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Effect of Elevated Ozone on Soybean (*Glycine max* L.) Cultivar: Role of Orange Juice and Synthetic Ascorbic Acid

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ABSTRACT

Ozone is a hazardous gas for the environment and negatively affects plant and human health. These days, phytoextracts are commonly used as a source of bioactive compounds for reducing the detrimental environmental effects on plants. In the presented study, soybean cultivar JS-335 was used to assess the protective role of synthetic ascorbic acid (SAA) and orange juice (25% orange juice, enriched ascorbic acid) under ozone stress conditions. The results showed that under ozone stress, soybean cultivar JS-335 reduced growth and biomass and negatively affected the biochemical properties of plants due to these changes, finally causing yield losses. Foliar-applied OJ >and SAA improved plant growth and development and increased crop yield. It was discovered that a 25% OJ coupled with ascorbic acid and other essential nutrients and biomolecules was almost as effective as a 100 ppm SAA in reducing the harmful effects of ozone stress on soybean plants. As a result, it was determined that OJ, a less expensive source of ascorbic acid, can improve ozone resistance in plants in ozone-prone areas.

INTRODUCTION

Anthropogenic activity causes environmental pollution, including air, water, and soil environment. Environmental pollutants, including gaseous and suspended particulate matter, cause injurious effects on plant growth and biomass (Chaudhary & Rathore 2018a, b, 2019). Ozone is a secondary pollutant that negatively affects plant and human health (Rathore & Chaudhary 2019, 2021c, Soni et al. 2021). The tropospheric ozone (O_3) is presently raised in widespread areas of the Northern Hemisphere (Feng et al. 2015, Sicard et al. 2017) and is possibly phytotoxic to ozone-sensitive vegetation (Saitanis et al. 2015). When plants uptake higher doses of ozone, they experience a chain of physiological and biological alterations alternating from a single cell to a whole plant (Jolivet et al. 2016). When exposed to higher oxygen levels, vegetation threatens food sources and affects ecosystem stability and biosphere survival (Lu et al. 2015, Wang et al. 2016).

The vegetation protection against destructive ozone effects is thus a significant problem. Several potential agrochemicals available in the market are tested as a protector of plants against ozone phytotoxicity (Saitanis et al. 2015, Chaudhary & Rathore 2022). A recent study by Chaudhary & Rathore 2020 states that the exogenous application of EDU, PU, and ascorbic acid protects against ozone stress. Exogenous application of ascorbic acid is considered to mitigate the extreme stress circumstances on the whole plants (Khalil et al. 2010). Exogenous ascorbic acid has been used to investigate the influence of exogenous ascorbic acid on many morphological, physiological, and biochemical processes in plants under stress, including wheat (Singh & Bhardwaj 2016), soybean (Amira & Qados 2014) and groundnut (Chaudhary & Rathore 2020).

Many plants are identified that naturally contain huge quantities of ascorbic acid in their fruit or other parts. The sweet orange (*Citrus sinensis* L.) is a common fruit (Etebu & Nwauzoma 2014) that is high in ascorbic acid (Galaverna & Dall'Asta 2014). It also contains trace amounts of minerals such as calcium, magnesium, potassium, polyphenols, niacin, thiamin, and folate (Yahia 2017, Chanson-Rolle et al. 2016). The Exogenous application of orange juice on soybean plants under ozone stress was not yet conducted. Therefore, these applications maybe develop a better tool for plants to survive against ozone stress and shield against agricultural loss.

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Soybean can grow as a substitute crop in areas where abiotic stresses, such as ozone stress, are a major constraint to agricultural production (Fita et al. 2015, Bazile et al. 2016). Thus, the exogenous application of organic substances containing ascorbic acid, which effectively improves the hostile effects of abiotic and biotic stress on plant species, can boost soybean plant tolerance under such conditions. Numerous studies have stated in the literature that ascorbic acid is a powerful mitigating feature against ozone and other abiotic stresses. However, evidence on the impact of foliar ascorbic acid treatment on the harmful effects of ozone stress in soybean plants is currently unavailable. As a result, this study aimed to compare the effects of pure synthetic ascorbic acid and OJ rich in ascorbic acid on soybean plants under ozone stress.

MATERIALS AND METHODS

Experimental Design and Protectants Application

Pots experiment was conducted with three replicates of soybean (Glycine max L) cultivar at the Central University of Gujarat Campus in 2018. Under OTC (Open top chamber) 75 ppb ozone was applied for 4 hours every day till harvesting and foliar spray of synthetic ascorbic acid and sweet orange phytoextract was applied. Four treatments were applied, such as Treatment T1 (Control), T2 (ozone fumigation 75 ppb), T3 (ozone fumigation + SAA), and T4 (ozone fumigation + OJ). Seeds of a soybean (variety JS-335) were obtained from the groundnut research station Junagadh. The pots were filled with 30.0 kg of sandy loam soil with a pH of 8.1 and a composition of 65% sand, 27.5% silt, and 7.5% clay. Twenty seeds were sown in equal size of each pot, and after two weeks of seed germination, thinning was performed. In addition to the control distilled water (DW) foliar spray, 100 ppm synthetic ascorbic acid (SAA) and 25% OJ rich in ascorbic acid (25 percent OJ) were applied 10 days interval after plant germination till maturity. Fresh sweet oranges (Citrus sinensis L.) were purchased from a nearby fruit market, and after extracting the peel, the pulp was used to remove juice using an electric juicer. The juice was then processed at 4 degrees Celsius for one day before use.

Before use, the content of ascorbic acid in 25 % OJ was estimated to be 20.7 mg/L using Keller & Schwager's (1977). In addition to ascorbic acid, the OJ produces a mixture of inorganic and organic nutrients (Chanson-Rolle et al. 2016). In distilled water, various solutions were prepared, including 25% OJ and 100 ppm synthetic ascorbic acid. The sample was collected for growth & physiology analysis after 25 DAS and 50 DAS, and final harvesting was done at 120 DAS.

Growth and Biomass

The growth and biomass of the plant part were estimated separately. A triplicate of each plant per pot was taken and washed with purified water before being used to assess growth and biomass. Although other plants were refrigerated, the following biochemical parameters were determined: Graphical methods were used for leaf area measurement, and a regular meter scale measured root and shoot length. The mass of plants, such as the fresh and dry weight of plant parts, was determined by electric balance. Plants' parts were dried in a hot air electric oven at 80 degrees centigrade till a constant weight was achieved.

Photosynthetic and Non-Photosynthetic Pigments

Method Arnon (1949) was used to check for photosynthetic pigments (chlorophyll a, b, carotenoid, and total chlorophyll). A 0.25 g leaf sample was mixed with 5 mL of an 80 percent acetone solution for chlorophyll analysis. The sample was crushed with the help of pestle mortar and kept overnight at 4°C, and their optical density was taken at 663 nm and 645 nm with the help of a spectrophotometer.

