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FOLIAR APPLICATION OF ASCORBIC ACID ALLEVIATES ADVERSE EFFECTS OF ZINC TOXICITY IN PEAS, *PISUM SATIVUM* L.

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ABSTRACT

The present study was designed to observe whether the hostile effects of zinc stress on *Pisum sativum* L. plants could be alleviated by exogenous application of ascorbic acid as a foliar spray and how far it regulates the plant antioxidant enzyme system. A pot experiment was conducted to study the effect of foliar application of ascorbic acid on morphological and antioxidants attributes of two *Pisum sativum* L. cultivars (Meteor and 2001-40) grown under zinc stress. Treatment of ZnSO4.7H2O (5 ppm and 10 ppm) through rooting medium and ascorbic acid (5 ppm and 10 ppm) as foliar spray were applied after three weeks of germination. In pea plants exposed to zinc stress, growth was significantly reduced in terms of shoot and root lengths, fresh and dry weights, leaf area and number of leaves per plant. However, foliar application of ascorbic acid (AsA) was able to alleviate the zinc induced and significantly improve the above growth traits. The chlorophyll a, b and carotenoids levels were decreased under zinc stress. However, these pigments were increased by follow up the application of AsA. The total soluble proteins were decreased in zinc stress. The foliar application of AsA and zinc increased the level of ascorbic acids. The enzymatic activities of catalase (CAT) and Peroxides (POD) were decreased under zinc and foliar application AsA. Ascorbic acid (5 ppm and 10 ppm) played significant role in the amelioration the zinc stress. Overall, cv. 2001-40 performed better morphological, biochemical and antioxidants attributes compared to cv. Meteor under zinc stress.

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INTRODUCTION

Heavy metal contamination is considered as one of the most severe problems worldwide and has main impact on environmental and human health. Metal concentrations in soil range from less than 1 mg Kg-1 (ppm) to high as 100,000 mg kg-1. Agricultural soils in several parts of the world are slightly to temperately polluted by heavy metal toxicity such as Zn, Cd, Cr, Ni, Co, Pb, Cu and As. The reason behind is the use of extensive phosphate fertilizers, industrial waste, sewage sludge application, and dirt from smelters, and ruthless irrigating practices in cultivated lands for longer time (Bell et al., 2001; Passariello et al., 2002; Schwartz et al., 2001). The phytotoxic effect of heavy metals in plants displays itself through visual symptoms such as chlorosis, necrosis and wilting (Gomes et al., 2011).

Zinc is one of the most abundant trace elements involved in several physiological processes and it becomes toxic to plant in excess concentration (Sinniah, 2004). Zn is redox-stable under physiological conditions, being considered as one of the least toxic heavy metal (Codina et al., 2000). The average concentration of this element is 25 to 150 mg kg-1 in plants. Deficiencies of Zn are generally related with concentrations of less than 20 mg kg-1, and poisonousness will occur when the Zn leaf concentration exceeds 400 mg L-1 (Doberman and Fairhurst, 2000). Zinc toxicity also causes chlorosis in the younger leaves which can extend to mature leaves after long exposure to zinc contaminated soil (Ebbs and Kochian, 1997).

Ascorbic acid (AsA) is a small and water-soluble antioxidant molecule. It is main source of vitamin C for human being (Pastori et al., 2003). AsA is a non-enzymatic complex which permits plants to defend against stresses by reducing oxygen free radicals (Shafiq et al., 2014). It is present in all living plant cells, the major quantities being generally in the flowers and leaves and actively growing parts (Smirnoff et al., 2001). AsA is usually dispersed in the cytosol of the plant and it acts as an antioxidant. Also, the plants with small ascorbate production are relatively sensitive to various environmental stresses which can harmfully distress their growth and development (Ahmed et al., 2013).

Foliar application of ascorbic acid has been very operative in improving plant growth and development by changing oxidative defence system, cell expansion, ion transports, phytohormone signalling, and other correlated processes under stress or non-stress environment (Pignocchi and Foyer, 2003). AsA plays a regulatory role in the plant growth by modifying the production of plant hormones such as ABA, GA and ethylene and plays significant roles under abiotic stress (Pastori et al., 2003). Foliar application of ascorbic acid enhanced plant tolerance to drought stress, salinity stress, chill and heavy metal stress (Chao and Kao, 2010; Shalata and Neumann, 2001).

Pisum sativum L. (Pea) belongs to family Fabaceae. It is a cool season crop grown in several parts of the world in winter to early summer depending on location (Naz et al., 2014). It is self-pollinated, annual herb, stem weak, alternate leaves and terminal branched vines leaflets oval or elliptic (Ghafoor et al., 2005). *P. sativum* is grown all over the world for various uses as food and forage. Pea is the world's third most important legume grain after soybean and common beans (Timmerman-Vaughan et al., 2005). It is one of the important vegetables in the world and ranks among the top ten vegetable crops. It is generally used in human food throughout the world and it is rich in proteins (21-25 %), carbohydrates, Calcium, phosphorous, vitamin A and C, and has high levels of amino acids lysine and tryptophan (Bhat et al., 2013).

The main objective of the present study was to observe the comparative effect of different concentration of Ascorbic acid (0, 5 and 10 ppm) on the performance of pea plants subjected to different concentration of Zinc stress (0, 5 and 10 ppm) and to develop easy ways to reduce the impact of zinc stress in crops for farmers.

