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Variable Effects of Silicon on Salt Tolerant Indices in Rice Genotypes at Seedling Stage

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Authors' contributions

This work was carried out in collaboration among all authors. Authors RS and SP designed the experiments. They performed the experiments and analyzed the data. Author RS wrote the draft paper. Author GRR finalized the manuscript. All authors have reviewed the manuscript and agreed to the manuscript contents. All authors read and approved the final manuscript.

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ABSTRACT

Silicon (Si) is known to improve salt tolerance in rice. However, the correlation of silicon with different physiological and biochemical indices of salt tolerance is not properly understood. Two rice genotypes with different silicon accumulation ability were evaluated along with two standard checks in response to 10 dS/m salinity stress (NaCl) and external Si source (1mM) during their seedling stage. All evaluated genotypes showed an evident decrease in biomass and chlorophyll content under salinity stress, while reporting an enhances in Si accumulation, Na^+/K^+ ratio, proline, electrolyte leakage, lipid peroxidation, hydrogen peroxide, and antioxidant activities. The external Si supplementation significantly improved rice tolerance to salinity through increased Si content, low Na^+/K^+ ratio, better osmolyte production, reduced membrane permeability, and improved antioxidant enzyme activities. Multivariate factor analysis with principal component factor statistically correlates

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and visualizes silicon accumulation with salt tolerance indices. The Hierarchical clustering in rice obtained based on the study of salt tolerance indices, distinguishes genotypes with different treatments into three clusters. In conclusion, the clustering grouped salt-tolerant Var.Lunishree and salt stress high silicon accumulating Var.Swarna together validates silicon mitigating effect on salinity in rice.

Keywords: Rice; silicon; salinity tolerance; ion content; antioxidant enzymes; factor analysis.

1. INTRODUCTION

The staple crop rice (*Oryza sativa* L.) plays a significant role in the world economy by providing food for two-thirds of the world's population. Therefore, several breeding programs were planned to bring a quantum jump in rice productivity. In India, rice production is marked very low with wide fluctuations in the yield due to various abiotic and biotic stresses. With climate change, an increase in sea level and global mean temperature increases the risk of saltwater intrusion in low-lying and coastal areas. The Indo-Gangetic Basin in India suffered huge losses of about 45% in rice production from soil salinity [1,2]. Rice is extremely sensitive to salt stress, particularly during seedling and reproductive stages showing complete crop loss [3]. The effect of salt toxicity was listed as ionic stress, osmotic stress, nutrient imbalance, hormonal imbalance, and high production of reactive oxygen species (ROS) [4]. Salt stress adversely reduces plant growth through ionic toxicity and osmotic stress by manipulating a chain of physiological processes, finally suppressing photosynthesis and yield [5]. Plant growth depends on numerous elements existing in the soil, which could be categorized into beneficial, essential, and toxic groups. Beneficial elements are crucial for some specific plant groups or species [6]. The silicon (Si) is a known beneficial element for several plant species, predominantly in rice. Silicon accumulation enhances rice resistance towards physical stress, biotic, and abiotic stress [7,8,9,10,11]. The accumulation of silicon is highly variable and ranges from 1-100 mg/g dry weight depending on the plant species and the growth medium [12,13]. The monocots are generally referred to as silicon accumulators as they accumulate Si in the same concentration range as the essential macronutrients. Some studies reported that silicon application increases tolerance to salinity and drought in plants [14]. It was also reported that Si application enhanced plant growth, biomass, and photosynthetic pigments in many plants [15,16]. The present study was to validate the alleviation of salinity with silicon supplementation in rice and establish a correlation between silicon and salt tolerance indices in rice.

2. MATERIALS AND METHODS

2.1 Plant Materials, Germination Conditions and Experimental Design

Seeds of four rice varieties i.e. Swarna, Jagbandhu, IR29, and Lunishree were procured from Rice Research Station, OUAT (Orissa University of Agriculture and Technology), and ICAR-NRRI (ICAR-National Rice Research Institute, Cuttack). Among the genotypes, Var.Lunishree is a local salt-tolerant genotype released by NRRI especially for the coastal saline belt of Odisha. Also, Var.IR29 is a known salt-sensitive genotype which is widely used in salinity tolerance screening programs. About 50 seeds were surface sterilized with 0.1% mercury chloride for two minutes and treated with 0.5% bavistin (w/v) for 15 minutes before germination in dark at 28℃ for five days. Seedlings were transplanted on nylon net frame fitted in plastic bowls containing Yoshida nutrient solution (YNS) and were exposed to 3000 lux for 12 hr photoperiod [17]. The experiment is conducted twice. About fourteen days after the start of the experiment, the nylon net frames were fitted into the styrofoam boards and floated on plastic pots filled with 3L of YNS (pH 5.0±0.5). These pots were placed in a glasshouse maintained at 28±2℃ with approximately 50% relative humidity. The culture solution was changed every 4 days interval, and the pH and reduced water level were also maintained. Salinity was induced for a week in YNS to obtain electrical conductivity (EC) of 10 dS/m, while non-saline control showed EC of 1 dS/m. Sodium chloride (NaCl) and diatomaceous earth (opal, SiO_2) were directly added to nutrient solutions and applied after four weeks of seedling growth. The experiment consists of four treatments;i) control (without Si and NaCl, C), silicon only (1.0mM, Si), NaCl only (10 dS/m, N) and Si-NaCl together (1.0mM+10 dS/m, NSi), arranged in completely randomized design (CRD). All samples were

weighed and stored at -20°C before analysis. Measurements were taken on dry weight, ion content, membrane permeability, osmolyte, and ROS content.

