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Effect of elevated ozone on soybean (Glycine max L.) cultivar: Role of orange juice and synthetic ascorbic acid

Indra Jeet Chaudhary (indrajeet.cug@gmail.com)
Central University of Gujarat

Bhavna Nigam
Central University of Gujarat

Dheeraj Rathore
Central University of Gujarat

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Abstract

Ozone is a kind of hazardous gas for the environment and negatively affects the 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 presented study, soybean cultivar JS-335 was used for assessment of 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, biomass and also negatively affects the biochemical properties of plants due to these changes finally caused yield losses. Foliar-applied OJ >and SAA were found to be very effective in improving plant growth and development and also increased the yield of the crop. 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 be used to improve ozone resistance in plants in ozone-prone areas.

1. Introduction

The anthropogenic activity caused environmental pollution including air, water, and soil environment. Environmental pollutants including gaseous and suspended particulate matters cause injurious effects on plant's growth and its biomass (Chaudhary and Rathore, 2018a,b, 2019). Ozone is secondary pollutants and it's also caused negative effect on the plant and human health (Rathore and Chaudhary, 2019, 2021; 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 experienced a chain of physiological and biological alterations alternating from a single cell to a whole plant (Jolivet et al., 2016). When vegetation is exposed to higher levels of oxygen, it poses a threat to food sources, as well as affecting ecosystem stability and biosphere survival (Lu et al., 2015; Wang et al., 2016).

The vegetation protection against ozone destructive effects is thus a significant problem. Several potential agrochemicals available in market are tested as a protectors of plants against ozone phytotoxicity (Saitanis et al., 2015; Chaudhary and Rathore, 2022). A recent study reported by Chaudhary and Rathore, 2020, the exogenous application of EDU, PU and ascorbic acid play protective role against ozone stress. Exogenous application of ascorbic acid is considered to be mitigating the extreme circumstances of stress 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 and Bhardwaj, 2016), soybean (Amira and Qados, 2014), and groundnut (Chaudhary and Rathore, 2020).

Many plants are identified which naturally content huge quantity of ascorbic acid in their fruit or other parts. The sweet orange (Citrus sinensis L.), is a common fruit (Etebu and Nwauzoma, 2014) that is high in ascorbic acid (Galaverna and 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 yet not conducted. Therefore, these applications maybe develop a better tool for plant to survive against ozone stress and shield agricultural loss.

Soybean has the ability to 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, exogenous application of organic substances containing ascorbic acid, which is effective in improving the hostile effects of abiotic and biotic stress on plant species, can be used to boost soybean plant tolerance under such conditions. Approximately in numerous studies, It has been stated in the literature that ascorbic acid is a potent 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, the aim of this study was to compare the effects of pure synthetic ascorbic acid and OJ rich in ascorbic acid on soybean plants under ozone stress.

2. Material And Method

2.1 Experimental design and protectants application

Pots experiment was conducted with three replicates of soybean (Glycine max L) cultivar at Central University of Gujarat Campus during 2018. Under ozone (75 ppb) stress conditions for 4 hours a day, the effect of foliar-applied synthetic ascorbic acid and sweet orange phytoextract was studied. Four treatment were applied such as Treatment T1 (Control), T2 (ozone fumigation 75ppb), T3 (ozone fumigation + SAA), 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 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 each pot, and after two weeks of germination, thinning was performed to keep each pot at ten seedlings of nearly equal size. 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 after plant germination. 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 using, the content of ascorbic acid in 25 percent OJ was estimated to be 20.7 mg/L using Keller and Schwager's process (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. Sample was collected for growth & physiology analysis after 25 DAS, 50 DAS and final harvesting was done at 120 DAS.

2.2 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 root and shoot length was measured by a regular meter scale. Mass of plants such as the fresh and dry weight of plant part was determined by electric balance. Plants parts were dried in a hot air electric oven at 80 degree centigrade till a constant weight achieved.

