ORIGINAL ARTICLE

Zinc-induced modulation of some biochemical parameters in a high- and a low-zinc-accumulating genotype of *Cicer arietinum* L. grown under Zn-deficient condition

Shadab Naseer Siddiqui · Shahid Umar · Muhammad Iqbal

Abstract This study presents a comparative account of biochemical responses evoked by a high-zinc-accumulating (HZnG) and a low-zinc-accumulating (LZnG) genotype of chickpea (*Cicer arietinum* L.) under conditions of zinc deficiency (0 mg $ZnSO_4 kg^{-1}$ soil) and on exogenous zinc supply (2.5, 5, and 10 mg $ZnSO_4 kg^{-1}$ soil), at different growth stages (pre-flowering, flowering, and post-flowering) of the crop. Both the genotypes exhibited significant variation in contents of chlorophyll, protein, nitrate, and leghemoglobin, and in the activity of enzymes (nitrate reductase, superoxide dismutase, and carbonic anhydrase), under zinc-limiting condition. The HZnG maintained a significantly higher level of biochemical parameters at deficient zinc levels, while LZnG was more affected by Zn deficiency (with reduced biochemical attributes), compared to HZnG. However, application of soil zinc alleviated the Zn-induced disturbance and improved the biochemical parameters in both genotypes. The differential biochemical response of plants to Zn deficiency indicated existence of genotypic variation in efficient utilization of the available Zn. The HZnG was more effective in overcoming Zn deficiency than the LZnG. This study suggests that metal-dependent biochemical parameters can be used as reliable indices for selection of genotypes with a better Zn utilization capacity, i.e., the Zn-efficient genotypes.

S. N. Siddiqui · S. Umar · M. Iqbal (✉)
Department of Botany, Faculty of Science, Hamdard University,
New Delhi 110062, India
e-mail: iqbalg5@yahoo.co.in

Introduction

The growth and yield potential of plants depend on their genetic background and environmental factors. Zinc (Zn) deficiency in soils represents a common environmental factor that limits crop production; it reduces not only the yield but also the nutritional quality of grain, affecting billions of people worldwide. The magnitude of Zn-deficient soils is increasing rapidly in many regions of the world including India. If this trend continues, the present level of nearly 50 % Zn-deficient soil in the country is likely to become about 63 % by the year 2025 (Das and Green 2013). Given the high prices of animal products, use of chickpea (*Cicer arietinum* L.) as a source of energy and high-quality dietary proteins is desirable, particularly in developing countries like ours. However, deficiency of zinc in these regions (Saxena 1993; Singh 2008) causes significant yield losses in chickpea crop, which is sensitive to zinc deficiency (Ahlawat et al. 2007). Chickpea genotypes differ in their requirement of zinc, and hence in their tolerance to Zn deficiency (Khan et al. 1998, 2000). Furthermore, genotypes with high Zn accumulation capacity can thrive with low level of zinc and maintain a better growth and yield (Khan et al. 1998; Siddiqui et al. 2013). Therefore, cultivating high-zinc-accumulating chickpea genotypes capable of maximizing their yield with low availability of Zn (Zn-efficient genotypes) seems to be a key approach for improving chickpea production under rapidly increasing Zn deficiency in the soil. However, a better understanding of zinc utilization mechanism is necessary to define the strategies adopted by the plant to combat Zn deficiency. Therefore, inclusion of available physiological Zn in criteria for selecting plant genotypes with enhanced utilization of Zn is warranted.

Zinc, an essential micronutrient for plants, is involved in synthesis of indole-3-acetic acid (IAA) and protein, carbohydrate metabolism, photosynthesis, and the nitrogen fixation in legumes (Broadley et al. 2007; Cherif et al. 2011). It is also a key constituent of enzymes like carbonic anhydrase (CA) and superoxide dismutase (SOD). As a cofactor of CA, Zn is important in photosynthesis; the activity of CA is highly correlated with photosynthetic rate (Ohki 1976; Rengel 1995). Evidence indicates that much of the injury to plants due to environmental stresses is associated with oxidative damage through direct or indirect formation of reactive oxygen species (ROS), which are very unstable molecules and interact nonspecifically with many cellular components, including photosynthetic pigments, proteins, nucleic acids, and lipids, and damage their normal functions. Zn modulates free radicals and their damaging effects by enhancing the plants' antioxidant systems (Zago and Oteiza 2001). It has a role in stabilization and protection of biomembranes against oxidative and peroxidative damage, loss of plasma membrane integrity, and alteration of membrane permeability (Bettger and O Dell 1981). Therefore, the Zn-deficiency-induced increase in membrane permeability and the decrease in detoxification mechanisms (Cakmak 2000; Pandey et al. 2012) seem to be responsible for impairment of various cellular functions in Zn-deficient plants. Furthermore, being involved in the regulation of cell metabolic pathways (Andreini et al. 2006), inadequate amount of Zn negatively affects several enzymatic systems and associated metabolic functions (Alloway 2004), and limits plant growth and development (Cakmak et al. 1999). Zinc deficiency retards photosynthesis and nitrogen metabolism, reduces flowering and fruit development, prolongs growth periods (resulting in delayed maturity), decreases yield and quality, and causes suboptimal nutrient use efficiency (Das and Green 2013). Therefore, exogenous supply of zinc to maintain its balanced level in plant cells is critical.

Since different chickpea genotypes differ in their response to Zn deficiency, genotypes with diverse Zn accumulation capacity might trigger biochemical responses differently under zinc-limiting conditions, and these responses might indicate genotypic difference in zinc utilization. Screening of crops not just for yield, but even for individual yield components, may provide adequate information on mechanisms underlying protection against low availability of plant nutrients. Since plant responses differ during different growth phases (Cao et al. 2013), induced biochemical responses in chickpea under Zn deficiency may also vary with developmental stages of the crop. Therefore, the present study with a high-Zn-accumulating (HZnG) and a low-Zn-accumulating (LZnG) chickpea genotype was carried out to understand how they cope with zinc deficiency at physiological level during different stages of plant growth, and how the soil Zn application works in modulating the biochemical processes and alleviating the effect of Zn deficiency.

Materials and methods

Plant material and growth conditions

Two chickpea genotypes, HZnG (IC269837) and LZnG (IC269867), differing in their Zn accumulation and sensitivity to Zn deficiency (Siddiqui et al. 2013), were evaluated for their performance in a pot experiment under natural field condition at Jamia Hamdard, New Delhi (India). The soil used for the experiment had a sandy loam texture (83.6 % sand, 6.8 % silt, and 9.6 % clay), with pH 7.1, and 0.78 mg diethylenetriamine pentaacetic acid (DTPA)-extractable Zn per kilogram of soil (Ankerman and Large 1974).