Table 1. Rice genotypes used in the experiment

Genotypes	Salinity tolerance	Abbreviation
1.IR29	Salt-susceptible	G1
2. Swarna	Moderately tolerant	G2
3.Jagbandhu	Moderately tolerant	G3
4.Lunishree	Salt-tolerant	G4

2.2 Biomass and Pigment Content

The decreases in the fresh and dry weights of samples in each treatment were recorded after drying plant parts for 3 days at 70°C. Then, the relative dry weight (DW) was calculated as Relative Shoot/Root DW (%) = DWT/DWC×100 (where DWT= dry weight of shoot/root under treatment and DWC=dry weight of shoot/root under control.

Chlorophyll a/b and carotenoids were determined by cutting fresh leaves into small pieces (0.1g) and extracting chlorophyll overnight with an 80% acetone solution at 4°C [4]. The samples were centrifuged for 5 minutes at 5000 rpm. The absorbance of the supernatant was read at 470, 645, and 663 nm for the determination of chlorophyll a/b and carotenoids contents using a spectrophotometer (LAMBDA 365, Perkin Elmer).

2.3 Silicon Uptake

Silicon uptake in rice was determined by the amino-molybdenum method [18]. The powdered samples (0.1g) of shoots and roots were digested separately with 3 mL of 50% NaOH in 50 mL polypropylene tube by autoclaving for 15 minutes at 1°C. About 0.5mL of the digested aliquot was transferred to a 25mL polyethylene tube. Then, 15mL of 20% acetic acid and 5mL ammonium molybdate (54 g/L, pH-7.0) were added in the tube. The mixture was shaken thoroughly, kept for 5 minutes, then supplemented with 2.5 mL of 20% tartaric acid and 0.5 mL reducing solution (prepared by mixing A and B solution adjusted to 250 mL with ddH_2O, where A=2 g of Na_2SO_3 and 0.4g of 1-amino-2-naphthol-4-sulfonic acid in 25 mL of ddH_2O and B=25 g of $NaHSO_3$ in 200 mL of ddH_2O). The total volume was made up to 25 mL with 20% acetic acid. The absorbance was recorded after 30 minutes of blue color formation at 650 nm using a spectrophotometer (LAMBDA 365, Perkin Elmer). Silicon content was estimated with a standard calibration curve.

2.4 Ion Concentration

The 3 days oven-dried leaf samples were used for the determination of ions concentration. The ions like sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) were estimated using 10 mg of dried leaves from each treatment. Leaves were cut into 1cm size pieces, placed in respective test tubes containing 20 mL ddH_2O, and placed in a boiling water bath for an hour [4]. Further, the tubes were autoclaved at 121°C for 20 minutes. The supernatants were filtered and diluted 10 times for ions quantification by atomic absorption spectrophotometer (iCE™ 3300 AAS).

2.5 Membrane Stability Index

Salinity causes membrane damage leading to loss of permeability potential. A standard protocol was used to determine the electrolyte leakage to assess the membrane stability index [4]. About 100mg fresh leaves were cut into small pieces and transferred into test tubes with 10mL of ddH_2O. The plugged tubes were incubated in a water bath having a constant temperature of 32°C for 2 hours. This was referred to as the initial EC of the sample (EC1) measured using an electrical conductivity meter. The same tubes were later autoclaved for 20 minutes at 121°C to release the electrolytes present in cells. After cooling to 25°C, the final EC of each sample (EC2) was measured. The electrolyte leakage potential (ELP) was calculated as = EC1/EC2×100.

Lipid peroxidation (LP) is another index to measure for membrane stability by calculating the concentration of the MDA (Malondialdehyde) content in leaf. The total reaction mixture consists of 4mL containing 1 mL of the sample, 3 mL of 2% 2-thiobarbituric acid (TBA, w/v) dissolved in 20% trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 minutes and then rapidly cooled on ice. The mixture was centrifuged at 12,000 rpm for 10 min and the absorbance of the collected supernatant was estimated at 532 nm. The absorbance at 600nm of supernatant was also recorded due to

turbidity. The lipid peroxidation was measured as nmol $MDAg^{-1}FW$ (Fresh weight) (extinction coefficient =155 $mM^{-1}cm^{-1}$).

2.6 Proline and Total Sugar Determination

A standard method was adopted for proline analysis using 200 mg fresh leaf tissues [19]. Tissue was homogenized using 4 mL of 3% aqueous sulfosalicylic acid (w/v). About 2 mL of the homogenized extract was added with 2 mL of ninhydrin acid and 2 mL of glacial acetic acid and boiled at 100°C for 1 hour. The reaction was quenched rapidly by putting the tubes in ice. Toluene was used for extracting proline, and the absorbance of the toluene fraction was measured at 520 nm. Proline concentration was estimated based on a calibration curve and expressed as µg/mL.