The amount of anthocyanin in soybean leaves was calculated using Beggs and Wellmann's process (1985). A 100 mg leaf sample was blended with 100 mL propanol, hydrochloric acid, and water (18:1:81 v/v) in a mixture of 100 mL propanol, hydrochloric acid, and water. The following formula was used to measure the total sum of anthocyanin:

$$\text{Anthocyanin (mg.g}^{-1}\text{ fresh leaf)} = A535 - 2.2\ A650/W \times V$$

Where V = mL volume of extract, W = g fresh weight of leaf

Estimation of Oxidative Stress

Hydrogen peroxide activity was analyzed by Velikova et al. (2000). The extraction solution makes with the help of 5 mL of 0.1% TCA (ice cold). In a pestle mortar takes, 0.25 g of fresh leaf and 5 mL of TCA were added, and the sample was crushed. After centrifuging the homogenate, 500 liters of supernatant is combined with 500 liters of 10 mM potassium phosphate buffer (7.0 pH). After mixing the solution with 1 mL of 1 M potassium iodide (KI) and leaving it at room temperature for 20 minutes, the OD was measured at 390 nm.

MDA content was measured using Heath & Packer's (1968) method. MDA was determined using 5% TCA and 0.5 percent TBA. In a pestle mortar, 0.25 g fresh leaf was mixed with 5 mL of 5% TCA solution, and the homogenate was centrifuged after crushing. After centrifugation, mix 500 mL of the supernatant with 2 mL of 0.5 percent thiobarbituric acid (TBA). The solution mixtures were then kept in a water bath at 95°C for 50 minutes before being cooled in

an ice bath. The solution's OD was estimated at 600nm and 532nm.

Membrane permeability was determined by the described method by Blum and Ebercon (1981). An electrical conductivity meter was used to calculate ion leakage from fresh leaves in deionized water (Eutech Instruments). A punching machine carved the leaf samples into 1 cm diskettes. After cutting 20 diskettes from each sample, 10 mL of deionized water was applied to a glass beaker. The conductivity of the solution was calculated after the beakers had been held at room temperature for 3 hours.

Estimation of Antioxidants

Non-enzymatic Antioxidants

Flavonoids: Cameron et al. (1943) proposed a method for estimating flavonoid material. A 0.1 g fresh leaf sample was put in 100 mL ethanol and acetic acid mixture (99:1, v/v) and boiled for 2 minutes. After cooling to room temperature, the solution was centrifuged for 10 to 15 minutes at 8000xg. The solution's absorbance was estimated at different wavelengths from 250 to 350 nm and represented as flavonoid absorbance (A mg^{-1} fresh wt).

Ascorbic acid: For the determination of ascorbic acid in a leaf sample of soybean, used method of Keller & Schwager (1977). Ascorbic acid contents were estimated with the help of extracting solution (extracting solution: Dissolved 5 g oxalic acid and 0.75 g EDTA in one liter of distilled water). In an ice bath, a 500 mg fresh leaf sample was homogenized with 20 mL of extracting solution, and the homogenate was centrifuged at 6000xg for 15 min. After centrifuging the sample, 1 mL of the supernatant was taken, and added 5 mL of 2, 6-dichlorophenol-indophenol solution in pink color developed. After constant shaking, the O.D. of the solution was taken (Es) at 520 nm wavelength. Then one drop of ascorbic acid 1% solution was added to bleach the pink color and obtain the OD of turbid solution (Et) at the same wavelength.

For blank (Eo), 1 mL of extracting solution and 5 mL of DCPIP solution were mixed, and O.D. was measured as mentioned above.

A 1% aqueous ascorbic acid solution was used for the calibration curve, which was diluted to obtain varying concentrations. The total amount of ascorbic acid was calculated by using the following formula.

Ascorbic acid (mg g^{-1} fresh leaf) = [{Eo – (Es – Et)} × V] / (v × W × 1000)

Where W = weight of leaf taken (g); V = total volume of the mixture (mL); v = supernatant taken for analysis (mL). The standard curve estimates the value of {Eo-(Es – Et)].

Total phenols: The amount of total phenols was estimated by Mallick and Singh (1980) using 70% acetone. For phenol determination, a 100 mg fresh leaf sample was crushed with 10 mL of 70% acetone, and the suspension was centrifuged at 6000xg for 10 minutes. Then, 1 mL of supernatant was taken in a test tube, 1 mL of folin-ciocalteu reagent was added, 2 mL of Na_2CO_3 (20% w/v) solution, and the final volume was made up of 10 mL with distilled water. This mixture was heated in a water bath for one minute and then cooled at room temperature. The blue color developed in a solution and the solution's OD was measured at 650 nm wavelength. A standard curve was prepared with known amounts of quinine for the phenol contents.

Enzymatic Antioxidants

For estimating antioxidative enzyme activity, fresh leaves sample (250 mg) was crushed in 5 mL (50 nM) of cool potassium phosphate buffer (7.8 pH). The homogenate was centrifuged for 20 min at 4°C at 12,000 xg. The supernatant was kept at -200°C to estimate the following antioxidative enzymes.

Catalase activity: Catalase activity was estimated by Chance & Maehly 1955, using 100 µL supernatant in 1.9 mL potassium phosphate buffer (50 mM, pH 7.8). 1 mL of 5.9mM H2O2 was also added to the mixture, and the OD was measured at 240 nm after every 20 seconds for two minutes.

SOD activity: For estimating SOD activity, 50µL supernatant was added with 400 µL distilled water, 250 µL (50 mM) potassium phosphate buffer (pH 7.8), 100µL L-methionine, 100 µLtritron-X, 50 µL nitro blue tetrazolium (NBT) and 50 µL riboflavin. The solution's optical density (OD) was recorded at 560 nm (Van Rossum et al. 1997).

POD activity: For estimating peroxidase activity, 100µL supernatant was added with 1.8 mL potassium phosphate buffer (50 mM, 7.8 pH), 100 µL guaiacol (20 mM), and 100 µL H_2O_2 (40 mM), and the OD of the solution was calculated at 470 nm after every 20 seconds for 3 min as defined in Chance & Maehly (1955).

APX activity: For the APX activity, 100 µL supernatant was mixed with a 3 mL solution containing 100 mM phosphate (pH 7), 0.1 mM EDTA-Na2, 0.3 mM ascorbic acid, and 0.06 mM H_2O_2. The OD was read at 290 nm for 30-second intervals until the ascorbic acid oxidized. One unit of APX forms 1 µ M of ascorbate oxidized per minute in assay conditions (Nakano & Asada 1981).

Primary Metabolites

Total soluble sugars, reducing sugars: Foliar sugar contents were estimated using the method described by Somogyi (1952). The leaf sample (50 mg) was crushed in

5 mL of 80% ethanol and centrifuged for 15 min at 3500xg. Pellets obtained were washed four-time using 80% ethanol and distilled water. The mixture was centrifuged at every washing. 1 mL of aliquot was mixed with 1 mL of copper reagent and boiled in the hot water bath for 10 minutes. After boiling, the solution was kept cooled to room temperature straightway, and 1 mL of arsenomolybdate was added. The solution was left for 30 minutes to complete the reaction before taking OD at 500 nm to estimate soluble sugars. For reducing sugar, 0.5 mL of diluted aliquot was mixed with 1 mL of 5% phenol reagent and left for 10 minutes to uphold room temperature. This solution was mixed in 5 mL of H_2SO_4. The solution was shacked well and left in a water bath for 10 minutes before measuring the OD at 480 nm. A standard curve obtained using purified glucose estimated total soluble sugar and total reducing sugar. The remaining pellet samples were washed twice with 52% perchloric acid and distilled water and then centrifuged to estimate starch content. The volume of supernatant was made to 50 mL with distilled water. 1 mL aliquot of pooled supernatant was taken to estimate the starch content.