MATERIALS AND METHODS

The experiment was set up in the Old Botanical Garden, University of Agriculture Faisalabad during the winter season in 2018-19. Seeds of both *P. sativum* cultivars were obtained from Oil Seed Department, Ayub Agriculture Research Institute Faisalabad. A pot experiment was carried out to check the influence of foliar application of ascorbic acid (AsA) on morphometric and physio-chemical attributes of two pea cultivars (2001-40 and Meteor) under zinc stress. The experiment was laid out in a completely randomized design (CRD) with three replicates. Twelve seeds of cultivar 2001-40 and Meteor were sown in plastic pots (20 cm diameter and 24 cm depth) containing 9 kg well mixed soil on 14th November, 2018. After one week of germination, six plants of uniform size were retained per pot. Treatment of zinc sulphate ($ZnSO_4.7H_2O$) in two different concentrations (5 ppm and 10 ppm) through rooting medium and ascorbic acid (5 ppm and 10 ppm) as foliar spray were applied after three weeks of germination. The growing plants were irrigated at regular interval till the termination of the experiment. The plants were harvested after ten days of treatment. The uprooted plants were blotted dry and their root and shoot lengths were measured by measuring tape. The number of leaves and number of branches per plant were calculated with common observation and mean value was recorded. Leaf area was measured by multiplying maximum length, maximum width and correction factor.

Total leaf area = Maximum leaf length × Maximum leaf width × C.F.

Where C.F. stands for Correction factor= 0.68

The fresh weights of root and shoot were measured by using the electrical balance. The roots and shoots were dried in oven at 80°C for three days. After three days, the dry weight of root and shoot was measured.

Photosynthetic pigments were measured by Arnon et al. (1956) method. Fresh leaf samples (100 mg) were reserved overnight in 10 mL of 80% acetone. The next morning, the extracts were centrifuged at 10,000 rpm for 5 minutes and the absorbance of supernatant was noted at 663, 645 and 480 nm using a Spectrophotometer (IRMECO UV-Vis Model U2020).

The total soluble proteins (TSPs) were measured by Bradford (1976) method. For the estimation of TSPs, fresh leaf samples were homogenized in 2.5 ml sodium phosphate buffer (pH 7). The homogenized mixture was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was used for the determination of TSPs at 595 nm. Fresh leaf samples (120 mg) were ground in 6% TCA by using pestle and mortar and then was filtered. Filtrate (2 mL) was mixed with 2% dinitrophanyl hydrazine in 9N H2SO4 (1 ml) and one drop of 10% thiourea that was prepared in 70% ethanol. The samples were boiled in water bath for 15 minutes and then cooled at room temperature. After this, 2.5 mL of 80% H2SO4 was added and absorbance was taken at 530 nm using Spectrophotometer following the method of Mukherjee and Choudhuri (1983).

The catalase and peroxidase were estimated by following the method of Maehly and Chance (1955). Fresh leaf material (100 mg) was ground in 2.5 ml of sodium phosphate buffer (pH 7). The ground material was centrifuged at 12000 rpm for 15 minutes at 4°C. The pellet was discarded and the supernatant was used for the assessment of CAT and POD. The reaction mixture for catalase contained 1 ml H2O2 (5.9 mM), 1.9 ml (2 ml) potassium phosphate buffer (pH 7) and 100 µl of enzyme extract. The decline in absorbance was read at 470 nm after 20 seconds on spectrophotometer. One unit of CAT was taken as an absorbance change of 0.01 units per min. The POD reaction mixture (3 ml) contained 100 µl H2O2 (40 mM), 100 µl guaiacol (20 mM), 750 µl potassium phosphate buffer (50 mM; pH 7.8) and 100 µl of enzyme extract. Then the change in absorbance at 470 nm was read after every 20 seconds. One unit of POD activity was based on increase in absorbance unit per min. MS Excel was used to calculate the means and standard errors while analysis of variance was estimated by using COSTAT computer software.

RESULTS AND DISCUSSION

Ascorbic acid is one of the abundantly occurring water-soluble antioxidant organic compound and is mostly distributed in the cytosol of the plant. It is required in trace amounts to sustain normal plant growth in higher plants. It is the chief source of vitamin C for humans and important compound for plants with significant roles as an antioxidant and as a modulator of plant growth through hormone signalling (Ahmed et al., 2013). The shoot length of pea plants was significantly ($P \leq 0.05$) reduced under zinc stress in comparison to control condition (Table 1). Maximum decrease in shoot length was determined in cv. Meteor under 10 ppm level of zinc stress. Though, maximum rise was observed in both cultivars at 10 ppm level of AsA applied in 5 ppm of zinc stress. Overall, cv. 2001-40 displayed more shoot length under zinc stress as associated to cv. Meteor as shown in figure 1a. Zinc stress reduced the root length significantly ($P \leq 0.05$) in both cultivar of pea (Table 1). However, the foliar application of AsA increased the root length significantly ($P \leq 0.05$) at 10ppm without zinc stress among the all treatments and foliar application of AsA ($P \leq 0.05$). Maximum decrease in root length was detected in Cv. Meteor under 10 ppm level of applied zinc. Overall the cultivar. 2001-40 revealed more root length as related to cv. Meteor as shown in figure 1b.