Ten healthy equal-sized chickpea seeds of each genotype were surface-sterilized with 0.1 % mercuric chloride for 5 min, washed thoroughly with deionized water, and then germinated in the dark in noncontaminated sand moistened with deionized water. After 1 week, five most vigorous and equally developed seedlings were transferred to 23-cm-diameter soil-filled earthen pots lined with polythene bags (to avoid contamination). The entire experiment was conducted under naturally illuminated field condition with $32/20\pm 4$ °C average day/night temperatures and relative humidity of 70–76 %. The soil used in pots was mixed homogenously with recommended basal doses of N, P, K, and S to get 25 kg N, 20 kg P, 30 kg K, and 20 kg S ha^{-1}. The sources of N, P, K, and S were urea, single super phosphate, muriate of potash (KCl), and gypsum, respectively. Zinc at concentrations of 0, 2.5, 5, and 10 mg kg^{-1}, symbolized as Zn_0, $Zn_{2.5}$, Zn_5, and Zn_{10}, respectively, was added to the soil as $ZnSO_4 \cdot 7H_2O$ and thoroughly mixed. The treatments were arranged in a randomized design with three replications. Growth was visually assessed at every 5 weeks until the final harvest. The necessary aftercare operations and plant protection measures were carried out as and when required to maintain a good and healthy crop. The crop was also given protective irrigation, depending upon water requirement.

The first sampling was done at pre-flowering stage, i.e., 40 days after sowing (DAS), and four plants were maintained in each pot up to 60 DAS. The second sampling was done at flowering stage (60 DAS), and three healthy plants of uniform size were maintained in each pot until the third sampling at post-flowering stage (90 DAS). Data on contents of chlorophyll, protein, and nitrate, and activity of nitrate reductase (NR), SOD, and CA enzyme in leaves were collected at each growth stage. For this, fully expanded fresh leaves on the upper one half to one third of the stem were sampled and cleaned with deionized water to remove any surface contamination before biochemical analysis. The leghemoglobin (LHb) content was determined from fresh root nodules, to find out the capacity of both chickpea genotypes to fix the atmospheric nitrogen. Plants were carefully uprooted with root

system intact. The roots were rinsed several times with deionized water, and the nodules were detached carefully with forceps.

Chemicals used

The chemicals of analytical grade were obtained from E. Merck, SRL, and Sigma-Aldrich (St. Louis, MO, USA), and a high-purity deionized water was used.

Leaf chlorophyll content

Chlorophyll was extracted from freshly sampled leaves following the DMSO method described by Hiscox and Israelstam (1979). The absorbance of leaf extract was recorded at 645 and 663 nm, using DMSO as a blank, by a spectrophotometer (model DU 640, Beckman, USA). Content of total chlorophyll (TChl) was calculated using the following formula given by Arnon (1949):

$$TChl = 20.2\,OD_{645} + 8.02\,OD_{663}$$

Soluble protein content

The total soluble protein content in leaf tissue was estimated following the method of Bradford (1976), using 0.5 g chopped fresh leaves, homogenized in 1.5 ml of phosphate buffer (0.1 M, pH 7.0) with a pre-chilled mortar and pestle. The homogenate was centrifuged at 5000 rpm for 20 min at 4 °C (REMI CPR24, New Delhi, India). An equal amount of chilled 10 % TCA was added to 0.5 ml of the supernatant, and then again centrifuged at 3300 rpm for 30 min. The supernatant was discarded and the pellet left was washed with acetone. It was then dissolved in 1 ml of NaOH (0.1 N). To a 0.2-ml aliquot, 1 ml of the Bradford's reagent was added and vortexed. The tubes were kept for 10 min for optimal color development. The absorbance was then recorded at 595 nm. Calculations for leaf protein contents were made using the calibration curve of bovine serum albumin (BSA). The protein content was expressed in milligrams per gram fresh weight (mg g^{-1} F.W.).

Nitrate content

Nitrate content of leaves was estimated using the method of Grover et al. (1978). For extraction of nitrate, 0.5 g fresh leaves were taken in a conical flask; 50 mg charcoal and 10 ml distilled water were added to it and boiled for 4–5 min. After filtration, the volume was made up to 50 ml by adding DDW. An aliquot (1 ml) of the extract was added with 0.5 ml CuSO4 solution, 0.25 ml hydrazine sulfate, and 0.25 ml of 0.1 N NaOH in sequence. The vials were kept in water bath incubator for 10 min at 33 °C and then transferred to ice, before adding 0.5 ml chilled acetone and 1.0 ml sulphanilamide and N-(1-napthyl) ethylene diamine dihydrochloride (NEDD) to it. The volume was increased to 6 ml by adding 1.5 ml DDW, and the vials were kept for 20 min for color development. Optical density of the pink solution was read at 540 nm. The nitrate content was expressed as micromoles per gram (μmol g^{-1}) F.W. The concentration of nitrate was determined against the standard curve prepared by using potassium nitrate (KNO_3) solution.

Leghemoglobin content

LHb content of nodules was determined by the method of Wilson and Reisenauer (1963). An amount of 0.5 g fresh and bold nodules was homogenized with 10 ml of precooled Drabkins solution (Wilson and Reisenauer 1963), using chilled mortar and pestle. The homogenate was squeezed through four layers of cheesecloth and then centrifuged at 5000 rpm in a cooling centrifuge at 4 °C for 30 min. The supernatant obtained was used for the assay of LHb. The absorbance of the supernatant was read at 540 nm (Wilson and Reisenauer 1963). LHb concentration was calculated by using bovine hemoglobin (Sigma, USA) as a standard, and values were expressed in milligrams per gram of the nodule mass (mg g^{-1} nodule) F.W.

Nitrate reductase (EC 1.6.6.1) activity

Activity of NR in leaf was estimated, as per the method of Jaworski (1971), by suspending 250-mg leaf material in 2.5 ml phosphate buffer (0.1 M, pH 7.5) containing equal amounts of potassium nitrate solution (0.2 M) and isopropanol (5 %), and two drops of chloramphenicol (0.5 %), in a screw-capped vial. After sealing, the vials were incubated at 30 °C in the dark for about 2 h. The aliquot (0.4 ml) was added with 0.3 ml of 3 N HCl solution containing 1 % sulphanilamide and 0.02 % aqueous NEDD. The final volume was made up to 5 ml by DDW. After 20 min, the absorbance was read at 540 nm. A standard curve was plotted, using varying concentrations of potassium nitrate (KNO_3), and used for calculation. The enzyme activity was expressed as micromoles of nitrite per hour per gram (μmol nitrite h^{-1} g^{-1}) F.W.

