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Influence of Si Supplementation on Growth and Some Physiological and Biochemical Parameters in Salt-Stressed Tobacco (Nicotiana rustica L.) Plants

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Abstract

Tobacco is a salt-sensitive glycophyte crop species. In this work effect of silicone (Si) supplementation (1 mM as Na2SiO3) was studied in Nicotiana rustica L. cv. Basmas grown hydroponically in growth chamber under control, low (25 mM) and high (75 mM) NaCl concentration for two weeks. Dry matter production of leaves was depressed by salinity level as low as 25 mM and higher salt concentration decreased plants dry weight by 52-82%. Si supplementation alleviated salt stress effect as could be judged by higher dry weight of shoot and roots in +Si plants compared with –Si counterparts. Leaf chlorophyll a and carotenoids concentrations and net assimilation rate were higher in Si-treated plants not only in salt-affected but also in control plants. Si treatment resulted in higher concentration of soluble carbohydrates but not proline. Leaf transpiration rate, unexpectedly, was not diminished by Si and water use efficiency was rather lowered by Si in salt-treated plants. Si application caused a slight reduction of Na concentration while increased that of K and Ca significantly and resulted in higher K:Na ratio in the leaves, stem and roots. Our results suggested that Si application improved tolerance to salt stress in tobacco due to an enhancement of photosynthesis, accumulation of organic osmolytes as well as improvement of K:Na selectivity but not limiting water loss. In addition, greater dry matter production of Si-supplemented plants in the absence of salt was associated with elevated photosynthesis rate, higher K and Ca uptake and proline content.

Keywords: Tobacco, Salinity, Silicone, K:Na ratio, Organic osmolytes.

Introduction

Salinity is among the most stressful soil factors that limits plants growth and productivity. Crop plants are mainly sensitive to salt and under arid and semiarid climatic conditions, soil salinity is the major constraint for growth and yield of crop species [10].

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Plant performance is adversely influenced by salinity via different mechanisms. There are two components of salt stress, include osmotic stress and toxic effect of ions, that influence differentially plant growth and metabolism depending on salt concentration [35]. Osmotic stress is linked to lower salt concentrations while ion toxicity effect impairs plants growth under higher salinity levels [16]. The threshold concentration of salt for these two contrastive effects is highly dependent on the extent of uptake and accumulation of salt and susceptibility of plant species. Glycophytes are salt-sensitive species that are affected even by low concentrations of salt and higher levels of salinity may disturb severely metabolism and growth and cause ultimately plants death [36].

Silicone (Si) is the second most abundant element in the earth's crust and soils, but it has not yet been categorized as an essential nutrient for higher plants [4, 37]. Regarding plants ability for Si uptake and accumulation, they are classified into Si-accumulator and non-accumulator species [4, 37]. In the absence of Si, growth and reproduction of accumulator species are severely depressed while non-accumulator species do not apparently require Si for optimum growth [4]. Nevertheless, Si in the growth medium of non-accumulator species ameliorates detrimental effects of various environmental stresses [4, 15, 22].

Effects of different abiotic stresses are alleviated in the presence of Si, include drought [13, 42], UV-radiation effects [42], chilling and freezing [27] and toxicity of heavy metals [19]. Effect of Si on the alleviation of salt stress effects has been reported for Si-accumulator species such as rice [31, 46] and cucumber [48] as well as in some non-accumulator species such as tomato [39].

Various mechanisms are involved in the ameliorative effect of Si on plant response to abiotic stresses include activation of antioxidative defense [21] and improvement of plants ability for water and nutrients uptake from soil [22, 39, 45]. Under salt stress, Si reduces Na uptake and increases K:Na ratio thus, alleviates ion toxicity effect in several plant species such as rice [31, 46] barley [23] and tomato [39].

Tobacco is the most important non-food crop species in the world and is highly susceptible to salt. Different cultivars of tobacco (*Nicotiana spp*.) belonging mainly to *N. tobacum* or *N. rustica* species are produced in the North and North-Western Iran with an estimated area of 12 500 ha of cultivated land and annual production of around 21 000 tons [10]. The most widely cultivated species in the North-Western Iran is *N. rustica*.

Land salinization is a major limiting factor for crop production in Iran [11] mainly as the consequence of low precipitation and lacking management strategies of saline soils. In North-Western Iran agricultural lands in proximity to the Salt Lake Urmia [9] are being faced with the rise of water tables causing salt accumulation in the surface soils. Consequently, improvement of salt tolerance in glycophyte crop species such as tobacco could be regarded as an important strategy for enhancement of productivity and yield of these species cultivated on salinized agricultural soils.

Research works on the effect of Si in tobacco has been limited to the studies on its effect on preventing infections by pathogens [47]. Information is lacking on the mitigation of environmental stresses include salt stress by Si in tobacco. In accumulator species, Si deposition in the leaf epidermis causes reduction of transpiration and improves capability of leaves for water retention [31]. In addition, due to deposition in the root endodermis Si reduces Na bypass flow [14, 46]. In non-accumulator species such as tobacco, however, Si concentration is lower than 5% [47] and thus, it may alleviate the effects of salt using other mechanisms.

This work was aimed at studying the effect of Si supplementation on growth, photosynthesis and ion relations in tobacco plants under salinity stress. In order to elucidate the effect of Si as influenced by the level of salt concentration, two contrastive salt levels, selected according to our preliminary experiment, were applied in this work.

Materials and Methods

Plants culture and treatment

Seeds of tobacco (*Nicotianarustica* L. cv. Basmas) plants provided by the Agricultural Research Center, Urmia, Iran, were surface-sterilized using sodium-hypochlorite at 5%and were germinated in the dark on perlite. Seven-day-old young seedlings were pre-cultured in Hoagland nutrient solution [18] for three weeks.