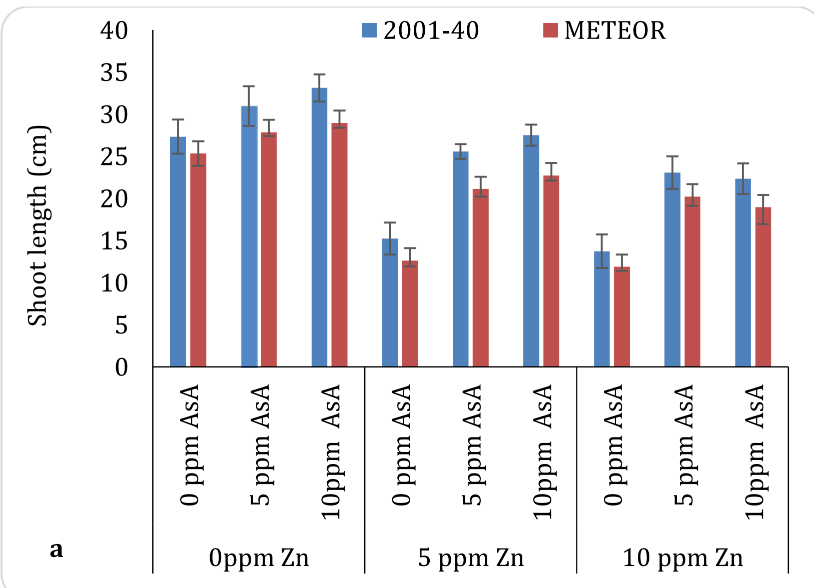
The fresh weight of shoot was significantly ($P \leq 0.05$) reduced under elevated level of zinc stress. However foliar application of AsA (10 ppm) enhanced the shoot fresh weight in zinc concentration 10 ppm in comparison to 0 and 5 ppm concentration of AsA as shown in figure 2a. The maximum fresh weight of shoot was observed in AsA (10 ppm) under control condition among the all. Elevated level of zinc stress significantly ($P \leq 0.05$) decreased shoot dry weight (Table 1). Maximum decline in shoot dry mass was detected in cv. Meteor under 10 ppm level of applied zinc (Figure 2b).

Zinc stress significantly ($P \leq 0.05$) reduced the fresh and dry weight of root of pea plants (Table 1).

Table 1: Analysis of variance for shoot length, root length, fresh weight of shoot, fresh weight of root, dry weight of shoot, dry weight of root, Number of branches/ plant, Number of leaves/plant and leaf area of two Pea cultivars (*Pisum sativum* L.) grown under zinc stress conditions with foliar application of ascorbic acid.

Source of variation	DF	Shoot length	Root length	Fresh weight of shoot	Fresh weight of root	Dry weight of shoot	Dry weight of root	No. of branches/plant	No. of leaves/plant	Leaf area
Cvs	1	141.13***	4**	1.12***	0.060***	0.12***	0.01***	18.96***	174.24***	0.86**
Zinc	2	550.74***	11.22***	2.88***	0.46***	0.20***	0.06***	18.90***	280.35***	12.10***
AsA	2	341.38***	2.09*	0.33***	0.05***	0.11***	0.01***	9.46***	112.57***	1.09**
Cvs × Zinc	2	1.84ns	0.01ns	0.08ns	2.07ns	0.00ns	3.35ns	0.24ns	0.01ns	0.00ns
Cvs × AsA	2	4.46ns	0.01ns	0.02ns	2.46ns	5.16ns	1.68ns	0.79ns	3.12ns	1.56ns
Zinc × AsA	4	25.14*	0.93ns	0.19**	0.00ns	0.00ns	0.00***	2.12ns	12.87ns	0.30ns
Cvs × Zinc × AsA	4	0.12ns	0.09ns	0.03ns	0.00ns	0.00ns	1.51ns	0.24ns	0.65ns	0.01
Error	36	6.54	0.42	0.03	0.00	0.00	0.00	0.88	6.11	0.08

ns = non-significant, *, **, *** significant at 0.05, 0.01 and 0.001 levels, respectively



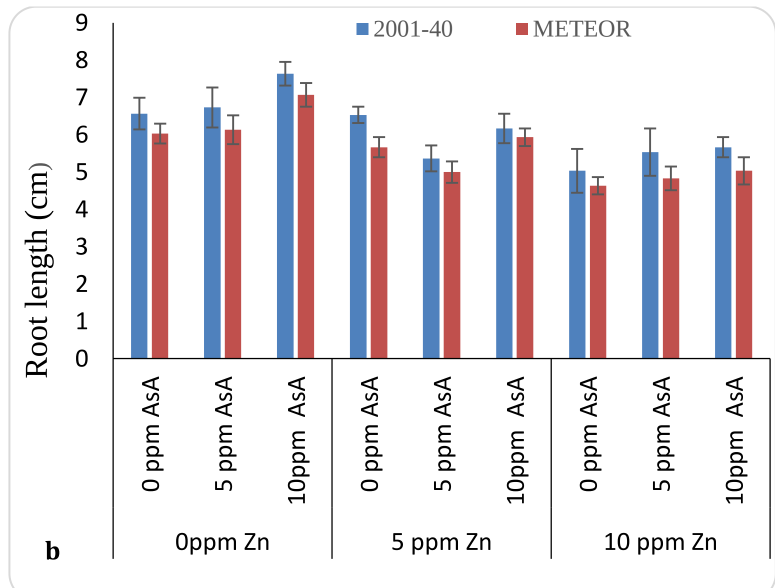


Figure 1: Shoot (a) and root (b) length of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