Amino acids and proteins: The amino acid was determined using Hamilton et al. (1943) method. 1 mL of the sample (used for antioxidants) was added with an equal amount of 10% pyridine and acidic ninhydrin in test tubes. The mixture was heated for 30 min at 100°C, cooled at room temperature, and upraised volume to 7.5 mL using distilled water. The OD was recorded at 570 nm. Protein was estimated by using the method of Lowry et al. (1951).

Yield Characteristics

Yield characteristics were calculated using the number of capsules plant^{-1}, number of seed plant$^{-1,}$ and weight of seed and pod plant^{-1}.

Statistical Analysis

The study involved a fully randomized two-factor ozone stress and exogenous ascorbic acid treatment. Using the HPSS software, the data collected for each parameter were subjected to Duncan's Multiple Range Test analysis. The least significant difference was estimated at the 0.05 percent likelihood stage to estimate the significant differences among the mean values. Using Origin Pro software, PCA was used to describe the homogeneous characteristics of a soybean cultivar and the association between each vector tested under different treatments at two sampling dates (2019).

RESULTS

Growth and Biomass

Leaf area and plant height: The leaf area and plant height of ozone-treated plants was highly affected compared to a control plant. At the same time, the application of natural ascorbic acid played a protective role against ozone stress than synthetic ascorbic acid as compared to control plants. The maximum increase in leaf area was found in treatment T4 (14.86%) at 25 DAS and plant height in the same treatment (28.17%) at 50 DAS (Fig. 1). The treatment-wise difference in leaf area and plant height was noted as maximum in treatments T4 > T3 > T1 > T2 (Fig. 1).

Total biomass and root shoot ratio: Plant biomass and root shoot ratio show variable treatment and age factors results. Total plant biomass was reduced by the ozone stress of the experimental crop (Fig. 1). However, exogenous application of SAA and OJ-treated plants neutralized the ozone effect. It enhanced the weight of dry leaf mass and total plant biomass of the experimental cultivar. A maximum increase in dry leaf weight was found at 70.13% under treatment T4 at 25 DAS, and a higher increment of total plant biomass (117.57%) was noted under the same treatment on the same day after the sowing of plants. The root-shoot ratio of the plant showed a negative percentage reduction in an enhanced ozone-treated plant at 25 DAS compared to control plants. In comparison, exogenous applied AA showed a positive value of percentage increments at 25 DAS and a negative value at 50 DAS.

Oxidative Stress

Hydrogen peroxide and MDA contents: Enhance ozone increases the production of hydrogen peroxide and MDA contents in plants. Higher production was found at 16.56% at 50 DAS in treatment T2. Application of natural and synthetic ascorbic acid reduced the production of Hydrogen peroxide in soybean cultivar (Fig. 2). Treatment-wise, hydrogen peroxide production was noted as T2 > T1 > T3 > and T4. Accumulation of MDA in ozone-stressed seedlings was higher than in control, whereas, in the presence of exogenous application of SAA and OJ, MDA contents were reduced significantly (Fig. 1). The maximum increase of MDA was observed in treatment T2 (25.35%) at 25 DAS (Fig. 2).

Membrane permeability: Membrane permeability showed a significant increase in ozone stress. The higher membrane injury was recorded under ozone stress. The maximum increase of membrane permeability was found under treatment T2 (12.53%) at 50 DAS (Fig. 2), and the lowest percentage of membrane permeability was found in treatment T4 (-20.22%) at 20 DAS as compared to control plants. Membrane stability shows reverse trends as membrane permeability.

Photosynthetic and Non-Photosynthetic Pigments

Total chlorophyll and carotenoids: Ozone-treated plants

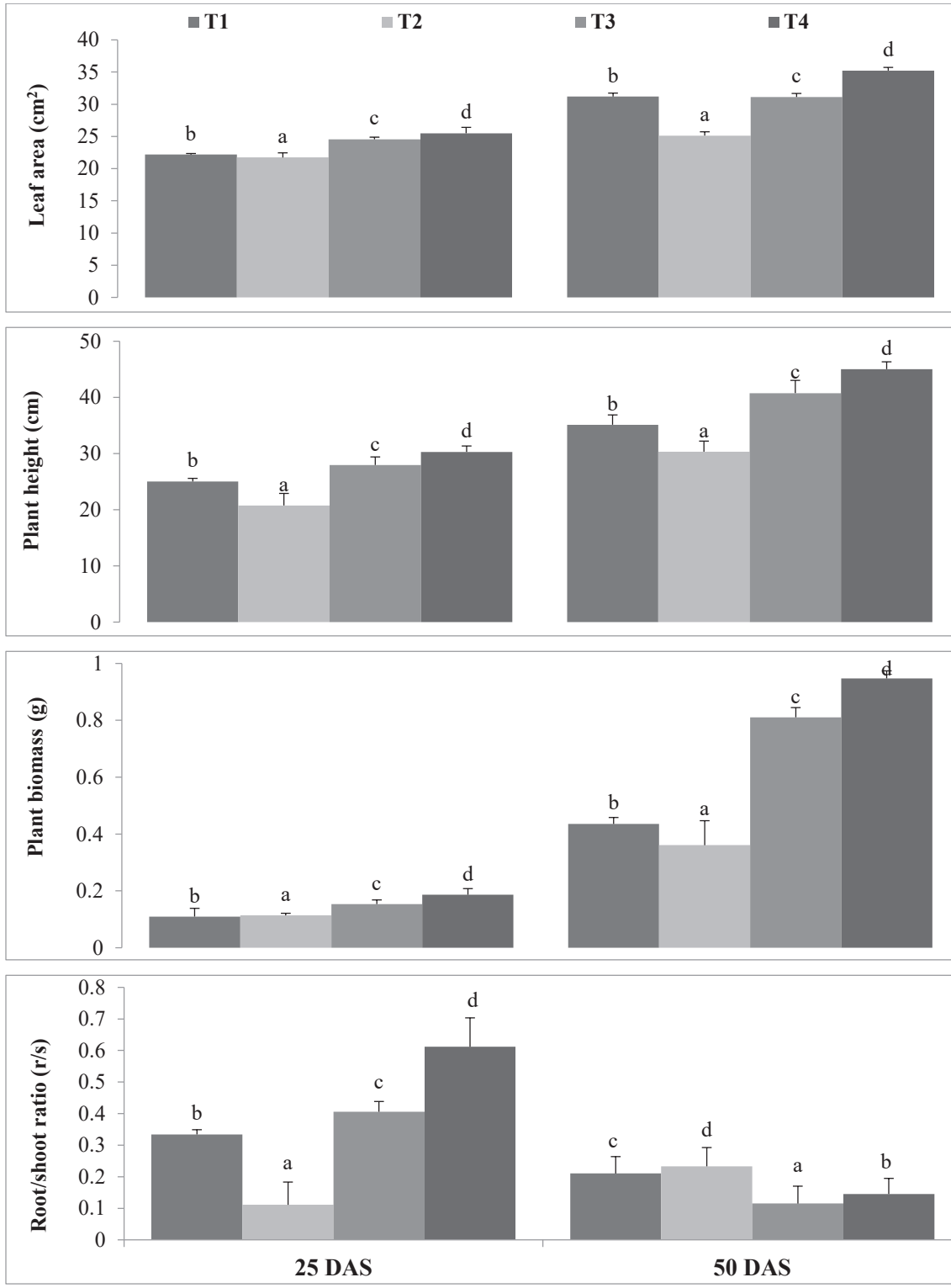

Fig. 1. Role of natural and synthetic ascorbic acid on leaf area (cm^2), plant height (cm), total plant biomass (g), root shoot ratio (R/S) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