Superoxide dismutase (EC 1.15.1.1) activity

Fresh leaf tissues (0.5 g) were ground in extraction buffer containing 0.05 % (v/v) Triton X-100 and 1 % (w/v) polyvinylpyrrolidone in potassium phosphate buffer (100 mM, pH 7.5) under cold condition (4 °C) with the help of pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant obtained

was used for determination of SOD. The activity of SOD was assayed according to Dhindsa et al. (1981) by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). The photoreduction of NBT (production of blue formazan) was measured at 560 nm by UV-Vis spectrophotometer; 50 % inhibition of NBT reduction was considered as one unit of enzyme activity. SOD activity was expressed in unit per milligram of protein per hour (U mg^{-1} protein h^{-1}).

Carbonic anhydrase (EC 4.2.1.1) activity

Freshly sampled leaf tissues (0.2 g) were homogenized in 10 mL of solution containing 50 mM HEPES-NaOH (pH 7.5), 0.5 mM EDTA, 10 mM DTT, and 10 % (v/v) glycerol, using chilled mortar and pestle. Triton X-100 was added to it in a final concentration of 0.1 % (v/v). The homogenate was then centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant obtained was used for the assay of CA activity, based on the method described by Makino et al. (1992). Activity of CA was determined in Wilbur-Anderson unit, following time-dependent reduction in pH from 8.25 to 7.45 at 0–4 °C. The unit (U) of enzyme activity was calculated according to the formula $U = 10 (T - T_0)/T_0$, where T and T_0 represent the time required to change the pH from 8.25 to 7.45, with and without the extract of crude enzyme, respectively. The enzyme activity was presented as units per milligram of protein (U mg^{-1} protein).

Statistical analyses

Analysis of variance (ANOVA) was performed using the GraphPad Prism software program (GraphPad Prism version 5, San Diego, CA, USA) to detect differences among the variables measured and to separate the main effects of Zn supply and genotypes and their interactive effects. Comparison of treatment means was carried out using the Duncan's multiple range test (DMRT) ($p < 0.05$).

Results

Soil Zn deficiency significantly hampered biochemical processes in both genotypes, which differ in their ability to respond to Zn-limiting conditions; LZnG exhibited more depression in vital biochemical activities than HZnG. Exogenous supply of soil Zn alleviated the decrease in metabolic activities and caused improvement in parameters studied.

Chlorophyll content

In general, chlorophyll content was the maximum at flowering stage and significantly depressed at post-flowering stage

(Table 1). The effect of applied Zn on total chlorophyll content was significant at each stage in both genotypes, although the flowering and post-flowering stages were more responsive ($p < 0.001$) than the pre-flowering stage ($p < 0.05$) (Table 1). Treatment Zn_5 was most effective, causing an increase of 2.05, 5.62, and 7.29 % in HZnG and 23.14, 13.17, and 16.66 % in LZnG at pre-flowering, flowering, and post-flowering stages, respectively, compared to the control. Although the amount of chlorophyll was maximum in HZnG, the improvement was more in LZnG. The difference between the Zn-treated plants of the two genotypes was significant ($p < 0.001$). Interaction effect of genotype and treatment (G × Zn) was nonsignificant ($p > 0.05$) at all stages of plant growth.

Total soluble protein content

Application of zinc had a positive and significant ($p < 0.001$) effect on total soluble protein content at each stage of plant growth (Table 2). Zn_5 dose was most beneficial, compared to the control of both the genotypes. A significant difference ($p < 0.001$) between genotypes was noted in control (Zn_0) as well as Zn-fertilized plants at all growth stages. HZnG had a higher content than LZnG in all Zn treatments. LZnG was more responsive to additional zinc supply. The maximum increase in protein content in LZnG (20.87 %) was recorded at the flowering stage with Zn_5 treatment. Plants of both genotypes grown at higher zinc level (Zn_{10}) exhibited a nonsignificant ($p > 0.05$) difference from those grown at Zn_5 treatment. The interaction (G × Zn) effect was significant at pre-flowering ($p < 0.01$) and flowering ($p < 0.001$) stages.

Nitrate content

The Zn effect on nitrate content in leaves differed significantly ($p < 0.001$) between the two genotypes at each growth stage (Table 3). A statistically higher value was recorded for HZnG than LZnG at all growth stages with each treatment. However, difference among Zn-treated plants in both genotypes remained nonsignificant ($p > 0.05$) at all growth stages. The nitrate content of plants grown at higher zinc level (Zn_{10}) was statistically similar to those grown at Zn_5 treatment in both genotypes. Flowering stage exhibited the highest nitrate content. Interactive (G × Zn) effect was nonsignificant ($p > 0.05$).

Leghemoglobin content

The two genotypes behaved differently under Zn-limited condition. In the control (Zn_0), genotype HZnG had a higher LHb content than LZnG in all growth stages, maintaining an average excess of 32.53 % (Fig. 1). Zinc deficiency significantly

Table 1 Effect of Zn fertilization on total chlorophyll content (mg g^{-1} F.W.) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.)

Treatments	Pre-flowering stage		Flowering stage		Post-flowering stage	
	HZnG	LZnG	HZnG	LZnG	HZnG	LZnG
Zn$_0$	0.878±0.020 a	0.648±0.050 c	1.510±0.016 b	1.192±0.003 e	0.822±0.013 b	0.618±0.016 d
Zn$_{2.5}$	0.895±0.012 a (1.93)	0.757±0.013 b (16.82)	1.579±0.016 a (4.56)	1.267±0.021 d (6.29)	0.854±0.011 ab (3.89)	0.681±0.023 c (10.19)
Zn$_5$	0.896±0.011 a (2.05)	0.798±0.023 b (23.14)	1.595±0.010 a (5.62)	1.349±0.010 c (13.17)	0.882±0.010 a (7.29)	0.721±0.010 c (16.66)
Zn$_{10}$	0.895±0.010 a (1.93)	0.783±0.020 b (20.83)	1.583±0.016 a (4.83)	1.329±0.022 c (11.49)	0.864±0.010 ab (5.10)	0.720±0.014 c (16.50)
ANOVA						
Genotype (G)	***		***		***	
Treatment (T)	*		***		***	
G × T	Ns		Ns		Ns	

Values in parenthesis indicate percent variation with reference to respective controls. Each value represents a mean±SE of three replicates. The data followed by different letters are significantly different at *p*<0.05 (Duncan's multiple range test). Sampling was done at three stages of plant growth

Ns not significant

**p*<0.05 and ***p*<0.001, significant

decreased the LHb content of nodules in the genotypes at all stages of plant growth. It increased from pre-flowering to post-flowering stage in the control as well as in Zn-treated plants. Differences in LHb content due to zinc application were significant (*p*<0.001) in both the genotypes at all the sampling stages. The LHb content was significantly higher under Zn$_5$ treatment, i.e., by 52.38 % at pre-flowering, 36.63 % at flowering, and 21.4 % at post-flowering stages with respect to their controls. Genotypic variation in LHb content due to Zn application was significant (*p*<0.05) at post-flowering stage only.

