



Research article

Silicon nutrition improves growth of salt-stressed wheat by modulating flows and partitioning of Na⁺, Cl⁻ and mineral ions

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ARTICLE INFO

Keywords:

Wheat
Sodium transport
Salt tolerance
Silicon
Mineral distribution
X-ray microanalysis

ABSTRACT

Silicon (Si) is reported to improve salt stress tolerance of cereals, but little is known about the effects of Si on flows and partitioning of sodium (Na⁺), chloride (Cl⁻), and essential mineral ions at the tissue and cellular level. Wheat (*Triticum aestivum* L.) was exposed to 200 mM NaCl for 30 d in hydroponics, with or without 2 mM Si. X-ray microanalysis coupled with scanning electron microscopy (SEM) was used to quantify the cell-specific ion profiles across root and leaf cells, paralleled by measurements of wheat growth and physiological responses. Under salt stress, higher Na⁺ and Cl⁻ concentrations were detected in root epidermal, cortical and stelar cells, eventually increasing their concentrations in different leaf cells, being highest in the epidermal cells and lowest in the vascular bundle cells. The potassium (K⁺) and magnesium (Mg²⁺) profiles were generally opposite to those of Na⁺ and Cl⁻. NaCl-dependent deregulation of essential nutrient homeostasis and excessive toxic ions accumulation in leaves was correlated with enhanced electrolyte leakage index (ELI), decreased chlorophyll contents, photosynthesis and other physiological parameters, and ultimately hampered plant growth. Conversely, Si addition improved the growth and physiological performance of salinized wheat by reducing Na⁺ and Cl⁻ concentration in root epidermal and cortical cells, and it improved root uptake and storage of K⁺ and Mg²⁺ ions and their loading into xylem for distribution to shoots. These results suggest that Si-mediated inhibition of Na⁺ uptake, maintained nutrient homeostasis and improved physiological parameters to contribute to wheat growth improvement under salt stress.

1. Introduction

Sodium (Na⁺) and silicon (Si) are among the most abundant elements found in the earth's crust (Flam-Shepherd et al., 2018). Though not listed as essential plant elements, both are nevertheless reported to be beneficial for plant growth under certain conditions (Epstein, 2009; Kronzucker et al., 2013). For instance, most plant species (excluding halophytes) are reported to benefit from low external Na⁺ concentrations in the soil, even though high concentrations hamper plant growth severely (Negrão et al., 2017). Reduced plant growth under salt stress is usually ascribed to cellular ion toxicity [mainly because of Na⁺ and chloride (Cl⁻) ions], and/or hyperosmotic stress, nutrient imbalance (due to competition of toxic Na⁺ and Cl⁻ ions with essential nutrients), thereby causing numerous physiological and metabolic dysfunctions and ultimately plant demise (Tester and Davenport, 2003; Munns and Tester, 2008).

Conversely, there are no reports of Si toxicity in plants despite its uptake and accumulation in plant tissues exceeding those of

macronutrients such as calcium (Ca²⁺), magnesium (Mg²⁺), and phosphorous (P) (Epstein, 1999). However, the well-documented benefits of Si nutrition are generally in the form of increased plant tolerance to biotic and abiotic stresses (Farooq and Dietz, 2015). Even though the mechanisms underlying these beneficial effects of Si have remained largely unknown, increased silica deposits in root and leaf tissues are long known to improve structural properties and hence physical defense against pathogen and herbivory attacks (Mehrag and Mehrag, 2015). Furthermore, our recent work has provided evidence that Si application can induce changes in the expression of stress-associated proteins at the transcript level in rice challenged with cadmium (Cd) stress (Farooq et al., 2016), but information about their physiological and metabolic consequences is limited at present.

Wheat (*Triticum aestivum* L.) – a dietary staple – is generally quite sensitive to salt stress and suffer severe yield losses, particularly in irrigated fields that are prone to salinization as compared to non-irrigated areas (Mass and Hoffman, 1977; Zhu, 2003). An extent of damage mainly depends on the salt concentrations, stress tolerance capacity of

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<https://doi.org/10.1016/j.plaphy.2019.06.010>

Received 30 January 2019; Received in revised form 9 June 2019; Accepted 10 June 2019

Available online 11 June 2019

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plants coupled with differences in the contribution of Na^+ translocation by apoplastic and symplastic pathways, and the consequent changes in micro-distribution and subcellular compartmentation of mineral ions in various cell types (Yeo et al., 1987; Munns and Tester, 2008). Further, wheat is categorized as Si-accumulating species (Casey et al., 2004) that greatly benefits from Si nutrition under stress conditions, including soil salinity (Tahir et al., 2012). Generally, protective role of Si in improving salinity tolerance of plants is ascribed to blockage of bypass route for Na^+ uptake due to silicate polymerization in the endodermis and exodermis (Gong et al., 2006; Shi et al., 2013), promotion of lignification and suberization, and formation of Casparian band (Fleck et al., 2011, 2015; Hinrichs et al., 2017), which all might alter properties of the membrane transport systems responsible for solute transport from roots to shoots (Flam-Shepherd et al., 2018). Despite a known reduction in Na^+ transport via bypass flow, very little is known about the precise relationships between Si and the fluxes of toxic Na^+ and Cl^- ions that cycle through different subcellular compartments in root and leaf cells, and particularly how Si influences ionic homeostasis of essential mineral elements under salt stress. Therefore, the present study was conducted to understand the influence of Si nutrition on minimizing Na^+ toxicity and improving growth in wheat by altering elemental distribution and compartmentalization within plant tissues under salt stress.

2. Materials and methods

2.1. Growth conditions

Seeds of wheat (*Triticum aestivum* L.) genotype 'Faisalabad-2008' [previously reported as moderately salt-tolerant genotype (Kanwal et al., 2013; Naz et al., 2015)], were obtained from Saline Agriculture Research Center, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan. After surface sterilization with 5% (v/v) sodium hypochlorite (NaOCl) solution for 10 min, and thorough rinsing in deionized water, healthy seeds were sown in polyethylene-coated iron trays with a 5-cm-deep layer of washed sand. The moisture content was optimized for seed germination and seedling establishment with distilled water. The 10-d-old uniform plants were shifted to 10 L plastic pots containing 0.5x Hoagland solution (Farooq et al., 2016). Seedlings were grown for one week in a growth chamber having 16 h photoperiod with light intensity of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $20 \pm 1^\circ\text{C}$ and $65 \pm 5\%$ relative humidity. At the age of 17 d, the plants were stressed with 200 mM NaCl (added in 100 mM d^{-1} increments to reach the final concentration), with or without 2 mM supplemental Si for another 30 d, while the control plants were maintained in 0.5x Hoagland medium alone. A supplementary Ca^{2+} dose was also added as CaCl_2 to the NaCl-containing pots to give a final $\text{Na}^+:\text{Ca}^{2+}$ of 15:1 (Läuchli et al., 2008). The solution was changed every week and the pH was adjusted to 6.0 ± 0.5 by using either 0.5 M KOH or 1 M HCl on a daily basis. At the end of the experiment, plants were harvested and separated into roots and shoots for length measurement. For root length, measurements were made on all seminal roots (including lateral roots). Subsequently, root and shoot tissues were kept in an oven at 65°C to constant dry weight for quantification of biomass yield.

