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Research Article

Effects of Silicon on membrane characteristics, photosynthetic pigments, antioxidative ability, and mineral element contents of faba bean (*Vicia faba* L.) plants grown under Cd and Pb stress.

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Abstract

In a previous study, we found that the application of silicon (Si) was beneficial in improving germination, growth and yield of faba bean (*Vicia faba* L.) plants under heavy metal (HM) stress. In the current study we try to clarify the mechanism that might be involved in the ameliorating effects of Si on faba bean plants grown under different levels of Cd and Pb. The effect of Si on cell membrane stability, photosynthetic pigments, carbohydrate contents, antioxidant enzyme activities, free proline and mineral elements were investigated in plants grown in soil supplemented with different Cd and Pb concentrations (0, 5, 50 and 100 mM for Cd and 0, 50, 500 and 1000 mM for Pb) and were sprayed with Si (in the form of Na2SiO3) at concentrations of 0, 1, 2 and 3 mM. The experiment was arranged in a factorial design with 5 replications. The HMs caused an increase in proline content and in some enzyme activities, Chl a, b and carotenoids were decreased at relatively high levels of Cd and Pb. Application of Si caused a significantly increase in the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) in plant leaves as compared to unstreated plants. Enhanced antioxidant activities helped to decrease oxidative damage and develop tolerance against HM stress in Si-treated faba bean plants. An increase in the degree of HM tolerance induced by silicon was indicated by the improvement of the membrane stability index, photosynthetic activity and consequently the carbohydrate pool. The data provided evidence that silicon treatment reduced the adverse effects of heavy metal stress on faba bean plants, and might play a key role in providing stress tolerance by stimulation of the antioxidant system as a stress protection mechanism.

Keywords: heavy metal, silicon, *Vicia faba*, antioxidant enzymes.

Introduction

Faba bean (*Vicia faba* L.) is widely grown in many regions as a source of protein for both human and animal nutrition (Crepon *et al.*, 2010). It is also traditionally used as a cover crop to recover nitrogen content and prevent erosion of the soil, and is appreciated for its good agronomic characteristics (Kopke and Nemecek, 2010). The nutritional value of faba bean has been attributed to its high protein content, which ranges from 25% to 35%. The seeds are also a good source of sugars, minerals and vitamins; being particularly rich in calcium and iron, and the contents of thiamin, tocopherols, niacin and folic acid are high as compared with other grains (Hassanein *et al.*, 2012). In Western countries, faba bean is one of the main sources of protein and energy for most poor people who consider faba bean is a good alternative to expensive meat and fish protein. In addition, cultivation of faba bean leads to an increase in the concentration of soil nitrogenous compounds (Hungria and Vargas, 2000). Growth and metabolism of faba bean plants are affected by many abiotic stresses such as salinity and water deficit.

Heavy metal stress is one of the major abiotic stresses that cause environmental pollution in recent decades (Castro *et al.*, 2011). These metals are unlike other

organic pollutants are not degraded and converted into harmless compounds via biological processes. Heavy metals persist for a long time in the environment. In addition, heavy metals can enter into the food chain. A common feature of environmental stress is their ability of production of toxic oxygen derivatives (Chiban *et al.*, 2011). Heavy metals make a significant contribution to environmental pollution as a result of human activities such as mining, smelting, electroplating, energy and fuel production, power trans-mission, intensive agriculture, sludge dumping and military operations (Nedelkoska and Doran, 2000). However, elevated concentrations of both essential and non-essential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants (Li *et al.*, 2010). Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure, or from displacement of an essential element, resulting in deficiency effects (Capuana, 2011). In addition, a heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Li *et al.*, 2011). Detailed studies indicate that heavy metals have effects on chlorophyll content in plants. Heavy metals are known to interfere with chlorophyll synthesis either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient (Meers *et al.*, 2010).

Some plant species have capacity to grow in the metal contaminated soil and accumulate elevated amount of heavy metals as an eco-physiological adaptation in metaliferous soil. Some legume species such as *Vicia faba* and Phaseolus vulgaris has been reported to be a good accumulator of lead and cadmium (Zhang *et al.*, 2010; Aldoobie and Beltagi, 2013). The mechanisms involved in heavy metal tolerance may range from exclusion, inclusion and accumulation of heavy metals depending on the plant species (Kaushik *et al.*, 2005). Distinct concentrations of metals induce different biochemical responses in plants. In sensitive plants, high concentration of these metals inhibits enzymes involved in photosynthetic reaction (Smirnoval *et al.*, 2006). Brassica juncea (Indian mustard), a high biomass producing plant can accumulate lead, chromium, cadmium, copper, nickel, zinc, boron and selenium (Palmer *et al.*, 2001; Akbaş *et al.*, 2009). Even trace elements have been shown to have toxic effect on different plant traits such as leaf, stem, root flower etc. (Sivakumar *et al.*, 2001). Cadmium and lead are common heavy metals in industrial discharge of aeronautic, metal and metallurgy, and refinery industries and show toxic effects on plants and animals. Previously, the Cd and Pb concentration in Saudi soils and water was usually low but it has increased during the last decade because of heavy industrialization. Among the pollution-producing metals, lead is a widespread heavy metal in the environment and it is regarded as non-essential elements and have a long half-life which is extremely persistent in the environment (Aldoobie and Beltagi, 2013), with high toxicity and easily taken up by plants (Wua *et al.*, 2003) and then enters the food chain, resulting in a serious health issue for animals and humans.

Based on current knowledge, Liang *et al.* (2006) concluded that Si is not inert, but acts as a physical or mechanical barrier in plants. It is not only deposited in the cell walls, but is also actively involved in the metabolic and/or physiological activities, especially in plants subject to environmental stresses (Liang *et al.*, 2006). Generally, the ameliorative effect of silicon sources varies genotypically among plant species (Kulikova and Lux 2010) and hence, we focus on the effect of silicon and its influence on faba bean physiology and metabolic defense compounds. In our previous study on the effect of silica Si in faba bean reveal improved growth parameters and increased seed germination attributes but the effect of Si on faba bean physiological components in plants is essential to gain the detailed functional properties of mineral fertilizers. Determination of essential regulatory and defense compounds in faba bean such as antioxidant system compounds is necessary to ascertain the biotic and abiotic stress tolerance mechanisms adapted through silica fertilization. Consequently, silicon-mediated alleviation of abiotic stress may substantially contribute to the adaptation of faba bean (Miao *et al.* 2010). The objective of the current study was to examine the utility of silicon, as a promising plant development regulatory substance to increase the tolerance of faba bean plants against heavy metal stress and to clarify the mechanism that might be involved in the ameliorating effects of Si on faba bean plants grown under HM stress conditions.

