogen. New Phytol., 55: 229-234.

29. Handel, E.V., 1968. Direct micro determination of sucrose. Anal. Biochem., 22: 280-283.

30. Rose, R., C.L. Rose, S.K. Omi, K.R. Forry, D.M. Durall and W.L. Bigg, 1991. Starch determination by perchloric acid vs. enzymes: evaluating the accuracy and precision of six colorimetric methods, J. Agric. Food Chem., 9: 3-11.

31. Lynch, A.L., R. Chen, P.J. Dominowski, E.Y. Shalaev, J.R. Yancey and N.K.H. Slater, 2010. Biopolymer mediated trehalose uptake for enhanced erythrocyte cryosurvival. Biomaterials, 31: 6096-6103.

32. Umbreit, W.W., R.H. Burris and J. Frederick, 1972. Manometric & Biochemical Techniques. 5th Ed. Minneapolis: Burgess.

33. Muting, D. and E. Kaiser, 1963. Spectrophotometric method of determining of amino-N in biological materials by means of the ninhydrin reaction. Seyler's Zschr. Physiol. Chem., 332: 276.

34. Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-207.

35. Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem., 72: 248- 54.

36. Snedecor, G.M. and W.C. Cochran, 1969. Statistical Methods. 6th Ed. Iowa Univ. Press. Ames. Iowa U.S.A.

37. Dolatabadian, A., S.A.M. Modarressanavy and F. Ghanati, 2011. Effect of salinity on growth, xylem structure and anatomical characteristics of soybean. Notulae Scientia Biologicae, 3(1): 41-45.

38. Sharma, I., E. Ching, S. Saini, R. Bhardwaj and P.K. Pati, 2013. Exogenous application of brassinosteroid offers tolerance to salinity by altering stress responses in rice variety Pusa Basmati-1. Plant Physiology and Biochemistry, 69: 17-26.

39. Kumar, V., V. Shriram, T.D. Nikam, N. Jawaliand and M.G. Shitole, 2009. Antioxidant enzyme activities and protein profiling under salt stress in Indica rice genotypes differing in salt tolerance. Arch. Agron. Soil Sci., 55: 379-394.

40. Bandeoglu, E., F. Eyidogan, M. Yuceland and H.A. Oktem, 2004. Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. Plant Growth Regul., (42): 69-77.

41. Deivanai S., R. Xavier, V. Vinod, K. Timalata and O.F. Lim 2011. Role of Exogenous Proline in Ameliorating Salt Stress at Early Stage in Two Rice Cultivars. J. of Stress Physiology and Biochemistry, 7(4): 157-174.

42. Gouffi, K., N. Pica, V. Pichereau and C. Blanco, 1999. Disaccharides as a new class of non accumulated osmoprotectants in *Sinorhizobium meliloti*. Appl. Environ. Microbiol., 65: 1491-1500.

43. Zeid, I.M., 2009. Trehalose as osmoprotectant for maize under salinity-induced stress. Research Journal of Agriculture and Biological Sciences, 5: 613-622.

44. Shahid, M.A., R.M. Balal, M.A. Pervez, T. Abbas, M. Ashfaq, U. Ghazanfar, M. Afzal, A. Rashid, F. Garcia-Sanchez and N.S. Mattson, 2012. Differential response of pea (*Pisum sativum* L.) genotypes to salt stress in relation to the growth, physiological attributes antioxidant activity and organic solutes. Australian Journal of Crop Science, 6(5): 828-838.

45. Jun, S.S., H.J. Choi, H.Y. Lee and Y.N. Hong, 2008. Differential Protection of Photosynthetic Capacity in Trehalose- and LEA Protein-producing Transgenic Plants under Abiotic Stresses. Journal of Plant Biology, 51(5): 327-336.

46. Garg, A.K., A.P. Ranwala, W.B. Miller and R.J. WU, 2007. HPLC analysis of trehalose and other soluble carbohydrates from the leaf tissue of Indica and Japonica rice varieties. Rice Genetics Newsletter, 24: 44-47.

47. Gupta, A.K. and N. Kaur, 2005. Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. J. Biosci., 30: 761-776.

48. Nemat, I., F. Moradi, S. Gholizadeh, M.A. Esmaeili and M.R. Bihamta, 2011. The effect of salinity stress on ions and soluble sugar distribution in leaves, leaf sheaths and roots of rice (*Oryza sativa* L.) seedlings. Plant Soil Environ., 57: 26-33.

49. Siringam, K., N. Juntawong, S. Cha-um and C. Kirdmanee, 2011. Salt stress induced ion accumulation, ion homeostasis, membrane injury and sugar contents in salt-sensitive rice (*Oryza sativa* L. spp. indica) roots under isoosmotic conditions. African Journal of Biotechnology, 10(8): 1340-1346.

50. Ranwala, A.P. and W.B. Miller, 2009. Comparison of the dynamics of non-structural carbohydrate pools in cut tulip stems supplied with sucrose or trehalose. Postharvest Biology and Technology, 52: 91-96.

51. Wingler, A., T. Fritzius, A. Wiemken, T. Boller and R.A. Aeschbacher, 2000. Trehalose induces the ADP-glucosepyrophosphorylase gene APL3 and starch synthesis in Arabidopsis. Plant Physiol., 124: 105-114.