2.2. Physiological measurements

Leaf chlorophyll contents were estimated with a handheld SPAD-502 chlorophyll meter (Minolta, Osaka, Japan). Due to ease in handling and measurement, 2–3 fully emerged young leaves from each treatment were selected, and each leaf was measured several times as described previously (Farooq et al., 2015a, b). Measurements of net photosynthetic rate (A), transpiration rate (E), and stomatal conductance (gs) were done using a portable infrared gas analyzer (IRGA; LCA-4 ADC, Hoddeson, England). These measurements were done on the youngest fully emerged leaves in the morning (0800–1000 a.m.) with the

following adjustments: leaf surface area 6.25 cm^2 , ambient CO_2 concentration (Cref) $371 \mu\text{mol mol}^{-1}$, temperature of leaf chamber (Tch) varying from $25\text{--}28^\circ\text{C}$, leaf chamber volume gas flow rate (v) 296 mL min^{-1} , leaf chamber molar gas flow rate (U) $400 \mu\text{mol s}^{-1}$, ambient pressure (P) 97.95 kPa, PAR (Qleaf) at leaf surface the maximum up to $770 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Ben-Asher et al., 2006).

Leaf relative water content (RWC) was determined according to the method described by Sairam and Tayagi (2004). 0.5 g leaf samples were weighed as fresh weight (FW) and immediately hydrated with distilled water for 4 h. Subsequently, the turgid leaves were quickly blotted to remove surface water and immediately weighed to obtain fully turgid weight (TW). The leaves were then dried in an oven at 65°C for 48 h to determine dry weight (DW). The RWC was calculated as:

$$\text{RWC} = [(\text{FW}-\text{DW})/(\text{TW}-\text{DW})] \times 100$$

The electrolyte leakage index (ELI) was measured by estimating the ions leached from wheat leaf tissue into the distilled water by the method described by Sairam and Tayagi (2004). 0.2 g leaf samples were placed in test tubes containing 10 mL double-distilled water in two sets. One set of test tubes was kept in a water bath at 40°C for 30 min and its electrical conductivity (C_1) was recorded using a conductivity meter. The second set of test tubes was kept at 100°C in boiling water for 15 min and its electrical conductivity (C_2) was also recorded. ELI was calculated by using the formula:

$$\text{ELI} = [1-C_1/C_2] \times 100$$

2.3. Sample preparation for micro-distribution and compartmentation of ions in root and leaf cells

The elemental distribution and compartmentation of ions were examined using a scanning electron microscope (SEM) equipped with an energy dispersive X-ray (EDX) analyzer. Samples were prepared according to Shi et al. (1987). Briefly, the plant roots and leaves were cut into segments of 1 cm in length with a blade, and immersed quickly into liquid nitrogen, followed by freeze-drying at -80°C for 24 h using a Genesis Pilot Lyophilizer (VirTis). Subsequently, samples were mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, West Chester, PA) and were coated with carbon ($\sim 30 \text{ nm}$ thickness) in a carbon string evaporator (Ernest F. Fullam, Inc., Latham, NY). Three separate samples were collected for X-ray microanalysis for each treatment, and the relative weight of the mineral ions in leaf epidermal, cortical and vascular bundle cells, and root epidermal, cortical and stelar cells was quantified automatically according to net K-shell X-ray peak counts after subtraction of background X-ray counts. The root cross-section and the transverse section of wheat leaves were examined in a JEOL 6610LV SEM (tungsten hairpin electron emitter) (JEOL Ltd., Tokyo, Japan). EDX spectroscopy (elemental analysis) was done using an Oxford Instruments AZtec system (Oxford Instruments, High Wycombe, Bucks, England), software version 2.1a, using a 20 mm silicon drift detector crystal (6610LV SEM) and an ultrathin window.

2.4. Statistical analysis

The data were subjected to statistical analysis using computer software STATISTIX 8.1 (Tallahassee, 2003). The completely randomized design (CRD) with two factors was employed for the analysis of variance (ANOVA), and Tukey's HSD (honest significant difference) test was used to compare treatment means differences at $P \leq 0.05$.

3. Results

3.1. Plant growth and physiological response

In order to test the hypothesis that Si supplementation improves the

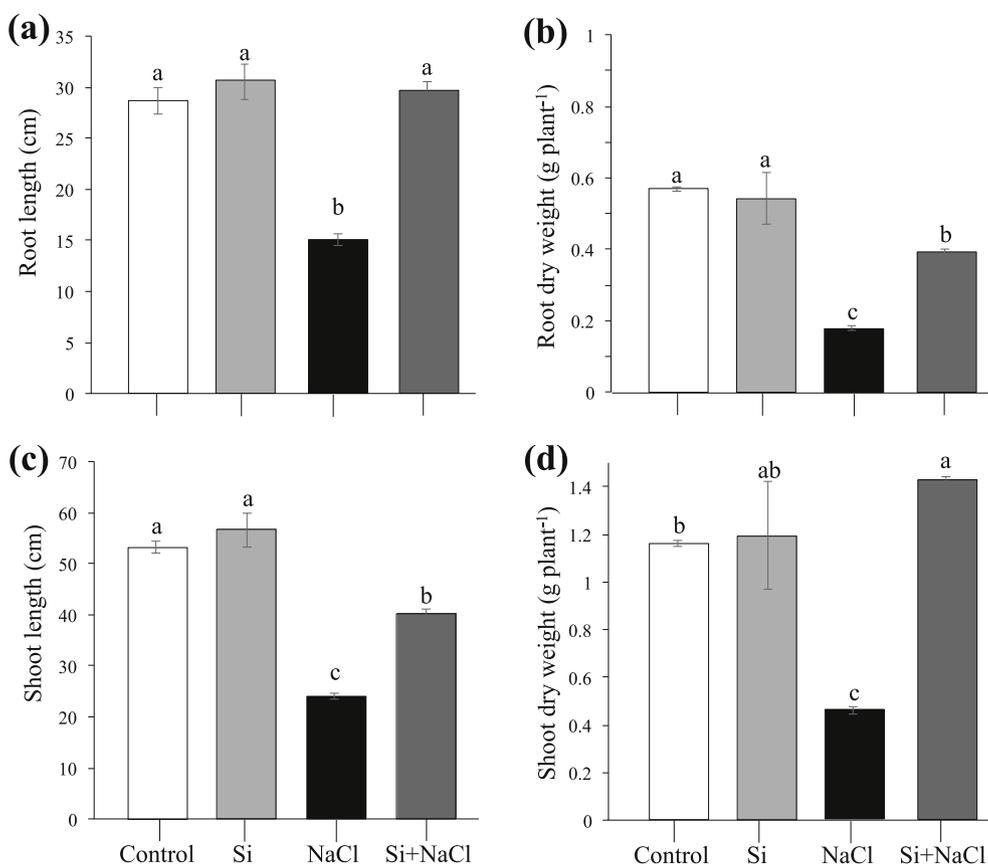


Fig. 1. Effect of Si on the length (a, c), and dry weight (b, d) of roots (a, b), and shoots (c, d) of wheat genotype 'Faisalabad-2008' stressed with 200 mM NaCl, with or without 2 mM supplemental Si for 30 d in 0.5x Hoagland solution. Significant differences were calculated by Tukey's (HSD) test, and are labeled with different letters ($P \leq 0.05$). The values are means \pm S.E. from three independent experiments.