Nitrate reductase

Application of zinc significantly improved (*p*<0.05) the NR activity in leaves of both the genotypes (Fig. 2). Differences between HZnG and LZnG were not significant (*p*>0.05). Growth stages affected the enzyme activity, as it increased up to flowering stage, but declined at post-flowering stage in both genotypes. It was highest at the flowering stage, irrespective of genotypes and treatments. Of all the treatments, Zn$_5$ was most effective. Interaction effect of genotype and treatment (G × Zn) was nonsignificant (*p*>0.05).

Table 2 Effect of Zn fertilization on protein content (mg g^{-1} F.W.) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.)

Treatments	Pre-flowering stage		Flowering stage		Post-flowering stage	
	HZnG	LZnG	HZnG	LZnG	HZnG	LZnG
Zn$_0$	15.22±0.176 b	12.11±0.093 c	19.07±0.101 b	14.23±0.255 d	14.16±0.140 b	11.94±0.046 d
Zn$_{2.5}$	15.80±0.113 a (3.81)	13.71±0.166 c (13.21)	19.77±0.243 a (3.67)	16.72±0.354 c (17.49)	14.42±0.154 ab (1.83)	13.45±0.150 c (12.64)
Zn$_5$	15.99±0.115 a (5.06)	13.88±0.180 c (14.61)	19.83±0.156 a (3.98)	17.2±0.257 c (20.87)	14.68±0.105 a (3.67)	13.77±0.079 c (15.32)
Zn$_{10}$	15.96±0.089 a (4.86)	13.75±0.083 c (13.54)	19.77±0.224 a (3.67)	16.96±0.139 c (19.18)	14.59±0.198 a (3.03)	13.57±0.055 c (13.65)
ANOVA						
Genotype (G)	***		***		***	
Treatment (T)	***		***		***	
G × T	**		Ns		***	

Values in parenthesis indicate percent variation with reference to respective controls. Each value represents mean±SE of three replicates. The data followed by different letters are significantly different at *p*<0.05 (Duncan's multiple range test). Sampling was done at three stages of plant growth

Ns not significant

***p*<0.01 and ***p*<0.001, significant

Table 3 Effect of Zn fertilization on nitrate content (μmol g^{-1} F.W.) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.)

Treatments	Pre-flowering stage		Flowering stage		Post-flowering stage	
	HZnG	LZnG	HZnG	LZnG	HZnG	LZnG
Zn_0	24.60±0.341 a	19.21±0.136 b	42.30±0.428 b	31.84±0.657 c	18.14±0.200 b	14.26±0.186 c
$Zn_{2.5}$	25.19±0.550 a (2.39)	19.48±0.275 b (1.40)	42.77±0.231 ab (1.11)	31.91±0.115 c (0.21)	18.73±0.218 a (3.25)	14.41±0.247 c (1.05)
Zn_5	25.37±0.225 a (3.13)	19.65±0.256 b (2.29)	43.82±0.159 a (3.59)	32.24±0.664 c (1.25)	18.79±0.170 a (3.58)	14.58±0.077 c (2.24)
Zn_{10}	25.26±0.312 a (2.68)	19.53±0.781 b (1.66)	43.45±0.390 ab (2.71)	32.18±0.175 c (1.06)	18.77±0.174 a (3.47)	14.49±0.124 c (1.61)
ANOVA						
Genotype (G)	***		***		***	
Treatment (T)	Ns		Ns		Ns	
G × T	Ns		Ns		Ns	

Values in parenthesis indicate percent variation with reference to respective controls. Each value represents mean±SE of three replicates. The data followed by different letters are significantly different at $p<0.05$ (Duncan's multiple range test). Sampling was done at three stages of plant growth

Ns not significant

*** $p<0.001$, significant

Superoxide dismutase

The activity of SOD increased significantly with growing plant age as well as zinc dose (Fig. 3). It was lowest at pre-flowering stage and highest at post-flowering stage in both the genotypes. In the control (Zn_0), SOD activity was about 34, 20, and 23 % higher in HZnG than in LZnG at pre-flowering, flowering, and post-flowering stages, respectively. With increase in Zn level, the activity increased significantly ($p<0.001$) in both HZnG and LZnG plants. Zn_{10} treatment was most effective, with 60.42 % increase in SOD activity over the control at the post-flowering stage. It is interesting to note that at high Zn treatment (Zn_{10}), SOD activity was about 11, 10, and 8 % higher in LZnG than in HZnG at pre-flowering, flowering, and post-flowering stages, respectively. Interaction effect (G × Zn) was also significant ($p<0.001$) at all stages of growth in both genotypes.

Carbonic anhydrase

In plants grown without zinc treatment (Zn_0), CA activity in leaves was significantly higher (by 41.17 %) in the HZnG than in LZnG. It increased in response to exogenous application of zinc and with increasing plant age (Fig. 4). Exposing plants to increased zinc levels resulted in significant ($p<0.001$) improvement in CA activity, compared to plants grown with low Zn in both genotypes. On an average, the maximum response was generated in plants treated with Zn_{10}, showing an increase of 51.23, 37.6, and 22.9 % over the control at the pre-flowering, flowering, and post-flowering stages, respectively. The highest enzyme activity was noticed at the post-flowering stage. Genotype HZnG had a greater CA activity than LZnG

in the untreated (Zn_0) as well as zinc-treated populations. In plants with high zinc (Zn_{10}) treatments, genotype HZnG had a higher CA activity than LZnG. Interaction (G × Zn) effect was significant ($p<0.001$) at all stages of plant development.

Discussion

Our study showed a significant reduction in the total chlorophyll, soluble protein, and leghemoglobin contents of the control plants than the Zn-treated plants of both the genotypes. The decrease in these attributes is linked to Zn-deficiency-induced metabolic damage within the plant. However, HZnG could maintain significantly higher levels in zinc-deficient condition (Tables 1, 2 and 3; Fig. 1). Nitrate content does not show a significant difference between the control and treated plants.