Total soluble sugar (TSS) was estimated by the modified anthrone method [20]. About 100mg samples were homogenized and incubated for 3 hours in boiling water with 5 mL of 2.5N HCl. The extract obtained was neutralized and made up to 100 mL. To 1 mL aliquot, 4mL anthrone solution was added to dehydrate glucose to hydroxymethylfurfural. The absorbance of the green-colored compound was recorded at 630 nm and total soluble sugar expressed as µg/mL.

2.7 Protein Estimation

Total protein was extracted by homogenizing 0.5g of leaf tissue in 4 mL of 50mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 2% PVP (w/v) and 0.1% triton X-100.The homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C to obtain a supernatant. About 500µL aliquot of the supernatant was used to determine the total protein content in the samples utilizing bovine serum albumin as the standard [21]. The protein (mg/g fresh weight) was calculated using the linear equation: $y = 0.0024x + 0.013$.

2.8 Quantification of Oxidative Damage

The spectrophotometric determination of H_2O_2 content was performed following the standard method[22]. About 0.1g of leaf sample was homogenized in 1mL of 0.1% TCA and centrifuged at 10,000 rpm for 15 minutes. Subsequently, 0.5mL of supernatant was mixed with 10mM phosphate buffer (0.5ml, pH-7.0) and 1ml potassium iodide (1M).The mixture was incubated at 25℃ for 30 mins and the absorbance was measured at 390nm. The H_2O_2 content was determined from a standard calibration curve.

All antioxidant assays were conducted by spectrophotometer (LAMBDA 65, Perkin Elmer) with a total reaction mixture solution of 3 mL, including sample extract. All enzyme activities were expressed as unit/ min/ mg of protein.

Superoxide dismutase (SOD) activity was determined by recording absorbance at 560 nm.100 µL of the enzymatic extract were added to 50 mM phosphate buffer (pH-7.8) containing 55 µM NBT (Nitro-blue tetrazolium), 1µM riboflavin, 9.9 mM methionine, and 2MEDTA. The reaction mixture without sample was taken as control and kept in light, while blank in dark. One unit activity is measured as the enzyme required for 50% inhibition of NBT [23].

Catalase (CAT) activity was measured in the reaction mixture consisting of 100 µL of sample extract, 50 mM phosphate buffer (pH-7.0) and 10 mM H_2O_2 solution [24,25]. The activity was estimated based on the initial rate of disappearance of H_2O_2 per minute at 240 nm ($E = 39.4\ mM^{-1}\ cm^{-1}$).

Ascorbate peroxidase (APX)activity was estimated in the reaction mixture consisting of 100 µL of the sample extract, 25 mM phosphate buffer (pH-7.0),1 mM EDTA, 5 mM ascorbate and 0.1 mM H_2O_2 [26].The oxidation of ascorbate is proportional to decrease in absorbance at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$) per minute.

Guaiacol peroxidase (GPX) activity was measured using a modified spectro-method [27,28]. The reaction mixture was comprised of 100 µL of sample extract, 50 mM (pH-7.0) phosphate buffer, 20 mM guaiacol (2-ethoxyphenol) and 0.042% H_2O_2. The increase in absorbance due to guaiacol oxidation was measured, per minute, at 470 nm ($E = 26.6 mM^{-1}\ cm^{-1}$).

2.9 Statistical Analysis

All experimental data were means of three replicates. ANOVA (analysis of variance) was performed for all parameters using a preloaded analysis package in Microsoft Excel and means were separated pairwise by Fischer's least significant difference (LSD) at a 5% significance level. Pearson's correlation test was performed to determine the relationships between salt

tolerance traits. The quantitative data were subjected to multivariate analysis by PCA (principal component analysis), factor analysis, and agglomerative hierarchical clustering (AHC) using XLSTAT for Windows software package.

3. RESULTS AND DISCUSSION

Here we discussed the effects of salt stress and Si treatment on key traits or salt-tolerance indices of rice under salinity.

3.1 Effect on Dry Weight and Pigment Content

A significant decrease in relative shoot and root dry weight was observed in seedlings when exposed to salt stress (Table 2). The salt susceptible Var. IR29 (50.1%) showed the highest reduction in relative shoot dry weight, followed by Jagbandhu (59.0%) and Swarna (81.6%). The salt-tolerant genotype Lunishree (90.4%) showed the lowest decrease in relative shoot dry weight.

The applications of Si under saline condition increased the relative dry weight of shoots in all genotypes and the highest increase was reported in Swarna (128.6%). A similar pattern was observed in the case of relative root dry weight. The contents of photosynthetic pigments, i.e. total chlorophyll a/b and carotenoids under salt stress and Si treatments are presented in Table 2. It is obvious from the table that salt stress decreased total chlorophyll and carotenoids accumulations in all genotypes. The addition of Si resulted in marginal increases in the levels of chlorophyll a/b and carotenoids under stress conditions, whereas, under unstressed conditions, it reduced all pigment levels. Scientists reported that Si application during stress resulted in increased plant development and yield [29]. In the present study, salinity caused a significant reduction in relative shoot and root dry weights of all genotypes. Similar findings were reported in rice seedling, a significant reduction in total dry matter was recorded under salinity [28]. In contrast, Si addition during salt stress significantly increased the relative dry weight of both shoot and root. The findings of our study were also consistent with the reports of Si alleviation of negative effects in salinity (100 mM NaCl) on plant dry matter yield and chlorophyll content by the application of 0.25 and 0.5 mM Na_2SiO_3 to wheat plants growing under complete nutrient solution [30,31].