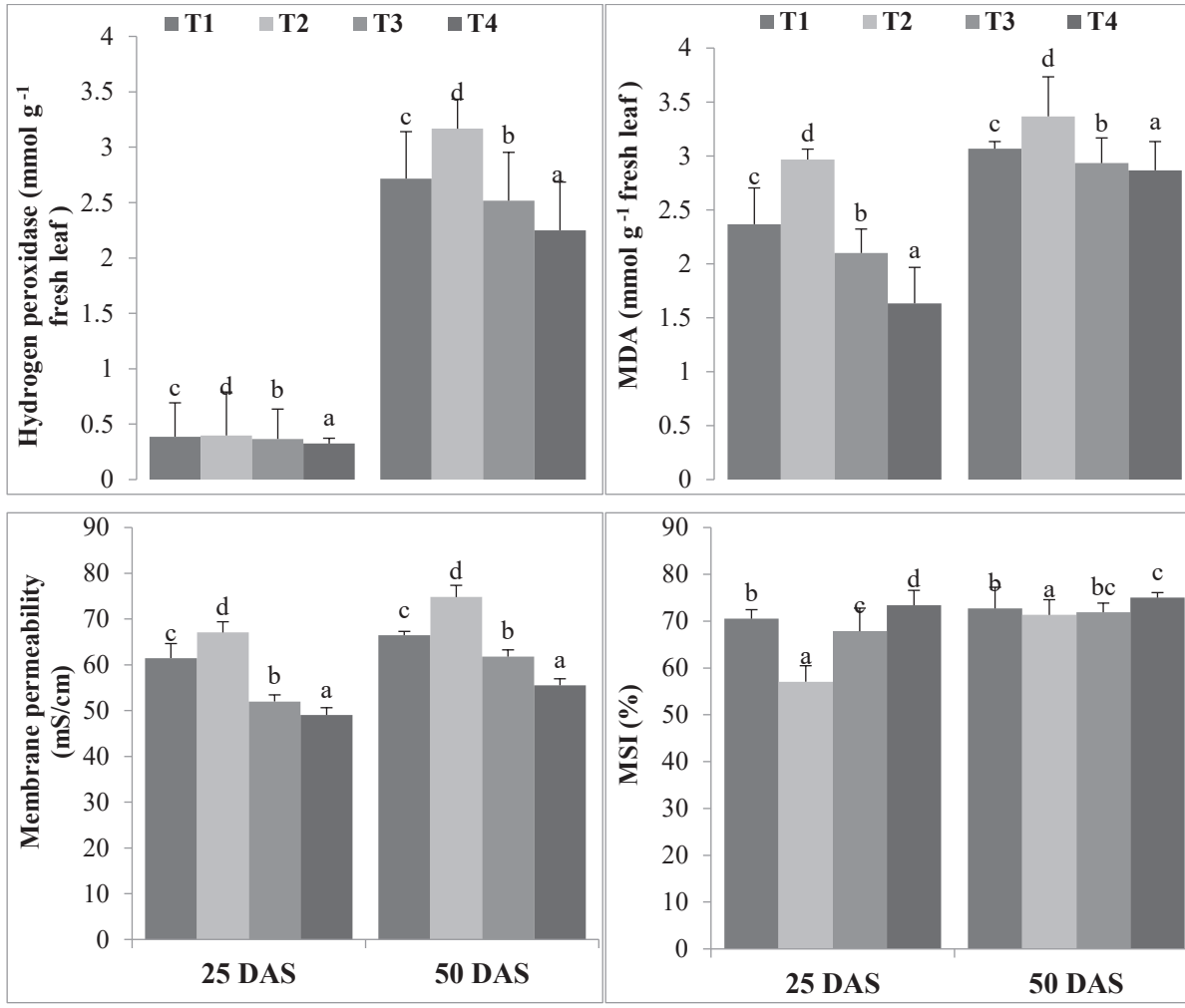

Fig. 2. Role of natural and synthetic ascorbic acid on hydrogen peroxide (mmol.g^{-1} fresh leaf), MDA contents (mmol.g^{-1} fresh leaf), membrane permeability (mS.cm^{-1}), and membrane stability (%) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

caused a significant decrease in chlorophyll a, b and total chlorophyll content compared to the control (Fig. 3). While application of SAA and OJ precipitated significant increases in chlorophyll a, b and total chlorophyll content in stressed plants. The maximum increase of total chlorophyll was noted under treatment T4 (35.84%) at 50 DAS and a minimum in treatment T2 (-33.38%) at 25 DAS compared to control plants. Increasing chlorophyll trends in treatments were T4>T3>T1>and T2. Carotenoid and anthocyanin contents were also reduced under ozone stress. The maximum carotenoid increase was found in treatment T4 (29.81%) at 50 DAS and a minimum in treatment T2 (-18.83%) at 25 DAS.

Anthocyanin: Ozone stress also negatively affects the anthocyanin of plants. While the application of OJ and SAA increased the anthocyanin concentration in plants. Anthocyanin of plant leaf also follows the same trends as carotenoids content and maximum values were noted in treatment T4 (19.12%) at 25 DAS as compared to control plants (Fig. 3). Treatment-wise, increasing trends of anthocyanin in plants were noted T4>T3>T1> and T2.

Antioxidants Activity

Flavonoids, Phenol and Ascorbic Acid

According to the data, the production of flavonoids decreases remarkably under ozone stress (Fig. 3). The flavonoid content maximum increased (97.96%) at 50 DAS under treatment T4. Ozone increased total phenolic compounds significantly (Fig. 2). The maximum increase of phenolic contents was 101.40% at 50 DAS under the T3 treatment. Ozone caused