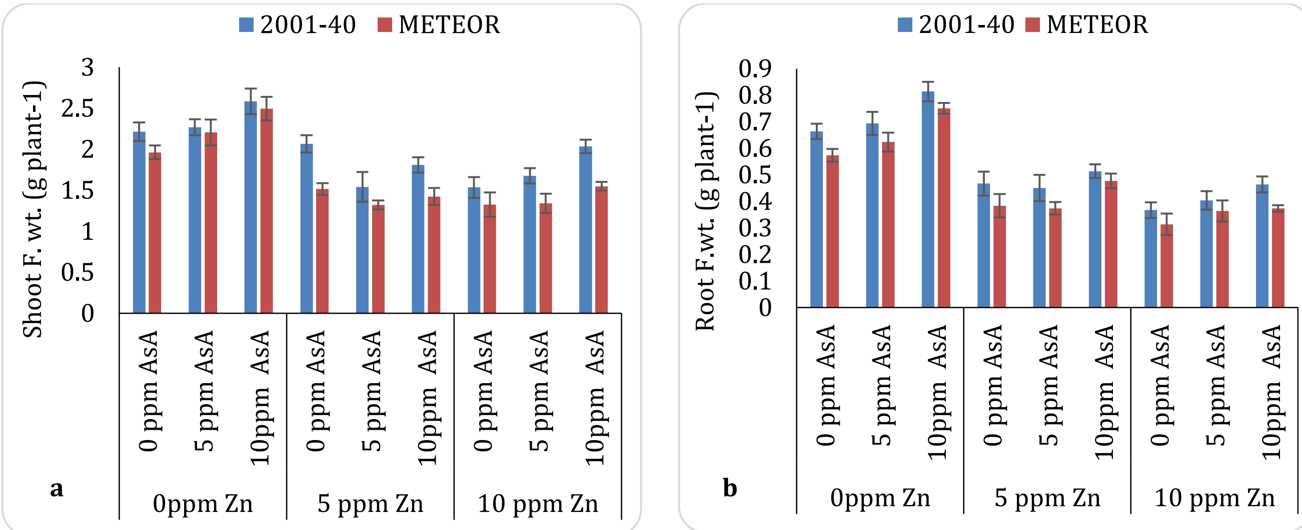


Figure 2 a, b: Fresh weight shoot (a) and root (b) of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

Maximum increase in root fresh and dry weight was noted in cv. 2001-40 at level of 10 ppm AsA applied. Maximum decrease in fresh and dry weight of root was noted in cv. Meteor under 10 ppm level of applied zinc. Generally, cv. 2001-40 responded well as compared to cv. Meteor as shown in figure 2c and d. Zinc stress significantly (P≤ 0.05) reduced the number of leaves and branches as well as leaf area. However, the foliar application of AsA significantly (P≤ 0.05) increased the number of leaves and branches as well as leaf area (Table 1).

