BIOLOGIA PLANTARUM 48 (2): 255-260, 2004

Zinc-induced changes in morpho-physiological and biochemical parameters in *Artemisia annua*

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Abstract

Responses of *Artemisia annua* to different concentrations of zinc [50, 100, 200, 300 and 400 μg g^{-1}(soil dry mass)] were studied during plant ontogeny. Total leaf area, dry mass of leaves, length and dry mass of shoots and roots increased with the age of the plant but the magnitude of increase declined significantly under the influence of Zn treatment. Net photosynthetic rate, intercellular carbon dioxide concentration and stomatal conductance were highest at flowering stage in control and treated plants and decreased at post flowering stage. Contents of chlorophyll *a*, chlorophyll *b*, carotenoids, proteins and nitrate reductase activity in leaves increased from pre-flowering to maximum level at flowering stage and decreased thereafter in both control and treated plants. Presence of Zn in the soil drastically decreased/inhibited all the parameters, and the magnitude of decline increased with increasing Zn concentration.

Additional key words: carotenoids**,** chlorophyll, dry matter, growth, leaf area, net photosynthetic rate, nitrate reductase activity, protein, stomatal conductance, zinc.

Introduction

Zinc is a major industrial pollutant of the terrestrial as well as aquatic environment (Foy *et al*. 1978). General symptoms of zinc toxicity are wilting, necrosis of old leaves, and reduced plant growth (Wallnofer and Engelhardt 1984). Although, zinc is needed as micronutrient by all living organisms (*e.g.* Arjunan and Samboornaraman 1994), however, it becomes hazardous at higher concentrations and inhibits growth (Rauser 1973, Dogar and Vanhai 1979, Bergmann 1992, Ali *et al*. 1999). Zinc toxicity inhibits chlorophyll formation in young leaves (Nag *et al*. 1984, Kaya *et al*. 2000). Photosynthesis and transpiration are also reduced by high concentrations of zinc (Van Assche *et al*. 1979). The observed reduction in growth is also a consequence of Zn^{2+} interference with nutrient uptake (Chaney 1993, Kaya *et al*. 2000) and certain enzyme activities (Quariti *et al*. 1997). The present study explores variations in growth, physiological and biochemical responses of *Artemisia annua*, a medicinal plant to varied concentrations of zinc at different stages of plant growth and development.

Materials and methods

Healthy seeds of *Artemisia annua* were germinated in the open, and seedlings were transferred in earthen pots (30 × 30 cm) containing sterilized soil and farm compost in 7:3 ratio. Pots were further fertilized with P and K (corresponding to 60 kg ha^{-1} each in the form of single super phosphate and muriate of potash). The seeds were sown in December, when the mean monthly temperature ranged from 5.3 °C (minimum) to 33.1 ºC (maximum). After a month of seedling growth individual pots were randomly treated with one of five concentrations of Zn [50, 100, 200, 300 and 400 μg g^{-1}(soil d.m.)]. Plants were sampled at the age of 4 months at (pre-flowering phase),

Received 28 June 2002, *accepted* 25 April 2003.
Abbreviations: Car - carotenoids, Chl - chlorophyll, c_i - intercellular carbon dioxide concentration, NEDD - naphthylethylene diamine dihydrochloride, NRA - nitrate reductase activity, P_N - net photosynthetic rate, g_s - stomatal conductance.
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5 months (flowering stage) and 7 months (post-flowering phase). The mean minimum and maximum temperature at the time of three samplings were 19.6 and 33.7, 23.5 and 37.9, 23.5 and 36.6 °C, respectively.