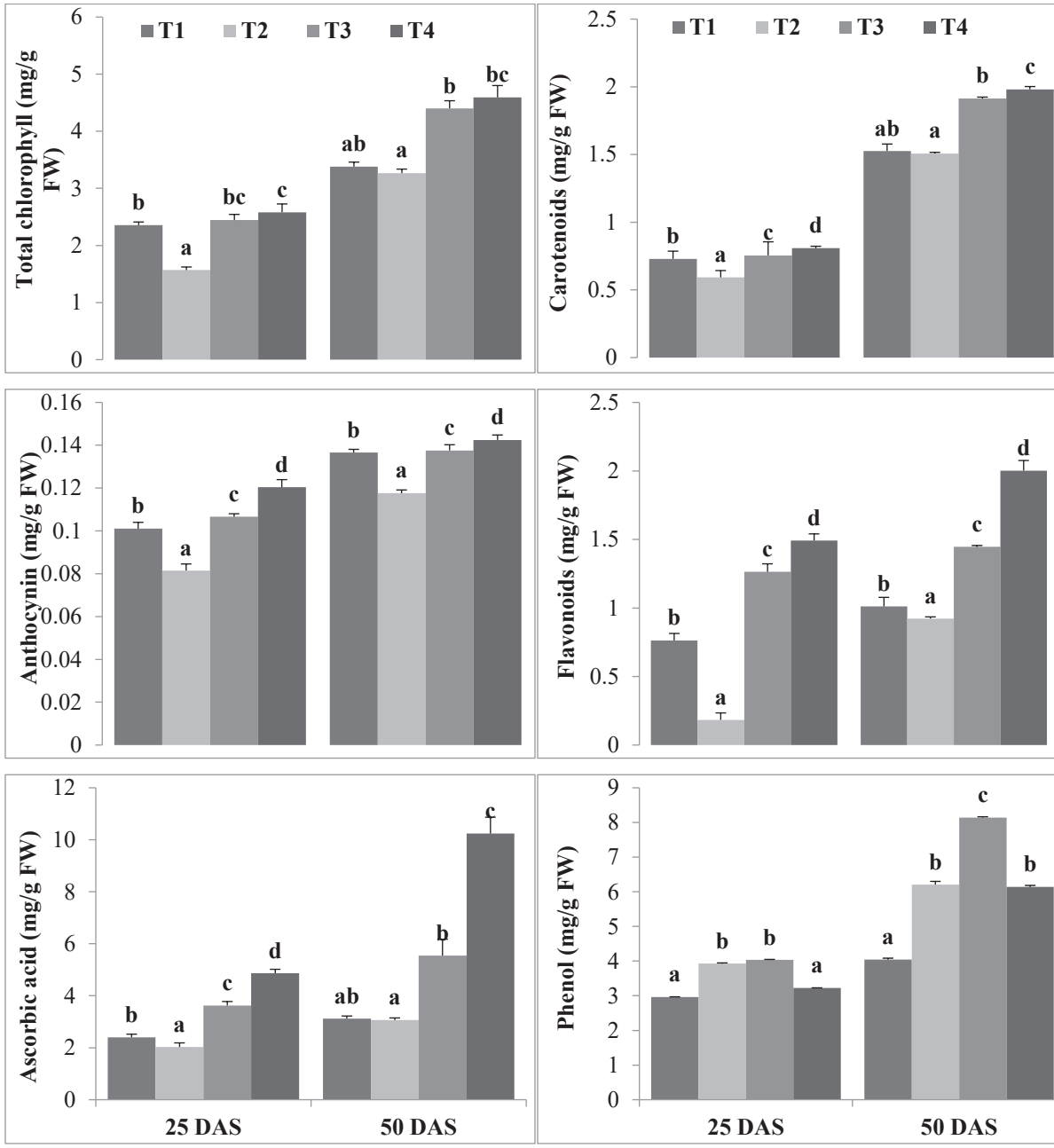

Fig. 3. Role of natural and synthetic ascorbic acid on total chlorophyll (mg.g^{-1} fresh wt.), carotenoids (mg.g^{-1} fresh wt.), anthocyanin (mg.g^{-1} fresh wt.), flavonoids (mg.g^{-1} fresh wt.), ascorbic acid (mg.g^{-1} fresh wt.) and phenol (mg.g^{-1} fresh wt.) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

a negative effect on ascorbic acid content in selected crops. A maximum increase in ascorbic acid (228.20%) was found under T4 treatment at 50 DAS and a minimum in treatment T2 (-15.55%) at 25 DAS (Fig. 3).

Enzymatic Antioxidants

CAT, POD, SOD, and APX: The antioxidant enzymes CAT showed deviation in their activities under ozone stress. A maximum increment of CAT (0.74%) was found under T2 treatment at 50 DAS and a minimum in treatment T4 at 25 DAS. POD (430.38%) under T2 treatment at 50 DAS. The maximum increase of SOD (143.77%) was noted at 50

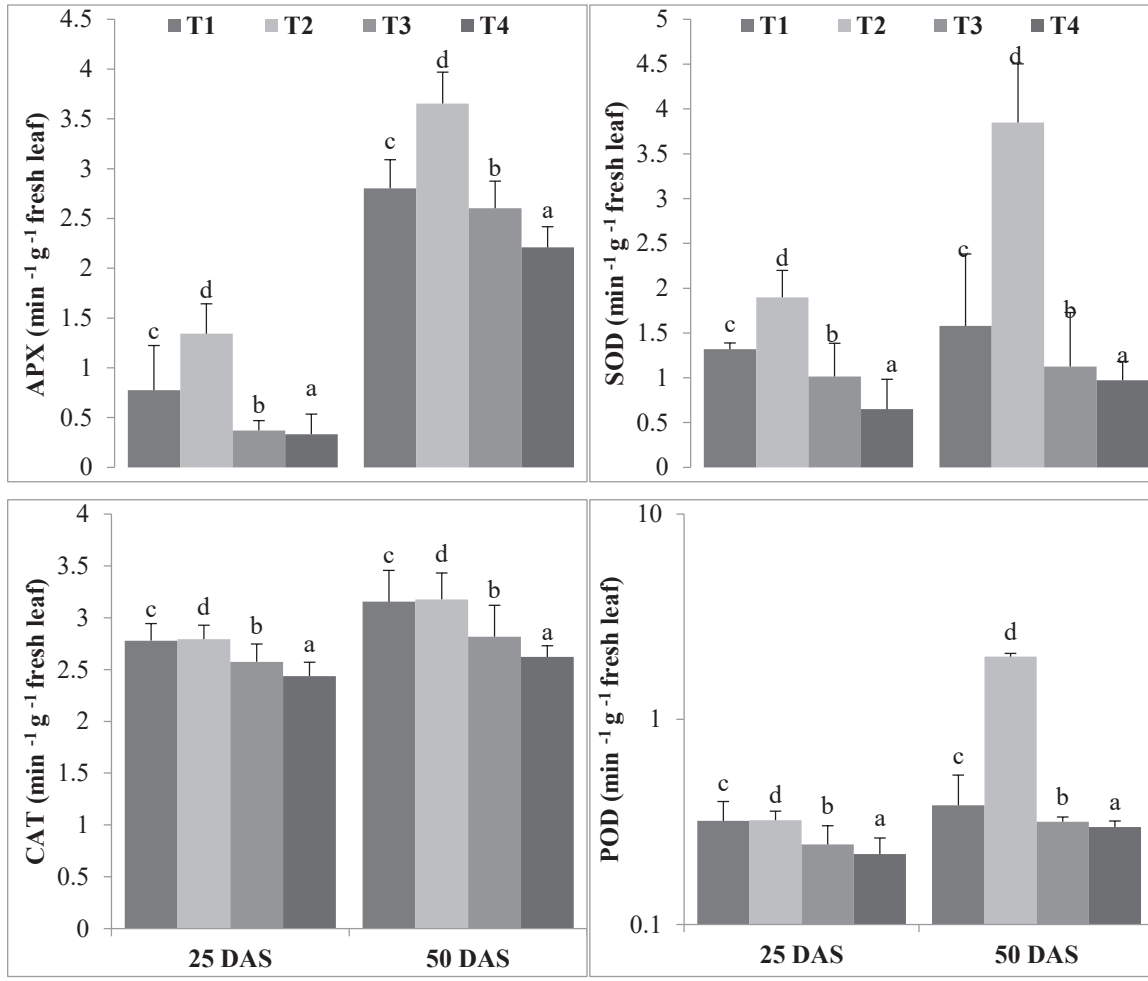

Fig. 4. Role of natural and synthetic ascorbic acid on CAT (min^{-1} .g^{-1} fresh leaf.), POD (min^{-1} .g^{-1} fresh leaf), SOD (min^{-1} g^{-1} fresh leaf), and APX (min^{-1} .g^{-1} fresh leaf) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

DAS under T2 treatment, and APX (72.90%) was noted at 25 DAS under T2 treatment (Fig. 4). Activity of POD was also increased due to the application of elevated ozone. At the same time, protectants applied to plants reduced POD activity at both sampling periods. Higher activity of POD was noted at 25 DAS of plants than at 50 DAS of plants. Maximum values of POD activity were found in treatment T2 at 50 DAS of plants compared to control plants. Ozone pollution increased the SOD activity in soybean plants, and higher values was observed at 50 DAS of plants. Treatment-wise trends of SOD activity were noted higher in T2 > T1 > T3 > and T4. Higher values of APX were also found in ozone-treated plants than in control and SAA > and OJ. The age-wise higher value of APX was estimated 50 days after the plant sowing in all selected treatments.