2.3. Photosynthetic and non-photosynthetic pigments

Method Arnon (1949) was used to check for photosynthetic pigments (chlorophyll a, b, carotenoid, and complete chlorophyll). For chlorophyll analysis, a 0.25 g leaf sample was taken and mixed with 5 mL of an 80 percent acetone solution. 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, 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:

Anthocyanin (mg g-1 fresh leaf) = A535 - 2.2 A650/W ×V

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

2.4 Estimation of oxidative stress

Hydrogen peroxide activity was analysed by method 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 added 5ml of TCA and the sample was crushed. After centrifuging the homogenate, 500 litres of supernatant is combined with 500 litres of 10 mM potassium phosphate buffer (7.0pH). After mixing the solution with 1 mL of 1M 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 and Packer's (1968) method. MDA was determine 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. Take 500 mL of the supernatant after centrifugation and blend it 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 Blum and Ebercon (1981). An electrical conductivity metre was used to calculate ion leakage from fresh leaves in deionized water

(Eutech Instruments). A punching machine was used to carve the leaf samples into 1 cm diskettes. After cutting 20 diskettes from each sample, 10 mL 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.

2.5. Estimation of antioxidants

2.5.1. Non enzymatic antioxidants

2.5.1.1 Flavonoids

Cameron et al. (1943) proposed a method for estimating flavonoids 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).

2.5.1.2 Ascorbic acid

For determination of ascorbic acid in leaf sample of soybean was used method of Keller and 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 distilled water). In an ice bath, 500 mg fresh leaf sample was homogenized with 20 ml of extracting solution, and the homogenate was centrifuged at 6000xg for 15 min. After centrifuged the sample 1 ml of the supernatant was taken and added 5 ml of 2, 6-dichlorophenol-indophenol solution pink color developed. After constant shaking O.D. of the solution was takes (Es) at 520 nm wavelength. Then one drop of ascorbic acid 1% solution was added in order to bleach the pink color and obtained OD of turbid solution (Et) at the same wavelength.

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

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) = $[\{Eo - (Es - Et)\} \times V] / (v \times W \times 1000)$

Where, W = weight of leaf taken (g); V = total volume of the mixture (ml); v = supernatant taken for analysis (ml). Value of $\{Eo-(Es - Et)]$ is estimated by the standard curve.

2.5.1.3 Total phenols

The amount of total phenols was estimated by the method Mallick and Singh (1980) using 70% acetone. For phenol determination, 100 mg fresh leaf sample was crushed with 10 ml of 70% acetone, and the suspension was centrifuged at 6000xg for 10 minutes. Then taken 1 ml of supernatant in a test

tube and added 1 ml of folin-ciocalteu reagent plus 2 ml of Na_2CO_3 (20% w/v) solution and the final volume was made up to 10 ml with distilled water. This mixture was heated in a water bath for one minute and then cooled at room temperature. Blue coloured developed in a solution and OD of the solution was measured at 650 nm wavelength. A standard curve was prepared with known amounts of quinine for the amount of phenol contents.

2.5.2 Enzymatic antioxidants

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

2.5.2. 1 Catalase activity

Catalase activity was estimated by Chance and 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 in the mixture and the OD was measured at 240 nm after every 20 seconds for two minutes.

2.5.2. 2 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 optical density (OD) of the solution was recorded at 560 nm (Van Rossum et al., 1997).

2. 5.2. 3 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 H2O2 (40 mM) and the OD of the solution was calculated at 470 nm after every 20 seconds for 3 min as defined in Chance and Maehly (1955).

2. 5.2. 4 APX activity

For the APX activity, 100 µL supernatant was mixed with 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 till the ascorbic acid oxidised totally. One unit of APX forms 1 µ M of ascorbate oxidized per minute in assay conditions (Nakano and Asada, 1981).