salt tolerance of wheat, growth responses such as root and shoot length, and dry weight of roots and shoots were recorded initially. The data showed that the 30-d NaCl stress significantly reduced root and shoot length and dry weight (Fig. 1). As compared to control, salt stress caused a significant decline in root length (48%), shoot length (55%), and dry weights of roots (69%) and shoots (61%), however, this inhibition was alleviated significantly by Si supplementation (Fig. 1). For instance, Si addition to the salt-stressed growth media significantly improved the root length of wheat plants that reached the level of untreated controls (Fig. 1a). Shoot length of salt-stressed plants receiving Si nutrition was significantly increased (by ~ 1.7 -fold) as compared to stressed plants without an extra Si supply (Fig. 1c). Root and shoot dry weight of salt-stressed plants was, respectively, 2.2- and 3.1-fold higher in the presence of Si compared to stressed plants lacking Si (Fig. 1b, d). However, differences were not detected between control plants grown with and without Si supply (Fig. 1).

Growing wheat for 30 d in nutrient solution supplemented with 200 mM NaCl significantly inhibited all physiological parameters studied, except for a significant increase in ELI under salt stress as compared to untreated controls (Table 1). The ELI was calculated to assess the membrane permeability and the data showed that salt stress

significantly damaged the cell membranes. However, Si supplementation to the stressed plants significantly reduced the ELI and improved the stomatal conductance of the salt-stressed wheat leaves compared to stressed plants lacking Si (Table 1).

The total chlorophyll contents of the stressed plants decreased significantly (35%) and hence the photosynthetic rate declined by $\sim 58\%$ compared with the control. In contrast, Si supplementation significantly increased the total chlorophyll contents and photosynthetic rate of salt-stressed plants by $\sim 50\%$ compared to stressed plants without Si (Table 1). Furthermore, the 30-d period of salt stress significantly decreased the leaf RWC (15%) compared with control plants. In contrast, RWC in the leaves of salt-stressed plants receiving Si nutrition improved significantly (by $\sim 16\%$) compared to stressed plants without an extra Si supply (Table 1). Concomitant with these data, transpiration rate of salt-stressed wheat leaves was also significantly reduced (by about 67%) compared to control, indicating that wheat plants grown under salt stress were severely influenced by a water potential gradient due to excess of salts in the growth medium (Table 1). The inclusion of Si in the NaCl-containing nutrient solution significantly increased RWC and, the transpiration rate was also increased (by ~ 1.5 -fold) compared to the stressed plants not fed with Si. Interestingly, addition of Si in the

Table 1

Physiological characteristics of wheat genotype 'Faisalabad-2008' stressed with 200 mM NaCl, with or without 2 mM supplemental Si for 30 d in hydroponics. The values are means \pm S.E. from three independent experiments. Significant differences were calculated by Tukey's (HSD) test, and are labeled with different letters for the treatment means within the same row ($P \leq 0.05$).

Parameters	Control	Silicon	NaCl	Silicon + NaCl
Electrolyte leakage index (%)	7.33 \pm 0.28c	3.30 \pm 0.45d	35.8 \pm 1.30a	12.9 \pm 0.59b
Stomatal conductance (mmol m ⁻² s ⁻¹)	0.27 \pm 0.01a	0.18 \pm 0.02b	0.05 \pm 0.01c	0.17 \pm 0.01b
Chlorophyll contents (SPAD value)	26.9 \pm 0.78b	33.9 \pm 1.21a	17.4 \pm 1.99c	36.1 \pm 0.61a
Photosynthetic rate (μ mol m ⁻² s ⁻¹)	19.2 \pm 0.09a	20.2 \pm 0.18a	8.1 \pm 0.02c	16.2 \pm 0.12b
Relative water contents (%)	91.2 \pm 0.01a	96.1 \pm 0.03a	77.5 \pm 0.01b	93.1 \pm 0.05a
Transpiration rate (mmol m ⁻² s ⁻¹)	2.09 \pm 0.03b	3.42 \pm 0.03a	0.69 \pm 0.05d	1.71 \pm 0.03c

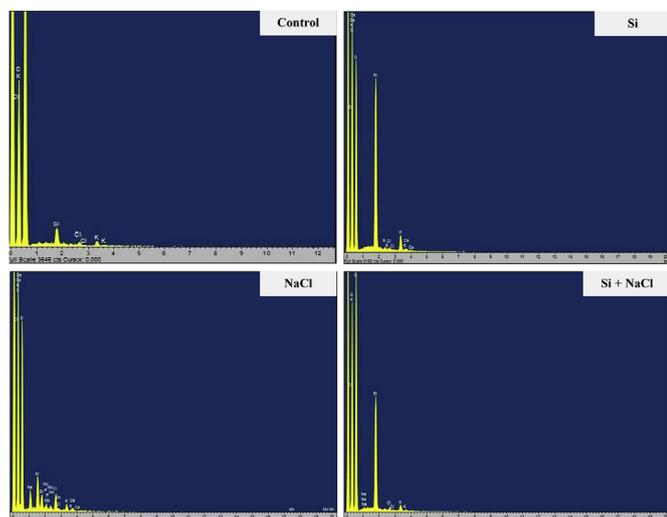


Fig. 2. Energy dispersive X-ray (EDX)-based microanalysis of mineral elements in root tissues of wheat genotype 'Faisalabad-2008'. Wheat seedlings were exposed to 200 mM NaCl in 0.5x Hoagland solution for 30 d, with or without 2 mM supplemental Si, while the control plants were maintained in 0.5x Hoagland solution medium alone. For each measurement, root samples from three independent experiments were selected to analyze the energy spectra of different mineral elements, and representative EDX microanalysis images are shown.

growth media of control wheat plants also substantially improved their physiological performance as indicated by reduced ELI and stomatal conductance (by 55% and 33% lower, respectively), as well as significantly enhanced chlorophyll contents (26%) and transpiration rate (63%) compared with the untreated control plants (Table 1).