Primary Metabolites

Total soluble and reducing sugar: The quantitative profile of total soluble sugar and reducing sugar varied significantly within the plants under ozone stresses (Fig. 5). Maximum increase of total soluble sugar and reducing sugar content (24.91% and 46.90%, respectively) was noted at 50 DAS under T4 treatment. The maximum increase of reducing sugar (18.05%) was found at 25 DAS under the T4 treatment. The trends of increasing concentration of TSS and TRS were found in treatment T4 than T3> T1 > and T2.

Total soluble proteins and free amino acids: Total soluble proteins and free amino acids decreased significantly under ozone stress conditions. A major difference was found in protein and amino acids under ozone stress conditions. At the same time, exogenous applied SAA and OJ increased

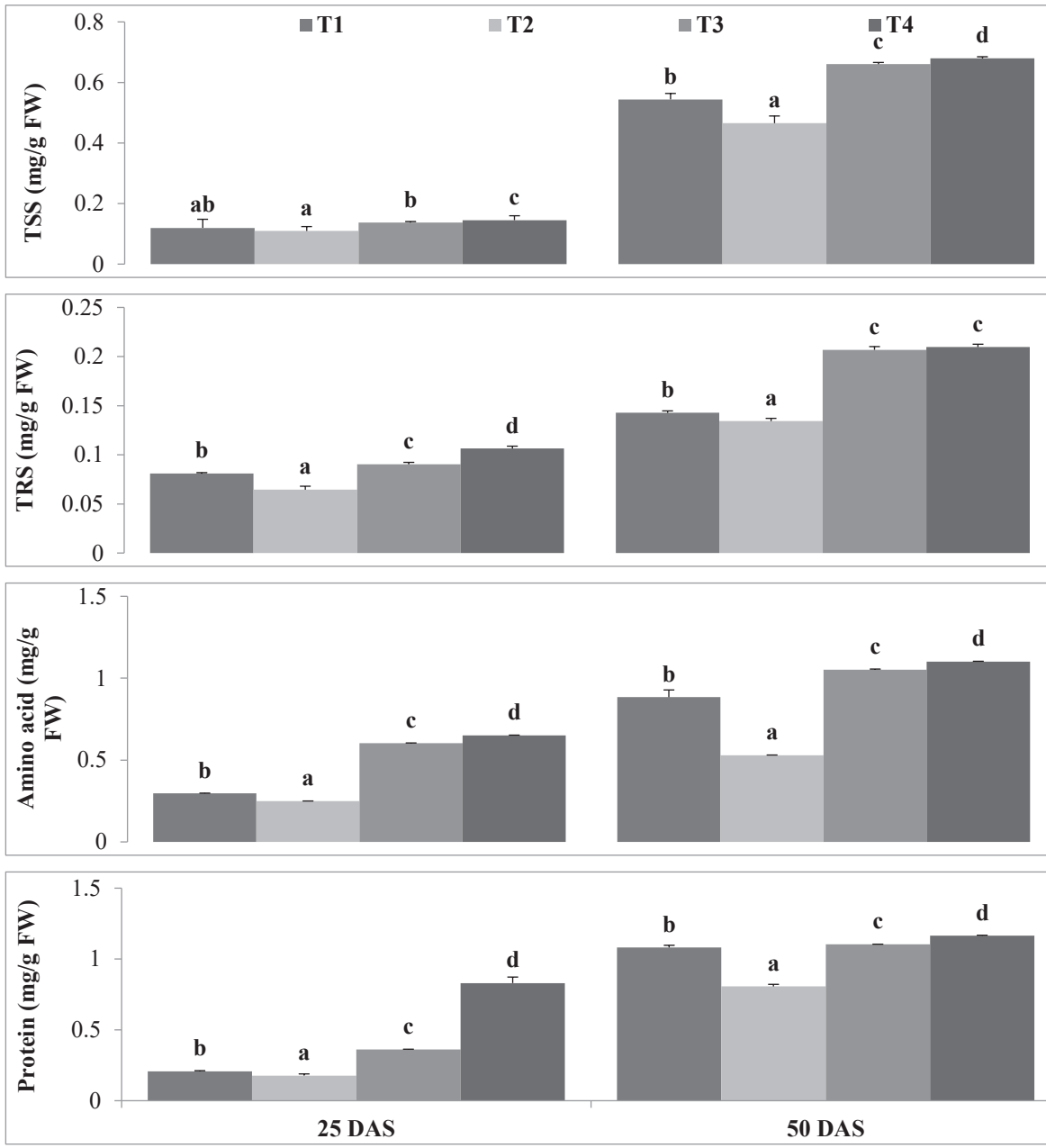

Fig. 5. Role of natural and synthetic ascorbic acid on TSS (mg/g fresh wt.), TRS (mg/g fresh wt.), amino acid (min^{-1} $.g^{-1}$fresh wt.) and protein (min^{-1} $.g^{-1}$ fresh wt.) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

these content in plants (Fig. 5). Maximum increase of total soluble protein was found in T4 treatment (301.71%) at 25 DAS and while a maximum increment of amino acid was also noted in same treatment (119.14%), at same DAS.

Yield Characteristics

Yield characteristics of soybean cultivars, such as the number of pods, number of seeds, seed weight, and total yield, are also affected by the application of elevated ozone. While the application of the protectant increased the yield of plants. Total yield reduction was observed under the T2 treatment (-19.16%). The maximum yield increase (19.46%) was noted under T4 treatment as compared to control plants (Fig. 6). While the application of SAA