2.7. Primary metabolites

2.7. 1 Total soluble sugars, Reducing sugars

Foliar sugar contents were estimated using the method described by Somogyi (1952). Leaf sample (50 mg) was crushed in 5 ml of 80% ethanol and centrifuged for 15 min at 3500xg. Pellets obtained was

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 hot water bath for 10 minutes. After boiling, the solution kept for cooled to room temperature straightway and then added 1 ml of arsenomolybdate. The solution was left for 30 minutes to complete the whole reaction before taking OD at 500 nm for estimating 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. Total soluble sugar and total reducing sugar was estimated by standard curve obtained using purified glucose. Remaining pellet samples was 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 for estimating the starch content.

2.7.2 Amino acid and proteins

Amino acid was determining using method Hamilton et al. (1943). 1mL of 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, and cooled at room temperature, upraised the 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).

2.8. Yield characteristics

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

2.9. Statistical Analysis

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

3. Results

3.1 Growth and Biomass

3.1.1 Leaf area and plant height

Leaf area and plant height of ozone treated plants was highly affected as compared to control plant while application of natural ascorbic acid play protective role against ozone stress than synthetic

ascorbic acid as compared to control plants. Maximum increased of leaf area was found in treatment T4 (14.86%) at 25 DAS and plant height in same treatment (28.17%) at 50 DAS (Fig. 1). The treatment wise difference of leaf area and plant height was noted maximum in treatments T4 > T3 > T1 > T2 (Fig. 1).

3. 1.2 Total biomass and root shoot ratio

Plant biomass and root shoot ratio shows variable result in treatment and age factors. Total plant biomass was reduced by ozone stress of experimental crop (Fig. 1). However, exogenous application of SAA and OJ treated plants not only neutralized the ozone effect but also enhanced the weight of leaf dry mass and total plant biomass of experimental cultivar. Maximum increased in leaf dry weight was found 70.13% under treatment T4 at 25 DAS and higher increment of total plant biomass (117.57%) was noted under same treatment at same day after sowing of plants. Root shoot ratio of plant showed negative percentage reduction in enhanced ozone treated plant at 25 DAS as compared to control plants while exogenous applied AA showed positive value of percentage increments at 25 DAS and negative value at 50 DAS.

3.2 Oxidative stress

3.2.1 Hydrogen peroxide and MDA contents

Enhance ozone increased the production of hydrogen peroxide and MDA contents in plants. Higher production was found 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 T2 > T1 > T3 > and T4. Accumulation of MDA in ozone stressed seedlings was higher than control, whereas in the presence of exogenous application of SAA and OJ MDA contents was reduced significantly (Fig.1). The maximum increase of MDA was observed in treatment T2 (25.35%) at 25 DAS (Fig. 2).

3.2.2 Membrane permeability

Membrane permeability showed significant increase in ozone stress. The higher membrane injury was recorded under ozone stress. Maximum increase of membrane permeability was found under treatment T2 (12.53%) at 50 DAS (Fig. 2) and 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.

3.4. Photosynthetic and non-photosynthetic pigments

3.4.1 Total chlorophyll and Carotenoids

Ozone treated plants 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. Maximum increase of total chlorophyll

was noted under treatment T4 (35.84%) at 50 DAS and minimum in treatment T2 (-33.38%) at 25 DAS as compared to control plants. Increasing trends of chlorophyll in treatments were T4>T3>T1>and T2. Carotenoid and anthocyanin contents were also reduced under ozone stress. Maximum increased of carotenoid was found in treatment T4 (29.81%) at 50 DAS and minimum in treatment T2 (-18.83%) at 25 DAS.

3.4.2 Anthocyanin

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

3.5 Antioxidants activity

3.5 .1 Non-enzymatic antioxidants activity

3.5 .1.1 Flavonoids, phenol and ascorbic acid

According to the data, production of flavonoid 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). Maximum increase of phenolic contents 101.40% at 50 DAS under T3 treatment. Ozone caused negative effect on ascorbic acid content in selected crop. Maximum increased in ascorbic acid (228.20%) was found under T4 treatment at 50 DAS and minimum in treatment T2 (-15.55%) at 25 DAS (Fig. 3).