3.2. Micro-distribution and compartmentation of ions in wheat root and leaf cells

The growth and physiological data described so far indicated importance of Si nutrition in improving salt tolerance of wheat plants. Further, EDX-based microanalysis of mineral elements was performed in order to understand the effect of Si on the distribution of ions in root and leaf cells grown under four different treatments. The distribution of elements was documented by analyzing energy spectra of Na^+ , Cl^- , K^+ , Ca^{2+} , Mg^{2+} and silica ions. Peak emissions for C, O, and P were also detected but have not been discussed in this paper. The representative EDX spectra of six mineral elements measured by X-ray technique indicated a considerable difference in cells of wheat roots treated with 200 mM NaCl with or without supplementary Si (Fig. 2). Exogenous application of Si to the growth media of control plants elevated the peaks of Si, K^+ , and Ca^{2+} . In contrast, Na^+ and Cl^- enrichments in root cells were detected in the presence of salt stress, as compared to non-stressed control. However, the peak of silica ions was clearly larger than that of toxic Na^+ and Cl^- ions in root cells of salt-stressed plants supplemented with Si which ultimately resulted in higher Ca^{2+} and K^+ peaks (Fig. 2).

Analysis of wheat root cross-sections by SEM indicated a scattered pattern of salt distribution in roots of NaCl-treated plants. The Si supplementation of salt-treated plants enhanced the accumulation of silica ions on the cell wall, potentially restricting the entry of toxic Na^+ and Cl^- ions in root cells and their subsequent distribution to aerial tissues (Fig. 3).

This was further investigated by the quantification of the relative percentage of mineral elements in root epidermal, cortical and stelar cells by SEM equipped with EDX analyzer, after subtraction of background noise. It should be noted that slight variations due to the relocation of some diffusible elements, e.g. Na^+ and K^+ , during the

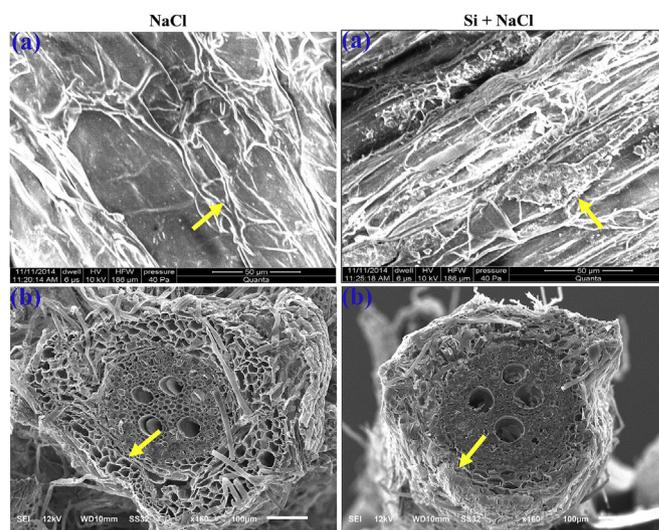


Fig. 3. Scanning electron microscope (a) and the corresponding root cross-section (b) images of typical cryo-planned transverse sections of roots of wheat genotype 'Faisalabad-2008' from which EDX analyses were obtained [Bars: a = 50 μm ; b = 100 μm]. Seventeen-day-old wheat seedlings were exposed to 200 mM NaCl, with or without 2 mM Si for 30 d in 0.5x Hoagland solution. Arrows indicate the differential pattern of salt deposition in these transverse sections of NaCl and Si + NaCl treated plants, highlighting restricted entry of salt entry in root cells due to the accumulation of silica ions on the cell wall.

tissue-embedding process cannot be completely excluded (Fig. 4). The relative weight percentage of mineral elements quantified in the cells usually represent their ionic states e.g. in this study, Na^+ , Cl^- , K^+ , Ca^{2+} , and Mg^{2+} ions, because they are absorbed, transported, and subsequently deposited in this form (Xu et al., 2015). However, Si is taken up by plants as silicic acid [$\text{Si}(\text{OH})_4$] and subsequently deposited in the form of amorphous silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) (Farooq and Dietz, 2015). The data in Fig. 4 demonstrate that the response characteristics of the relative weight of mineral elements quantified in the epidermal, cortical and stelar cells of salt-stressed wheat roots was almost similar except for Na^+ and Cl^- . As expected, under salt stress, the relative weight of Na^+ and Cl^- was significantly increased in different cells of wheat root, and the extent of the percent increase, particularly in root epidermal cells was larger than any other element (Fig. 4a and b). On the other hand, salt stress caused a significant reduction in relative K^+ and Mg^{2+} weight in root epidermal and cortical cells, and relative Ca^{2+} weight in root epidermal, cortical and stelar cells as compared to non-stressed control plants (Fig. 4c–e). Among the six mineral elements quantified in three different cell types in roots exposed to salt stress, the maximum relative accumulation of Na^+ and Cl^- was observed in the epidermal cells, followed by cortical and stelar cells (Fig. 4a and b). However, there was no significant difference in relative Na^+ weight of salt-treated root cortical and stelar cells (Fig. 4a). Furthermore, Si supplementation in the growth media of salt-stressed plants caused a significant decline in the relative weight percentage of Na^+ and Cl^- in root epidermal and cortical cells, but no significant change in relative weight percentage of Na^+ and Cl^- was observed in root stelar cells compared to stressed plants lacking Si. Meanwhile, there was a significant increase in the relative weight of K^+ in root epidermal, cortical and stelar cells due to Si nutrition under both the saline and non-saline conditions (Fig. 4c). Moreover, with Si supplementation under salt stress, though the relative weight of Ca^{2+} , and Mg^{2+} remained stable in stelar cells, their weight increased significantly in root epidermal and cortical cells compared to stressed plants not fed with Si (Fig. 4d and e). Furthermore, the quantification of the relative weight of Si in different cells of Si-treated control plants revealed that maximum Si accumulated in root epidermal cells followed by cortical and stelar cells. Similarly,