3.2 Silicon Accumulation

Under unstressed conditions, 'Swarna' variety reported a high accumulation of Si in the shoot as 1.97 mg/g. During salt stress on Si supplementation, all genotypes showed higher Si accumulation. In the root, an irregular pattern was seen in Si accumulation. In general, Si accumulation increases continuously in root

Table 2. Effects of salinity and Si treatment on relative dry weight and photosynthetic pigments activities in rice varieties

Varieties	Treatments	Relative fresh weight (%)		Chl a	Chl b	Carotenoids
		Shoot	Root	(µg/g Fresh weight)		
IR-29	Control	100.0	100.0	2.96	1.49	1.02
	Si	90.2	100.0	1.74	1.41	0.62
	Salt	50.1	63.3	1.15	0.52	0.39
	Salt + Si	59.7	79.6	1.96	0.64	0.43
Swarna	Control	100.0	100.0	2.66	1.10	0.86
	Si	138.7	90.4	2.48	1.05	0.88
	Salt	81.6	87.0	2.05	0.81	0.70
	Salt + Si	128.6	97.2	2.68	1.18	0.86
Jagbandhu	Control	100.0	100.0	3.63	1.72	1.24
	Si	109.4	69.2	2.93	1.34	0.97
	Salt	59.0	66.2	1.34	0.36	0.29
	Salt + Si	93.2	70.8	1.98	0.55	0.37
Lunishree	Control	100.0	100.0	4.06	2.39	1.48
	Si	104.2	88.3	3.80	1.81	1.23
	Salt	90.4	89.0	4.02	2.31	1.45
	Salt + Si	97.6	82.9	4.18	1.82	1.37
*$LSD_{0.05}$		16.4	10.5	0.75	0.55	0.34

**All values are means (n=3), separated by Fisher's Least Significant Difference (LSD) at P = 0.05*

under all treatments, except in 'Swarna' and 'Lunishree'. Overall, the Si accumulated in roots is higher than the Si accumulation in the shoot. The Var. Swarna showed high shoot Si accumulation in comparison to the other three genotypes under both stressed and unstressed conditions. Var. Swarna also showed the highest increase in relative dry weight and higher pigment accumulation during salinity. Hence, the data suggested that Si accumulation was the primary cause of the reduction in salt stress.

3.3 Ion Concentration

Three ions (Na^+, K^+, Ca^{2+}) were involved in ion regulation during stress (Table 3). The salt susceptible Var.IR29 showed the highest accumulation of Na^+ (69.08 mg/kg), while the salt-tolerant Var. Lunishree showed a very small increment in Na^+ content under stress (4.21 mg/kg). The inclusion of Si under stress decreases the uptake of Na+ ions in plant cells preventing toxicity. Salinity also decreases the uptake of K^+ and Ca^{2+} ions in the crop system. With the addition of Si under both stressed and unstressed conditions, the concentration of K^+ and Ca^{2+} ions increases in rice. The ion regulation under salinity is an indispensable measure deciding the tolerance of rice crops. It had been stated that salt tolerance in rice varies considerably across genotypes due to their dissimilarity to cope up with the excess Na^+ level in maintaining ion homeostasis [32]. The author also explained that for salt-tolerance, rice is required to maintain a low Na^+/K^+ ratio. The salt-tolerant 'Lunishree' showed low Na^+ content under salinity indicating tolerance. On the other hand, Si inclusion resulted in reduced Na^+/K^+ ratio improving homeostasis and tolerance in rice. The low Na^+/K^+ ratio was found in Var. Lunishree followed by Var. Swarna and lowest in Var.IR29 as well as in Var. Jagbandhu. Na^+ ion exclusion from the shoots is often considered as the most essential feature for salt tolerance in plants and also explains the prime reason behind Si-induced salt tolerance [33]. A similar result was demonstrated in stressed maize, Si application (75mM Na_2CO_3) increased the K^+ and K^+/Na^+ ratio in stressed maize in comparison to Si-deprived stressed maize [34]. Similarly, the results showed a decrease in K^+, Ca^{2+} concentration under salinity; however, on the addition of Si, their concentration increases under salinity (Table 3). Some reports showed no significant ($P = 0.05$) increase in Ca^{2+} concentration on Si addition in leaves [35].