Root, shoot and leaf samples were separated and oven dried at 70 ºC for 48 h to determine dry masses. Total foliar area was estimated with a digital leaf area meter (*LI-COR*, Lincoln, NE, USA). Net photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular carbon dioxide (c_i) were calculated using an open gas exchange system with *LI-3100* (*LI-COR*). Chlorophyll (Chl) content was estimated by the method of Hiscox and Israelstam (1979). Fresh leaf material (100 mg) and 10 cm^3 DMSO were taken in vials and kept in oven at 65 °C for 4 h. Absorbance was read at wavelengths 663, 645, 510 and 480 nm using spectro-photometer *Beckman DU 640 B* (Fullerton, USA). The amounts of Chl *a* and Chl *b* and carotenoids (Car) were determined by the formulae of Duxbury and Yentsch (1956) and MacLachlan and Zalik (1963). Nitrate reductase activity (NRA) in fresh leaves was determined by the hydrazine reduction method (Klepper *et al.* 1971). Fresh leaves (0.3 g) were kept in vials with 3 cm^3 phosphate buffer (0.1 M, pH 7.2) and 3 cm^3 of KNO_3 (0.4 M). The vials were kept in vacuum desiccators and vacuum infiltration was done till the leaves settled down. The vials were kept in water bath incubator for 1 h at 33 °C and then in hot water for 5 min to stop the reaction. To 1 cm^3 of aliquot, 0.2 cm^3 of sulphanylamide (1 % in 0.1 M HCl) and 1 cm^3 of naphthylethylene diamine dihydrochloride (NEDD) (0.02 %) were added and the final volume was made up to 6 cm^3. The vials were kept in dark for 20 min for colour development. Absorbance was measured at 540 nm on *Beckman DU 640 B* spectrophotometer. Concentration of protein was determined by using bovine serum albumin as standard (Bradford 1976). Fresh leaves (0.5 g) were ground with 1 cm^3 phosphate buffer (0.1 M, pH 7.0) in mortar and pestle and kept in ice. The material was centrifuged at 5 000 *g* for 10 min. Supernatant (0.5 cm^3) was mixed with 0.5 cm^3 TCA and again centrifuged at 3 300 *g* for 30 min. The supernatant was discarded and the pellet left was washed twice by double distilled water (DDW) and dissolved in 0.1 M NaOH and mixed with 0.5 cm^3 Bradford reagent and kept for 1/2 h. Absorbance was measured at 595 nm on *Beckman DU 640 B* spectrophotometer. The data were analyzed statistically to estimate the significance of the variations observed.