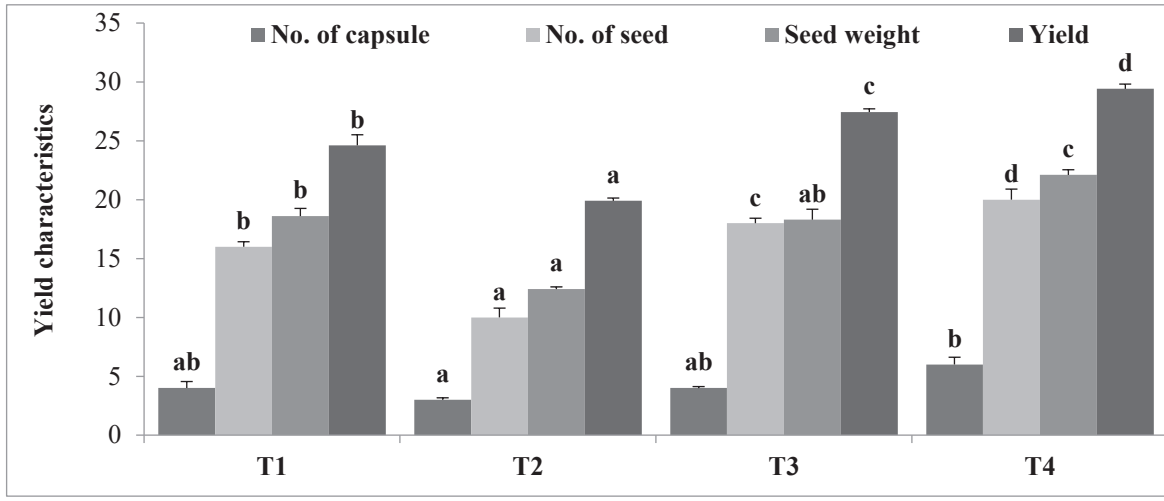

Fig. 6. Role of natural and synthetic ascorbic acid on no. of the capsule (plant^{-1}), number of seeds (plant^{-1}), seed weight (g.plant^{-1}), and total yield (g.plant^{-1}) of soybean cultivar (Mean ± standard deviation of three replicates presented by thin vertical bars, Value within each column followed by the same letter are not significantly different ($p< 0.05$) using Duncan's Multiple Range Test).

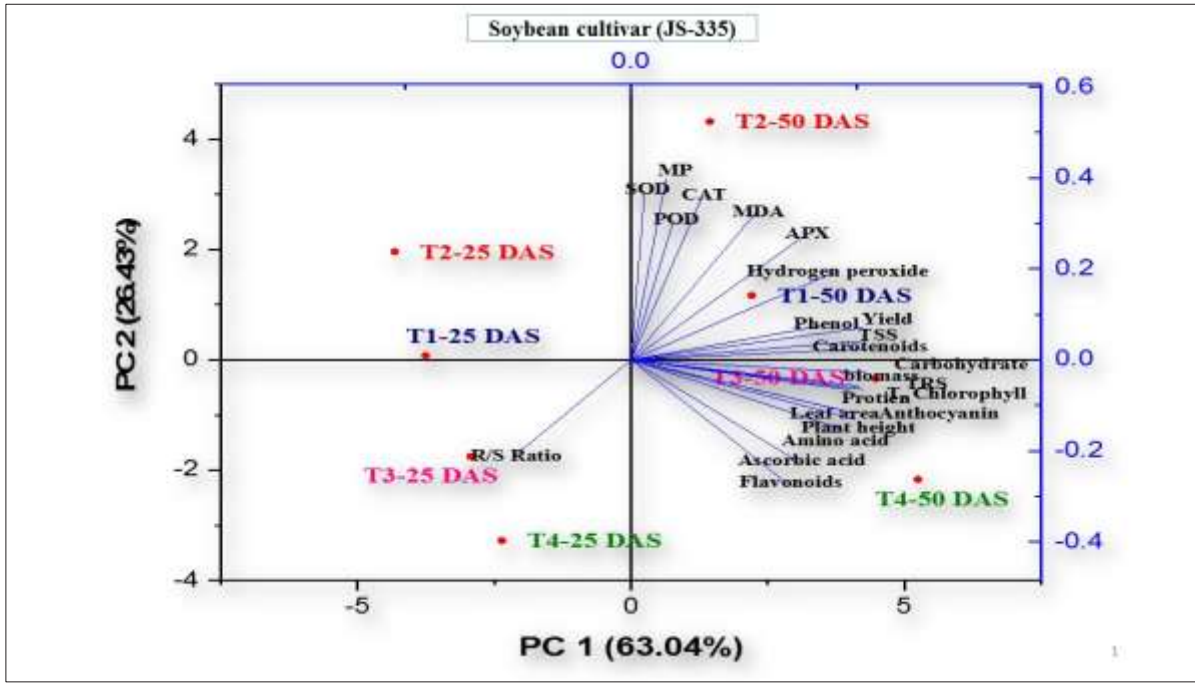

Fig. 7. Principle component analysis (PCA) correlation bi-plot of growth, biomass, and biochemical responses to ozone stress. Symbols represent the standardized scores on PC1 (x-axis) and PC2 (y-axis) for the ozone stress and ascorbic acid protectants on soybean cultivars (cv. JS-335). Vector coordinates represent the correlations between standardized variables and principal components (PCs).

shows a moderate increment in the yield of soybean plants.

Principle Component Analysis

PCAs analysis shows that the protectant application positively correlated with each parameter (Fig.7). The total percentage variance of cultivar JS-335 was found to be 63.04% and 26.43% at PC1 and PC2 with Eigenvalue 14.49 and 6.04. Percentage variation at PC3 was noted at 5.43% and eigenvalue 1.24. Leaf area, plant height, and protein content were highly correlated, while plant biomass, total chlorophyll, and TRS also showed strong relationships among the parameters.

All selected treatments showed a negative value 25 days after the sowing of the plant, while 50 days after the sowing of the plant represented a positive value at PC1. Treatment, wise highest positive score value of the cultivar was noted in T4 (5.52) than in T3 (4.47) >T1 (2.21) > and T2 (1.47). Antioxidant defense, such as non-enzymatic and enzymatic antioxidants, showed positive values at both PCs. Therefore, PCA analysis data confirmed that OJ is more effective than SAA compared to control plants, and elevated ozone caused a negative effect on soybean cultivar JS-335.

DISCUSSION

For plant growth and development, ozone is a toxic pollutant. A higher concentration of ozone caused agricultural losses and created food crises worldwide. Therefore a current study was carried out to improve plants' growth and yield using phytoextract enriched with ascorbic acid. The presented study showed that 100 ppm SAA and 25% OJ (enriched with ascorbic acid) could improve the ozone resistance of soybean plants. A study also reported that the ambient level (13.89 to 22.42 ppb day^{-1}) of ozone caused a negative effect on groundnut cultivars while applying synthetic ascorbic acid improved plant growth and yield (Chaudhary & Rathore 2020). In the present work, leaf area, plant height, and total plant biomass of soybean were reduced under ozone stress. While the application of exogenous OJ >and SAA increases the plant's leaf area and the plant's height and total biomass of the plant, its means that natural ascorbic acid is a more effective protectant against ozone stress. Various studies also reported that the elevated ozone caused a negative impact on leaf area and plant height and reduced plant biomass (Agathokleous et al. 2018, Rathore & Chaudhary 2019, 2021). However, exogenous application of ascorbic acid is attributed to ozone resistance by oxidative resistance organization, photosynthesis, and Osmo protection metabolism. The photosynthetic rate of chlorophyll a,b, and carotenoids was reduced under ozone stress, either due to reduced synthesis of key chlorophyll complexes (Agathokleous et al. 2018) or due to the destruction of pigment and protein molecules (Amira & Qados 2014). Ozone stress significantly reduced the contents of photosynthetic pigments such as chlorophyll a, b, and carotenoids in the current study. Foliar-applied 100 ppm OJ >and SAA improved soybean plants' chlorophyll and carotenoid contents.