Four-weeks-old plants were transferred to hydroponic medium containing Hoagland nutrient solution and precultured for further one week. Thereafter, Si treatments including without (–Si) or with (+Si) 1 mM Si (as Na_2SiO_3) were applied. One week after starting Si application, salinity treatments as three levels of NaCl at 0 (control), low (25 mM) and high (75 mM) concentrations were started. Tobacco plants exposed to 100 mM NaCl and higher salinity died one week after treatment, therefore, 75 mM NaCl was set as the maximum salinity level for the cultivar used in this work.

In order to determine the possible effect of Na as accompanying ion in the Si salt applied to plants, an experiment was conducted in parallel with the main

experiment with control (without addition of salt or Si) and 2 mM NaCl containing an equivalent Na with 1 mM Na_2SiO_3. Dry weight (mg plant^{-1}) of plants under control (2.35±0.16) and 2 mM salt (2.66±0.64) was not different significantly (Tukey test, $P<0.001$).

Plants were grown under controlled environmental conditions with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and at a photon flux density of about 400 µmol $m^{-2}s^{-1}$.

Plants harvest

Two weeks after starting salinity treatment (3 weeks after Si treatment, 8 weeks after sowing) plants were harvested. Leaves, stem and roots were separated, washed with distilled water and blotted dry on filter paper. Plants dry weight was determined after drying in 60°C for 48 h. Subsamples were taken for biochemical and ion analyses before and after drying, respectively. Before harvest, chlorophyll fluorescence and gas exchange parameters were determined in attached leaves.

Determination of chlorophyll fluorescence and gas exchange parameters

Chlorophyll (Chl) fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK). Measurements were carried out on the second youngest, fully expanded and attached leaves of 4 plants per treatment. An average of 4 records from different parts of each individual leaf was considered for each replicates. Leaves were acclimated to dark for 30 min using leaf clips before taking the measurements for dark-adapted leaves. Maximum quantum yield of PSII (*Fv/Fm*) was calculated using initial (F_0), maximum (F_m) and variable ($F_v=F_m-F_0$) fluorescence parameters. Calculations for light-adapted leaves were undertaken using initial (*Ft*), steady-state (F_s), maximum (F_m'), variable ($F'v=F'm-Ft$) and $F_0'[F_0'=F_0/(F_v/F_m)+(F_0/F_m')]$ fluorescence for excitation capture efficiency of open PSII ($F'v/F'm$),photochemical quenching (qP) $[(F_m'–F_s)/(F_m'–F_0')]$ and non-photochemical quenching (qN) $[1– (F_m'–F_0')/(F_m–F_0)]$ [32].

CO_2 assimilation and transpiration rates were measured in parallel for chlorophyll fluorescence measurements in the same leaf with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 A.M. and 13:00 P.M. at harvest. The measurements were conducted with photosynthetically active radiation (PAR) intensity at the leaf surface of 400 µmol m^{-2} s^{-1}. The net photosynthesis rate by unit of leaf area (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and the stomatal conductance to water vapor (g_s, mol m^{-2} s^{-1}) were calculated using the values of CO_2 and humidity variation inside the chamber, both measured by the infrared gas analyzer of the photosynthesis system.

Determinations of leaf pigments

Leaf concentration of Chl*a*, *b* and carotenoids (Car) were determined according to Lichtenthaler and Wellburn [26]. Leaves were homogenized in 80% cold acetone in the dark at 4°C. After 24 h, the absorption of samples was determined at 663 (Chl a), 646 (Chl b) and 470 (Car) nm using spectrophotometer (Specord 200, Analytic Jena, Jena, Germany). Determination of anthocyanins was performed using a pH differential method at pH 1 and pH 4.5 in the methanol/HCl (98:2, v/v) extract and was expressed as mg of cyanidine-3-glucoside g^{-1} FW [12]. Total flavonoids content was determined in the methanol extract of leaves. An aliquot of 1 ml of the solution containing 1 mg extracts in methanol was added to test tubes containing 0.1 ml of 10% $Al(NO_3)_3$, 0.1 ml of 1 M potassium acetate and 3.8 ml of methanol. After 40 min at room temperature, the absorbance was recorded using spectrophotometer at 415 nm. Quercetin was used as a standard [40].

Determinations of organic solutes

For determination of nonstructural carbohydrates, samples were homogenized in 100 mM phosphate buffer (pH = 7.5) at 4ºC. After centrifugation at 12000 *g* for 15 min, an aliquot of the supernatant was mixed with anthrone-sulfuric acid reagent and incubated for 10 min at 100°C. After cooling, the absorbance was determined at 625 nm. Standard curve was created using glucose (Merck) [29].

Proline was extracted and determined according to Bates et al. [2]. Leaf tissues were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 *g* for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Proline (Sigma) was used for production of a standard curve.

Determination of elements concentration

For determination of Na, K, and Ca content, oven-dried samples were weighed and ashed in a muffle furnace at 550 ºC for 8 h, resolved in HCl, and made up to volume by distilled water. Concentrations of Na, K, and Ca were determined by flame photometry (PFP7, Jenway, UK).

Experimental design and statistical analyses

This experiment was undertaken in randomized

block design with four replications as four independent pots and two factors including salinity (S) and Si application (Si). Two-way ANOVA was performed using Sigma Stat 2.03. Differences between the means were detected using the Tukey test ($P<0.05$).

Results

Plants growth was inhibited by both applied levels of salt. Reduction of dry mass by low (25 mM) salt concentration was significant only for the leaves, while growth impairment by higher salt concentration (75 mM) was significant for all three plant fractions (Fig. 1). Si supplementation improved plants dry weight not only in salt-affected plants, but also in control ones.