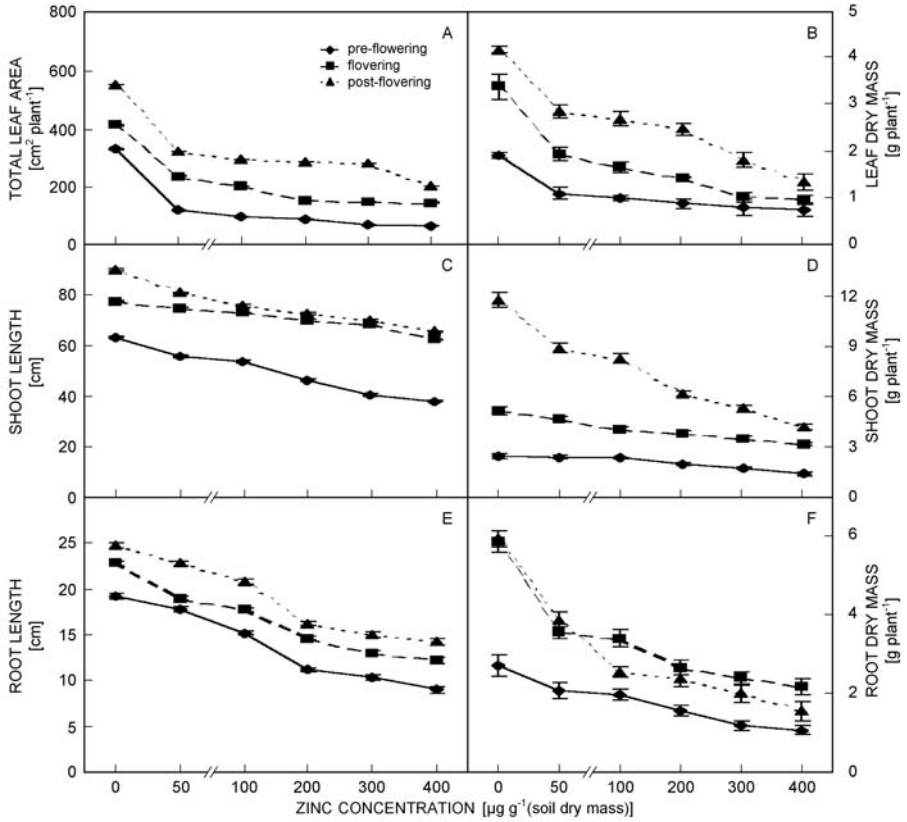

Fig. 1. Effect of zinc concentrations on total leaf area (*A*), leaf dry mass (*B*), shoot length (*C*), shoot dry mass (*D*), root length (*E*), and root dry mass (*F*) in *Artemisia annua*. *Vertical bars* show ± SE of mean, $n = 4$. Differences between control and treated plants are significant at $P = 0.05$.

Results

Total leaf area and dry mass increased with age in control as well as treated plants. However, the rate of increase was significantly lower under the influence of Zn^{2+} treatment (Figs. 1, 2*A*). The maximum retardation about 80.8 % in total leaf area and 71.51 % in leaf dry mass were detected at pre-flowering and flowering stages at 400 µg g^{-1} Zn treatment. Shoot length, root length increased with the age of the plant. Zinc application at all the concentrations significantly decreased the shoot and root lengths, though the highest reduction of 39.9 and 43.0 %, respectively, were observed at 400 µg g^{-1} Zn treatment (Fig. 1). Dry mass of shoot, root and total plant (Fig. 2*A*) also increased with plant age, but the rate of increase dropped significantly up to 64.2, 78.7 and 67.6 % at 400 µg g^{-1} Zn treatments. Nitrate reductase activity (Fig. 2*B*) and total soluble protein content (Fig. 2*C*) were higher at flowering stage in both control and treated plant but the magnitude of increase was decreased significantly under Zn treatment. The maximum reductions were about 62.8 % in case of NR activity and 56.3 % in case of protein content at 400 µg g^{-1} Zn treatment (Fig. 2). P_N, c_i and g_s increased from pre-flowering to flowering stage and decreased thereafter (Fig. 3*A*,*B*,*C*). The decrease in P_N, g_s and c_i were 58.3, 85.9, and 55.2 %, respectively, at highest Zn concentration (Fig. 3). Concentrations of Chl *a*, Chl *b* and Car contents were lower at pre- and post-flowering stages and were maximum at flowering stage in control and treated plants but the rate of increase was significantly reduced under Zn treatment (Fig. 3*D*,*E*,*F*). The maximum reductions were 34.0, 57.2, 39.1, and 35.0 % in Chl *a*, Chl *b*, Chl *a*+*b* and Car, respectively, at 400 µg g^{-1} Zn.

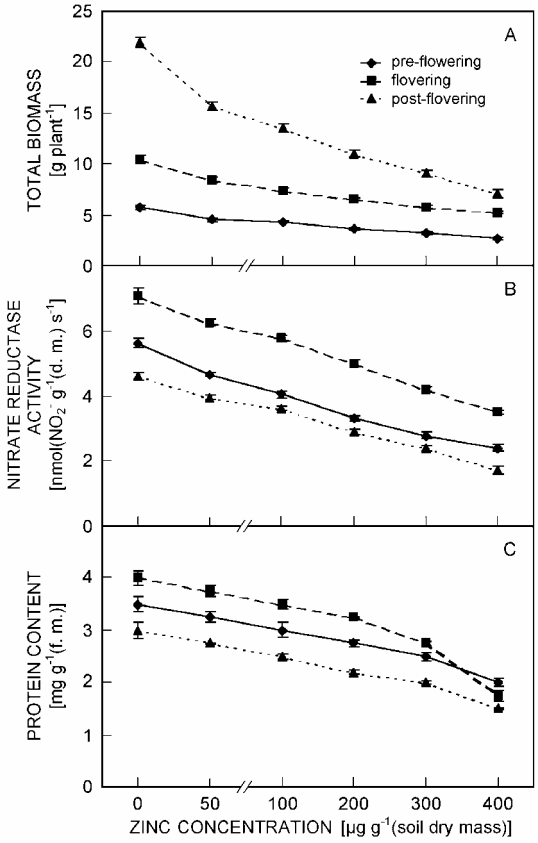

Fig. 2. Effect of zinc concentrations on total biomass (*A*), nitrate reductase activity (*B*), and protein content (*C*) in *Artemisia annua. Vertical bars* show ± SE of mean, n = 4. Differences between control and treated plants are significant at P = 0.05.