The differential biochemical status of the genotypes under Zn-limiting condition is attributed to the availability of Zn and its utilization at cellular level. The maintenance of normal functioning in HZnG under Zn-limiting conditions is related to its capability to secure adequate amounts of Zn in plant cells through its inherent ability of extracting zinc efficiently from the low-Zn growth medium, possibly due to an efficient ion-transport system (Khan et al. 1998), and to its better Zn use efficiency at the cellular level. Since zinc is a functional, structural, or regulatory cofactor for photosynthesis, protein production, and maintenance of membrane structure (Marschner 1995), zinc-deficient plants underwent a functional decline (Brown et al. 1993; Cakmak and Marschner 1993; Gurmani et al. 2012). Increase in Zn supply significantly enhanced the above parameters, except nitrate content (Tables 1, 2 and 3;
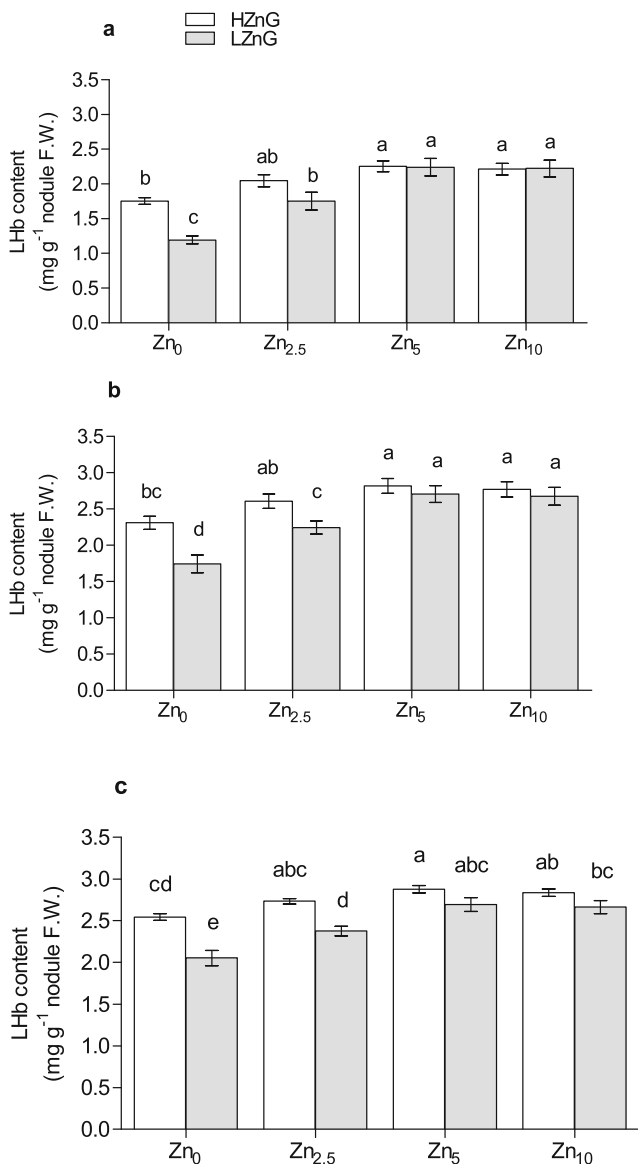
Fig. 1 Effect of different rates of applied Zn on leghemoglobin (LHb) content (mg g^{-1} nodule F.W.) of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.) at **a** pre-flowering, **b** flowering, and **c** post-flowering stages of plant growth. Vertical bars represent±standard errors of means of three replicates. The data followed by different letters are significantly different at $p<0.05$ (Duncan's multiple range test)

Fig. 2 Effect of different rates of applied Zn on nitrate reductase (NR) activity (μmol nitrite h^{-1} g^{-1} F.W.) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.) at **a** pre-flowering, **b** flowering, and **c** post-flowering stages of plant growth. Vertical bars represent±standard errors of means of three replicates. The data followed by different letters are significantly different at $p<0.05$ (Duncan's multiple range test)

Fig. 1) almost at each stage of plant growth. In general, values for biochemical parameters were the maximum at flowering stage and minimum at post-flowering stage. Since Zn has a positive influence on plant metabolism (Zhao et al. 2012), its exogenous supply alleviated the decline caused by Zn deficiency. Plants treated with Zn had significantly greater amounts of pigment than the control (Table 1). The rise in the chlorophyll and protein contents was the maximum with Zn$_5$, the lowest being in the control. Zinc may likely maintain chlorophyll synthesis through sulphydryl group protection (Cakmak 2000) and participates in chlorophyll synthesis (Li
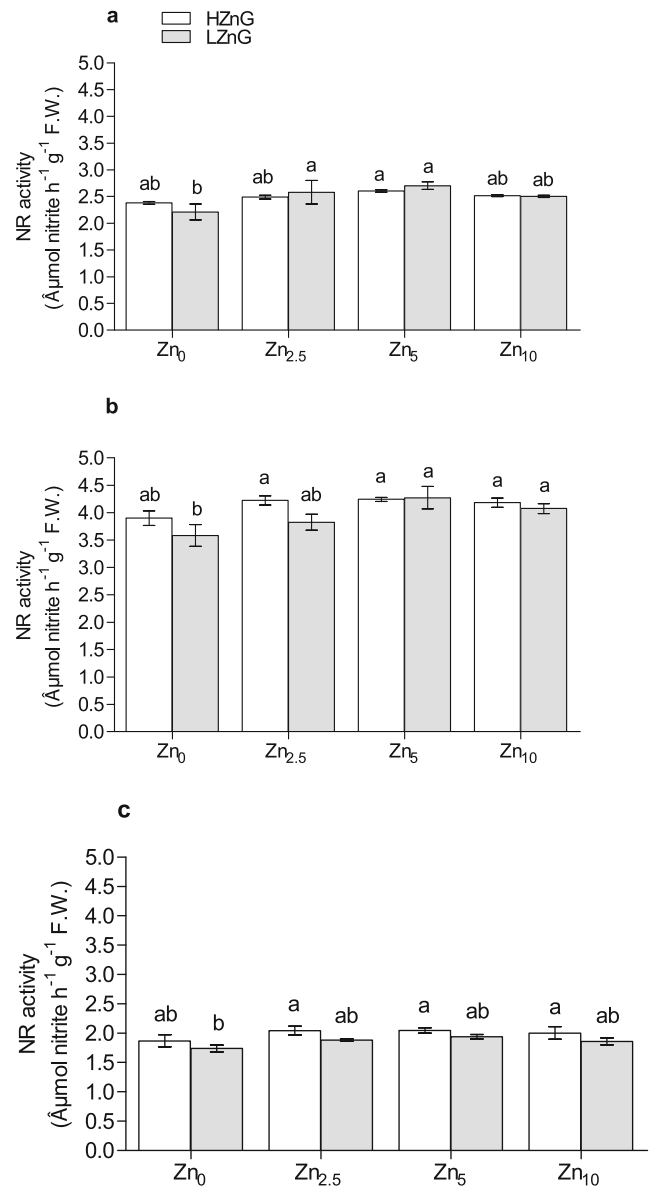
et al. 2006) and in activation of several proteins (Broadley et al. 2007). Earlier works also confirm that zinc application improves chlorophyll content (Chaab et al. 2011; Weisany et al. 2011; Gurmani et al. 2012).