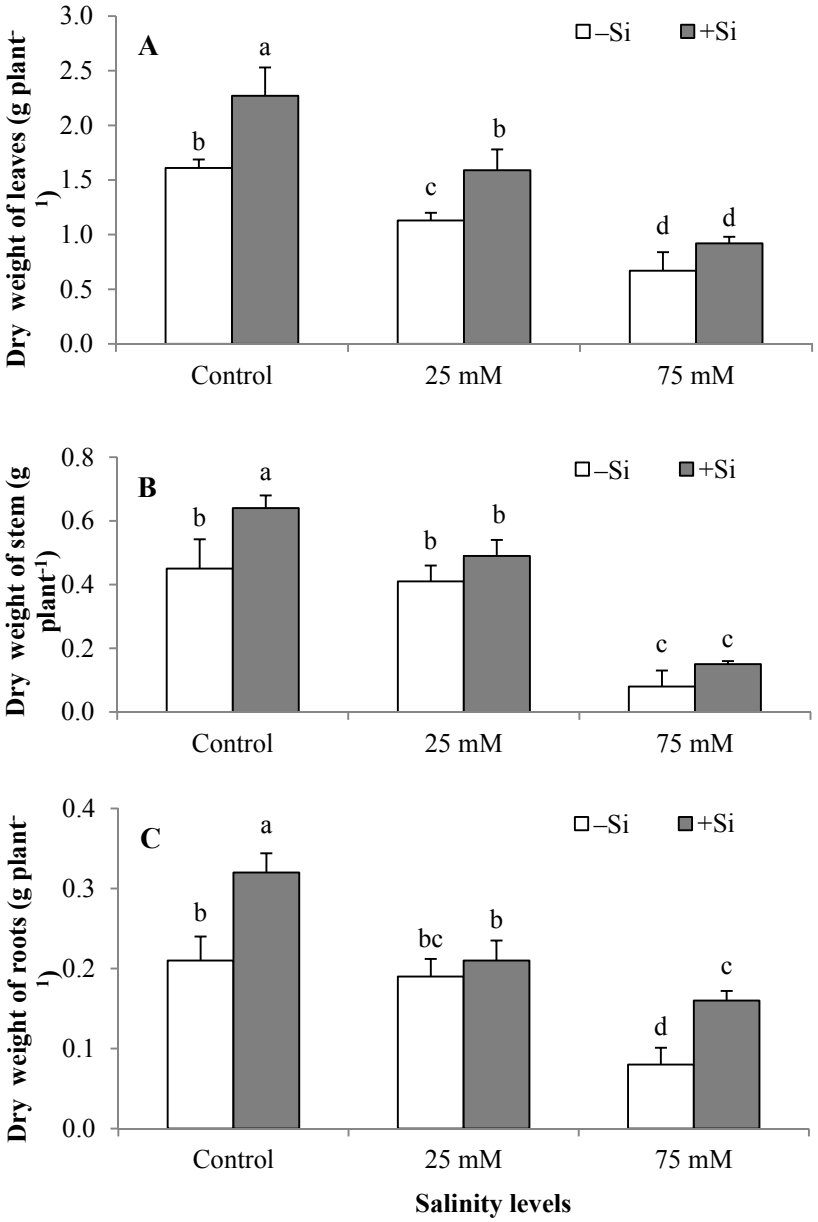

Figure 1. Dry weight of leaves (A), stem (B) and roots (C) in tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si) or presence (+Si) of 1 mM Na_2SiO_3 in hydroponic medium. Bars indicated by the same letter are not significantly different ($P<0.05$).

Table 1. Leaf concentration of chlorophyll (Chl) a, b, carotenoids (Car), anthocyanins and flavonoids (mg g^{-1} FW) in tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si) or presence (+Si) of 1 mM Na_2SiO_3 in hydroponic medium. Data of each column followed by the same letter are not significantly different (P<0.05).

Treatments		Chl a	Chl b	Car	Anthocyanins	Flavonoids
–Si	Control	0.49±0.03 ^{e}	0.16±0.03 ^{c}	0.17±0.00 ^{d}	7.11±1.02 ^{b}	4.96±0.06 ^{a}
	25 mM	0.72±0.04 ^{d}	0.18±0.02 ^{c}	0.15±0.00 ^{d}	9.21±1.66 ^{ab}	4.85±0.06 ^{a}
	75 mM	0.55±0.04 ^{e}	0.13±0.02 ^{c}	0.15±0.00 ^{d}	11.6±0.44 ^{a}	4.91±0.05 ^{a}
+Si	Control	1.41±0.01 ^{c}	0.42±0.01 ^{a}	0.34±0.01 ^{c}	8.25±1.14 ^{ab}	4.76±0.08 ^{a}
	25 mM	1.61±0.08 ^{b}	0.39±0.08 ^{a}	0.38±0.02 ^{b}	10.2±2.61 ^{ab}	4.81±0.05 ^{a}
	75 mM	2.09±0.02 ^{a}	0.51±0.06 ^{a}	0.43±0.02 ^{a}	11.5±2.02 ^{a}	4.78±0.05 ^{a}

Table 2. Chlorophyll fluorescence parameters including F_v/F_m (photochemical efficiency of PSII), F'_v/F'_m (excitation capture efficiency of open PSII), q_P (photochemical quenching) and q_N (non-photochemical quenching) in the leaves of tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si) or presence (+Si) of 1 mM Na_2SiO_3 in hydroponic medium. Data of each column followed by the same letter are not significantly different (P<0.05).

Treatments		F_v/F_m	F'_v/F'_m	qP	qN
–Si	Control	0.81±0.03 ^{a}	0.75±0.03 ^{a}	0.94±0.01 ^{a}	0.12±0.02 ^{a}
	25 mM	0.84±0.02 ^{a}	0.76±0.02 ^{a}	0.95±0.01 ^{a}	0.10±0.05 ^{a}
	75 mM	0.86±0.01 ^{a}	0.76±0.01 ^{a}	0.94±0.01 ^{a}	0.10±0.04 ^{a}
+Si	Control	0.85±0.01 ^{a}	0.75±0.01 ^{a}	0.93±0.02 ^{a}	0.09±0.02 ^{a}
	25 mM	0.84±0.01 ^{a}	0.75±0.01 ^{a}	0.92±0.03 ^{a}	0.10±0.08 ^{a}
	75 mM	0.85±0.01 ^{a}	0.75±0.01 ^{a}	0.93±0.01 ^{a}	0.12±0.01 ^{a}

Ameliorative effect of Si, however, was not significant for roots under low salt concentration and for stem under both applied salinity levels (Fig. 1).