3.4 Proline and Total Soluble Sugar (Tss)

The concentration of the osmolytes (proline and total soluble sugar) significantly increased in all genotypes under stressed conditions. Table 3 shows the variation in proline and TSS content in rice under different treatments. Salt stress at 10 dS/m led to about a 2-fold increase in proline level when compared to the control. Var.IR29 (125.51µmole/g FW) and Var. Jagbandhu (132.77µmole/g FW) showed high proline content, a further increase in proline level was recorded with the addition of Si during stress. However, in the salt-tolerant Lunishree (62.30µmole/g FW) and Swarna (66.66µmole/g FW), the proline level declined after Si addition. Similar to proline, a considerable increase was observed in total soluble sugar content of all genotypes under Si treatments (Table 3). However, the amount of TSS was found to same as control even after the addition of Si in non-saline media. Together with maintaining homeostasis, a frequent salt tolerance mechanism in plants is overproduction of osmolytes. The osmolytes like proline and soluble sugars help to adjust the distorted osmotic concentration, prevent membrane integrity, and are involved in stabilization of enzyme activities and thus contribute to stress tolerance [36]. Proline plays a key role in protecting the subcellular structures from damage and thus its content increases under stress in each genotype [37]. With the application of Si under salinity, the high Si accumulating Var. Swarna and salt-tolerant Var.Lunishree showed a reduction in proline content. The above outcomes were in accordance with reports presented in wheat; they stated that Si addition decreased the proline content and membrane permeability of leaves [37]. With the increase in salinity, the TSS concentration increased in the seedling of all rice genotypes. The stressed seedlings without Si were compared to the Si-treated stressed seedling, showed significantly higher TSS content with exception of Var. Swarna. This observation was quite similar to the previous findings in which higher soluble sugar levels were reported in Si treated drought-stressed wheat [38]. Another study reported significantly lower soluble sugar in Si treated lentils when compared to Si-deprived stressed lentils under drought stress [39]. As an explanation, the authors proposed that adding Si decreases the anabolism of soluble sugar content under stress.

Table 3. Effect of salinity and Si supplementation on Ion concentration (Na^+, K^+, Ca^{2+}), Si accumulation and osmolyte content (i.e. proline and total soluble sugar) activities in rice varieties

Varieties	Treatments	Ion concentration (mg/kg)			Si concentration Si (mg/g)		Osmolyte balance	
		Na^+	K^+	Ca^{2+}	Shoot	Root	Proline (µmoles/g FW)	Total soluble sugar (mg/g FW)
IR-29	Control	7.46	15.61	14.48	1.05	6.11	25.09	2.4
	Si	15.71	21.26	17.48	0.86	7.00	38.38	2.72
	Salt	69.08	11.73	21.55	1.28	8.32	95.47	2.88
	Salt + Si	46.52	37.78	23.87	1.94	8.68	125.51	3.27
Swarna	Control	3.05	3.05	14.87	1.47	3.40	78.02	1.01
	Si	3.34	3.34	15.84	1.97	4.64	66.88	1.20
	Salt	41.33	38.97	19.03	1.78	3.66	101.02	3.06
	Salt + Si	34.97	47.33	22.91	2.26	5.25	66.66	2.74
Jagbandhu	Control	8.33	8.33	23.68	0.75	1.46	54.84	1.91
	Si	8.83	8.83	17.77	0.89	1.65	72.1	1.38
	Salt	51.18	48.75	24.16	0.68	3.39	120.55	2.56
	Salt + Si	48.75	51.18	16.42	1.24	4.02	132.77	2.84
Lunishree	Control	2.93	15.98	10.02	1.27	2.30	26.95	1.78
	Si	3.18	13.13	16.80	1.28	3.19	30.53	1.48
	Salt	4.21	12.77	16.22	1.50	2.88	62.99	2.94
	Salt + Si	4.70	17.06	10.99	1.73	4.48	62.3	2.99
*$LSD_{0.05}$		18.88	20.13	0.013	0.28	0.86	32.00	0.56

**All values are means (n=3), separated by Fisher's Least Significant Difference (LSD) at P = 0.05*

3.5 Membrane Permeability

In all genotypes, salinity considerably increased the ELP and LP levels causing plant cell damage (Table 4). The increase in ELP and LP under stress was very evident and was reduced with the application of Si. The percentage of electrolyte leakage intolerant Lunishree variety (95.07%) was very high, but the amount of peroxidation (13.34 nmoles/g Fresh Weight) was very low under salinity. Under stress conditions, the lowest ELP was found in Swarna (91.78%) which was further reduced with Si addition (70.96%). However, in Swarna, the LP value was very high (47.35 nmoles/g FW). Also, high salinity significantly increased the leakage potential and accumulation of MDA content in cells of stressed plants [34,40]. The inclusion of Si in nutrient solution appreciably reduced both ELP and LP levels of all genotypes. With Si addition under salinity, Var. Swarna showed lower ELP value, while Var. Lunishree showed lower LP value. Thus, we could conclude that Si induces a reduction in either ELP or LP or both parameters, to provide salt tolerance in rice.

3.6 Protein Content

The variations in the total protein content of leaves were also determined during salt-Si interaction (Table 4). With the addition of Si, total protein content was improved in all genotypes, except Lunishree. Such improvement in soluble protein content was attributed to Si playing a crucial role in binding amino acids to form specific proteins and its active involvement in configuring DNA and functioning of mRNA [41,42].