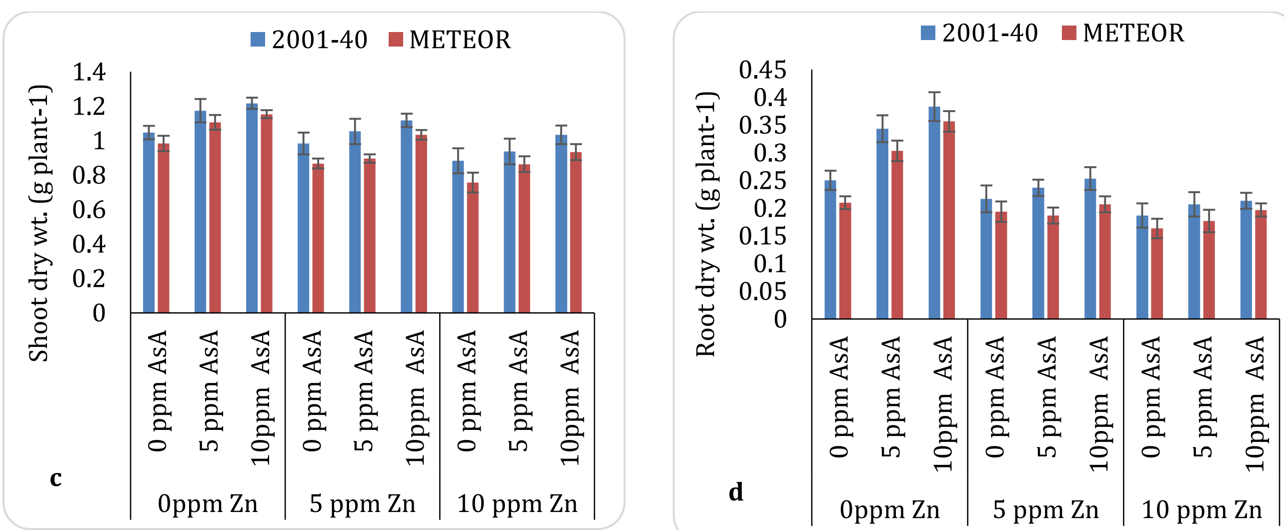


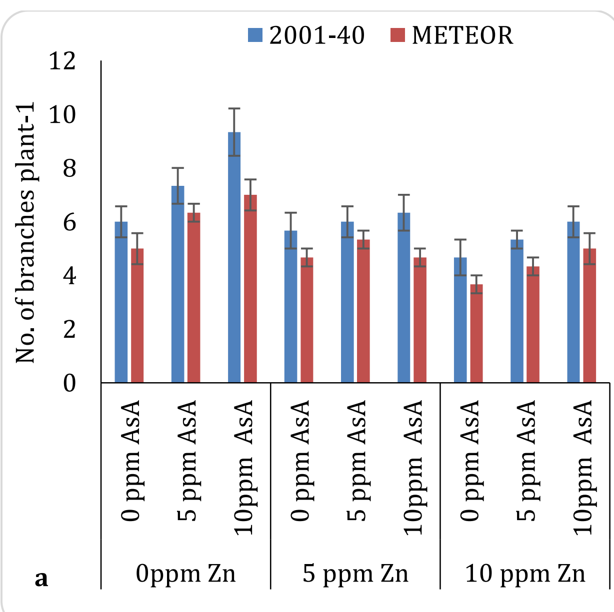
Figure 2 c, d: Dry weight of shoot (c) and root (d) of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

In general, the cultivar 2001-40 performed better in comparison to another cultivar Meteor (figure 3a, b and d). Maximum decrease in number of branches/plants was detected in cv. Meteor under 10 ppm level of applied zinc. In previous study, it was noticed that the morphological attributes of tomato plant like (root and shoot length, dry and fresh weights of root and shoot and leaf area) decreased with an increase in zinc concentration. Zinc at high level may obstruct the root growth directly by reticence of cell division or cell elongation or combination of both, resulting in the incomplete exploration of the soil volume for uptake and translocation of nutrients and water and induced mineral deficiency. The decline in leaf area at higher concentration of zinc can be attributed to either a decrease in the numbers of cells (Vijayarengan and Mahalakshmi, 2013).

Analysis of variance of the data for chlorophyll a of the two pea cultivars grown under zinc stress conditions with foliar application of ascorbic acid is presented in Table 2. The statistical data showed significant ($P \leq 0.05$) results between cultivars and treatment means (Zinc and Ascorbic Acid) while non-significant results regarding the interaction between cultivars and treatment means. Imposition of zinc stress caused a significant ($P \leq 0.05$) reduction in chlorophyll a of pea cultivars (Table 2). Application of AsA caused a significant ($P \leq 0.05$) increased in chlorophyll a of both the pea cultivars (Table 2). Maximum increase was observed in both the cultivars at 5 ppm and 10 ppm levels of AsA applied (Figure 4a). Maximum decline was observed in cv. Meteor under 10 ppm level of applied zinc. However, foliar spray of AsA at 5 ppm and 10 ppm minimized the effect of zinc stress in both the cultivars (Figure 4a). Overall, under zinc stress and non-stress conditions, cv. 2001-40 performed better as compared to cv. Meteor.

Maximum increase in chlorophyll b was noted in both the cultivars at 5 ppm and 10 ppm levels of AsA applied (Figure 4b) However, foliar spray of AsA at 5 ppm and 10 ppm minimized the effect of zinc stress in both the cultivars. Overall, under zinc stress and non-stress conditions, cv. 2001-40 performed well as compared to cv. Meteor (Figure 4b and Table 2). Maximum increase in chlorophyll a/b was noticed in both the cultivars at 5 ppm and 10 ppm levels of ascorbic acid applied (Figure 4c).

Overall, under zinc stress and non-stress conditions, cv. 2001-40 performed well as compared to cv. Meteor. Maximum increase in total chlorophyll was noticed in both the cultivars at 5 ppm and 10 ppm levels of AsA applied (Figure 4d). Overall, under zinc stress and non-stress conditions, cv. 2001-40 showed better response as compared to cv. Meteor (Figure 4d). In previous research, it was revealed that chlorophyll a and b contents decreased under zinc stress. Likewise, decrease of photosynthetic pigment contents under zinc stress were also detected in tea and red cabbage. The decline in chlorophyll contents in plants exposed to heavy metal stress is believed to be due to inhibition of enzymes of chlorophyll. Decrease in pigments may be a primary result of distressed biosynthesis or greater deprivation of thylakoids under zinc stress. The excess supply of zinc seems to induce iron and magnesium shortage and dropping of chlorophyll biosynthesis (Subba et al., 2014).