Leaf concentration of Chl a increased by low but not high salt concentration in the absence of Si, while increased by both salinity levels in Si-treated plants. Si supplementation resulted in higher Chl a, b and Car in control as well as in salt-affected plants. Concentration of anthocyanins increased by salinity treatments, while Si did not affect it significantly. Leaf concentration of flavonoids was not influenced either by salt or Si addition (Table 1).

Chl fluorescence parameters were not influenced by salt or Si treatments significantly (Table 2). In contrast, leaf gas exchange parameters were affected by both applied treatments (Fig. 2). Net assimilation rate (A) remained stable under salt treatments, while transpiration rate (E) and stomatal conductance (g_s) both were reduced by higher salt concentration significantly (Fig. 2). Supplementation of plants with Si caused a consistent increase of all three gas exchange parameters, slightly or significantly. In the absence of salt net assimilation rate was significantly higher in Si-treated plants, while it was less affected by Si in salt-treated ones. In plants grown under higher salt concentration, Si addition increased significantly transpiration rate and stomatal opening of leaves (Fig. 2).

Na concentration of all three plant fractions increased continuously by increasing salt concentration in the medium irrespective to the Si treatment. Applied Si reduced slightly Na concentration, the significant effect, however, was observed only for stem Na concentration under high salt concentration (Table 3).

In contrast to Na, K concentration was continuously decreased by both applied salinity levels in all three plant fractions without Si addition. In Si-supplemented plants, K concentration was higher as compared with their –Si counterparts irrespective to the salinity treatment. This effect was observed in the leaves and stem more pronouncedly than in the roots (Table 3). On the other hand, in Si-supplemented plants leaf and stem K concentration was increased with increasing salt concentration (Table 3).

Ca concentration decreased in the leaves upon salinity treatments in both –Si and +Si plants. Si-treated plants, however, had significantly higher Ca concentration compared with their –Si counterparts irrespective to salinity treatment. In the stem, salt treatment did not affect Ca concentration in the –Si plants. Similar with the leaves, Si application increased stem Ca concentration. Similar with the leaves, Ca concentration declined by salinity treatment in the roots, however, in contrast to the leaves and stem, Si-treated plants had lower Ca concentration in the roots compared with their –Si counterparts (Table 3).

The ratio of K:Na was greatly decreased by both salt treatments in all three plant fractions, however, comparison of means using t-test revealed that this ratio at each level of salt was higher in +Si plants as compared with –Si counterparts (Table 3).

Concentration of soluble sugars in the leaves, but not in the roots, was steadily increased by increasing salt