3.5 .2 Enzymatic antioxidants

3.5 .2.1 CAT, POD, SOD and APX

The antioxidant enzymes CAT showed deviation in their activities under ozone stress. Maximum increment of CAT (0.74%) was found under T2 treatment at 50 DAS and minimum in treatment T4 at 25 DAS. POD (430.38%) under T2 treatment at 50 DAS. Maximum increase of SOD (143.77%) noted at 50 DAS under T2 treatment and APX (72.90%) noted at 25DAS under T2 treatment (Fig. 4). Activity of POD was also increased due to application of elevated ozone while protectants applied plant showed reducing activity of POD at both sampling periods. Higher activity of POD was noted at 25 DAS of plant than 50 DAS of plants. Maximum values of POD activity was found in treatment T2 at 50 DAS of plants as compared to control plants. Ozone pollution increased the SOD activity in soybean plant and higher values was observed at 50 DAS of plants. Treatment wise trends of SOD activity was noted higher in T2 > T1 > T3 > and T4. Higher values of APX was also found in ozone treated plant than control and SAA > and OJ. Age wise higher value of APX was estimated at 50 day after sowing of plant in all selected treatments.

3.6. Primary metabolites

3.6.1 Total soluble and reducing sugar

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) noted at 50 DAS under T4 treatment. Maximum increase of reducing sugar (18.05%) was found at 25 DAS under T4 treatment. The trends of increasing concentration of TSS and TRS was found in treatment T4 than T3> T1 > and T2.

3.6.1 Total soluble proteins and free amino acids

Total soluble proteins and free amino acids decreased significantly under ozone stress conditions. There was prominent different was found in protein and amino acids under ozone stress condition while exogenous applied SAA and OJ increased these content in plants (Fig. 5). Maximum increase of total soluble protein was found in T4 treatment (301.71%) at 25 DAS and while maximum increment of amino acid was also noted in same treatment (119.14%), at same DAS.

3.6. Yield characteristics

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

3.7 Principle component analysis

PCAs analysis shows that the application of protectant positively correlated with each parameters (Fig.7). Total percentage variance of cultivar JS-335 was found 63.04% and 26.43% at PC1 and PC2 with Eigenvalue 14.49 and 6.04. Percentage variation at PC3 was noted 5.43% and eigenvalue 1.24. Leaf area, plant height and protein content was highly correlated with each other's while biomass of plant, total chlorophyll and TRS was also shows strong relation among the parameters. All selected treatments showed negative value at 25 day after sowing of plant while at 50 day after sowing of plant represent positive value at PC1. Treatment wise highest positive score value of cultivar was noted in T4 (5.52) than T3 (4.47) >T1 (2.21) > and T2 (1.47). Antioxidants defence such as non-enzymatic and enzymatic antioxidant showed positive values at both PCs. Therefore PCA analysis data was confirmed that the application of OJ is more effective than SAA as compared to control plants and elevated ozone caused negative effect on soybean cultivar JS-335.

4. Discussion

For plant growth and development, ozone is a toxic pollutant. A higher concentration of ozone caused agricultural losses and create food crises worldwide. Therefore current study carried out for improving the growth and yield of plants using phytoextract enrich 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 was also reported that the ambient level (13.89 to 22.42 ppb day^{-1}) of ozone caused negative effect on groundnut cultivar while application of synthetic ascorbic acid improved plant growth and yield (Chaudhary and Rathore, 2020). In present work leaf area, plant height and total plants biomass of soybean was reduced under ozone stress condition. While the application of exogenous applied NAA > and SAA increases the leaf area of the plant and also increases the height and total biomass of the plant its means that natural ascorbic acid is a more effective protectant against ozone stress. Various studies were also reported that the elevated ozone caused negative impact on leaf area, plant height and also reduced the total biomass of plants (Agathokleous et al., 2018; Rathore and Chaudhary, 2019; 2021). However, exogenous application of ascorbic acid attributed to ozone resistance by oxidative resistance organisation, 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 and 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 the chlorophyll and carotenoids contents in soybean plants.