52. Bae, H., E. Herman, B. Bailey, H.J. Bae and R. Sicher, 2005. Exogenous trehalose alters Arabidopsis transcripts involved in cell wall modification, abiotic stress, nitrogen metabolism and plant defense. Physiol. Plant., 125: 114-126.

53. Mishra, M.K. and V. Prakash, 2010. Response of non-enzymatic antioxidants to zinc induced stress at different pH in *Glycine max* L. cv. Merrill. Acad. J. Plant Sci., 1: 1-10.

54. Zhang, J.L., T.J. Flowers and S.M. Wang, 2010. Mechanisms of sodium uptake by roots of higher plants. Plant Soil., (326): 45-60.

55. Kaya, C., A.L. Tuna, M. Ashraf and H. Altunlu, 2007. Improved salt tolerance of melon (*Cucumis melo* L.) by the addition of proline and potassium nitrate. Environ Exp Bot., (60): 397-403.

56. Hajihashemi, S., K. Kiarostami, A. Saboora and S. Enteshari, 2007. Exogenously applied paclobutrazol modulates growth in salt-stressed wheat plants. Plant Growth Regul., 53: 117-128.

57. Siddiqui, M.H., F. Mohammad, M.N. Khan and M.M.A. Khan, 2008. Cumulative effect of soil and foliar application of nitrogen, phosphorus and sulfur on growth, physico-biochemical parameters, yield attributes and fatty acid composition in oil of erucic acid-free rapeseed-mustard genotypes. Journal of Plant Nutrition, (31): 1284-1298.

58. Talaat, N.B. and B.T. Shawky, 2014. Protective effects of arbuscular mycorrhizal fungi on wheat (*Triticum aestivum* L.) plants exposed to salinity. Environ. & Exp. Bot., 98: 20-31.

59. Delavari, P.M., A. Baghizadeh, S.H. Enteshari, K.H.M. Kalantari, A. Yazdanpanah and E.A. Mousavi, 2010. The Effects of Salicylic Acid on Some of Biochemical and Morphological Characteristic of *Ocimum basilicucm* under Salinity Stress. Australian Journal of Basic and Applied Sciences, 4(10): 4832-4845.

60. Abdalla, H. and E. Selem, 2014. Fast Neutrons Irradiation Induced Changes in Active Ingredients, Amino Acids and Chlorophyll Contents in *Ambrosia maritime* (Damsisa) Influenced by Soil Water Stress. Life Science J., 11(6): 24-30.

61. Tounektia, T., A.M. Vadela, M. O˜nateb, H. Khemiraa and S. Munné-Bosch, 2011. Salt-induced oxidative stress in rosemary plants: Damage or protection? Environ. & Exp. Bot., 71: 298-305.

62. Nounjana, N., P.T. Nghia and P. Theerakulpisut, 2012. Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. J. of Plant Physio., 69: 596-604.

63. Ali, Q. and M. Ashraf, 2011. Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defence mechanism. Journal of Agronomy and Crop Science, 197: 258-271.

64. Rajaravindran, M. and S. Natarajan, 2012. Effects of Salinity Stress on Growth and Biochemical Constituents of the Halophyte *Sesuvium portulacastrum*. International Journal of Research in Biological Sciences, 2(1): 18-25.

65. Joshi, A.J. and H. Misra, 2000. Halophytic grasses as vital components of crop halophytes. *Sporobolus madraspatanus* Borproc. Seminar on Sustainable Halophytes Utilization in the Mediterranean and Subtropical Dry Region, Osnabrueak University, Osnabrueck, pp: 32.

66. Ge, L.F., D.Y. Chao, M. Shi, M.Z. Zhu, J.P. Gao and H.X. Lin, 2008. Overexpression of the trehalose-6-phosphate phosphatase gene OsTPP1 confers stress tolerance in rice and results in the activation of stress responsive genes. Planta, 228(1): 191-201.

67. Chary, S.N., G.R. Hicks, Y.G. Choi, D. Carter and N.V. Raikhel, 2008. Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol., 146: 97-107.

68. Van Dijck, P., J.O. Mascorro-Gallardo, M. De Bus, K. Royackers, G. Iturriaga and J.M. Thevelein, 2002. Truncation of Arabidopsis thaliana and *Selaginella lepidophylla* trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochem J., 366(1): 63-71.

69. Müller, J., R. Aeschbacher, A. Wingler, T. Boller and A. Wiemken, 2001. Trehalose and trehalase in Arabidopsis. Plant Physiology, 125: 1086-1093.

70. Almeida, A.M., L.A. Cardoso, D.M. Santos, J.M. Torné and P.S. Fevereiro, 2007. Trehalose and its applications in plant biotechnology. *In Vitro* Cell. Dev. Biol. Plant., 43: 167-177.

71. Jiang, W., F.L. Fu, S.Z. Zhang, L. Wu and W.C. Li, 2010. Cloning and Characterization of Functional Trehalose-6-Phosphate Synthase Gene in Maize. J. Plant Biol., 53: 134-141.

72. Fernandez, J.M.G., C.O. Mellet, J.L.J. Blanco, J.F. Mota, A. Gadelle, A.C. Sarguet and J. Defaye, 2010. Isothiocyanates and Cyclic Thiocarbamates of α, α'-Trehalose, Sucrose and *Cyclomalto oligosaccharides*. Cheminform, 26(28): DOI: 10.1002/chin.199528236.