Materials and Methods

Pot experiments were carried out in the greenhouse during winter season to investigate the mechanisms

that might be involved in the ameliorating effects of Si on faba bean plants grown under Cd and Pb stress conditions. The effect of Si on cell membrane stability, photosynthetic pigments, carbohydrate contents, antioxidant enzyme activities, free proline and mineral elements were investigated in HM stressed and non-stressed faba bean plants. The experiments were arranged in a factorial design with 5 replications at Cd levels of 0, 5, 50 and 100 mM or Pb levels of 0, 50, 500 and 1000 mM and Si concentrations of 0, 1, 2 and 3 mM.

Preparing and sowing of seeds

Seeds of faba bean (cv. RM) were obtained from authorized agriculture company and were sterilized with 10% sodium hypochlorite solution for 10 minutes, washed three times with distilled water, and coated with N-fixing bacteria (*Rhizobium leguminosarum*) using Tween 20 agent as an adhesive and scattering material. Identical seeds were then sown in plastic pots (30 cm inner diameter) filled with 10 kg sandy soil artificially polluted with Cd or Pb. Physical and chemical properties of the soil used in the study were recorded in Table (1). After sowing, irrigation was applied to supply seedlings with 100% available water, at two-day intervals until the seedlings reached the third leaf stage. Seedlings were then thinned to 3 plants/pot and pots were divided into two main groups for Pb and Cd treatments, with each group divided into three subgroups for Si foliar application. Plants were fertilized with Sangeral complete fertilizer (Sinclair Horticulture LTD, England), in two equal portions; the first during the seedling stage and the second at the start of flowering stage. The fertilizer consists of macro elements, total nitrogen 20% N (4.4% Ammonia - 5.8% Nitrate - 9.8% Urea), Phosphorus (20% P2O5), Potassium (20% K2O), Mg (0.012%) Sulphur (0.04%), and microelements (as ppm) Fe (70), Zn (14), Cu (13), Mn (13), B (12) and Mo (12))

Table 1. Physical and chemical analyses of the soil used in the experiment.

<table>
<tr><td>Physical properties</td><td colspan="2">Chemical properties</td></tr>
<tr><td rowspan="3">Particle size distribution:
Sand (%) 92.3
Silt (%) 6.2
Clay (%) 1.5
Soil texture (Sandy)</td><td>CaCO3 (%) 0.41
OM (%) 0.26
ES (dSm^{-1}) 0.53</td><td>Soluble anions (meq/l)
CO32- 0.22
HCO3- 0.86
Cl- 1.83</td></tr>
<tr><td rowspan="3">Soluble cations (meq/l)
Ca2+ 2.96
Mg2+ 1.68
Na+ 2.04
K+ 0.21</td><td rowspan="3">Avail. elements (mg/kg)
N 19.2
P 8.3
Fe 2.4</td></tr>
<tr><td></td></tr>
<tr><td>pH 8.01</td></tr>
<tr><td colspan="3">Soil suspension (1 soil : 2.5 water)</td></tr>
</table>

At the third leaf stage, and two weeks after then, foliar application of Si (as Na_2SiO3) in concentrations of 1, 2, 3 mM was performed using a small pressure pump after adding Tween 20 (0.5%) as a wetting agent. The experiment consisted of 28 treatments (2 heavy metal treatments in 3 concentrations each with 3 Si treatments and water as control) and arranged in a factorial completely randomized design with 5 replicates for each treatment to make a sum of 140 pots.

2- Measurements

Three weeks after the second application of Si (about 100 days after germination), three replicates were taken from each treatment, and the following parameters were measured:

a) Determination of membrane characteristics

Lipid peroxidation (LP), electrolyte leakage (EL), and memberane stability index (MSI) were determined as follows:

Lipid Peroxidation

Lipid peroxidation was determined by measuring the amount of MDA according to Unyayar *et al.* (2006). About 0.5 g of leaf tissues from control and treated groups were cut into small pieces and homogenized by the addition of 5 ml of 5% trichloroacetic acid (TCA) solution. The homogenates were then transferred into fresh tubes and centrifuged at 12,000 rpm for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid (TBA) in 20% TCA solution were added into a new tube and boiled at 96 oC for 25 min. The tubes were transferred into ice-bath and then centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm, 0.5% TBA in 20% TCA solution was used as the blank. MDA contents were calculated using the extinction coefficient of 155 M^{-1} cm^{-1}. Values of MDA contents were taken from measurements of three independent samples, and SD of the means were calculated.

Electrolyte leakage

Ion leakage was determined as electrical conductivity (EC%) according to **Hassanein** *et al.* (2012). Leaf samples were cut into discs of uniform size and placed in 10 ml of double-distilled water at 40°C for 30 min, and its conductivity recorded (C1) using conductivity meter (Jenway 470 portable conductivity meter). Then it was kept in a boiling water bath (100°C) for 15 min and its conductivity also recorded (C2). The percentage of electrolyte leakage was calculated according to this formula: EC (%) = (C1/C2) × 100. Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

Membrane stability index

The membrane stability index (MSI) was estimated by placing 200 mg of leaves in 10 ml double distilled water in two sets. One set was heated at 40°C for 30 min in a water bath and the electrical conductivity (C1) was measured. The second set was boiled at 100°C in a boiling water bath for 10 min and the conductivity (C2) was measured; both conductivities were measured using a conductivity meter (ME977-C, Max Electronics, India). The MSI was calculated using the formula described by Premchandra et al (1990):

$$MSI = [1 – (C1/C2)] \times 100$$

b) Determination of Photosynthetic pigments

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined spectrophotometrically according to Metzner *et al.* (1965). A known fresh weight of leaves was homogenized in 85% aqueous acetone for 5 min. The homogenate was centrifuged and the supernatant was made up to known volume with 85% acetone and measured against a blank of pure 85% aqueous acetone at 3 wavelengths of 452.5, 644 and 663 nm. Taking into consideration the dilution made, it was possible to determine the concentrations of the pigment fractions (chlorophyll a, chlorophyll b and carotenoids) as *g* /ml using the following equations:

Chlorophyll a = 10.3 E 663 - 0.918 E 644
Chlorophyll b = 19.7 E 644 - 3.87 E 663
Carotenoids = 4.2 E 452.5 - (0.0264 chl a + 0.426 chl b).
Pigments then were calculated on the bases of mg/g fwt.