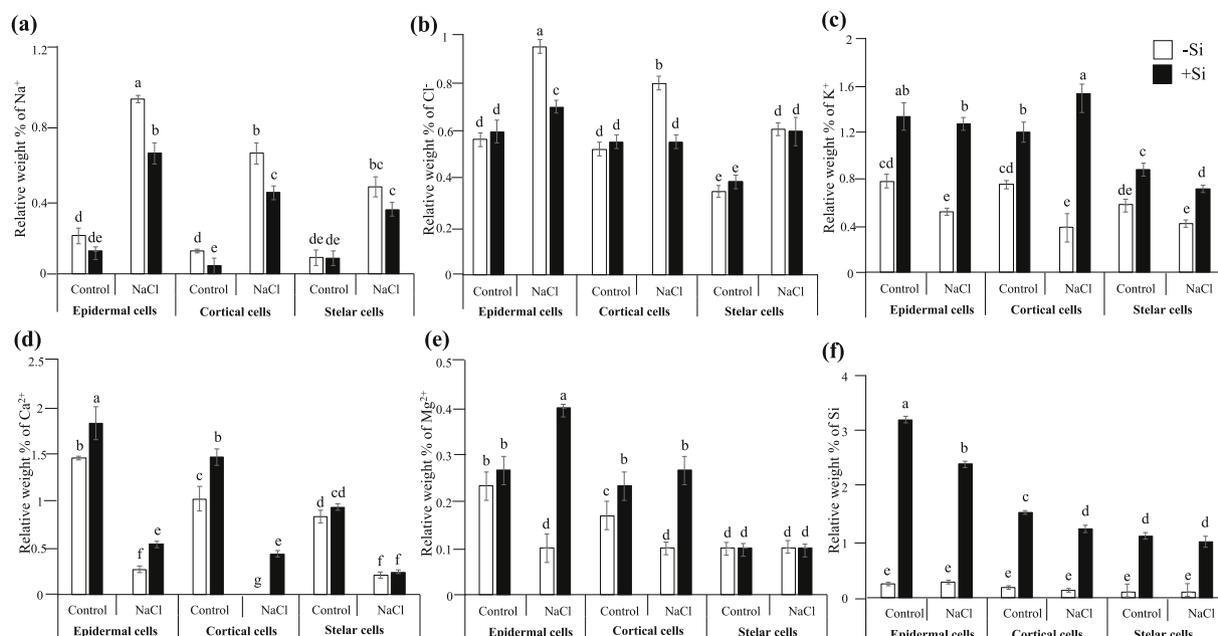


Fig. 4. Relative weight (%) of different elements in epidermal, cortical and stellar cells of wheat genotype ‘Faisalabad-2008’ roots stressed with 200 mM NaCl, with or without 2 mM supplemental Si for 30 d in 0.5x Hoagland solution. Data groups of significant difference were calculated by Tukey’s (HSD) test, and are labeled with different letters ($P \leq 0.05$). The values are means \pm S.E. from three independent experiments.

due to Si application under salt stress, maximum Si accumulated in root epidermal cells, with relatively lower accumulation in cortical and stellar cells. But there was no significant difference between relative weight of Si in cortical and stellar cells of salt-stressed wheat roots (Fig. 4f).

The XRD based energy spectra for the analysis of ions in leaf cells of wheat grown in salt-treated medium revealed a distinct and comparatively stronger signal of toxic Na^+ and Cl^- ions than those of root cells (Fig. 5). However, similar to root cells, the peak area for Na^+ was generally bigger than that of Cl^- ions in saline conditions. Conversely, the addition of Si in the growth medium of NaCl-treated plants substantially lowered the Na^+ and Cl^- peaks with a relative improvement

in the signal of K^+ , Ca^{2+} , and Mg^{2+} ions compared to stressed plants without Si supply (Fig. 5). Furthermore, similar to the root cells, SEM-based analysis of leaf tissues and cross-sections indicated the accumulation of Si along the layers of the epidermal cells and around stomatal cells (Fig. 6).

Regarding the quantification of relative weight of six mineral elements in leaf epidermal, cortical and vascular bundle cells, a trend similar to that recorded in root cells was observed, but the extent of ion accumulation (on relative weight percentage basis) in different cells of wheat leaves was higher compared with those of root cells (Fig. 7). As

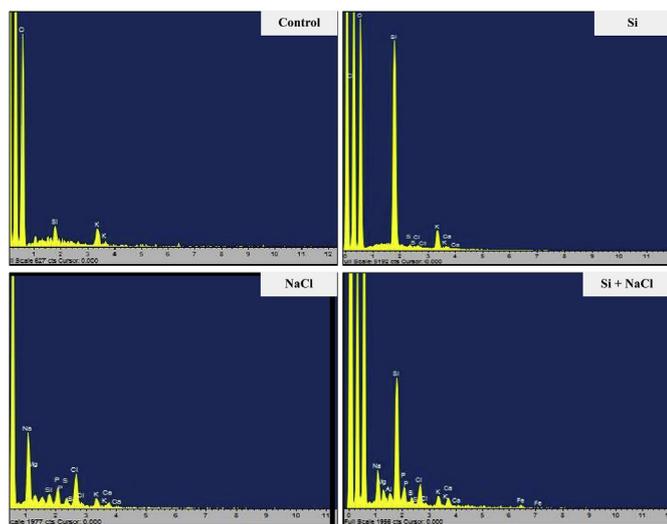


Fig. 5. Energy dispersive X-ray (EDX)-based microanalysis of mineral elements in leaves of wheat genotype ‘Faisalabad-2008’. Wheat seedlings were exposed to 200 mM NaCl in 0.5x Hoagland solution for 30 d, with or without 2 mM supplemental Si, while the control plants were maintained in 0.5x Hoagland solution medium alone. For each measurement, leaf samples from three independent experiments were selected to analyze the energy spectra of different mineral elements, and representative EDX microanalysis images are shown.

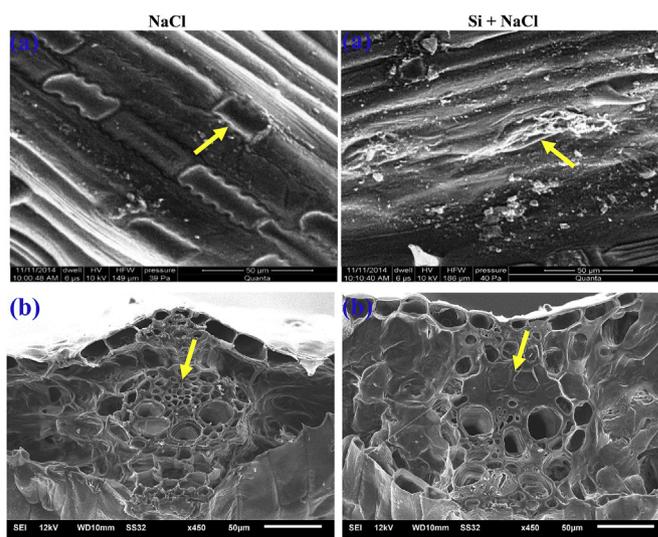


Fig. 6. Scanning electron microscope (a) and the corresponding leaf cross-section (b) images of typical cryo-planned transverse sections of leaves of the wheat genotype ‘Faisalabad-2008’ from which EDX analyses were obtained [Bars = 50 μm]. Seventeen-day-old wheat seedlings were exposed to 200 mM NaCl, with or without 2 mM Si for 30 d in 0.5x Hoagland solution. Arrows indicate differential accumulation of silica along the epidermal and stomatal cells of NaCl and Si + NaCl treated wheat leaves that might help plants improve water status under salt stress due to enhanced leaf thickness.