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 the lipid peroxidation and finally caused leaf membrane damage (Chaudhary and Rathore, 2019; Rathore and Chaudhary, 2019; 2021). In this study H_2O_2 production, MDA, and membrane damage were also higher in elevated ozone while application of NAA > and SSA control the production rate of H_2O_2 and membrane damage in soybean plants. Malondialdehyde (MDA) is a signalling 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 are controlled the leaf membrane injury (Chaudhary and Rathore, 2020; Rathore and Chaudhary, 2021).

Antioxidant defence systems, including enzymatic (SOD, POD, APX, and CAT) and non-enzymatic (phenolics, carotenoids, ascorbic acid, and flavonoids) antioxidant defence systems, protect cells from oxidative stress (Akram et al., 2017; Chaudhary and Rathore 2018a, 2019; Rathore and Chaudhary, 2021). The behaviours of CAT, SOD, APX, and POD were found to be enhanced in soybean plants when exposed to ozone. Although 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 noted to be higher in elevated ozone as compared to control plants. Rathore and Chaudhary (2021) were reported that ozone pollution increased the activity of these enzymes, and one more study was also reported by Darvishan et al., (2013) under water shortage regime of the corn plants.

Non-enzymetic antioxidants were 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, internal ascorbic acid content improved in soybean plants when applied 100 ppm SAA or NAA under ozone conditions. Numerous previous reports show that oxidative stress reduced ascorbic acid contents in plants (Chaudhary and Rathore, 2018a; 2019; 2020), and foliar application of ascorbic acid effectively improved the inherent ascorbic acid content under drought and ozone stress (Singh and Bharadwaj, 2016; Chaudhary and Rathore, 2020).

The synthesis of flavonoids is more under ozone stress conditions. The elevated ozone gradually reduced flavonoids contents while phenolic content was increased in plants. phenolic groups consume the flavonoids and proteins formation in plants (Rathore and Chaudhary, 2021). According to some research, the synthesis of flavonoids is thought to be increased in most plants when they are water-stressed (Ma et al., 2014; Nichols et al., 2015). Total flavonoids decreased in soybean plants under ozone stress, in comparison to our observations, 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 plant 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 play an important role in increasing plant tolerance to abiotic stresses like ozone because higher sugar levels can reduce water loss, sustain turgor, and reduce membrane destruction, both of which improve 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 report was also published that the ozone reduced the sugar content in cotton and groundnut cultivars (Chaudhary and Rathore, 2021b; Rathore and Chaudhary, 2021). The content of total soluble sugars and reducing sugars in soybean plants was increased by foliar application of ascorbic acid. Amira and Qados (2014) reported that ascorbic acid increased the sugar content in okra and soybean plants, respectively, under water stress conditions, close to our findings. Reduction of total carbohydrate in plants was also higher in elevated ozone treated plants than control plants and application of NAA > and SAA were increased the total carbohydrate in plants.

Amino acid and protein contents were also decreased under 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 and Agrawal, 2003; Chaudhary and Rathore, 2020). Water stress affects amino acid metabolism, and their content usually rises as a result, 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 also increased MDA contents, and damage the leaves membrane (Chaudhary and 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 found to be 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 NAA (25 percent OJ) could increase soybean plant ozone resistance.

Agricultural productivity is a major part of the economy of the world's develop and developing countries. In the present investigation, ozone pollution caused a negative impact on yield of soybean plant. A recent study was also reported that the ozone pollution reduced the growth and yield of castor bean and groundnut (Rathore and Chaudhary, 2019; Chaudhary and Rathore, 2021a). While the application of exogenous protectants such as NAA > and SAA improve the plant growth and yield of soybean cultivar. The higher effectiveness was found in the natural ascorbic acid than synthetic ascorbic acid. Its means that 25% of orange juice enriched with AA will have a useful tool for agricultural sustainability against ozone stress. Data was analyse by PCAs and application of OJ and SAA showed strong relation with yield and plant physiological characters its means applications of OJ is the used full tool for agricultural productivity at ozone prone area.