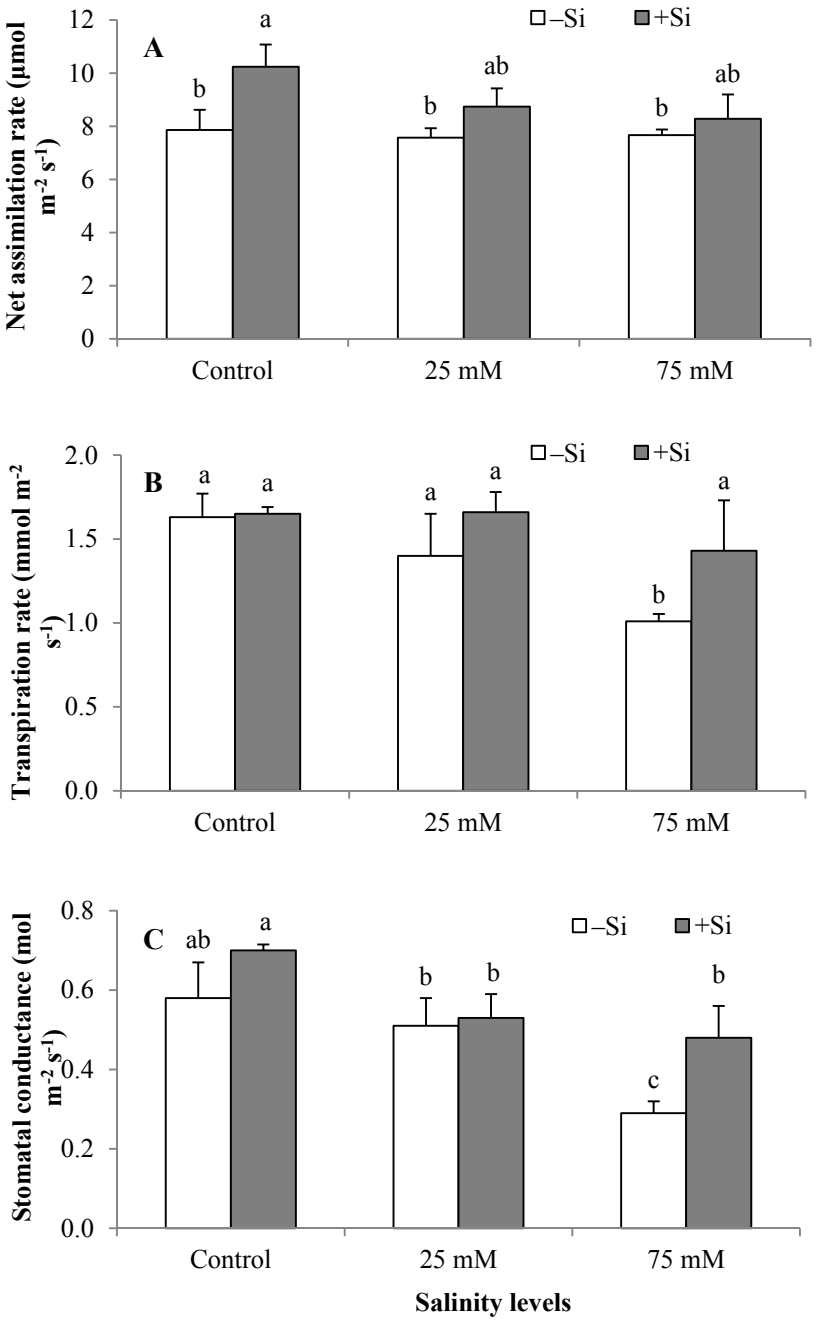

Figure 2. Gas exchange parameters including net assimilation rate (A), transpiration rate (B) and stomatal conductance (C) in the leaves of tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si) or presence (+Si) of 1 mM Na_2SiO_3 in hydroponic medium. Bars indicated by the same letter are not significantly different ($P<0.05$).

concentration in the medium (Fig. 3). Si treatment increased further leaf soluble sugar concentrations, so that the highest level was found in the leaves of plants treated with 75 mM salt and supplemented with Si. In the roots, in contrast, reduction of soluble sugars was observed in plants under higher salt concentration and Si did not affect it (Fig. 3).

Concentration of free proline increased by salt being significant at high salinity level in the leaves and roots. Si treatment, however, rather decreased free proline concentration in the leaves and roots of salt-affected plants. In the leaves of control plants, in contrast, Si

Table 3. Concentration of Na, K and Ca (mg g^{-1} DW) in the leaves, stem and roots of tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si) or presence (+Si) of 1 mM Na_2SiO_3 in hydroponic medium. Data of Na, K and Ca concentrations within each column followed by the same letter are not significantly different ($P<0.05$). Data of K:Na ratio were compared between –Si and +Si plants using t-test ($P<0.05$).

Treatments		Leaves	Stem	Roots
			Na concentration (mg g^{-1} DW)	
–Si	Control	2±0.4 ^{c}	9±0.9 ^{c}	9±1.5 ^{c}
	25 mM	23±5 ^{b}	24±5 ^{c}	16±2 ^{b}
	75 mM	46±13 ^{a}	54±9 ^{a}	32±6 ^{a}
+Si	Control	1.4±0.5 ^{c}	10±3.7 ^{d}	7±1.1 ^{c}
	25 mM	17±2 ^{b}	21±5 ^{c}	16±7 ^{b}
	75 mM	40±12 ^{a}	34±8 ^{b}	30±2 ^{a}
			K concentration (mg g^{-1} DW)	
–Si	Control	43±2 ^{d}	57±2 ^{c}	51±1 ^{b}
	25 mM	34±4 ^{e}	54±3 ^{cd}	42±1 ^{d}
	75 mM	28±2 ^{f}	49±2 ^{d}	41±2 ^{d}
+Si	Control	52±3 ^{c}	66±2 ^{b}	57±3 ^{a}
	25 mM	61±1 ^{b}	80±6 ^{a}	52±2 ^{b}
	75 mM	72±2 ^{a}	78±1 ^{a}	46±3 ^{c}
			Ca concentration (mg g^{-1} DW)	
–Si	Control	69±3 ^{b}	17±2 ^{c}	16±2 ^{ab}
	25 mM	31±2 ^{c}	18±1 ^{c}	18±3 ^{a}
	75 mM	32±6 ^{c}	17±6 ^{c}	10±1 ^{c}
+Si	Control	102±9 ^{a}	29±1 ^{b}	11±1 ^{c}
	25 mM	63±12 ^{b}	40±6 ^{a}	13±3 ^{bc}
	75 mM	62±3 ^{b}	29±4 ^{b}	11±2 ^{c}
			K:Na	
–Si	Control	19±2.4 ^{b}	6.5±0.5 ^{a}	7.7±1.4 ^{a}
	25 mM	1.5±0.5 ^{b}	2.3±0.5 ^{b}	2.7±0.3 ^{b}
	75 mM	0.6±0.2 ^{b}	0.9±0.2 ^{b}	1.3±0.2 ^{a}
+Si	Control	45±14 ^{a}	7.1±0.3 ^{a}	6.9±1.3 ^{a}
	25 mM	3.2±0.4 ^{a}	3.6±0.5 ^{a}	3.9±0.7 ^{a}
	75 mM	1.9±0.5 ^{a}	2.4±0.6 ^{a}	1.6±0.2 ^{a}

treatment increased free proline concentration (Fig. 3B).

Results of two-way ANOVA revealed that the majority of parameters determined in this work were influenced by both salt and Si treatments with the exception of Chl fluorescence parameters. The lack of interaction between these two factors on plant growth parameters emphasized that effect of Si was not limited to the stress conditions (Table 4).

Discussion

Plants growth under different salt concentrations and Si supplementation

Tobacco is a salt-sensitive glycophyte species. Salt tolerance of crop plants is typically expressed as reduction of yield associated with a given level of soil salinity i.e. threshold salinity, as compared with yield under non-saline conditions [41]. According to this method, crop species are classified into salt-sensitive, moderately-sensitive, moderately-tolerant and tolerant species [41]. The yield of sensitive crop species starts to decline at a soil electric conductivity (EC) of 3 dSm^{-1} (30 mM NaCl) compared with3-6 dSm^{-1} for moderately-sensitive, 6-8 dSm^{-1} for moderately-tolerant and >8 dSm^{-1} in tolerant species [41]. In this work, reduction of growth was observed for leaves at salinity level of 25 mM. Under such low salinity condition, dry matter of leaves was depressed significantly up to 30% while stem and roots did not respond to this salinity level. It indicated that leaves were the most susceptible plant part in tobacco and even low salt concentrations may depress the yield of leaves and thus, production of this non-food forage crop. Higher sensitivity of leaves was likely resulted from reduction of leaf expansion under lower water potentials. Positive turgor is necessary for expansion growth of cells, thus, plants respond immediately to osmotic stress as reduction of the rate of leaf expansion [35]. In agreement with this, leaf area and leaf expansion rate was the most sensitive parameter to salt in tobacco. From the two components of salt stress, i.e. hyperosmotic effect and disturbance of ionic equilibrium, the former is the main reason for growth reduction under lower salt concentrations [16].