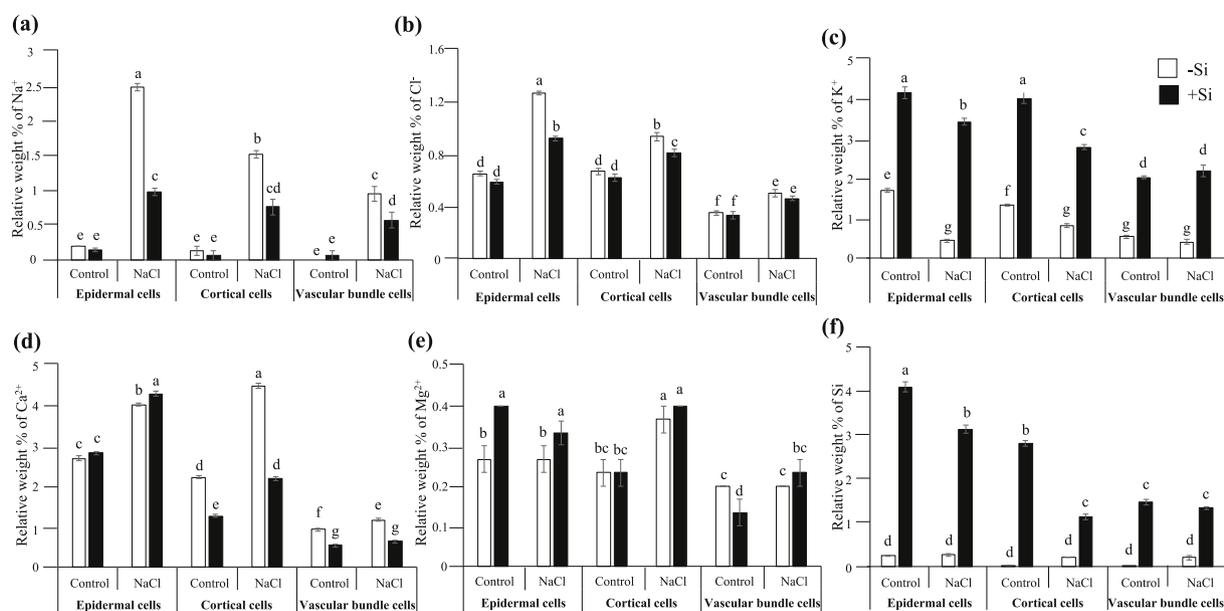


Fig. 7. Relative weight (%) of different elements in epidermal, cortical and vascular bundle cells of wheat genotype ‘Faisalabad-2008’ leaves stressed with 200 mM NaCl, with or without 2 mM supplemental Si for 30 d in 0.5x Hoagland solution. Data groups of significant difference were calculated by Tukey's (HSD) test, and are labeled with different letters ($P \leq 0.05$). The values are means \pm S.E. from three independent experiments.

compared to control plants, salt stress caused a significant increase in the relative weight of both Na^+ and Cl^- in three different cells of wheat leaves (Fig. 7a and b). While the presence of Si in the growth media of stressed plants resulted in significant decline in relative weight of Na^+ in different wheat leaf cells analyzed as compared to stressed plants lacking Si supply (Fig. 7a). A significant reduction in Cl^- accumulation in leaf epidermal and cortical cells was also observed due to Si nutrition under salt stress, but with no further change in vascular bundle cells (Fig. 7b). Similarly, no significant difference was detected in the relative weight of Na^+ and Cl^- in leaf epidermal, cortical and vascular bundle cells of control plants grown with and without Si supply (Fig. 7a and b). For the relative K^+ weight, a significant decline was observed in the leaf epidermal and cortical cells of salt-treated wheat as compared to control plants. However, no significant difference was recorded between the relative K^+ weight in vascular bundle cells of control and stressed plants (Fig. 7c). Moreover, the relative weight percentage of K^+ quantified in epidermal, cortical and vascular bundle cells of salt-stressed wheat leaves did not differ significantly from each other. Importantly, the Si treatment of control and stressed plants significantly increased the relative K^+ weight with the largest extent compared with those not treated with Si (Fig. 7c). A variable response was recorded for the relative weight of Ca^{2+} in the epidermal, cortical and vascular bundle cells of stressed plants treated with and without Si. Compared to non-Si control, salt stress caused a significant increase in relative Ca^{2+} weight in the leaf epidermal, cortical, and vascular bundle cells, however to variable extents (Fig. 7d). Further, Si nutrition of stressed plants caused a marginal but significant increase in relative Ca^{2+} weight in leaf epidermal cells than in stressed plants lacking Si. While the relative Ca^{2+} weight in the cortical and vascular bundle cells of Si supplemented control as well as salt-stressed plants decreased significantly compared with their respective controls not given an extra Si supply (Fig. 7d). Furthermore, compared to the non-stressed control, the relative Mg^{2+} weight did not change with the salt stress in the epidermal and vascular bundle cells, but, it increased significantly in the cortical cells of wheat leaves. The Si application under salt stress caused a significant increase in relative Mg^{2+} weight quantified in the epidermal cells, with a slight but non-significant increment in cortical and vascular bundle cells compared to stressed plants without Si supply (Fig. 7e). Due to Si supplementation under control and salt stress, the relative weight of Si was increased maximally in the leaf epidermal

cells, followed by cortical and vascular bundle cells of wheat leaves (Fig. 7f).

4. Discussion

Silicon is known to modulate numerous plant biological functions, particularly under stress conditions including salt stress (summarized by Farooq and Dietz, 2015). However, the mechanisms of Si-mediated improvement in salt stress tolerance have not been analyzed in detail. This study investigated the influence of Si nutrition on minimizing Na^+ toxicity and improving growth in wheat by altering distribution and compartmentalization of mineral ions within plant tissues under salt stress condition. Addition of 200 mM NaCl to the hydroponic nutrient solution caused a significant reduction in the growth of salt-stressed wheat after 30 d of treatment, but Si application alleviated the negative effects of salt stress and prevented further development of damage and consequently improved root and shoot growth of wheat to variable but significant extents (Fig. 1).

Analysis of physiological parameters provided deep insights into the growth responses of wheat plants exposed to salt stress with or without Si supplementation (Table 1). Under salt stress, total leaf chlorophyll contents were significantly decreased by the high internal salt concentration (Table 1). Exposure of sunflower to salt stress reportedly caused a substantial loss of leaf chlorophyll due to inhibition of 5-aminolaevulinic acid (a chlorophyll precursor), together with fast degradation of chlorophyll pigments by the enzyme chlorophyllase during the early stress period (Santos, 2004). In the present study, the consequent decrease in photosynthetic rate under salt stress may either be related to inhibition of chlorophyll biosynthesis (Santos, 2004), or a significant reduction in stomatal conductance (Table 1), that in turn decreases internal CO_2 concentration and activity of numerous key enzymes such as RuBisCO (Chaves et al., 2009), and therefore limits the carboxylation efficiency and photosynthetic performance of plants. Contrarily, we found significant improvements in leaf stomatal conductance, chlorophyll contents and photosynthetic rate of Si supplemented salt-stressed wheat (Table 1). Although the beneficial effects of Si are usually known in terms of enhanced plant stress tolerance (reviewed in Farooq and Dietz, 2015), but in the present work, we noticed that Si significantly reduced the ELI and stomatal conductance, but increased the leaf chlorophyll contents even in the absence of salinity