c) Determination of soluble sugars

Soluble sugar was extracted from dried leaf tissue with 80% ethanol. One gram of the dried tissues was homogenized with 80% ethanol then put in a boiling water bath for 15 minutes. After cooling, the extract was filtered and the filtrate was oven dried at 60^{o}C then dissolved in a known volume of water to be ready for soluble sugars determination. The soluble sugars were determined by the anthrone sulfuric acid method described by Scott and Melvin (1956). Briefly, One ml of the extract was mixed with 9 ml of anthrone sulphuric acid reagent in a test tube and heated for 7 min at 100°C. The absorbance was read spectrophotometrically (Shimadzu, RF-5301PC, Japan) at 620 nm, against a blank containing only distilled water and anthrone reagent.All data were calculated as mg 100 g^{-1} DW of leaves.

d) Determination of free Proline

Free proline content was determined colorimetrically in aqueous sulfosalicylic acid as described by Bates *et al.* (1973). Briefly, lyophilized plant material (0.1 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered

through Whatman #2 filter paper. Two ml filtrate was reacted with 2 ml acid-ninhydrin ($C_9H_6O_4$) and 2 ml of glacial acetic acid ($C_2H_4O_2$) in a test tube for 1 h at 100oC, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15–20 s. After 1 h, toluene was added and absorbance at 520 nm was measured by using spectrophotometer (Shimadzu, RF-5301PC, Japan). The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range 0.5–10 µg ml-1. The proline concentration of the extract was determined from the standard curve and calculated on a dry weight basis.

e) Determination of antioxidant Enzyme activities

Enzyme extraction: The samples were prepared as described by Mukherjee and Choudhuri (1983). Fresh leaf samples were submersed for 5 min in liquid nitrogen.The frozen leaves were kept at -80°C for further analyses. Enzymes were extracted from 0.5 g leaf tissue using a mortar and pestle with 5 ml extraction buffer containing 50 mM potassium phosphate buffer pH 7.6 and 0.1 mM Na-EDTA. The homogenate was centrifuged at 15,000 *g* for 15 min and the supernatant fraction was used to assay for the various enzymes. All steps in the preparation of enzyme extracts were performed at 4°C.

Superoxide dismutase (SOD) was assayed according to Karanlık (2001), by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT. Catalase (CAT) activity was determined by monitoring the disappearance of H2O2 according to the method of Cakmak and Marschner (1992).
Ascorbate peroxidase (APX) activity was determined by measuring the consumption of ascorbate by following absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme required to consume 1mol ascorbate min^{-1} (Cakmak and Marschner, 1992). Glutathione reductase (GR) activity was determined by measuring the enzyme-dependent oxidation of NADPH by following absorbance at 340 nm. One unit of GR activity was defined as the amount of enzyme that oxidized 1 mol NADPH min^{-1} (Cakmak and Marschner, 1992).

f) Determination of nutrient elements

Nitrogen (N), phosphorus (P), sodium (Na), potassium (K), and calcium (Ca) analysis, dried shoot samples were ground to pass a 20-mesh sieve and digested with a mixture of H_2SO_4-$HClO_4$ using microwave energy, a modified technique of Lachica *et al.* (1973).

In a mixture of sulfuric and perchloric acid total nitrogen was determined by the micro-Kjeldahl method (Bremner 1996). For total inorganic phosphorus estimation, 0.5 g of plant material was extracted in 8 ml trichloroacetic acid (6%) and centrifuged for 15 min at 18000 X g. phosphate in supernatant was determined colorimetrically after adding 5 mol sulphuric acid, 2.5% ammonium molybdate and 0.25% 1,2,4-aminonaphtholsulphonic acid solution. After 15 min incubation at 37^{o}C the absorbance was measured at 660 nm (Sacala *et al.*, 2008). For Measurements of Na+ and K+ concentrations, the leaves were dried in 60oC for 48 h. Then 1 gr of leaves was powdered and burned in 560oC to obtain ash then ashes digested in 10 ml of 1N HCL. The concentration of Na+ and K+ in the digested samples was determined using a flame photometer (Model 420, Sherwood, Cambridge, UK) (Yousufinia *et al, 2013).* Calcium was measured on acid-digested samples by atomic absorption spectrophotometry in a Perkin Elmer Analyst 800 (Perkin Elmer Inc.,Wellesley, MA) spectrophotometer equipped with a PE6017 lamp, and measured at 422.7 nm (Tejera *et al.*, 2005).

3- Statistical analysis

The collected data were analyzed statistically using factorial completely randomized design and analysis of variance according to Gomez and Gomez (1984) with the aid of COSTAT computer program. Treatment means were compared using the least significant difference test (LSD) at 5% level.

Results and Discussion

Lipid peroxidation and Electrolyte Leakage:

Electrolyte leakage (EL), lipid peroxidation (LP) and the membrane stability index (MSI) of *V. faba* plants showed different patterns of response when plants were treated with Cd, Pb or Si (Table 2). Application of different concentrations of Cd and Pb heavy metals

caused a significant increase in EL and LP, represented by the amount of Malon dialdehyde (MDA), compared with the control plants. The results illustrated remarkable increase in Malon dialdehyde content and ion leakage level in response to 100 mM of Cd and 1000 mM of Pb, compared to control. Thus, maximum values of EL and LP were recorded in plants exposed to 100 and 1000 mM of Cd and Pb, respectively. However, treatment of the stressed plants with Si caused a significant decrease in the EL and LP compared with those of the reference controls. In contrast, exposure of the plants to Cd and Pb caused a decrease in the MSI. While Si caused significant increases in the MSI of faba bean plants. In this concern, it was obvious that Cd treatments had a more harmful effect on membrane characteristics than Pb, therefore, MSI values of Cd were less than those of Pb treatments.

Membrane damage can be evaluated indirectly by measuring solute leakage (electrolyte leakage) from cells and the MSI (Ali *et al.*, 2008). The stimulation effect of stress on MDA values and EL% might be attributed to injury of plasma membrane. As an active redox metals, HMs are able to induce the overproduction of ROS, such as hydrogen peroxide hydroxyl radical, and superoxide anion directly, which in turn lead to lipid peroxidation and oxidative stress (Zhang *et al.* 2009). HM-caused oxidative damage was demonstrated by Vantová *et al.* (2013) and Dong *et al.* (2014). However, addition of Si alleviated the oxidative stress under HM-treatments, and the influence protected cell membrane from peroxiding and decreased the accumulation of MDA. Many studies reported that Si inhibited the plant from oxidation damage by regulating general mechanisms for cellular redox homeostasis and promoting the transformation of super oxide to H2O and O2 and also by enhancing the H2O2-scavenging enzyme activities (Dong *et al.*, 2014).