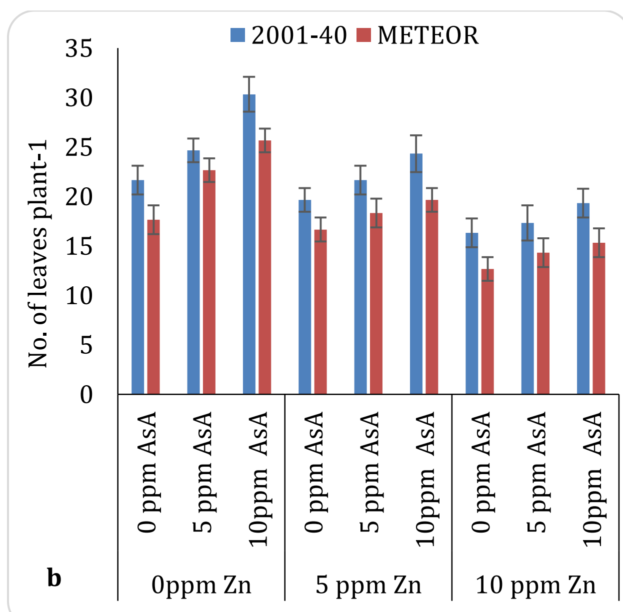


Figure 3 a, b: Number of branches (a), and leaves (b) of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

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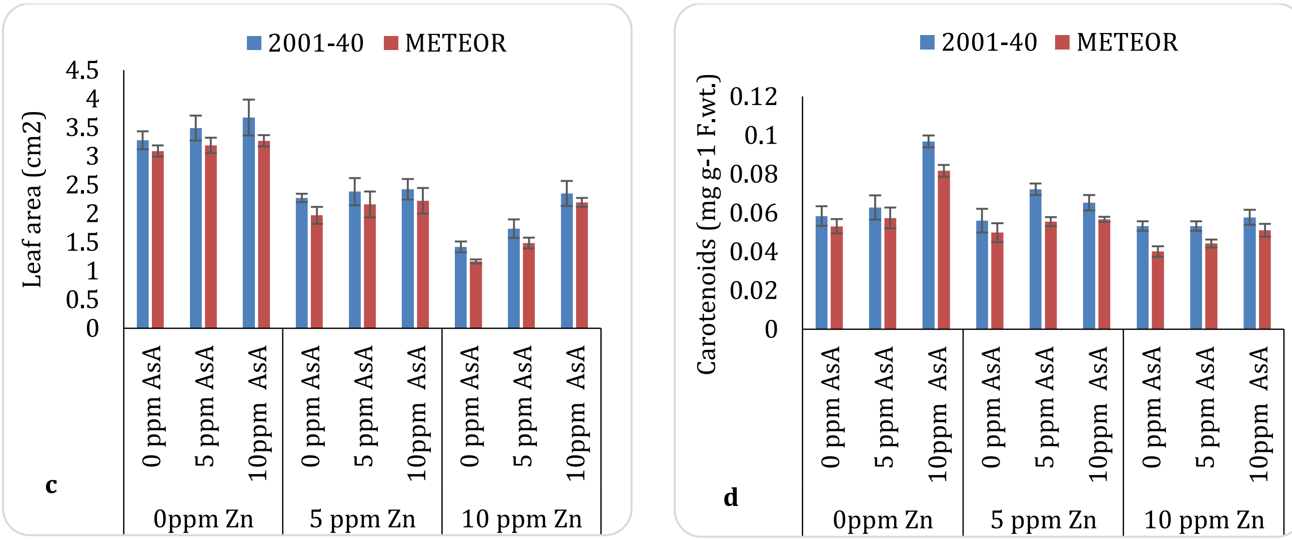

Figure 3 c, d: Leaf area (c), and carotenoids (d) of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