3.7 H_2O_2 content and Antioxidative Enzyme

The alteration in the level of H_2O_2 content and antioxidant enzyme (SOD, CAT, APX, and GPX) are shown in Table 4. The H_2O_2 produced was significantly higher in response to salt stress in all genotypes, but the concentration decreased with the addition of Si. The genotype Jagbandhu and IR29 represented the maximum accumulation of H_2O_2 under stress with concentrations of 0.65 nmole/g FW and 0.43 nmole/g FW, respectively. In response to increasing H_2O_2 content during stress, antioxidant activities also increased, i.e. the activity of SOD, APX, and GPX increased drastically. The enzyme concentration is further increased by the addition of Si under stress conditions. The CAT activity was increased during stress but was not much affected by Si

application under stress. In salt stress conditions, an appreciable difference is seen in SOD and POX enzyme levels by addition in media. Beside ionic and osmotic stress, salinity stress induces oxidative stress as a consequence of ROS burst, causing oxidative damage to plant proteins. The salt stress resulted in overproduction of H_2O_2in salt-sensitive rice during the seedling stage, causing oxidation of the plasma membrane, photosynthetic pigments, proteins, and nucleic acids. Silicon application induces a reduction in H_2O_2 content in all genotypes, so the findings of H_2O_2 accumulation were in good agreement with other reports [43]. To counteract the ROS species, plants possess enzymatic antioxidants, which are involved in defense mechanisms for ROS scavenging. The prime enzyme SOD converts superoxide into H_2O_2 in cell organelles preventing cellular damage, while CAT causes the breakdown of H_2O_2. Additionally, APX and GPX through the ascorbate-glutathione pathway lead to H_2O_2 scavenging. It was stated that enzymes like POX and CAT play a significant role in plant adaptation to stress conditions. The results of our study revealed that the SOD, CAT, and GPX activities increase under salinity stress, but decreases with Si addition. It was interesting to note that in the case of salt-sensitive cultivar, the SOD activity decreases with Si supplementation, while salt-tolerant showed an increase in activity under salinity. The obtained results were in harmony with Si seed-priming results of maize that a significant increase in antioxidant activities SOD, CAT, and POD was observed in stressed maize plants than the Si-treated stressed maize [38]. Some studies reported that the salt-sensitive cultivars tended to have higher POX value, whereas the salt-tolerant likely to have higher CAT value [44,45]. Our findings were supported by the statement that higher POX activities in the plant cells (both APX and GPX) and hence they are principally accountable for an H_2O_2 breakdown by ascorbate-glutathione pathway rather than CAT breakdown.

3.8 Pearson's Correlations Matrix

Correlation coefficients amongst the salt tolerance traits were analyzed through Pearson's correlation and presented in form of a matrix in Fig. 1. The Si accumulation in shoot displayed significant negative correlations to the H_2O_2 (r = -0.62, p=0.011) and CAT (r = -0.67, p=0.004). While, the Si accumulation in root showed significant positive correlation among Na+ content (r = 0.52, p =0.037), TSS (r = 0.53, p=0.035) and negative correlation with relative shoot dry weight (r = -0.66, p=0.005), Chlorophyll a (r = -0.58, p=0.019) and finally carotenoids

Table 4. **Effect of salinity and Si supplementation on membrane integrity (ELP and LP), protein content, oxidative burst (H_2O_2), and antioxidant enzyme (SOD, CAT, APX, and GPX) activities in rice varieties**

Varieties	Treatments	ELP	LP	Protein	H_2O_2	SOD	CAT	APX	GPX
IR-29	Control	84.74	13.46	5.47	0.23	4.84	0.02	1.09	0.81
	Si	80.15	14.07	6.23	0.34	5.11	0.02	3.30	1.00
	Salt	94.71	28.03	4.18	0.43	3.48	0.06	0.52	2.06
	Salt + Si	75.46	19.92	5.57	0.24	4.24	0.04	2.06	2.17
Swarna	Control	92.69	11.74	3.96	0.18	4.97	0.02	1.19	0.55
	Si	84.78	8.59	4.85	0.19	5.51	0.01	0.21	0.47
	Salt	91.78	47.35	4.56	0.37	5.85	0.05	0.60	0.98
	Salt + Si	70.96	35.04	4.87	0.22	5.49	0.04	0.82	1.54
Jagbandhu	Control	81.37	10.36	5.20	0.41	3.21	0.04	1.28	0.61
	Si	90.41	17.43	5.75	0.53	4.78	0.03	0.48	0.55
	Salt	96.03	28.23	4.35	0.65	5.29	0.13	2.85	3.33
	Salt + Si	92.70	24.57	4.97	0.49	6.94	0.04	0.55	3.44
Lunishree	Control	86.74	12.01	5.45	0.17	5.73	0.02	0.22	5.73
	Si	82.79	15.33	4.79	0.21	5.64	0.01	0.47	5.64
	Salt	95.07	13.83	4.93	0.27	6.01	0.01	1.19	6.01
	Salt + Si	83.41	13.34	4.85	0.15	3.99	0.02	0.33	3.99
*$LSD_{0.05}$		8.93	12.01	0.015	0.05	0.063	0.015	5.22	1.44

**All values are means (n=3), separated by Fisher's Least Significant Difference (LSD) at P = 0.05*

[ELP: Electrolyte Leakage Potential (%), LP: Lipid peroxidation (nmoles/g FW), Protein (mg/g FW), H_2O_2: Hydrogen Peroxide (nmole/g FW), SOD: Superoxide Dismutase (unit/ml/mg protein), CAT: Catalase (µmole H_2O_2 reduced/min/mg Protein), APX: Ascorbte Peroxidase (µmole Ascorbate oxidized /min/mg Protein), GPX: Guaiucol Peroxidase (µmole Guaiucol oxidized/min/mg Protein)]

(r = -0.56, p=0.025). The analysis of Na^+ accumulation and salt tolerance attributes revealed a strong positive correlation with other ions such as K^+ (r = 0.86, p=<0.001), Ca^{2+} (r = 0.63, p=0.008), lipid peroxidation (r = 0.73, p=0.001), H_2O_2 content(r = 0.56, p=0.024), proline (r = 0.77, p=<0.001) and TSS level (r = 0.61, p=0.011). The electrolyte leakage potential showed significant positive correlation with CAT (r = 0.51, p=0.045), while protein showed positive correlation with APX (r = 0.56, p=0.023). On the other hand, H_2O_2 depicted a strong correlation with SOD (r = 0.79, p=<0.001). Interestingly, all the three pigments (chlorophyll a/b and carotenoids) showed strongly negative correlation with lipid peroxidation and H_2O_2 content. The correlation coefficient gives an insight into the significant relationship between the quantitative variables or traits studied [46].