Table 2. Effects of silicon (Si) on electrode leakage (EL), lipid peroxidation (LP) and membrane stability index (MSI) of faba bean plants grown under different concentrations of Cd and Pb.

HM treatments	Silicon treatments											
	00	Si1	Si2	Si3*	00	Si1	Si2	Si3	00	Si1	Si2	Si3
	Electrolete leakage (%) (EL %)				Lipid peroxidation (MDA µg/g fwt)				Memberane stability index (MSI %)			
Cont	11.8	11.4	10.5	9.6	6.54	6.35	5.44	4.56	67.3	72.5	85.6	87.8
Cd1	13.2	12.7	11.3	10.6	7.86	7.11	6.23	5.45	63.5	70.6	76.4	80.5
Cd2	16.5	14.2	13.3	11.5	10.12	9.55	8.25	7.11	57.6	64.3	70.5	75.9
Cd3	22.6	21.4	19.6	17.8	13.24	11.76	9.44	8.32	48.9	52.2	68.5	71.4
Pb1	12.1	11.2	10.8	9.9	7.16	6.76	6.14	5.11	65.2	71.8	80.5	83.2
Pb2	14.7	13.6	11.5	10.3	8.54	8.13	7.38	6.65	60.4	68.5	74.3	78.8
Pb3	18.5	17.4	15.2	12.8	10.26	9.67	9.08	7.58	55.7	62.2	70.6	73.5
LSD (5%)	3.25	2.60	2.16	1.35	1.57	1.25	1.16	1.24	4.48	3.66	3.14	4.24

*Si1= 1 mM, Si2= 2 mM, Si3= 3 mM of silicon.

In early studies, Kassab *et al.* (2012) showed that damage of beet cells caused by ROS could induce Lipid peroxidation and consequently Electrolyte leakage. Reduction of MDA levels and EL% in response to Si treatments might be attributed to induction of antioxidant responses that protect the plants from oxidative damage, increased membrane stability and tolerance of plants which in turn enhanced scavenging of harmful free radicals (Sharhrtash *et al.*, 2011; Rubinowska *et al.*, 2014) and elevated Ca uptake that protects the plant from the oxidative damage by silicon treatments (Salwa et al, 2013). On the other side, application of Si could correct the stress-mediated damage to the plasma membrane, as was evident from the significant increase in MSI and the significant decrease in EL of treated plants compared with those of the reference controls. Similar results were obtained by Hamada (1986), who found that brassinolide also modifies membrane structure/ stability under stress conditions. In the present study one of the possible mechanisms for the improved membrane stability in response to silicon treatments was the detected decrease in lipid peroxidation in plants sprayed with Si, compared with untreated plants. Lower lipid peroxidation and higher membrane stability (lower ion leaching) have also

been reported in salt-stressed *Zea mays* (Hassanein *et al.*, 2009) and sugarcane (Gomathi and Rakkiyapan, 2011).

Photosynthetic pigments

The present results showed that the contents of photosynthetic pigments including chl *a*, chl *b* and carotenoids (Table 3), total chlorophyll (Fig.1) were significantly reduced with increasing heavy metal concentration in faba bean plants compared with those of HM-untreated plants in the absence of Si. Data in the table indicated clearly that the 5 mM of Cd and 50 mM of Pb treatments caused a slight increase in chl a and chl b content as compared with salt untreated control (Table 4). This observation was true either with or without Si treatments. At these concentrations of HMs, the most observed increase in total chlorophyll (Chl a + b) content was recorded under Si treatments (Fig. 1). In this regard, Heuer (2005) found that chlorophyll content increased in tomato under low levels of salinity. One reason of that was the thicker leaves produced under salt stress. Increases in leaf thickness tended to compensate slightly for the negative effects on leaf chlorophyll as response of salinity (Longstreth *et al.*, 1984) and heavy metal (Manios *et al.*, 2003) stresses.

Table 3. Effects of silicon (Si) on chlorophyll a (Chl a), chlorophyll a (Chl a) and carotenoids (Carot.) of faba bean plants grown under different concentrations of Cd and Pb.

HM treatments	Silicon treatments											
	00	Si1	Si2	Si3*	00	Si1	Si2	Si3	00	Si1	Si2	Si3
	Chl a (mg/g fwt)				Chl b (mg/g fwt)				Carot. (mg/g fwt)			
Cont	1.35	1.42	1.66	1.72	1.12	1.17	1.19	1.22	0.54	0.67	0.82	0.84
Cd1	1.39	1.45	1.52	1.58	1.14	1.15	1.17	1.19	0.57	0.60	0.66	0.72
Cd2	1.12	1.26	1.44	1.52	0.82	1.01	1.11	1.14	0.62	0.65	0.70	0.75
Cd3	1.06	1.17	1.18	1.22	0.63	0.68	0.84	0.93	0.43	0.46	0.52	0.54
Pb1	1.42	1.48	1.58	1.66	1.14	1.16	1.18	1.21	0.59	0.62	0.68	0.77
Pb2	1.28	1.35	1.41	1.48	1.05	1.08	1.11	1.16	0.65	0.69	0.74	0.75
Pb3	1.16	1.22	1.30	1.36	0.86	0.92	0.97	1.01	0.50	0.56	0.63	0.68
LSD (5%)	0.11	0.12	0.15	0.25	0.37	0.11	0.13	0.12	0.10	0.06	0.11	0.12

*Si1= 1 mM, Si2= 2 mM, Si3= 3 mM of silicon.

Increasing Cd and Pb levels higher than 5 and 50 mM, respectively, caused a progressive and significant decrease in chlorophyll a, chlorophyll b and carotene contents. It was clear that Cd showed more harmfull effect on photosynthetic pigments than Pb. At 1000 mM of Pb, Chla, Chl b and carotene decreased by about 14%, 23% and 7%, respectively, while the corresponding values at 100 mM of Cd were about 22%, 44% and 20%, respectively, as compared with control treatment. The application of Si as foliar spray seemed to alleviate the deleterious effect of HM stress on the chlorophyll content (Table 4). It seems that, at any salt treatment Si resulted in decreasing the harmful effect of salinity stress on chlorophyll content. In this regard, Yuvakkumar *et al.* (2011) found that total chlorophyll content of maize was increased by (13–17%) when treated with Si.