Our results, showing increase in the soluble protein content with increase in the soil Zn level, find support from similar findings of Feng-Juan et al. (2005). However, the link was weak for leaf nitrate content, as the effect was nonsignificant at each growth stage (Table 3). Zinc application improved leghemoglobin content, as was also observed by Shukla and Yadav (1982). Any alteration in LHb content may cause poor
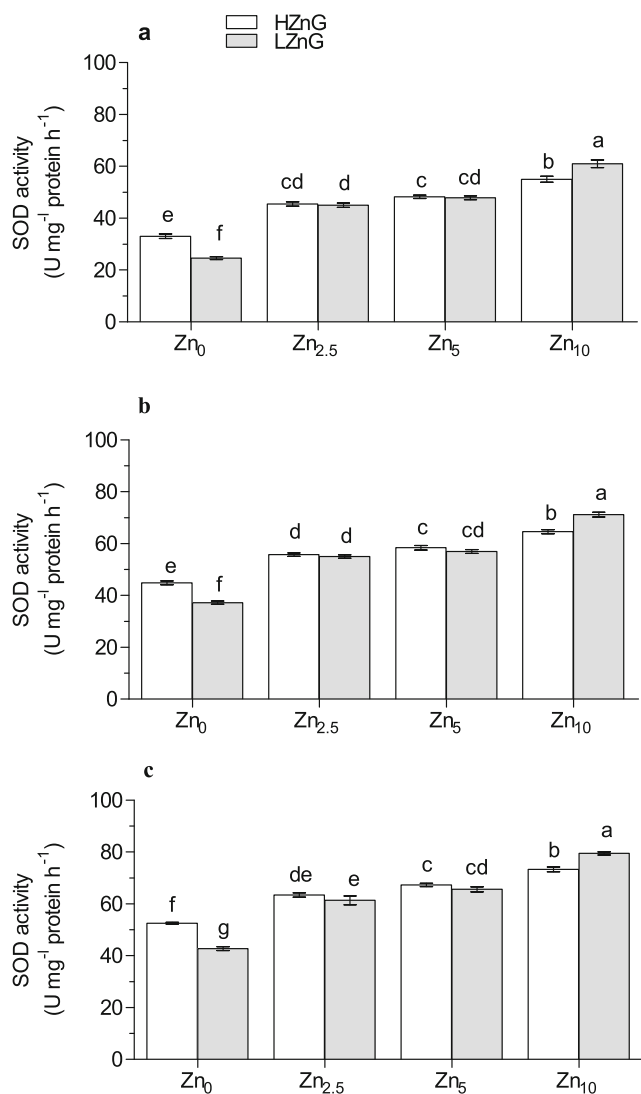
Fig. 3 Effect of different rates of applied Zn on super oxide dismutase (SOD) activity (U mg^{-1} protein h^{-1}) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.) at **a** pre-flowering, **b** flowering, and **c** post-flowering stages of plant growth. Vertical bars represent±standard errors of means of three replicates. The data followed by different letters are significantly different at *p*<0.05 (Duncan's multiple range test)

Fig. 4 Effect of different rates of applied Zn on carbonic anhydrase (CA) activity (U mg^{-1} protein) in leaves of high-zinc-accumulating (HZnG) and low-zinc-accumulating (LZnG) genotypes of chickpea (*Cicer arietinum* L.) at **a** pre-flowering, **b** flowering, and **c** post-flowering stages of plant growth. Vertical bars represent±standard errors of means of three replicates. The data followed by different letters are significantly different at *p*<0.05 (Duncan's multiple range test)

root nodulation, which would retard the capacity of chickpea plants to fix the atmospheric nitrogen (Ahlawat et al. 2007), and hence to suppress crop yield. Although additional Zn supply stimulated the biochemical activities more in LZnG than in HZnG, the latter showed a significantly higher level of all biochemical parameters.

Within the cell, efficient metabolism of nitrogen ensures a proper utilization of the available nitrogen (N) resources, which in turn depend on efficiency of enzymes involved. Therefore, any alteration in the activity of the key nitrogen assimilatory enzymes, such as NR, severely affects nitrogen metabolism. NR activity was significantly lower in Zn-deficient condition than in the Zn-sufficient plants of both
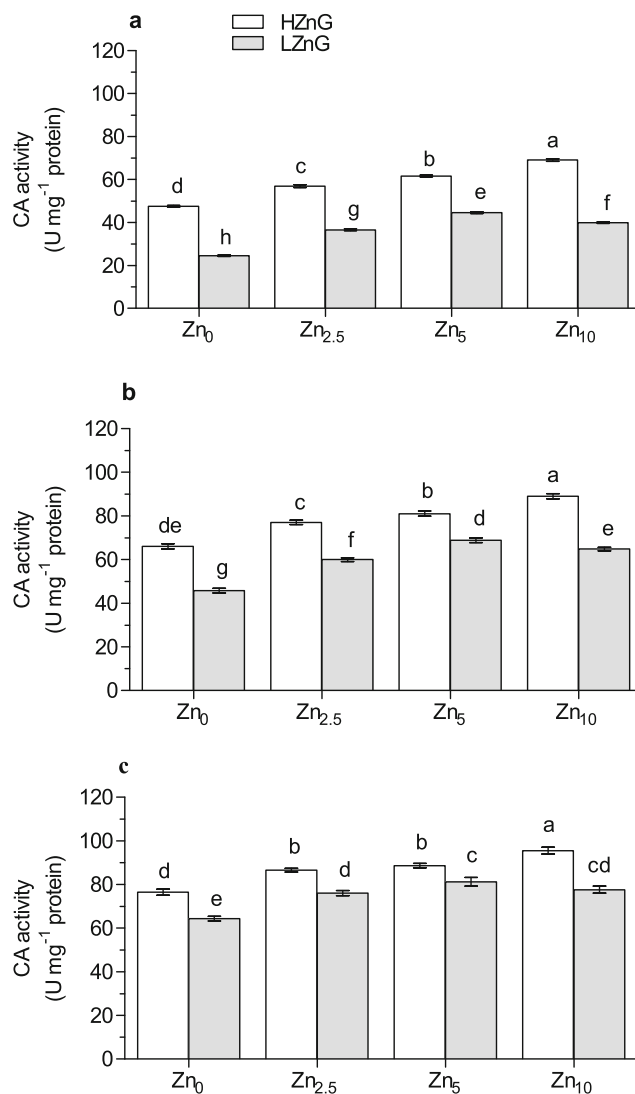
genotypes. However, control plants of HZnG could maintain a significantly higher NR activity possibly due to their better ability of Zn and nitrate uptake from soil solution, and efficient utilization potential of available tissue zinc for normal functioning of biomembrane, enzyme activation, and gene expression (Kim et al 2002). The observed increase in response to zinc fertilization (Fig. 2) could be due to an increase in its respective substrate (NO$_3^-$). The supply of N, either through organic or inorganic sources, affects the NR activity in plants, as do the environmental factors. Thus, supply of nitrate from roots to leaves must have a regulatory role for NR activity (Alguacil et al. 2006). The improved Zn level possibly stabilizes membrane structure and its fluidity, which

might facilitate the uptake of nutrients, including nitrate, thereby increasing the nitrate content, which is likely to induce NR (Campbell 1999).