3.9 Multivariate Data Analysis

The multivariate factor analysis was performed using the PCA component to make the result more complete. The PCA results obtained from the four rice genotypes (G1, G2, G3, and G4) grown under control (C), Si (Si) salinity (N) and salinity stress supplemented with Si (NSi), including the twenty salinity tolerance traits are described in Fig. 2. The data analysis showed principle component dimension-1(F1) describing 39.07% and principal component dimension-2 (F2) describing 14.21% of the original information. The total cumulative percentage of two PC dimensions F1 and F2 describes 53.28% variability in data. When we study the correlation circle, we found that F2 is closely related to Si accumulation in a shoot with high value and good representation quality. Silicon accumulation was positively loaded with APX, Si accumulation in the root, TSS, and protein with narrow angles in a group, while negatively correlated to H_2O_2, ELP, and CAT with prominent obtuse angles. However, the F1 showed a close connection with Na^+, Ca^{2+}, LP, K^+, and proline in right, i.e. these attributes are positively correlated with the first PCA dimension and to each other. Nonetheless, the F1 also presented a strong correlation with Chlorophyll a/b, carotenoids, RDM, SDM, and GPX on left and are grouped. The factor analysis of twenty traits gives an eigenvalue of 7.82 and produces 39.07% cumulative variability. In the PCA, the correlations between different physiological variables to Si accumulation in shoot and root were depicted. The close association of Si accumulation to salinity tolerance was attributed to APX activity, TSS, H_2O_2, ELP, and CAT, and strongly supports the idea of Si induce salt tolerance.

The PCA in combination with Agglomerative Hierarchical clustering (AHC) was used to classify rice genotypes into homogeneous classes based on their description by a set of physiological parameters to distinguish their level of salt tolerance (Fig. 3). Finally, based on their description of variables, the four genotypes with different treatments were clustered into three groups (I-III) based on their dissimilarity using Agglomerative Hierarchical clustering (AHC). The cluster-I consisted of control treatments (C) and only Si treatments (Si) of two genotypes IR29 (G1) and Lunishree (G4). Then, the cluster-II comprised of the salt-sensitive treatments (N), i.e. varieties IR29 (G1), Jagbandhu (G3), and

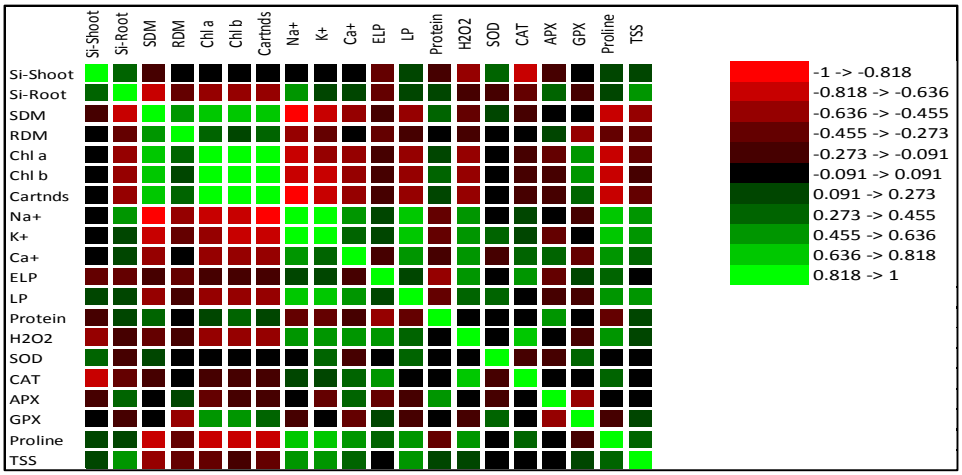

Fig. 1. The covariance matrix for the salt-tolerance related traits studied in four rice varieties