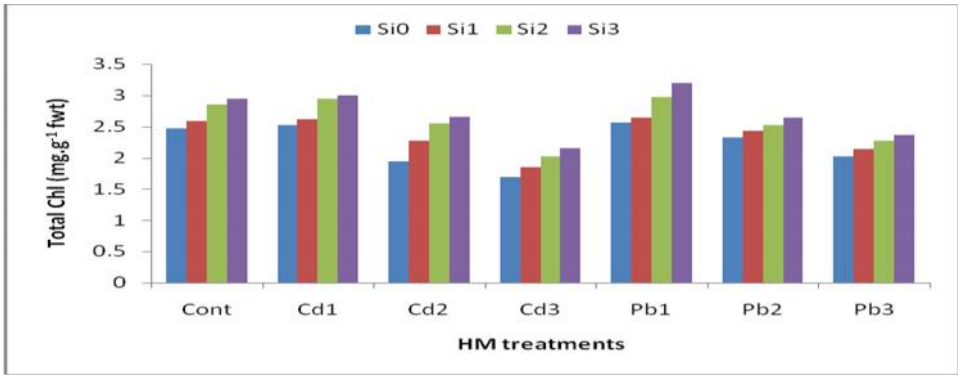

Fig. 1: Effects of silicon (Si) on total chlorophyll (Chl a +b) content of faba bean plants grown under different concentrations of Cd and Pb.

The decrease in chlorophyll content under stress conditions was reported by Vassilev *et al.* (2005), Kusvuran (2010), and Nazarbeygi *et al.* (2011) and might have been due to: (1) inhibition of enzymes associated with chlorophyll biosynthesis (Dong *et al.* 2014); (2) inhibition of uptake and transportation of other metal elements such as Mn, Zn, and Fe by antagonistic effects (John *et al.* 2009). Similar decrease in chlorophyll content under HM stress was reported in *Atriplex halimus* (Brahim and Mohamed 2011) and mangrove plant seedlings (Zhang *et al.* 2007). The results obtained in this study are in agreement with those of Rahimi *et al.* (2012) on fennel and Azooz *et al.* (2013) on *V. faba*. The decrease in chlorophyll content in stressed faba bean plants concomitant with the increase in proline content (discussed later) is consistent with the suggestion that nitrogen might be redirected to the synthesis of proline instead of chlorophyll. In addition, Bajguz (2011) ascribed the suppressed pigment content in HM-stressed plants to increased activity of chlorophyllase or disruption of the fine structure of the chloroplast, as well as instability of the chloroplast membrane and pigment protein complex. In this regard, Sevengor *et al.* (2011) and Emamverdian *et al.* (2015) attributed the reduction in leaf chlorophyll content under heavy metal and salt stress to the destruction of chlorophyll pigments and the instability of the pigment protein complex. **Chakraborty** *et al.* (2015) reported that decreased chlorophyll content associated with heavy metal stress is the result of inhibition of the enzymes responsible for chlorophyll biosynthesis in tomato plants. In this regard, HM was reported to affect chlorophyll biosynthesis and inhibit protochlorophyll reductase and aminolevulinic acid (ALA) synthesis (Appenroth *et al.*, 2010).

It was clear that the application of exogenous Si at moderate concentrations alleviated Cd and Pb-induced decrease in chlorophyll content. The enhancement of uptake and transportation of nutrient elements such as N, P, and K (discussed later) might contribute to chlorophyll synthesis. In addition, Chen *et al.* (2010) detected that stability and integrity of the subcellular structure under Cd or Pb stress contributed to the effective role of some treatments in preventing HM-induced leaf chlorosis and inhibition of photosynthesis in plant seedlings. Our results indicated that Si mediated recovery of chlorophyll contents and may played a role in the enhancement of photosynthesis and transpiration in faba bean, which might be responsible for the increase of Cd and Pb tolerance. Our results showed that application of Si alleviated the damaging effects of Cd and Pb on photosynthetic pigment contents by increasing the membrane stability index MSI (Table 1) compared with those of the reference controls. Moreover, chlorophyll content may be protected because of the high antioxidant enzyme activities that increased with Si treatments which prevented degradation of leaf chlorophyll (Sevengor *et al.*, 2011; Siddiqui *et al.*, 2014). The results showed that Si could stabilize the integrality of chloroplast membrane and protect the chloroplasts from heavy metal stress. Therefore, with Si treatment, total chlorophyll was higher than that of control (Fig. 1). In fact, Si was found to improve structure of chlorophyll and can facilitate formation of pigments (Morteza *et al.*, 2013) and protect chloroplasts from ageing (**Aldoobie and Beltagi, 2013)**.

Soluble sugar contents

The effects of Cd, Pb and Si on total soluble sugars content of *V. faba* plants were shown in (Fig. 2). The results revealed that low concentration of Cd (Cd1 treatment) as well as low and moderate concentrations of Pb (Pb1 and Pb2 treatments) increased the soluble sugar content ; however, higher concentrations of Cd and Pb showed a decrease in soluble sugar content. It was clear that Cd showed more negative effect on sugars than Pb. In this concern Pb3 treatment caused a decrease of about 13% in soluble sugars, while Cd3 treatment caused about 33% in soluble sugars, as compared with control. Early studies by Saleh and Al-Garni (2006) indicated that carbohydrates got inhibited if Cd concentration is more than 5 mg/kg soil. The present results corroborate with the findings of Ahmad *et al.* (2006) who found that an increase in soluble sugars at low concentrations of salt stress and decrease at higher concentrations in *Pisum sativum*. The decrease in total sugar content of stressed leaves probably corresponded with the photosynthetic inhibition or stimulation of respiration rate. The negative effect of heavy metals on carbon metabolism is a result of their possible interaction with the reactive centre of ribulosebisphosphate carboxylase (**John *et al.*, 2008).**

Soluble sugar, is an important constituent manufactured during photosynthesis and breakdown during respiration by plants. All metals have decreased the content with increasing concentration as reported

in agricultural crops (Rascio and Navari-Izzo, 2011). Such inhibition of photosynthesis in higher plants by heavy metals has been reported (John *et al.*, 2008). The low sugar levels may be due to lowered synthesis or diversion of the metabolites to other synthesis processes (Aldoobie and Beltagi, 2013).