High salt concentration, in contrast, impaired growth

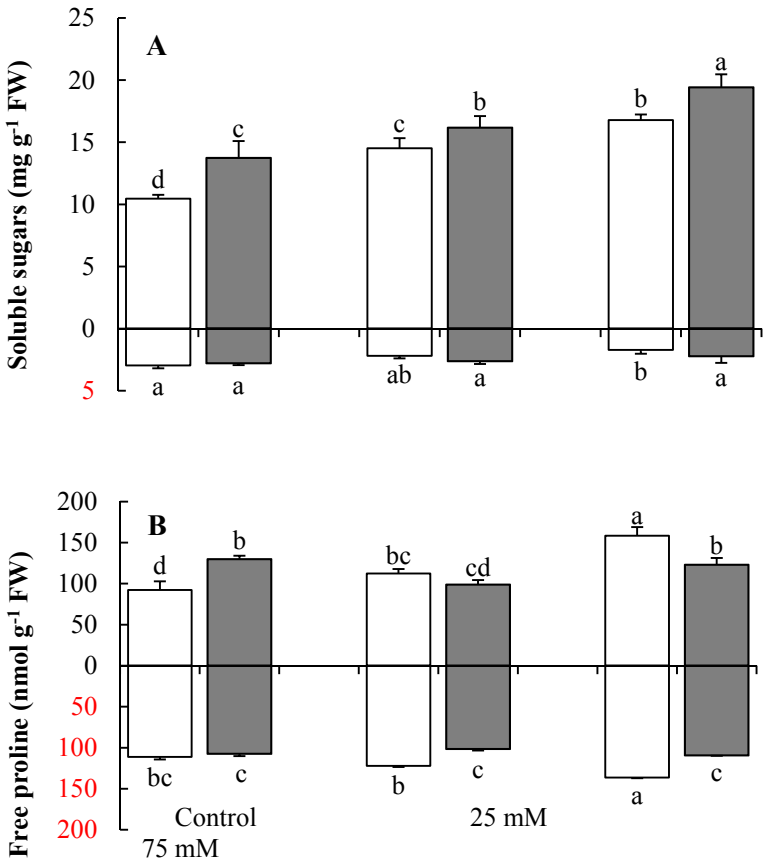

Figure 3. Concentration of total soluble sugars (A) and free proline (B) in the leaves (above of the horizontal axis) and roots (below of the horizontal axis) in tobacco (*Nicotiana rustica* L.) plants grown for two weeks under different levels of NaCl in the absence (–Si, open bar) or presence (+Si, dark bar) of 1 mM Na_2SiO_3 in hydroponic medium. Bars within each plant organ indicated by the same letter are not significantly different ($P<0.05$).

of all three plant parts and decreased dry weight of leaves, stem and roots up to 58%, 82% and 62%,respectively, indicating again higher susceptibility of this crop species to salt. Depression of plant growth under higher salt concentrations is related to toxic effects of Na ions and ionic disequilibrium but the threshold tissue Na concentration is dependent on plant species [16]. In this work Na concentration of about 40-50 mg g^{-1} DW was apparently toxic for tobacco as could be judged by significant growth reduction of plants. Salt concentrations higher than 75 mM caused death of plants in this work.

Silicon supplementation improved plants growth significantly regarding biomass of all three fractions not only under salinity but also under control conditions. Tobacco is not classified as a Si accumulator species [4] and its positive response to Si supplementation under optimum growth conditions has not been reported so far. Under saline conditions, in contrast, beneficial effect of Si on plants growth observed in this work was in agreement with the results obtained for other non-accumulator species such as tomato [39]. The possible involving mechanisms for Si effect under non-stress conditions as well as Si-mediated alleviation of salt stress in tobacco will be discussed later in this section.

Leaf pigment concentrations, photochemistry and gas exchange as affected by salt and Si supplementation

Leaf Chl a concentration was higher under low salt concentration that may be attributable to the concentration effect due to the higher reduction of leaf area compared with less inhibition of Chl synthesis. Under higher salt concentration, however, reduction of Chl a concentration surpassed apparently that of leaf growth and thus, its reduction was obvious. Two possible mechanisms are involved in the effect of salt

Table 4. Results of two-way ANOVA test (mean of squares) for the effect of different treatments including salinity (S) and silicon (Si) and their interactions (S×Si) on various physiological parameters in tobacco plants.
*** P<0.001, ** P<0.01, * P<0.05, ns: non-significant, according to Tukey test.

Parameters	S	Si	S×Si	Parameters	S	Si	S×Si
Leaf DW	2.622 ***	1.21 ***	0.084 ns	g_s	0.129 *	0.055 *	0.0207 ns
Stem DW	0.387 ***	0.052 *	0.014 ns	Leaf Na	3448 ***	113 *	18.44 ns
Root DW	0.043 ***	0.027 ***	0.004 ns	Stem Na	2405 ***	303 **	248 **
Chl a	0.276 ***	7.09 ***	0.030 ***	Root Na	1079 ***	1.84 ns	8.98 ns
Chl b	0.007 *	0.431 ***	0.021 ***	Leaf K	10.57 *	4314 ***	555 ***
Carotenoids	0.004 *	0.271 ***	0.007 *	Stem K	12.32 *	2312 ***	203.21 **
Anthocyanins	34.83 ***	9.70 ns	1.68 ns	Root K	235.59 ***	288.6 ***	17.91 ns
Flavonoids	0.00 ns	0.084 ns	0.013 ns	Leaf Ca	3724 ***	6789 ***	21.95 ns
Fv/Fm	0.00 ns	0.00 ns	0.00 ns	Stem Ca	90.1 **	1377 ***	74.92 *
F'v/F'm	0.00 ns	0.00 ns	0.00 ns	Root Ca	66.58 ***	27.22 *	18.59 *
qP	0.00 ns	0.00 ns	0.00 ns	Leaf sugars	70.09 ***	36.35 ***	1.081 ns
qN	0.00 ns	0.00 ns	0.00 ns	Root sugars	0.187 ***	0.718 **	0.097 ns
A	2.65 **	11.58 ***	1.652 *	Leaf proline	54.82 *	3074 ***	2644 ***
E	0.374 ***	0.320 **	0.076 ns	Root proline	444 ***	1752 ***	275 ***