Nitrate reductase activity in plant tissue is proportional to NO_3^- contents of the soil; whereas its synthesis is regulated by its flux rather than the contents of NO_3^- within the leaf (Shaner and Boyer 1976). Although we observed no significant increase in leaf nitrate content due to zinc fertilization (Table 3), NR activity improved significantly (Fig. 2). This concentration-based effect of Zn could be because NR activity was induced and/or its degradation was prevented at a given concentration of Zn, which might induce NR synthesis by mobilization of intracellular NO_3^-, and provide protection to in vivo NR degradation in the absence of NO_3^- (Singh et al. 1997). Zn was stimulatory for NRA up to a level, above which it became inhibitory or has no significant effect. Since the content of any active protein (enzyme) represents a fine balance between its synthesis/activation and degradation/ inactivation (Jain and Srivastava 1981), Zn concentration might play an active role in such a regulation where Zn concentration till certain level increased the NR activity. Moreover, an efficient N assimilation is likely to be favored by a high rate of CO_2 assimilation (Ferrario et al. 1998). Since increasing Zn level improves chlorophyll formation and CO_2 assimilation, NR activity should also increase accordingly. This study indicates that zinc has an impact on utilization of nitrogen available within the plant. Therefore, zinc fertilization might improve nitrogen utilization efficiency of chickpea genotypes.

Zn supply to the soil low in available Zn was positively correlated to the major Zn-requiring enzymes (SOD and CA) in the two genotypes (Figs. 3 and 4) differing in their Zn accumulation pattern. The low Zn content of the control plants was attributed to low availability of Zn to plants, which leads to a low enzyme activity (Singh et al. 2005).

SOD, which acts as the first line of defense against ROS, protects the cell from oxidative damage (Alscher et al. 2002), and zinc is a cofactor of SOD. An appropriate Zn fertilization has a protective role against photo-oxidative damage of tissues (Cakmak 2000; Wang and Jin 2005). In the present study, increase in zinc levels as well as the plant age increased the SOD activity in the chickpea genotypes differing in zinc accumulation capacity (Fig. 3). In Zn-deficient condition, HZnG maintained a significantly higher SOD activity, compared with LZnG (Fig. 3). This difference between the two genotypes is attributed to the zinc status of the plant (Cakmak et al. 1997). A similar, differential activity of SOD was reported in Zn-efficient and Zn-inefficient pea (*Pisum sativum*) genotypes grown under Zn-deficient condition (Pandey et al. 2012). Zinc deficiency in general suppressed SOD activity in both genotypes (Fig. 3), as observed also in some earlier studies (Sharma et al. 2004; Pandey et al. 2012). In our study, most of the parameters responded up to Zn_5 in both the genotypes,

while leaf SOD activity continued to increase up to Zn_{10} treatment.

CA is one of the most abundant Zn-containing metalloenzymes in chloroplasts of C_3 plants. Zinc addition, particularly at Zn_{10}, improved its activity in chickpea genotypes differing in Zn accumulation capacity (Fig. 4). The content and activity of the enzyme are under fine regulation at the level of transcription and/or translation (Okabe et al. 1980); either of these processes was favored by Zn_{10} that increased the CA activity (Fig. 4). It is known that a decline of CA activity due to Zn deficiency substantially reduces the net photosynthetic rate (P_N) in plants (Cakmak and Engels 1999; Hacisalihoglu et al. 2003), and our results approved it. Genotypic differences for CA activity were significant (Fig. 4). Earlier, Fischer et al. (1997) showed a higher P_N in Zn-deficiency-resistant wheat cultivars than in a sensitive cultivar. CA activity was reported to be high in tissues containing high levels of zinc in wheat (Hacisalihoglu et al. 2003). The activity was well correlated to zinc content of plants in the present study; it increased with increasing zinc level and/or plant age (Fig. 4). This suggests that CA activity could be used as a laboratory tool for assessing the zinc status and hence the zinc efficiency of chickpea genotypes.

In conclusion, there exists a genotypic variation for plant capacity to utilize the available zinc. This study suggests that by maintaining efficient antioxidant defense system and vital metabolic processes, the HZnG of chickpea shows a lower sensitivity toward Zn deficiency in comparison to LZnG. Thus, HZnG is more effective than LZnG in overcoming the Zn-related nutritional disorders in crops. Moreover, the metal-associated biochemical processes may be taken as a criterion while selecting Zn-efficient genotypes. Biochemical probing is therefore necessary for identifying the efficient crop plants and strengthening the potential genetic resources.

Conflict of interest There is no conflict of interest.

References

Ahlawat IPS, Gangaiah B, Zahid MA (2007) Nutrient management in chickpea. In: Yadav SS, Redden R, Chen W, Sharma B (eds) Chickpea breeding and management. CABI, Wallingford, pp 213–232

Alguacil M, Caravaca F, Díaz-Vivancos P, Hernández J, Roldan A (2006) Effect of arbuscular mycorrhizae and induced drought stress on antioxidant enzyme and nitrate reductase activities in *Juniperus oxycedrus* L. grown in a composted sewage sludge amended semi-arid soil. Plant Soil 279:209–218

Alloway BJ (2004) Zinc in soils and crop nutrition. International Zinc Association Publications, Brussels

Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Biol 531: 1331–1341

Andreini C, Banci L, Bertini I, Rosato A (2006) Zinc through the three domains of life. J Proteome Res 5:3173–3178

Ankerman D, Large R (1974) Soil and plant analysis. A&L Agricultural Laboratories Inc, New York

Arnon DI (1949) Copper enzymes in isolated chloroplasts, polyphenoxidase in beta vulgaris. Plant Physiol 24:1–15

Bettger WJ, O'Dell BL (1981) A critical physiological role of zinc in the structure and function of biomembranes. Life Sci 28:1425–1438

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–254

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. New Phytol 173:677–702

Brown PH, Cakmak I, Zhang Q (1993) Form and function of zinc in plants. In: Robson AD (ed) Zinc in soils and plants. Kluwer, Dordrecht, pp 90–106

Cakmak I (2000) Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytol 146:185–205

Cakmak I, Engels C (1999) Role of mineral nutrients in photosynthesis and yield formation. In: Rengel Z (ed) Mineral nutrition of crops. Haworth, New York, pp 141–148

Cakmak I, Marschner H (1993) Effect of zinc nutritional status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves. Plant Soil 156:127–130

Cakmak I, Ozturk L, Eker S, Torun B, Kalfa HI, Yilmaz A (1997) Concentration of zinc and activity of copper/zinc-superoxide dismutase in leaves of rye and wheat cultivars differing in sensitivity to zinc deficiency. J Plant Physiol 151:91–95