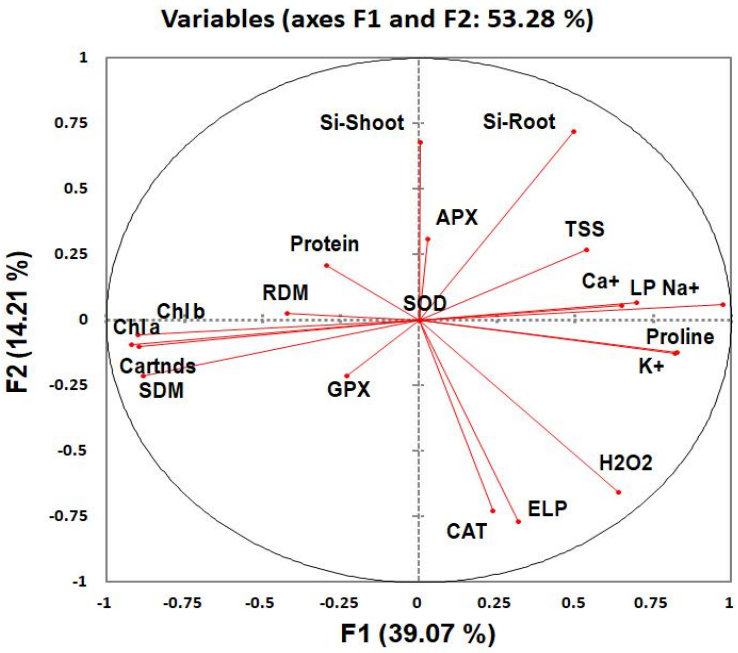

Fig. 2. Loading factors of principal components 1 and 2 of the PCA results obtained for salt-tolerance related traits as vectors

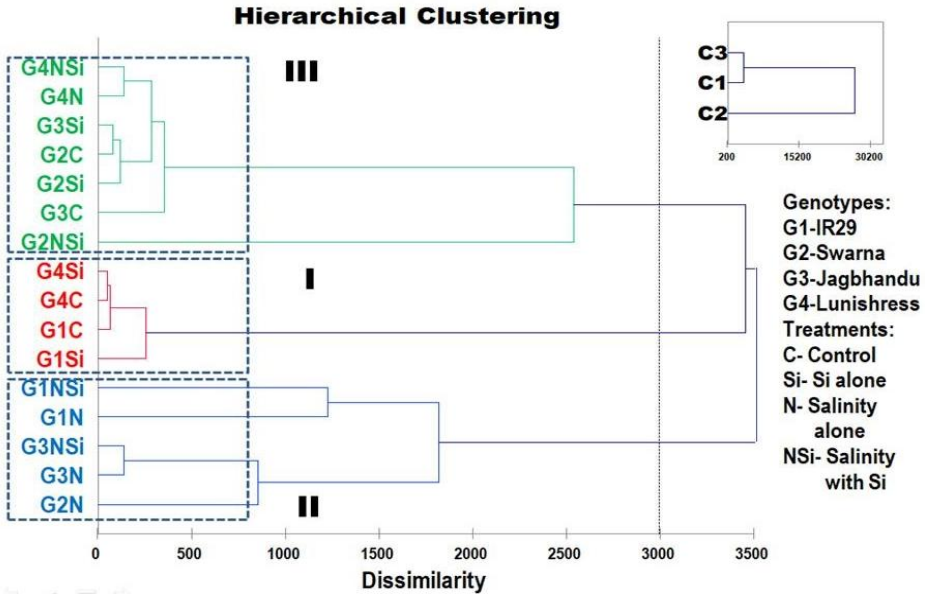

Fig. 3. Hierarchical Cluster analysis of four rice genotypes according to the effect of salinity and Si interaction under control (C), salt stress (N), Si alone (Si), and salt stress+Si (NSi)

The abbreviations used in this figure for varieties were explained in Table 1 and legend

Swarna (G2). This cluster is also comprised of Si and salinity combination treatment (NSi) of IR29 (G1) and Jagbandhu (G3) varieties. The result verified the idea that the low Si accumulation showed minimal salinity mitigation effect. The final and last cluster, cluster-III comprise of the salt-tolerant groups, as its lead by salt-tolerant variety Lunishree (G4). The cluster showed Var. Lunishree (G4) under only saline (N) and Si-saline combination treatment (NSi). Interestingly,

the PCA with AHC clustering included Swarna genotype (G2) in Si-saline combination treatment (NSi) in the salt-tolerant cluster (III), hence proved that high Si accumulation can impart salt tolerance to genotype. Some control treatments and Si treatment groups are also included in this group. Similar results were obtained in multivariate data analysis conducted on drought-tolerance traits due to Si application [47].

4. CONCLUSION

In conclusion, our study suggested that Si influences several physiological and biochemical traits in rice. With the comparison of variations among salt tolerance traits in two different Si accumulating rice varieties under Si supplemented saline condition gave a brief idea about the mechanism of Si-induced salt tolerance. The Si induced changes in Na^+/K^+ ratio, antioxidant enzyme activities (especially APX and SOD), total soluble sugar, lipid peroxidation, H_2O_2 content, electrolyte leakage potential, and proline content was a reliable indicator of salt stress tolerance. Si-induced tolerance was primarily due to Si accumulation and Na^+ ion exclusion. Besides the Na^+/K^+ ratio, antioxidant enzymes APX enhanced activity was also a crucial step towards Si-induce salt tolerance by operating the alternative pathway for H_2O_2 breakdown. The genetically high Si accumulator, Var.Swarna was identified as a salt-tolerant genotype as indicated through the cluster analysis. Hence, an external application of silicon in fields would lead to increased accumulation and improved salt tolerance in rice. Also, it's very essential to evaluate the genetic nature, i.e. to identify the ability of a variety to accumulate Si for more effective salinity mitigation and growth by Si application in rice. In the future, such varieties could be used as a parent genotype for selection and breeding programs to improve salt tolerance in rice.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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