stress (Table 1). However, Si supplementation to salt-stressed wheat greatly reduced the magnitude of injury to the cell membrane as indicated by a significant decrease in ELI (by ~64%) compared to stressed plants not treated with Si. In barley, Si nutrition reduced the electrolyte leakage and membrane lipid peroxidation of salt-stressed plants, thus maintained the membrane integrity resulting improved growth response (Liang et al., 1996). Further, we found that Si provision to salt-stressed wheat significantly increased the leaf chlorophyll contents to a value exceeding than those of unstressed controls (Table 1). Our results of Si-mediated complete restoration of chlorophyll level under salt stress are in agreement with Tuna et al. (2008) who found that with optimum Si supplementation to salt-stressed wheat, the chlorophyll level was even higher than that of controls. Earlier, Si nutrition was reported to enhance the chlorophyll contents and photosynthetic rate of barley both under non-saline and saline conditions (Liang et al., 1996; Liang, 1998). In this study, the beneficial effects of Si on the chlorophyll pigments and increased stomatal conductance and photosynthetic activity of salt-stressed wheat could be partly ascribed to our results of Si-mediated inhibition of Na^+ and Cl^- uptake and accumulation to be discussed in the next section (Table 1, Figs. 4a and 7a). Nonetheless, Si-mediated protection of photosynthetic apparatus and improved physiological performance are suggested to partially increase biomass yield of salt-stressed wheat by two diverse mechanisms: (i) Si nutrition may exert a feed-forward effect on dry matter production by stimulating photosynthetic rate linked to enhanced mesophyll conductance and altered primary metabolism (Detmann et al., 2012; Flam-Shepherd et al., 2018), and, (ii) probably also due to high Si accumulation in wheat shoots itself i.e. ~10% on DW basis (Casey et al., 2004). Furthermore, improvement in the growth related parameters of salt-treated wheat due to Si addition to the growth medium can also be related to enhanced leaf RWC and prevention of excessive transpiration rate (Fig. 1, Table 1), that ultimately reduces salt-induced osmotic stress (Negrão et al., 2017; Hussain et al., 2018).

Under salt stress, plant cellular damages and the resultant growth defects are primarily caused by the excessive uptake and accumulation of Na^+ and Cl^- ions (Flowers and Colmer, 2015). In general, maintenance of low cytosolic Na^+ concentration either by increased Na^+ efflux to the external solution or by compartmentalizing it in the vacuoles have been suggested as major salt tolerance mechanisms (Munns and Tester, 2008). Moreover, homeostatic maintenance of intracellular pools of essential nutrients like K^+ , Ca^{2+} , and Mg^{2+} play crucial roles in determining plant metabolic, growth and developmental responses to salt stress (Kronzucker et al., 2013). Application of Si is known to improve salt stress tolerance by decreasing Na^+ accumulation but increasing the uptake, and long-distance transport of essential mineral ions in various plant species (reviewed by Zhu and Gong, 2014). In the present work, analysis of micro-distribution of ions in salt-stressed wheat roots revealed significantly higher Na^+ and Cl^- contents in the epidermal, cortical and stelar cells that subsequently reduced K^+ accumulation in the root epidermal and cortical cells compared to unstressed control plants (Fig. 4a, b, c). Excess of Na^+ in the growth media competes with K^+ for binding sites and to inactivate enzymes that inhibit associated essential metabolic functions of the cell and, subsequently, salt-stressed plants suffer the dual injury of Na^+ toxicity and low K^+ concentrations (Munns and Tester, 2008). In the present study, it is noteworthy that a major proportion of Na^+ ions absorbed by wheat roots was translocated to aerial tissues (Figs. 4a and 7a), thereby causing significant physiological impairments by 200 mM NaCl (Table 1); these symptoms are probably indicative of salt sensitivity of the tested wheat genotype (Colmer et al., 2005). However, we found that Si supplementation to salt-stressed wheat significantly reduced Na^+ and Cl^- contents in root epidermal and cortical cells, but increased K^+ uptake in root epidermal, cortical and stelar cells compared to stressed plants without Si supply (Fig. 4a, b, c). Earlier, in roots of salt-stressed barley, addition of Si has been shown to maintain low Na^+

concentration in the cytosol by differentially modulating the expression of genes encoding Na^+/H^+ antiporters (Liang, 1999; Liang et al., 2005, 2006). The authors reported that Si supplementation stimulate the activities of root plasma membrane H^+ -ATPase, and tonoplast H^+ -ATPase and H^+ -pyrophosphatase (H^+ -PPase), which generate electrochemical H^+ gradient and act as driving force for the operation of Na^+/H^+ antiporters. These changes in H^+ pump activities were suggested to increase Na^+ exclusion from the cytosol through the Na^+/H^+ antiporters HvSOS1 and HvNHX1 localized at plasma membrane and tonoplast, respectively (Liang, 1999; Liang et al., 2005, 2006). Later, it was reported that increase in H^+ -ATPase activity by Si application reduces Na^+ uptake with concomitant improvement in K^+ uptake and accumulation in wheat (Mali and Aery, 2008). Taken together, this is suggested as a potential mechanism of Si-mediated enhanced salt tolerance of plants.

In addition to that, Si-induced physical barrier in roots is proposed as another potential salt tolerance mechanism by which Si reduces apoplastic Na^+ transport (also called transpirational bypass flow) and consequently decreases its accumulation in above-ground tissues (Gong et al., 2006; Shi et al., 2013). Our results of scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) spectroscopy analysis of frozen dried root samples of wheat indicated that Si accumulated in many parts of roots, making deposits on the root surface, strengthening the idea of improvement in root anatomical properties, thereby resulting in better root proliferation and nutrient uptake (Fig. 3). The scanned images of root cross-sections clearly indicated that NaCl (Na^+ and Cl^-) was found accumulated throughout the cross-section of root under salt stress while the salts were found accumulated outside the cell wall in the image of root section of Si treated plants (Fig. 3b). In silica-accumulating species such as wheat, Si is taken up actively and bind to epidermal cells and cell walls, thereby contributing to cross-linking of cell wall components (Farooq and Dietz, 2015). Previously, such silica deposits in wheat roots and the resultant structural changes were reported to promote Na^+ complexation to the cell walls, that reduces Na^+ transport to the above-ground tissues (Ahmad et al., 1992; Saqib et al., 2008; Tuna et al., 2008); nevertheless, direct evidence of Si-mediated enhanced Na^+ complexation that may trigger this potential salt tolerance mechanism in plants is still lacking. However, increasing evidence from rice indicate that Si treatment triggers the expression of lignin and suberin biosynthesis related genes in roots, thereby causing enhanced lignification and suberization of sclerenchyma in these tissues (Fleck et al., 2011; Suzuki et al., 2012), which may act as barrier to apoplastic Na^+ transport, a phenomenon associated with improved salt tolerance in rice (Krishnamurthy et al., 2011). Similarly, Si application was also reported to promote the development of Casparian band in the root exodermis and endodermis of rice (Fleck et al., 2015), which forms physical barrier to Na^+ and Cl^- translocation, coinciding with X-ray localization patterns of silica deposition in rice roots (Gong et al., 2006). These results of Si-induced blockage of apoplastic bypass flow were subsequently confirmed by significantly reduced translocation of the apoplastic dye trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS) in Si-treated rice roots (Yeo et al., 1999; Gong et al., 2006; Shi et al., 2013). Further work is required to understand if this mechanism of reduced Na^+ and Cl^- bypass flow by Si application is distinctive to rice, or is taxonomically widespread.