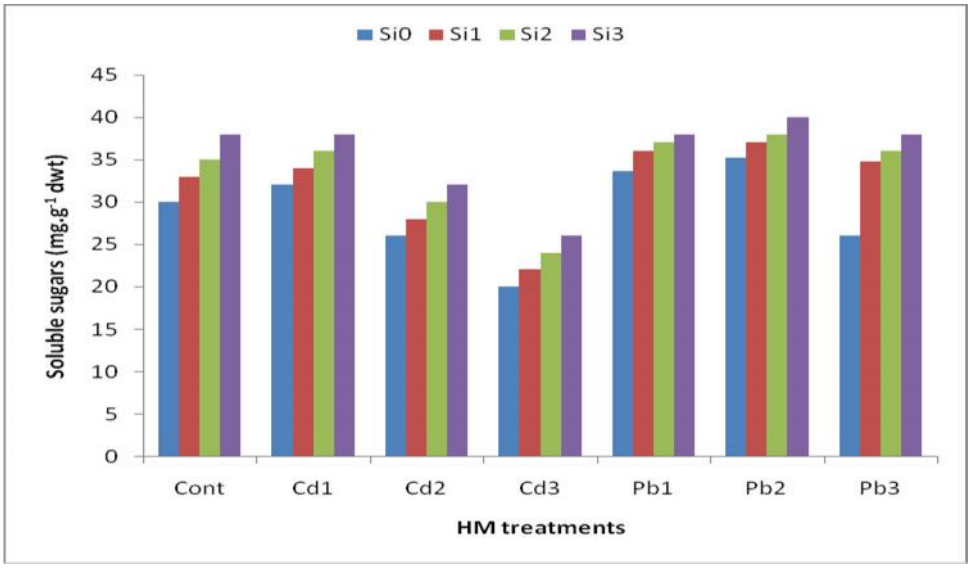

Fig. 2: Effects of silicon (Si) on total soluble sugars in faba bean plants grown under different concentrations of Cd and Pb.

Application of Si resulted, generally, in significant increases in the contents of soluble sugars in leaves of faba bean plants. The maximum total sugar content was estimated at low and moderate levels of Cd and Pb, respectively, particularly with Si3 treatments compared with that of the reference control. In this concern, Si3 treatment resulted in an increase of about 27% and 33% in plants treated with Cd1 and Pb2, respectively, as compared with control.

The inhibition in sugar accumulation in heavy metal-stressed plants was recorded by other authors (karimi *et al.*, 2012; Aldoobie and Beltagi, 2013). The decrease in soluble sugars and photosynthetic pigment contents were directly proportional to the applied concentration of Cd or Pb. These results led to the conclusion that heavy metals may inhibit photosynthetic activity or increase partial utilization of carbohydrates in other metabolic pathways. Application of Si generally stimulated the accumulation of sugars in HM-treated or untreated plants and the inhibitory effects of HM stress were partially alleviated. These findings were in accordance with those of Siddiqui *et al.* (2014). The enhancement effect of silicon on carbohydrate biosynthesis, especially soluble sugars, is considered to be the principle organic osmotica in a number of glycophytes subjected to stress conditions (Hassanein *et al.*, 2012). This effect highlights another possible mechanism by which silicon plays a positive role in alleviation of the harmful effects of HM stress. Subjecting water stressed faba bean plants to silicon synergistically increased the amounts of soluble sugars than in Si-untreated stressed ones which indicated that accumulation of these compounds by silicon plays a key role in retaining the water capacity of stressed cells which thereby can tolerate severe drought and HM stress (Abdalla, 2011; Rubinowska *et al.*, 2014)

Antioxidant enzyme activities

The results presented in Tables (4 and 5) show the effect of Cd and Pb as well as Si treatments on the activities of the antioxidant enzymes SOD, CAT, POD, APX and GR in *V. faba* plants at the vegetative stage. The activities of enzymes SOD, CAT, POD and APX showed progressive increase with increasing Cd and Pb concentrations, whereas the activity of GR significantly decreased with increasing HM concentrations, compared with those of the unstressed plants. Adding Si had significant positive effects on the activity of all enzymes under HM stress (Table 4 and 5). With increasing Si treatments a gradual increase in the activity of SOD, CAT, POD and APX was observed until Si2 treatment then tended to decrease at Si3, regardless of salinity treatment. Contrary, the addition of Si markedly decreased the GR activity as compared with control treatment.

Table 4. Effects of silicon (Si) on SOD, CAT and POD antioxidant enzyme activities of faba bean plants grown under different concentrations of Cd and Pb.

HM treatments	Silicon treatments											
	00	Si1	Si2	Si3*	00	Si1	Si2	Si3	00	Si1	Si2	Si3
	SOD (unit g^{-1} fwt)				CAT (μM H_2O_2 oxidized g^{-1} fwt)				POD (O.D g^{-1} fwt min^{-1})			
Cont	25.4	29.2	35.5	36.6	3.55	4.11	5.22	3.82	2.36	3.11	4.24	2.75
Cd1	28.5	31.6	36.4	31.7	4.23	4.85	5.15	4.32	2.75	3.34	4.42	3.25
Cd2	32.7	35.5	38.6	33.4	5.12	5.90	6.55	5.40	3.82	3.95	4.88	3.62
Cd3	21.6	24.2	26.4	13.6	3.42	3.87	4.14	3.66	2.54	3.25	3.85	2.74
Pb1	29.4	32.3	36.2	37.5	4.82	4.95	5.58	5.12	2.58	3.44	4.56	3.52
Pb2	33.6	36.6	40.5	38.4	6.18	6.77	7.28	6.54	3.11	3.65	4.64	3.25
Pb3	35.8	37.2	41.7	39.6	5.35	5.85	6.23	5.35	3.40	3.82	4.71	3.52
LSD (5%)	2.24	2.55	3.32	2.11	1.22	1.11	1.02	105	0.45	0.26	0.15	0.22

*Si1= 1 mM, Si2= 2 mM, Si3= 3 mM of silicon.

It is well known that heavy metal stress causes generation of excessive reactive oxygen species (ROS), which leads to cell toxicity, membrane disfunction and cell death (Shahid *et al.*, 2014). Plants have developed enzymatic and nonenzymatic mechanism to scavenge ROS (Hassanein *et al.*, 2012). Among the active oxygen species superoxide is converted by SOD enzyme to H2O2, which is further scavenged by CAT and APX. Overexpression of the APX gene in plants has showed improvement in protection against oxidative stress (Yasar *et al.*, 2008). In the present study, metal stress induced activation of antioxidant enzymes, such as SOD, POD and APX, in plant leaves. These results are in agreement with those of Gill (2014), who observed that HM stress increased the activities of antioxidant enzymes in leaves of many plant species. Increased activity of these antioxidant enzymes was considered to be a HM-tolerance mechanism in most plants (Nadgórska-Socha *et al.*, 2013; Gill, 2014).