Ozone enters through stomata and generates ROS in plants. It is a natural process, but due to elevated ozone, the production of H_2O_2 was higher and also increased lipid peroxidation and finally caused leaf membrane damage (Chaudhary & Rathore 2019, Rathore & Chaudhary 2019, 2021). In this study, H_2O_2 production, MDA, and membrane damage were higher in elevated ozone. The application of OJ >and SSA controlled the production rate of H_2O_2 and membrane damage in soybean plants. Malondialdehyde (MDA) is a signaling molecule that reflects oxidative stress-induced membrane damage (Shafiq et al. 2015). Moreover, exogenous application of protectants such as ethylene diurea, ascorbic acid, and phenyl urea controlled the leaf membrane injury (Chaudhary & Rathore 2020, Rathore & Chaudhary 2021).

Antioxidant defense systems, including enzymatic (SOD, POD, APX, and CAT) and non-enzymatic (phenolics, carotenoids, ascorbic acid, and flavonoids) antioxidant defense systems, protect cells from oxidative stress (Akram et al. 2017, Chaudhary & Rathore 2018a, 2019, Rathore & Chaudhary 2021). When exposed to ozone, CAT, SOD, APX, and POD behaviors were enhanced in soybean plants. Although the foliar application of natural ascorbic acid > and synthetic ascorbic acid has reduced the activities of CAT, SOD, POD, and APX in plants, the increasing incidence was higher in elevated ozone as compared to control plants. Rathore & Chaudhary (2021c) reported that ozone pollution increased the activity of these enzymes. Darvishan et al. (2013) also reported one more study under a water shortage regime of corn plants.

Non-enzymic antioxidants also played a vital role in defense, contrary to stress. Non-enzymatic antioxidant ascorbic acid (AA) plays a key function in stress safety by enzymatically detoxifying hydrogen peroxide and directly scavenging reactive oxygen species (ROS) (Hemavathi et al. 2011, Ye et al. 2012). Under ozone stress, the content of endogenous ascorbic acid in Soybean plants decreased in the current research. However, when applied 100 ppm SAA or NAA was under ozone conditions, internal ascorbic acid content improved in soybean plants. Previous reports show that oxidative stress reduced ascorbic acid contents in plants (Chaudhary & Rathore 2018d, 2019, 2020), and foliar application of ascorbic acid effectively improved the inherent ascorbic acid content under drought and ozone stress (Singh & Bharadwaj 2016, Chaudhary & Rathore 2020).

The synthesis of flavonoids is more under ozone stress conditions. The elevated ozone gradually reduced flavonoid contents while plants' phenolic content increased. Phenolic groups consume plants' flavonoids and protein formation (Rathore & Chaudhary 2021). According to some research, flavonoid synthesis is thought to increase in most plants when water-stressed (Ma et al. 2014, Nichols et al. 2015). Compared to our observations, total flavonoids decreased in soybean plants under ozone stress, while foliar application of 100 ppm synthetic ascorbic acid and 25% OJ enhanced with ascorbic acid increased flavonoid content in soybean

plants under ozone stress. Phenol contents of soybean plants were increased due to elevated ozone, and applied natural and synthetic ascorbic acid reduced the production of phenol in plant leaves.

Primary metabolites such as sugars, carbohydrates, and proteins were also affected due to ozone pollution. Sugars are important in increasing plant tolerance to abiotic stresses like ozone because higher sugar levels can reduce water loss, sustain turgor, and reduce membrane destruction, improving plant growth (Rodziewicz et al. 2014). Total soluble sugars and declining sugars in soybean plants exposed to ozone stress decreased dramatically in this research. Earlier reports also revealed that the ozone reduced the sugar content in cotton and groundnut cultivars (Chaudhary & Rathore 2021b, Rathore & Chaudhary 2021). The content of total soluble sugars and reducing sugars in soybean plants was increased by foliar application of ascorbic acid. Amira & Qados (2014) reported that ascorbic acid increased the sugar content in okra and soybean plants under water stress conditions, which is close to our findings. The reduction of total carbohydrates in plants was also higher in elevated ozone-treated plants than in control plants. Applying OJ> and SAA increased the total carbohydrate in plants.

Amino acid and protein contents were also decreased under the elevated ozone of the plant. Applied exogenous protectants increased the amino acid and protein contents in plant leaves. Enzymes that catalyze the hydrolysis of protein increasing the concentration of the phenolic compound in plants due to ozone stress may increase the synthesis of amino acids and proteins, resulting in reduced protein content in plants (Ambasht & Agrawal 2003, Chaudhary & Rathore 2020). Water stress affects amino acid metabolism, and their content usually rises, potentially causing protein synthesis (Zonouri et al. 2014). Ozone stress decreased the content of free amino acids and total proteins in soybean plants in this study. Furthermore, when foliar OJ and SAA were implemented under ozone stress, both sources of ascorbic acid increased the content of amino acids and total proteins in soybean plants. Similar findings have recently been reported in a variety of plants, including corn (Dolatabadian et al. 2010), wheat (Malik et al. 2015), and grapes (Zonouri et al. 2014), with the authors claiming that an increase in amino acid and protein content is positively associated with stress tolerance mechanisms in plants.

Overall, under ozone stress conditions, plants increased the production of H_2O_2 and MDA contents, damaging the leaves membrane (Chaudhary & Rathore, 2021a,b,c). The over-production of H_2O_2 in plant cells negatively affects plant growth, photosynthetic pigments, protein content, and, finally caused, yield loss. While increasing activities of antioxidants such as enzymatic (CAT, SOD, APX, and POD) and non-enzymatic (carotenoids, flavonoids, phenol, ascorbic acid) properties play a defensive role against ozone stress. In soybean plants, 25 percent OJ was more effective than 100 ppm SAA. OJ's effectiveness can be expected because it contains a variety of biomolecules and nutrients other than AA that could help plants grow and perform key metabolic functions. As a response, growth promotion by OJ may have been possible due to all nutrients in OJ rather than only AA. The foliar application of OJ (25 percent OJ) could increase soybean plant ozone resistance.

Agricultural productivity is a major part of the economy of the world's developed and developing countries. In the present investigation, ozone pollution caused a negative impact on the yield of soybean plants. A recent study also reported that ozone pollution reduced the growth and yield of castor beans and groundnut (Rathore & Chaudhary 2019, Chaudhary & Rathore 2021a). While the application of exogenous protectants such as OJ >and SAA improves the plant growth and yield of soybean cultivars. Higher effectiveness was found in natural ascorbic acid than in synthetic ascorbic acid. This means that 25% of orange juice enriched with AA will have a useful tool for agricultural sustainability against ozone stress. PCAs analyzed data, and the application of OJ and SAA showed strong relation with yield and plant physiological characteristics. This means OJ applications are a potent tool for agricultural productivity in ozone-prone areas.

CONCLUSION

Ozone is a burning problem for the agriculture of developed and developing countries. Overall, ozone enters through stomata in plant cells and causes negative effects in membrane damage, loss of relative water contents, and reduced plant growth and yield. However, to protect plants from oxidative stress, the exogenous application of protectants will be an enormous tool for agricultural loss. In the present study, foliar-applied synthetic or natural ascorbic acid improved plant growth, physiology, and yield of soybean cultivars. It was observed that 25% of OJ-enriched ascorbic acid was more effective than 100 ppm SAA in soybean plants subjected to ozone stress. Since OJ comprises several biomolecules and nutrients other than AA that could help promote plant growth and main metabolic activities, growth promotion by OJ may have been possible due to all nutrients found in OJ rather than by AA working slowly. Thus, foliar application of 100 ppm SAA and OJ (25 percent orange juice enriched) could increase soybean plant ozone resistance.

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