on reduction of leaf Chl concentration include enhancement of degradation as the consequence of oxidative damage [33] and reduction of nitrogen uptake [30] as the most important leaf mineral component for Chl synthesis [17] in salt-affected plants.

Leaf anthocyanins concentration, in contrast, was higher under both salinity levels implied likely higher synthesis in salt-affected plants. Anthocyanins are important players in the antioxidative defense of leaves under stress conditions [5]. Under salt stress, elevated levels of leaf anthocyanins have been reported in plants species such rice [7] and tomato [3].

Si-supplemented plants had higher Chl a, b and Car. Higher pigments concentration of Si-supplied plants has been also reported by other authors [45]. This may be the result of lower destruction of pigments as the consequence of higher protection of leaves against reactive oxygen species generated under salt stress. It has been observed that activity of antioxidative enzymes and concentration of related metabolites increased in Si-treated plants and membrane damage is mitigated considerably [21, 27]. Enhancement of leaf Car upon Si treatment may be of great importance to salt tolerance of plants. Under stresses such as drought and salinity the imbalance between photosynthetic light capture and NADPH utilization in carbon fixation may lead to excess excitation energy and damage to photosynthetic apparatus [16]. Heat dissipation of excess light energy mediated by Car is an effective mechanism for quenching excess photons and protecting leaves from damaging effects of excess excitation energy [34].Unexpectedly, leaf Chl fluorescence parameters were not influenced by salt or Si. It may suggest that changes of these parameters were under detection limit in this work and/or these parameters were not the critical components of leaf photosynthesis in tobacco plants under salinity.

In contrast, leaf gas exchange parameters were apparently the major factors in plants response to both salt and Si application. Salt at higher concentration depressed stomatal opening that caused in turn lower transpiration rate. Salinity affects stomatal opening immediately, firstly because of perturbation in the water relations and afterward due to the synthesis of ABA [35]. In consequence, salinity may restrict net assimilation rate primarily because of reduction of CO_2 supply due to a partial stomatal closure. In tobacco, however, reduction of stomatal conductance was not apparently effective in depression of CO_2 fixation rate, likely because of sufficient internal CO_2concentration. The ratio of C_i/C_{an} (ratio of internal CO_2 to reference CO_2) was changed slightly from 0.92±0.03 in control to 0.87±0.01 in plants treated with 75 mM salt (data not shown). Nevertheless, regarding a great depression of leaf area in salt-treated tobacco plants, a significant decline of net CO_2 fixation is expected on a whole-plant basis.

Leaf stomatal conductance was higher in Si-treated

tobacco plants similar with the reports on non-accumulator species such as tomato [39]. This effect may be the result of an improvement in plants water status (see below) and/or enhancement of the activity of proton pumping and K^+ currents in the guard cells. Higher plasma membrane H^+ATPase (25) and tonoplast V-ATPase and V-PPiase activities [24] has been reported in barley plants under Si treatment.

Following elevation of stomatal conductance, transpiration rate increased in Si-treated plants being significant under 75 mM salt. Contrastive response to added Si was reported in Si-accumulator species likely because of Si deposition in the leaf epidermis that reduces cuticular transpiration and water loss [31]. Leaf Si concentration in tobacco has been reported to be in the range of 100-450 μg g^{-1}DW [47], much less than the foliar concentrations found in accumulator species (20-40 mg g^{-1}DW) with epidermal Si depositions [4]. Similar with tobacco, higher water loss has been reported upon Si treatment in tomato plants [39]. However, in tomato simultaneously higher photosynthesis rate resulted in higher water use efficiency (the ratio of photosynthesis:transpiration) in Si-supplemented plants [39]. Here water use efficiency increased by salt (75 mM) from 4.86±0.99 to 7.57±0.49 in –Si plants while reduced from 6.19±0.21 to 5.63±0.51 in +Si ones (data not shown). Our data indicated that effect of Si on the alleviation of salt stress in tobacco under high salt concentration was not mechanistically related either to reduction of water loss or to elevation of water use efficiency. It may imply also that Si-treated plants had higher ability for water uptake likely because of greater root surface area. A significant increase of tobacco root biomass by Si application (Fig. 1C) may confirm this suggestion.

Moreover, an improvement in plants water uptake may enable them to have more open stomata and thus, to reach higher photosynthesis rate. In agreement with this, under control and low salinity levels, Si effect on the stomatal conductance was well correlated ($r=0.85$, $P<0.05$) with that of CO_2 fixation rate, suggesting the fundamental role of stomata-related mechanisms in the enhancement of photosynthesis in Si-supplemented plants. It could be speculated that, higher salt tolerance in Si-supplemented tobacco plants was closely linked to Si effect on stomatal opening, thus, maintaining CO_2 fixation under salt and providing plants with carbohydrates for osmotic adjustments and dry matter production.