The transverse cross-section images of wheat leaf blades examined under SEM indicated that Si accumulated around the stomata, in between the veins and along the layers of the epidermal cells of Si treated plants both under normal and saline conditions (Fig. 6). Enhanced silica accumulation in plant leaves has been reported to form a silica-cuticle double layer below the epidermis, that improves the physical defense power of plants against multiple stresses (Farooq and Dietz, 2015). In this study, Si deposition in wheat leaves improved growth and physiological performance of salt-stressed wheat (Fig. 1; Table 1), correlating with relatively reduced uptake of toxic Na^+ and Cl^- ions in roots of Si supplemented stressed plants and subsequently lowered their

accumulation in shoots (Fig. 4a and b and 7a, b), despite increasing stomatal conductance and transpiration rate as compared to stressed plants lacking Si (Table 1), suggesting that Si does not act to decrease Na^+ translocation by decreasing transpiration rate *per se*, but rather by minimizing bypass flow as discussed previously for rice (Yeo et al., 1999; Gong et al., 2006; Shi et al., 2013). Altogether, these processes contributed to root Na^+ uptake and transport inhibition with added Si, that consequently improved the uptake and microdistribution of metabolically essential ions like K^+ (Figs. 4 and 7).

The relative Ca^{2+} weight was also increased significantly in root epidermal and cortical cells due to Si nutrition of both control as well as salt-stressed plants (Fig. 4d). Similarly, root epidermal and cortical cells also displayed significant increase in relative Mg^{2+} weight due to Si presence in the growth media of salt-treated plants as compared to stressed plants without Si supply (Fig. 4e). However, Si application did not change the relative weight of Ca^{2+} and Mg^{2+} in root stelar cells both under control and salt stress (Fig. 4d and e). The Si– Ca^{2+} interactions vary considerably with the plant species and the type of stress to which plants are exposed (Ma and Takahashi, 1993; Liang, 1999). Better maintenance of Ca^{2+} ion homeostasis under salt stress is essential for maintaining plant membrane integrity, as well as regulation of cell wall enzyme activities (Munns and Tester, 2008). In this study, a large proportion of the Ca^{2+} absorbed by salt-stressed roots was translocated to wheat leaves. Although, Si provision to the salt-stressed plants significantly improved the Ca^{2+} accumulation in root epidermal and cortical cells (Fig. 4d), however, it slightly but significantly increased relative Ca^{2+} weight in salt-stressed leaf epidermal cells compared to those lacking Si (Fig. 7d). In contrast, Si supply significantly reduced the relative Ca^{2+} weight in leaf cortical and vascular bundle cells both under control and salt stress conditions (Fig. 7d). As described above, the Si accumulation in roots induces structural changes and promotes blockage of apoplastic pathways that are reported to increase Si and Ca^{2+} binding with the lignin- or phenol-carbohydrate complexes in cell walls, thereby making insoluble compounds that subsequently reduces Ca^{2+} transport to the above-ground tissues (Inanaga and Okasaka, 1995; Wang and Han, 2007). This is proposed as a possible mechanism for our results of Si-mediated significant decrease in relative Ca^{2+} weight in leaf cortical and vascular bundle cells of both control and salt-stressed plants as compared to their respective controls lacking Si (Fig. 7d). Similar results of repressed Ca^{2+} delivery to leaves of salt-stressed rice were also reported due to reduced transpiration rate in the presence of Si (Ma and Takahashi, 1993). Altogether, these results suggest that the growth stimulatory effect of Si on the above-ground tissues is probably caused by the local effects of silica accumulation in leaves. Improved plant physiological performance as discussed above for chlorophyll contents and photosynthesis, for instance in Si-supplemented salt-stressed plants, are possibly involved in this process (Table 1). In this context, Mg^{2+} is required for chlorophyll biosynthesis in plants, thus plays a key role in photosynthetic activity (Farhat et al., 2016). However, in this study, salt stress did not alter the relative Mg^{2+} level in leaf epidermal and vascular bundle cells but significantly increased it in cortical cells, while, competition with Na^+ at the root uptake sites caused a significant reduction in relative Mg^{2+} weight in epidermal and cortical cells of salt-stressed wheat roots as compared to non-stressed control plants (Figs. 4e and 7e). With these effects combined with those previously discussed reductions in K^+ ion homeostasis, a severe loss of cell viability and plant growth is evident which was significantly reversed by Si nutrition (Figs. 4 and 7). It should be noted that Si-induced maintenance of cell ionic homeostasis of essential elements with a parallel decrease in Na^+ and Cl^- concentrations and the consequently improved physiological performance are suggested as major mechanisms for improving salt tolerance in wheat.

5. Conclusions

Exogenous application of Si plays a significant role in improving plant tolerance against multiple biotic and abiotic stresses. In this study, NaCl-induced cellular damages and the resultant growth defects in wheat were significantly reversed by Si addition to the growth medium. Analyses of physiological attributes indicated that this amelioration was correlated with the improved gaseous exchange, photosynthetic rate, relative water contents and ultimately osmotic adjustment in plants under salt stress. Furthermore, the presence of Si in the nutrient formulation of salt-stressed wheat minimized specific ion toxicity mainly by immobilization of toxic Na^+ and Cl^- ions at the root surface, while increasing the uptake of essential elements such as K^+ , and Mg^{2+} . Because the availability of plant essential ions can alter salinity responses, the differences in wheat growth at the end of the experiment were strongly associated with differences in ion accumulation. One of the mechanisms of Si action was by altering the uptake and micro-distribution of mineral ions, thus indicate a decisive role of Si for readjusting nutrient ion homeostasis. Based on these results, Si fertilization of crop grasses is suggested as a viable strategy to improve stress tolerance (e.g. against salt toxicity) of plants at field level and need to be tested in future studies.

Conflicts of interest

None declared.

Author contribution

Tahir Javaid conducted all the experiments. Muhammad Ansar Farooq designed the experiments, discussed and analyzed the data and wrote the paper. Javaid Akhtar designed the experiments, supervised the study and commented on the paper. Zulfiqar Ahmad Saqib advised on experimental layout and participated in preparing initial paper draft. Muhammad Anwar-ul-Haq co-supervised the study and advised on experimental layout. The final version was read and approved by all authors.

Acknowledgments

Financial support by the Higher Education Commission (HEC), Pakistan (SRGP; Project # 1624) is gratefully acknowledged. We wish to thank Prof. Dr. Zed Rengel (Department of Soil Science and Plant Nutrition, University of Western Australia) for proofreading the manuscript. We also thank the anonymous reviewers for the careful review of our paper, and for the comments, corrections and suggestions that ensued.

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