Table 5. Effects of silicon (Si) on APX and GR antioxidant enzyme activities and proline content of faba bean plants grown under different concentrations of Cd and Pb.

HM treatments	Silicon treatments											
	00	Si1	Si2	Si3*	00	Si1	Si2	Si3	00	Si1	Si2	Si3
	APX (mM ascorbate g^{-1} fwt min^{-1})				GR (μg g^{-1} fwt)				Proline (mg per 100 g dwt)			
Cont	0.32	0.37	0.42	0.35	0.68	0.66	0.64	0.58	2.85	2.82	2.80	2.75
Cd1	0.36	0.41	0.46	0.40	0.65	0.62	0.59	0.53	3.55	3.56	3.48	3.45
Cd2	0.39	0.48	0.52	0.43	0.60	0.56	0.54	0.52	4.25	4.11	3.68	3.52
Cd3	0.30	0.40	0.44	0.38	0.56	0.50	0.47	0.46	3.11	3.05	3.00	3.00
Pb1	0.37	0.39	0.44	0.41	0.66	0.64	0.60	0.55	3.45	3.40	3.32	3.30
Pb2	0.40	0.46	0.48	0.43	0.65	0.62	0.58	0.52	4.22	4.00	3.42	3.40
Pb3	0.42	0.47	0.49	0.44	0.60	0.57	0.54	0.50	4.35	4.30	4.00	3.65
LSD (5%)	0.04	0.05	0.04	0.05	0.04	0.05	0.04	0.03	0.55	0.64	0.50	0.15

*Si1= 1 mM, Si2= 2 mM, Si3= 3 mM of silicon.

The Enzyme GR activates the glutathione-ascorbate cycle and converts GSSG to reduced glutathione (GSH) (Vega *et al.*, 2003). In addition, GR regulates GSH/GSSG ratio and supplies GSH for GPX and DHAR, which convert H_2O_2 to H_2O and reduce oxidized ascorbate, respectively. The present results showed that HM stress caused a decrease in GR activity. GR deactivation by stress was explained as a result of prevention of new enzyme synthesis (Liang *et al.*, 2006). The changes in GR activity (Table 5)

and lipid peroxidation (LP), as indicated by the accumulation of MDA (Table 2), in *V. faba* plants subjected to different levels of Cd and Pb as well as Si are recorded. The GR activity gradually decreased with increasing HM concentration, whereas a gradual increase in lipid peroxidation was observed, compared with those of unstressed plants. The maximum reduction in GR activity and the maximum increase in MDA (LP) content were detected at the highest levels of Cd and Pb.

It seems that silicon could alter the activity of antioxidative enzymes in plant organs to improve the stress tolerance. Results in Tables (4 and 5) showed that application of Si caused an increase in the activity of SOD, POD, APX and CAT in *V. faba* plants and this could ameliorate the effect of HM stress. High activity of CAT in Si-treated plants under HM stress suggests that the treated plants possess a better scavenging ability. The present study was consistent with the results reported by Shahid *et al.* (2014). The effect of Si on the antioxidant enzymes activity under HM stress has been reported by Gill (2014) who described an increase in SOD, GPX, CAT activity in HM-stressed plants. In an early study, Siddiqui *et al.* (2014) found that Si could increase antioxidative enzymes activity which played great role to counterbalance stress damages. In this regard Helaly *et al.* (2014) reported that SOD, CAT and POX activities were increased significantly in banana plants when Si doses increased.

Proline content

Obtained results show that proline accumulation increased as metal level(s) increased (Fig. 4). Significant differences (P ≤0.05) in proline content were observed between Si-treated and Si-untreated plants grown under different levels of Cd and Pb. Higher proline accumulation rate was found in Si-untreated plants. Maximum proline accumulation of 32.9% of control was observed at Cd2 treatment without Si. While in the case of Pb, maximum accumulation of 52.6% of control was registered at Pb3 treatment without Si. Under Si treatments, proline contents of plant leaves markedly decreased.

Current results showed that proline accumulation increased as Cd and Pb levels increased with the highest amount found in SI-untreated plants. Early studies showed that growing plants under HM stress increased proline content (Aly and Mohamed, 2012; Karimi *et al.*, 2012). Accumulation of free proline in response to heavy metal exposure seems widespread among plants (Thounaojam *et al.*, 2012). Various earlier studies reported that proline contents significantly increased in many species including common bean (Khadri *et al.* 2006), corn (Yoon *et al.* 2005) and soybean (Chon *et al.* 2003) under stress conditions. The exposure to heavy metals, especially Cd and Pb, is known to disturb the plant water balance. It is well known that proline prevents membrane damage and had a protective role in lipid proxidation induced by metals (Thounaojam *et al.*, 2012). Proline accumulation in plants under Cd and Pb stress is induced by a HM-imposed decrease of the plant water potential, and the functional significance of this accumulation would lie in its contribution to water balance maintenance; proline-mediated alleviation of water deficit stress could substantially contribute to Cd and Pb tolerance (Zengin and Munzuroglu, 2005).

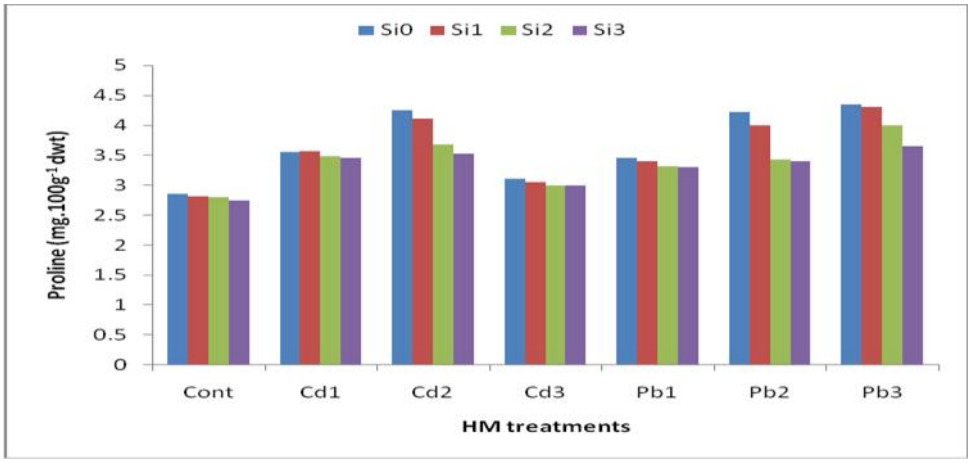

Fig. 4: Effects of silicon (Si) on free proline content of faba bean plants grown under different concentrations of Cd and Pb.