Discussion

Treatment by Zn brings about significant decrease in growth parameters at different developmental stages of *Artemisia annua* plants. Zn caused a significant reduction in plant height of *Artemisia annua* even at 50 and 100 µg g^{-1}(soil d.m.). Root length also decreased significantly at all Zn concentrations, except at 50 µg(Zn) g^{-1}. The difference in growth was significant at all developmental stages and Zn concentrations, except the lowest one.

Although Zn is essential for plant growth, its excess is inhibitive as was reported for a number of leguminous plants (Khudsar 1999). Elongation of shoot and root is retarded by Zn application in green grass (Veer and Lata 1989), *Phaseolus mungoo* (Chaoui *et al*. 1997), *Vigna radiata* and *Sorghum bicolor* (Balashouri 1995) and *Bacopa monniera* (Ali *et al*. 1999). Reduced shoot growth at higher concentration could be a consequence of its interference with certain essential metabolic events (Tripathy and Mohanty 1980, Van Assche and Clijsters 1990, Alia *et al*. 1995).

Zinc reduced leaf growth in *Artemisia annua* similarly as in *Phaseolus vulgaris* (Polson and Adams 1970). This may involve inhibition of cell division as well as cell elongation (Arduini *et al.* 1994). Inhibition in growth because of metal toxicity leads to a reduction in biomass production (Balashouri 1995, Chaoui *et al*. 1997, Quariti *et al*. 1997).

In the present investigation P_N, g_s and c_i were reduced significantly at each concentration of Zn. Reduced P_N with high concentration of zinc was also observed in *Phaseolus vulgaris* (Van Assche *et al*. 1979, 1980). Zn was found to inhibit electron transport (Tripathy and Mohanty 1980, Baker *et al.* 1982) and possibly acts at oxidizing site of PS 2 (Miller and Cox 1983, Van Assche and Clijsters 1986). Baker *et al*. (1982) proposed a site for zinc action between PS 2 and PS 1 in the electron

transport chain. Ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) activity decreased under Zn treatments (Van Assche *et al.* 1980). Plants with supraoptimal concentration of zinc have reduced g_s of leaves and a retarded activity of some chloroplastic and peroxisomal enzymes, compared to plants receiving the optimal Zn concentrations (Van Assche *et al*. 1979, 1980). The photosynthetic electron transport and Rubisco activity were inhibited in *Phaseolus vulgaris* treated with toxic amounts of Zn (Van Assche and Clijsters 1983). Zn inhibits Rubisco carboxylation activity without affecting its oxygenation activity (Van Assche and Clijsters 1986). The P_N was inhibited while CO_2 compensation concentration increased in the intact *Phaseolus vulgaris* plants after Zn treatment (Van Assche *et al.* 1988).