Under high salt concentration, however, an about 66% increase in stomatal opening was not associated with correspondingly higher net CO_2 fixation indicating likely involvement of limiting factors, i.e. metabolic impairment. Biochemical limitation of photosynthesis is likely in turn, resulted from Na toxicity and ionic imbalance [6, 38] as the common response of salt-sensitive glycophytes to higher salt concentrations [16]. Expression of numerous genes and activity of several enzymes related with photosynthesis are depressed under these conditions [6]. Ion cytotoxicity is caused by replacement of K^+ by Na^+ in biochemical reactions and conformational changes and loss of function of proteins [6]. Although ameliorative effect of Si on tobacco leaf photosynthetic activity was not observed under high salt concentration and plants had higher water loss under these conditions, dry weight of shoot organs and roots increased by Si slightly or significantly. It implies function of additional mechanisms for growth amelioration by Si under high salinity level (see below).

Organic osmolytes and ion relations as affected by salt and Si supplementation

In the leaves, higher soluble carbohydrates and free proline concentrations were observed in salt-affected plants. In contrast to the leaves, root soluble carbohydrates concentration was not higher in salt-treated plants while proline was equally increased in the leaves and roots by salinity. It indicated different importance of these organic osmolytes for the osmotic adjustment of the leaves and roots. In plants subjected to salt, in order to accommodate the ionic balance in the vacuoles, cytoplasm accumulates low-molecular-weight compounds, i.e. compatible solutes because they do not interfere with normal biochemical reactions [36]. Accumulation of organic osmosolutes protects structures and osmotic balance and supports continued water influx [16, 35]. It has been suggested that the majority of carbonand nitrogen assimilated under salt conditions are allocated to the osmotic purposes [16].

Si addition resulted in further increase of soluble carbohydrate in the leaves. This effect in the roots was significant only under high salt concentration. Higher net assimilation rate observed in Si-treated plants may provide excess reduced carbon sources for synthesis of soluble sugars. Soluble carbohydrates such as glucose, fructose, sucrose, fructans accumulate under salt stress and play a leading role in osmo-protection, osmotic adjustment, carbon storage, and free radical scavenging [16]. Sugars contribute up to 50% of the total osmotic potential in glycophytes subjected to saline conditions [1].

In contrast to carbohydrates, proline concentration rather lowered by Si application in the leaves and roots of salt-affected plants. Lower proline content in Si-treated plants implied likely that they were less affected by osmotic stress and thus, needed lower proline

concentrations compared with –Si counterparts. It has been proposed that accumulation of proline in plants grown under salt stress was due to salt injury and not as an indication of salt tolerance [28]. In two sorghum genotypes contrasting in salt tolerance proline accumulation was a reaction to salt stress and not a plant response associated with tolerance [8]. Higher concentration of proline was accumulated in sensitive rice cultivars than in tolerant genotypes under salt stress [28]. In view of these reports, the role of proline in salt tolerance and its use as a selection criterion in crop species has been questioned [1].In tobacco, regarding a constitutively low proline concentration, i.e. in the nanomolar range, its role as an organic osmoticum does not seem to be a critical component of plant response to added Si. Similar with our results, reduction of proline concentration in salt-stressed plants after treatment with Si has been reported for wheat [45] and grapevine [43].

In contrast, function of proline as free radicals scavenger was considered its main physiological role under salt stress in some species [30, 44]. Here, control plants without salt had higher proline concentration in the leaves that may contribute to the enhancement of plant defense capacity against free radicals. Beneficial elements are effective in the improvement of plants growth even under non-stress conditions because of activation of antioxidative defense that could mitigate effect of latent stress factors [4, 15].

Concentration of Na was diminished only slightly by Si treatment. In contrast, K concentration was significantly higher in Si-supplemented plants as compared with –Si counterparts. In Si-treated plants, concentration of K in the stem and particularly in the leaves was rather increased with increasing salt concentration (Table 3). It is obviously a concentration effect as the consequence of lower biomass in salt-treated plants in the absence of reduction of K content.

Si supplementation resulted in a significant increase of K:Na ratio, even though it remained much less than control plants. Because of toxic effects of Na on cell metabolism, the maintenance of K and Na homeostasis is important for the activities of many cytosolic enzymes and for maintaining membrane integrity [20]. The K:Na ratio is an important criterion for salt tolerance of plants and maintenance of photosynthesis and other metabolic processes is mainly dependent on the higher K:Na ratio in the salt-affected plants [36]. Higher K uptake upon Si treatment may be the result of an improved membrane integrity and selectivity process in Si-supplemented plants. Higher defense capacity against reactive oxygen species in Si-treated plants that have been reported in some plant species [21] is also highly probable in our experimental plants. In contrast to our results, differences were not observed in foliar content of K and Ca or K:Na ratio in salinized tomato plants treated with Si [39]. In wheat as a Si-accumulator species, however, reduction of Na in the leaves and roots and increase of K was observed by Si [45].

The same effect of Si was found on Ca concentration in the leaves and stem but not in the roots. Higher Ca concentration in the shoot organs of Si-treated plants may be in turn another mechanism for improved membrane integrity and higher K:Na selectivity [16]. Elevated transpiration rate may be responsible for higher shoot Ca concentration in Si-treated plants regarding the well-known dependency of Ca transport on transpiration rate [17]. This could be confirmed by correspondingly lower root Ca concentration in Si-treated plants compared with their –Si counterparts. The latter indicated also that only root-shoot Ca transport but not root uptake was affected by Si.

Conclusion

Si-mediated alleviation of salt stress effects in tobacco plants was related to the higher photosynthesis rate, an improved osmotic adjustment because of higher concentration of organic osmolytes and higher K, Ca and K:Na concentration ratio in Si-supplemented plants.

Similar mechanisms were involved in the effect of Si on plants growth under non-stress conditions. Elevated levels of CO_2 fixation rate that may support plants dry matter production, higher uptake of K and Ca and likely other nutrients and higher proline synthesis as an antioxidative compound, were mechanisms for growth enhancement in Si-treated plants in the absence of salt.

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