The accumulation of proline concomitant with increasing stress in faba bean plants was in agreement with the results obtained by John et al (2008) and Rahimi *et al.* (2012). These authors reported that proline accumulation in response to environmental stress protected the cell by balancing the osmotic strength of the cytosol with that of the vacuole and external environment. Proline accumulation could be a protective response, not only because of the osmoprotectant role of proline that prevents water-deficit stress under high HM stress, but also as a result of the radical scavenger and protein stabilization properties of proline (Ben Ahmed *et al.*, 2010). In addition, proline accumulation was reported to serve as a nitrogen storage compound and thus protect cellular structure (Rahimi *et al.*, 2012).

In our case, accumulation of proline in response to Cd and Pb was seen in Si-untreated plants much more than Si-treated plants, this may be due to decreased activity of enzyme responsible for proline degradation under stress that leads to increase proline accumulation in Si-untreated plants or increase activity of some enzymes that were responsible for proline biosynthesis (Al Khateeb and Al-Qwasemeh, 2014). The amount of free proline in sole Si treated plants (Si1, Si2 and Si3) were however similar to that of control (Si0) treatment in the absence of HM treatments (Fig 4). Current results suggest that proline contents decreased with Si application, which show the favorable role of Si in mitigating the adverse effects of salt stress on plants. It was concluded that addition of Si is beneficial to improve growth attributes and to mitigate the adverse effects of heavy metal stress. However, further studies are needed for understanding the mechanism of physiological and biochemical roles of Si in higher plants. Crusciol *et al.* (2009) found that silicon increased proline (a key solute in osmotic adjustment) content in stressed plant tissue. In this regard, Helaly *et al.* (2014) found that total proline was increased significantly in banana plants when Si doses increased. However, Lee *et al.* (2010) and Shen *et al.* (2010) found the opposite. Sonobe *et al.* (2011) also suggest a silicon-induced effect of osmotic adjustment in sorghum roots.

Nutrient elements content

Data in Table (6) indicated clearly that Cd and Pb treatments decreased N, P and K content of faba bean plants. The decrease in nutrient content was gradual and linear with the increase in HM concentration. The reduction in element concentration was more pronounced at the highest stress level with Cd being more negatively effective than Pb treatments. In this regard, Pb3 treatment reduced the N, P and K by about 14.8%, 25% and 16.3%, respectively, while the Cd3 treatment caused reductions of 30.5%, 41.6% and 31.3%, respectively, as compared with control. The negative effect of salinity stress on nutrient elements of bean plants was reported by Matijević *et al.* (2012) and Chibuike and Obiora (2014).

Table 6. Effects of silicon (Si) and nano-silicon (NSi) on N, P and Ca percentages in faba bean plants under different levels of salinity stress.

HM treatments	Silicon treatments											
	00	Si1	Si2	Si3*	00	Si1	Si2	Si3	00	Si1	Si2	Si3
	N (%)				P (%)				K (%)			
Cont	3.24	3.65	3.76	3.82	0.36	0.44	0.50	0.48	3.25	3.45	3.65	3.57
Cd1	3.04	3.45	3.55	3.65	0.31	0.37	0.42	0.40	3.18	3.28	3.33	3.31
Cd2	2.65	2.84	3.12	3.36	0.26	0.30	0.34	0.32	2.67	2.82	3.11	3.02
Cd3	2.25	2.56	2.82	2.95	0.21	0.22	0.27	0.25	2.22	2.28	2.30	2.30
Pb1	3.12	3.45	3.68	3.75	0.33	0.41	0.45	0.42	3.21	3.31	3.66	3.61
Pb2	2.85	3.25	3.56	3.62	0.30	0.36	0.40	0.38	3.06	3.15	3.26	3.24
Pb3	2.76	2.83	2.95	3.04	0.27	0.31	0.36	0.33	2.72	2.82	2.95	2.92
LSD (5%)	o.35	0.40	0.54	0.45	NS	0.12	0.15	0.06	0.35	0.26	0.25	0.30

*Si1= 1 mM, Si2= 2 mM, Si3= 3 mM of silicon.

It is obvious from the data in Table (6) that all Si treatments caused an observed increase in N%, P% and K% in plant shoots. The increase may be attributed to the enhancing effect of Si on the uptake of nutrients. These results agreed with those reported by Hanafy Ahmed *et al.* (2008) on wheat. The stimulating effect of Si on the absortion of elements might consider as an adaptation mechanism developed by the plants to overcome the stress caused by heavy metals (Bulut and Akıncı, 2010).

The most effective treatment of Si was Si2, which produced the highest percent increase in N, P and K of HM-stressed shoots.

Application of Si significantly increased the contents of nutrient element contents of HM-stressed plants. These findings agreed with experimentations with faba bean by (Abdelhamid *et al.* 2010) which indicate that stress tolerance is associated with an enhanced nutrient uptake. The ability of plant to increase nutrient transport into the shoot is critically importance for the maintenance of high growth rates and protection of the metabolic processes in elongation cells from the toxic effects of HM (**Karimi** *et al.*, 2013). Potassium content was higher in plants grown under Si treatments. Increased K content in shoot may be one of the possible mechanisms of increased HM-tolerance by Si application in faba bean plants (Hellal *et al.*, 2012). In this regard, it has been found that silicon when deposited in exodermis and endodermis of roots increase K and reduces Na uptake in plants (Matichenkov and Kosobrukhov, 2004; Chibuike and Obiora, 2014).

Conclusion

Results obtained from this study demonstrated that in the pot experiment, the Si treatments provided the tolerance toward Cd and Pb and had an ameliorating effect on HM-stressed faba bean. Tolerance in plants grown under Cd and Pb may be through (1) increasing chlorophyll content and photosynthesis as well as carbohydrate synthesis, (2) improving antioxidant enzyme activities against oxidative stress, and (3) maintaining intracellular ion equilibrium under HM stress by increasing the uptake and translocation of nutrient elements. The results may have a potential value on repairing heavy metal contamination and increasing crop production.

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