The amounts of Chl *a*, Chl *b* and Car in *Artemisia*

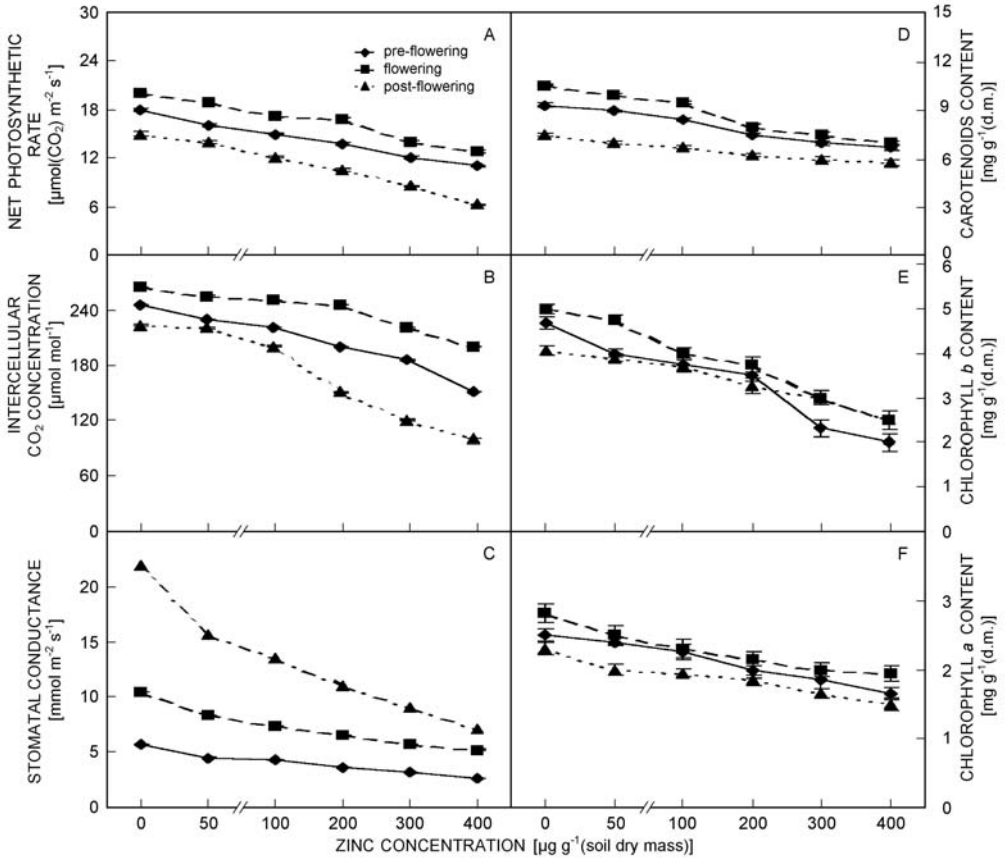

Fig. 3. Effect of zinc concentrations on net photosynthetic rate (*A*), intercellular CO_2 concentration (*B*), stomatal conductance (*C*), chlorophyll *a* (*D*), chlorophyll *b* (*E*), and carotenoids (*F*) contents in *Artemisia annua*. *Vertical bars* show ± SE of mean, $n = 4$. Differences between control and treated plants are significant at $P = 0.05$.

annua were relatively low under Zn stress. High concentration of Zn inhibited Chl *a* and Chl *b* contents in *Chlorella vulgaris* (Rai and Kumar 1980) and *Oryza sativa* seedlings (Nag *et al*. 1984). Chl content of primary needles of *Picea abies* tended to decline after Zn exposure (Schlegel *et al*. 1987).

NRA was also significantly lower in the leaves of Zn treated *Artemisia annua* plants. A small decrease in the leaf NRA may be attributed to a restricted translocation of the metal to the site of enzyme action (Bharti and Singh 1993). Inhibition of *in vivo* NRA at higher metal ion concentration could be due to a disorganization of chloroplast (Rebechini and Hanzely 1974) or lesser NO_3^- supply at the site of enzyme synthesis because of water stress created by the metal (Burzynski and Grabowski 1984). It may also be a direct effect of the metal ions on the enzyme/protein synthesis or activity as it has a strong affinity for the functional SH group of the enzyme (Prasad and Prasad 1987, Sinha *et al*. 1988).

The protein content normally declines under heavy metal stress (Vyas and Puranik 1993, Bhattacharya and Choudhuri 1994). *Artemisia annua* leaves had a significantly low protein content throughout the plant life in the zinc treated plants. Concentration of protein was significantly reduced in *Phaseolus vulgaris* roots by Cd and Zn treatments (Chaoui *et al.* 1997). Normally the roots were more affected by stress than shoots in terms of growth. The decline in proteins and RNA content and the corresponding rise in the activity of hydrolytic enzymes,

such as protease and RNAase due to heavy metal stress strongly suggest the catabolic activities. It is likely that heavy metal stress induces senescence through enhancement of catabolism of key metabolites such as chlorophyll, protein and RNA. In fact a decrease in protein level may be a consequence of decrease in NR activity, as the enzyme is believed to be rate limiting in the overall assimilation of nitrate (Beevers and Hageman 1969), consequently affecting total protein and growth of the plant.

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