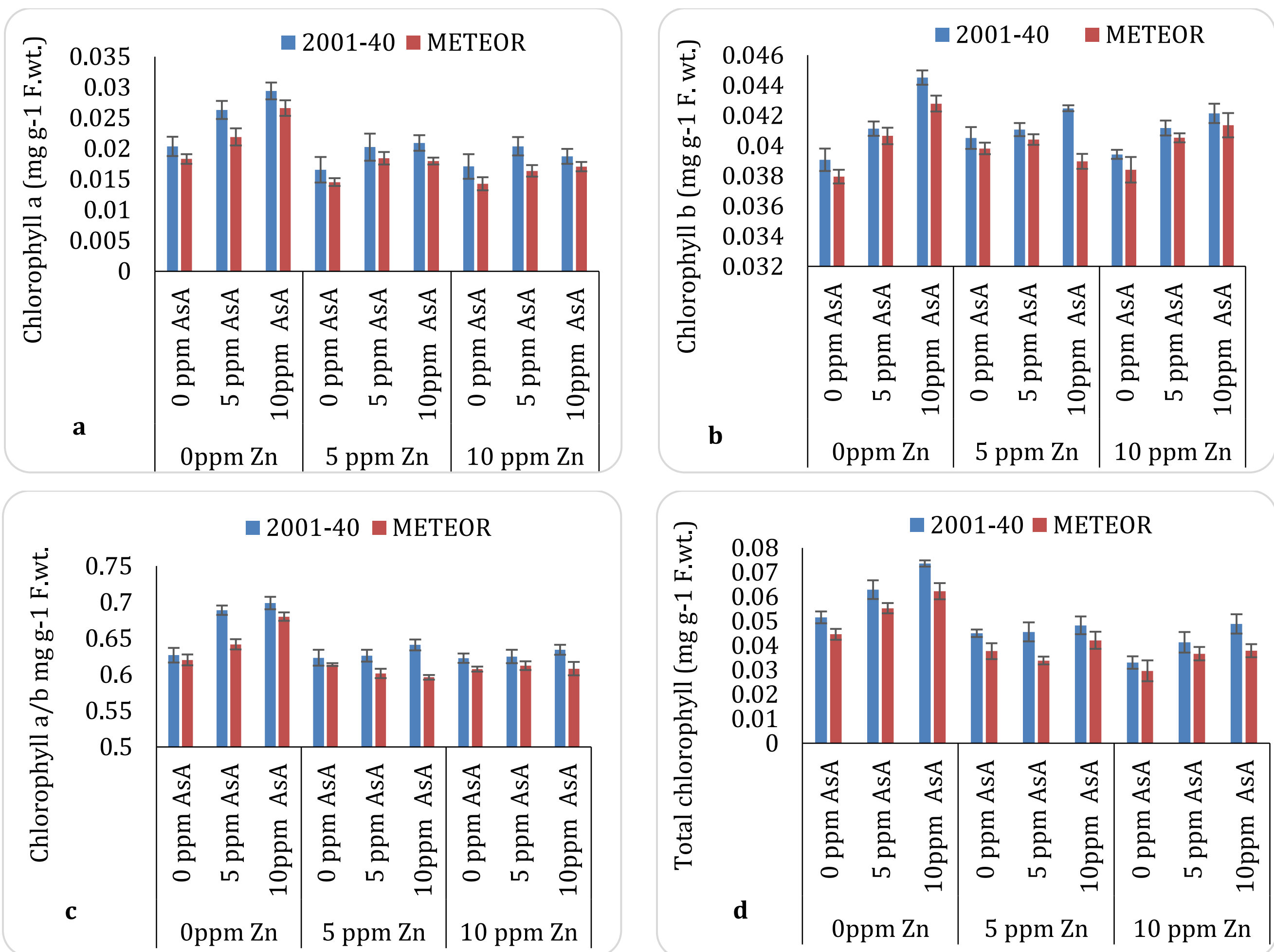

Figure 4: Chlorophyll a (a), b (b), a/b (c) and total chlorophyll (d) contents of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

Table 2: Analysis of variance for Carotenoids, chlorophyll a, b, a/b, total chlorophyll, total soluble proteins, catalase, peroxidase and ascorbic acid of two Pea cultivars (*Pisum sativum* L.) grown under zinc stress conditions with foliar application of ascorbic acid.

SOV	Carote.	Chl. A	Chl. B	a/b	a+b	TSP	CAT	POD	AsA
Cvs	0.00***	1.00***	1.88***	0.00***	8.22***	0.50***	8.60**	0.00***	28.58**
Zinc	0.00***	2.26***	1.48ns	0.01***	0.00***	0.89***	0.00***	0.01***	1.83ns
AsA	0.00***	1.19***	3.66***	0.00***	6.37***	0.26***	0.00***	0.00***	42.94***
Cvs × Zinc	3.87ns	8.11ns	7.61ns	8.44ns	7.26ns	0.00ns	4.30ns	2.10ns	0.02ns
Cvs × AsA	6.13	1.57ns	2.45ns	5.23*	1.48*	0.02ns	7.85ns	7.81ns	0.11ns
Zinc × AsA	5.18***	1.79*	8.12***	0.00***	1.13**	0.11**	1.54**	5.99**	14.59***
Cvs × Zinc × AsA	5.18ns	1.54ns	1.11ns	3.39ns	1.59ns	0.00ns	7.39ns	2.01ns	0.33ns
Error	4.45	5.64	8.65	1.58	2.84	0.02	3.20	1.35	2.28

ns = non-significant, *, **, *** significant at 0.05, 0.01 and 0.001 levels, respectively.

SOV= Source of variance, Carote. = Carotenoids, Chl. a= Chlorophyll a, Chl.b=Chlorophyll b, a/b= Chlorophyll a/b, a+b= Total Chlorophyll, TSP= Total soluble proteins, CAT=Catalase, POD=Peroxidase, AsA=Ascorbic acid

Application of AsA caused a significant ($P \leq 0.05$) increase in total soluble proteins (TSPs) of both the pea cultivars (Table 2). Maximum reduction was observed in cv. Meteor under 10 ppm level of applied zinc (Figure 5a). However, maximum increase in TSPs was observed in both cultivars at 5 ppm and 10 ppm levels of AsA applied (Fig 5a). Overall, foliar application of AsA diminished the harmful effect of zinc stress in both the cultivars as compared to control (Fig 5a). Cv. 2001-40 showed more TSPs as compared to Meteor. Maximum increase in ascorbic acid was observed in both cultivars at 10 ppm level of AsA applied (Fig 5b). However, foliar application of ascorbic acid combined with zinc treatments enhanced the ascorbic acid in both the pea cultivars as compared to control. Overall, cv. 2001-40 gave better results as compared to cv. Meteor. Maximum increase in catalase was observed in both cultivars at 5 ppm and 10 ppm levels of zinc applied (Figure 5c and Table 2). Foliar application of AsA diminished the harmful effect of zinc stress in both cultivars as compared to control. Overall, Cv. 2001-40 performed better as compared to cv. Meteor. Maximum increase in peroxidase was observed in both the cultivars at 5 ppm and 10 ppm levels of zinc applied (Figure 5d and Table 2). Overall, foliar application of AsA diminished the harmful effect of zinc stress in both cultivars as compared to control. Overall, Cv. 2001-40 gave better results as compared to cv. Meteor. Previous studies indicated that the contents of antioxidant enzymes increased under zinc stress. CAT and POD are redox metalloenzymes involved in cell protection against oxidative stress. Plant PODs, which are coded through small or big multigenic families, are involved in many significant physiological and developmental processes. POD can also be considered suitable indicators for environmental stresses since their action is affected by heavy metal, salt and other environment conditions. The initiation of catalase under zinc stress showed that it supports in preventing the oxygen radical accumulation (Luo et al., 2010).