Cakmak I, Kalayci M, Ekiz H, Braun HJ, Kilinc Y, Yilmaz A (1999) Zinc deficiency as a practical problem in plant and human nutrition in Turkey: a NATO-science for stability project. Field Crops Res 60: 175–188

Campbell HW (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu Rev Plant Physiol Plant Mol Biol 50:277–303

Cao Y, Zhang Q, Chen Y, Zhao H, Lang Y, Yu C, Yang J (2013) Identification of differential expression genes in leaves of rice (Oryza sativa L.) in response to heat stress by cDNA-AFLP analysis. BioMed Res Int. doi:10.1155/2013/576189

Chaab A, Savaghebi GR, Motesharezadeh B (2011) Differences in the Zn efficiency among and within maize cultivars in a calcareous soil. Asian J Agric Sci 3:26–31

Cherif J, Mediouni C, Ben Ammar W, Jemal F (2011) Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (Solanum lycopersicum). J Environ Sci 23: 837–844

Das S, Green A (2013) Importance of zinc in crops and human health. J SAT Agric Res 11:1–7

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93–101

Feng-Juan Y, Shi-Qi L, Xiu-Feng W, Jin-Bo Z (2005) The effect of zinc on physiological-biochemical indexes and nutrition quality of garlic. Soils Fert 1:30–36

Ferrario S, Valadier MH, Foyer CH (1998) Over-expression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. Plant Physiol 117:239–302

Fischer ES, Thimm O, Rengel Z (1997) Zinc nutrition influences the CO_2 gas exchange in wheat. Photosynthetica 33:505–508

Grover HL, Nair TVR, Abrol YP (1978) Nitrogen metabolism of the upper three leaf blades of wheat at different soil nitrogen levels. I. Nitrate reductase activity and content of various nitrogenous constituents. Physiol Plant 42:287–292

Gurmani AR, Din JU, Khan SU, Andaleep R, Waseem K, Khan A, Hadyat-Ullah A (2012) Soil application of zinc improves growth and yield of tomato. Int J Agric Biol 14:91–96

Hacisalihoglu G, Hart JJ, Wang YH, Cakmak I, Kochian LV (2003) Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiol 131:595–602

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334

Jain A, Srivastava HS (1981) Effect of salicylic acid on nitrate reductase activity in maize seedlings. Physiol Plant 51:339–342

Jaworski EG (1971) Nitrate reductase assay in intact plant tissue. Biochem Biophys Res Commun 43:1274–1279

Khan HR, McDonald GK, Rengel Z (1998) Chickpea genotypes differ in their sensitivity to Zn deficiency. Plant Soil 198:11–18

Khan HR, McDonald GK, Rengel Z (2000) Response of chickpea genotypes to zinc fertilization under field conditions in South Australia and Pakistan. J Plant Nutr 23:1517–1531

Kim T, Mills HA, Wetzstein HY (2002) Studies on the effect of zinc supply on growth and nutrient uptake in pecan. J Plant Nutr 25: 1987–2000

Li WYF, Wong FL, Tsai SN, Phang TH, Shao GH, Lam HM (2006) Tonoplast-located GmCLC1 and GmNHX1 from soybean enhance NaCl tolerance in transgenic bright yellow (by)-2 Cells. Plant Cell Environ 29:1122–1137

Makino A, Sakashita H, Hidema J, Mae T, Ojima K, Osmond B (1992) Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nutrition and their possible relationship to CO_2-transfer resistance. Plant Physiol 100:1737–1743

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London

Ohki K (1976) Effect of zinc nutrition on photosynthesis and carbonic anhydrase activity in cotton. Physiol Plant 38:300–304

Okabe K, Lindlar A, Tsuzuki M, Miyachi S (1980) Effects of carbonic anhydrase on ribulose 1,5-bisphosphate carboxylase and oxygenase. FEBS Lett 114:142–144

Pandey N, Gupta B, Pathak GC (2012) Antioxidant responses of pea genotypes to zinc deficiency. Russ J Plant Physiol 59:198–205

Rengel Z (1995) Carbonic anhydrase activity in leaves of wheat genotypes differing in Zn efficiency. J Plant Physiol 147:251–256

Saxena MC (1993) The challenge of developing biotic and abiotic stress resistance in cool-season food legumes. In: Singh KB, Saxena MC (eds) Breeding for stress tolerance in cool-season food legumes. John Wiley, New York, pp 3–14

Shaner DL, Boyer JS (1976) Nitrate reductase activity in maize (Zea mays L.) leaves. I. Regulation by nitrate flux. Plant Physiol 58:499–504

Sharma PN, Kumar P, Tewari RK (2004) Early signs of oxidative stress in wheat plants subjected to zinc deficiency. J Plant Nutr 27:449–461

Shukla UC, Yadav OP (1982) Effect of phosphorus and zinc on nodulation and nitrogen fixation in chickpea (Cicer arietinum L.). Plant Soil 65:239–248

Siddiqui SN, Umar S, Iqbal M (2013) Searching for chickpea genotypes with high Zn-accumulating capacity: a way to identify nutrient-efficient crop plants. Int J Agron Plant Prod 4:1697–1705

Singh MV (2008) Micronutrient deficiencies in crop and soils in India. In: Alloway BJ (ed) Micronutrient deficiencies in global crop production. Springer, New York, pp 93–123

Singh PK, Koul KK, Tiwari SB, Kaul RK (1997) Effect of cinnamate on nitrate reductase activity in isolated cucumber cotyledons. Plant Growth Regul 21:203–206

Singh B, Natesan SKA, Singh BK, Usha K (2005) Improving Zn efficiency of cereals under Zn deficiency. Curr Sci 88:36–44

Wang H, Jin JY (2005) Photosynthetic rate, chlorophyll fluorescence parameters, and lipid peroxidation of maize leaves as affected by zinc deficiency. Photosynthetica 43:591–596

Weisany W, Sohrabi Y, Heidari G, Siosemardeh A, Ghassemi-Golezani K (2011) Physiological responses of soybean (*Glycine max* L.) to zinc application under salinity stress. Aust J Crop Sci 5:1441–1447

Wilson DO, Reisenauer HM (1963) Determination of leghaemoglobin in legume nodules. Anal Biochem 6:27–30

Zago MP, Oteiza PI (2001) The antioxidant properties of zinc: interactions with iron and antioxidants. Free Rad Bio Med 31:266–274

Zhao LI, Yuan L, Wang Z, Lei T, Yin X (2012) Phytoremediation of zinc-contaminated soil and zinc-biofortification for human nutrition. In: Yin X, Yuan L (eds) Phytoremediation and Biofortification. Springer, New York, pp 33–57