Foliar application of ascorbic acid showed a positive effect on biomass production and chemical attributes of both cultivars. Foliar spray by ascorbic acid improved the growth of both cultivars under stress and non-stress conditions. Foliar spray of ascorbic acid significantly alleviated the adverse effects of zinc stress on growth and biochemical attributes of pea plants. Overall, cv. 2001-40 showed better results as compared to cv. Meteor.

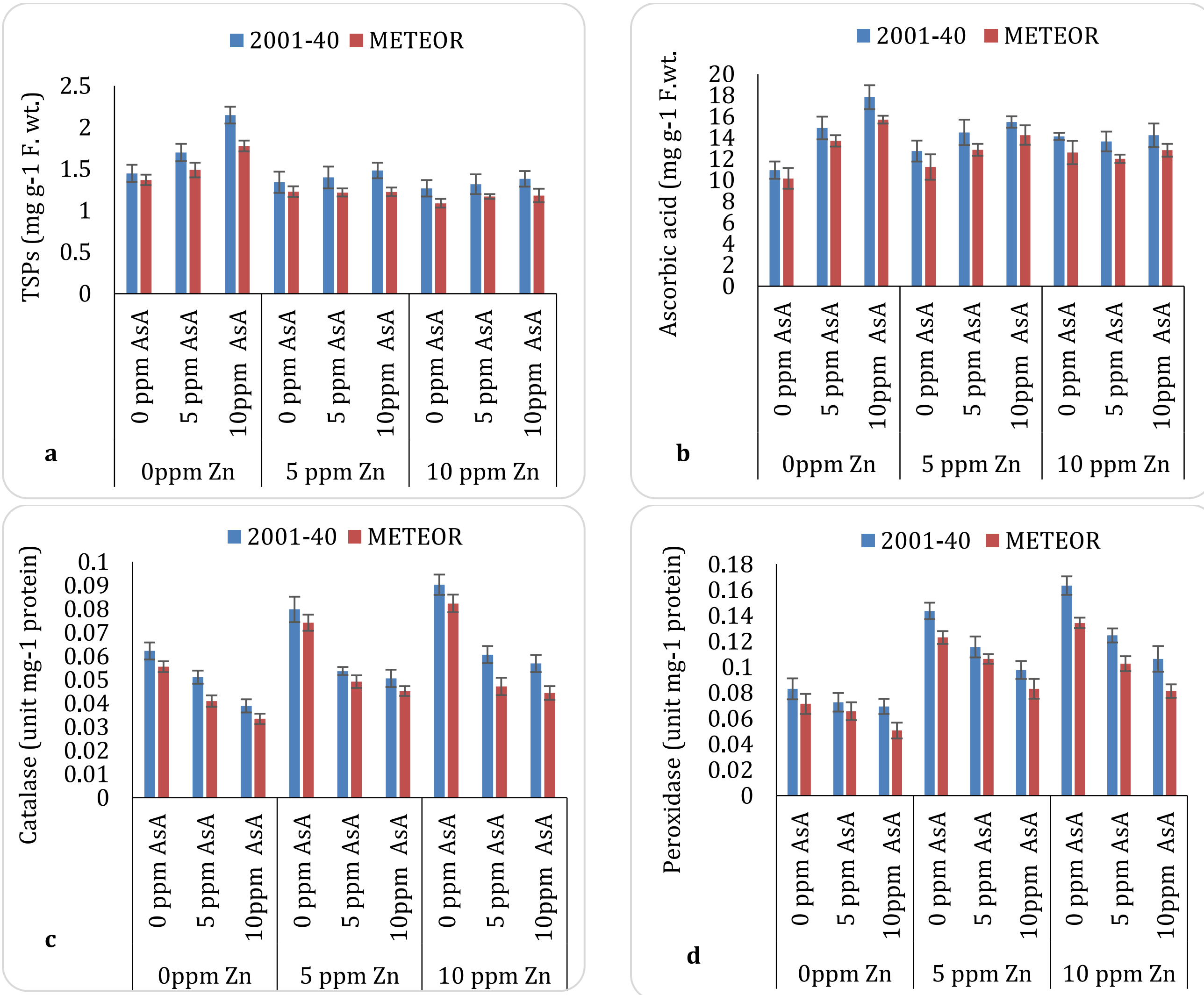

Figure 5: Total soluble proteins (a), ascorbic acid (b), catalase (c) and peroxidase (d) contents of two pea cultivars grown under zinc stress conditions with foliar spray of ascorbic acid.

Author contribution

All the authors have contributed equally in conducting the experiment, collecting and analyzing the data and writing the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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