5. Conclusion

Ozone is the burning problem for the agriculture of the world develop and developing countries. Overall, ozone enters through stomata in plant cells and caused negative effects in form of membrane damage, loss of relative water contents, and reduced plant growth and yield. However, for the protection of plants by oxidative stress, exogenous application of protectant will be an enormous tool of agricultural loss. In the present study foliar-applied synthetic or natural ascorbic acid improved the plant growth, physiology, and yield of soybean cultivar. 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 a number of biomolecules and nutrients other than AA that could be helpful for promoting 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.

Declarations

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Author contribution:

Indra Jeet Chaudhary: Conceptualization, Methodology, Software, Data curation, Writing – original draft, Investigation. Dheeraj Rathore: Conceptualization, Resources, Supervision, Software, Validation, Writing – review & editing, Project administration.

Conflict of Interest

This work does not include any human subject. This manuscript has not been published and is not under consideration for publication elsewhere. Authors have no conflicts of interest to disclose.

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Figures

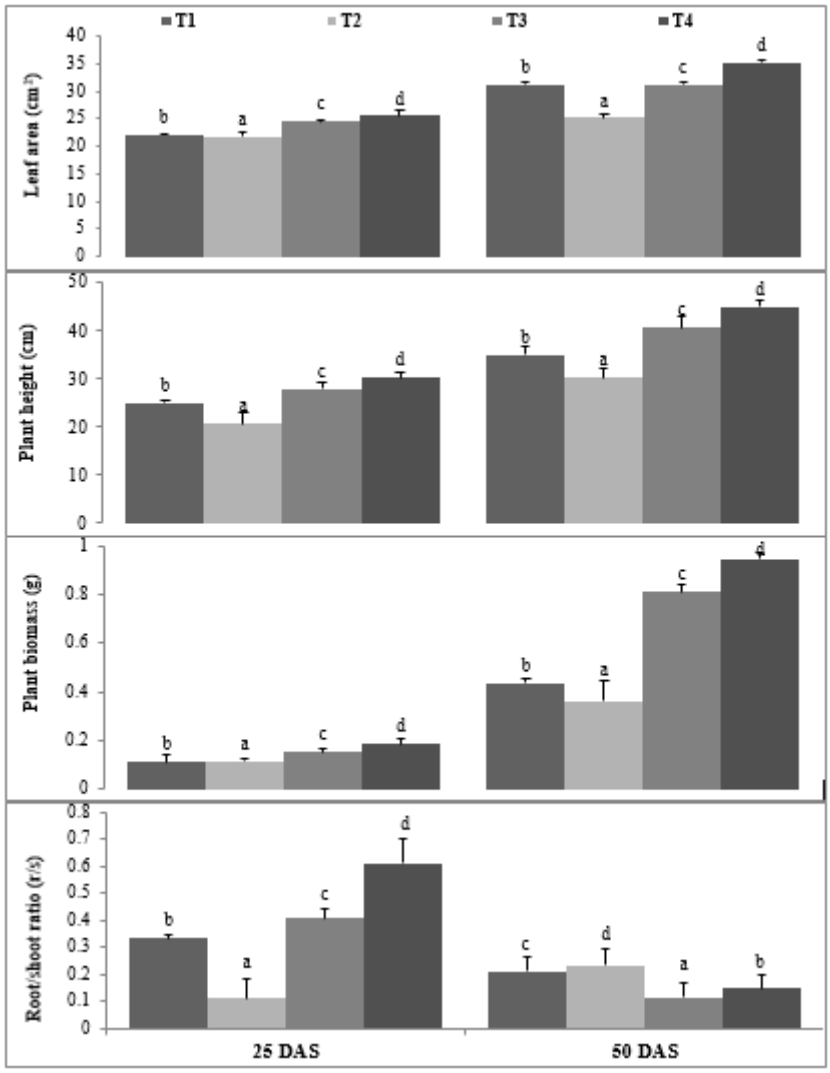

Figure 1

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).

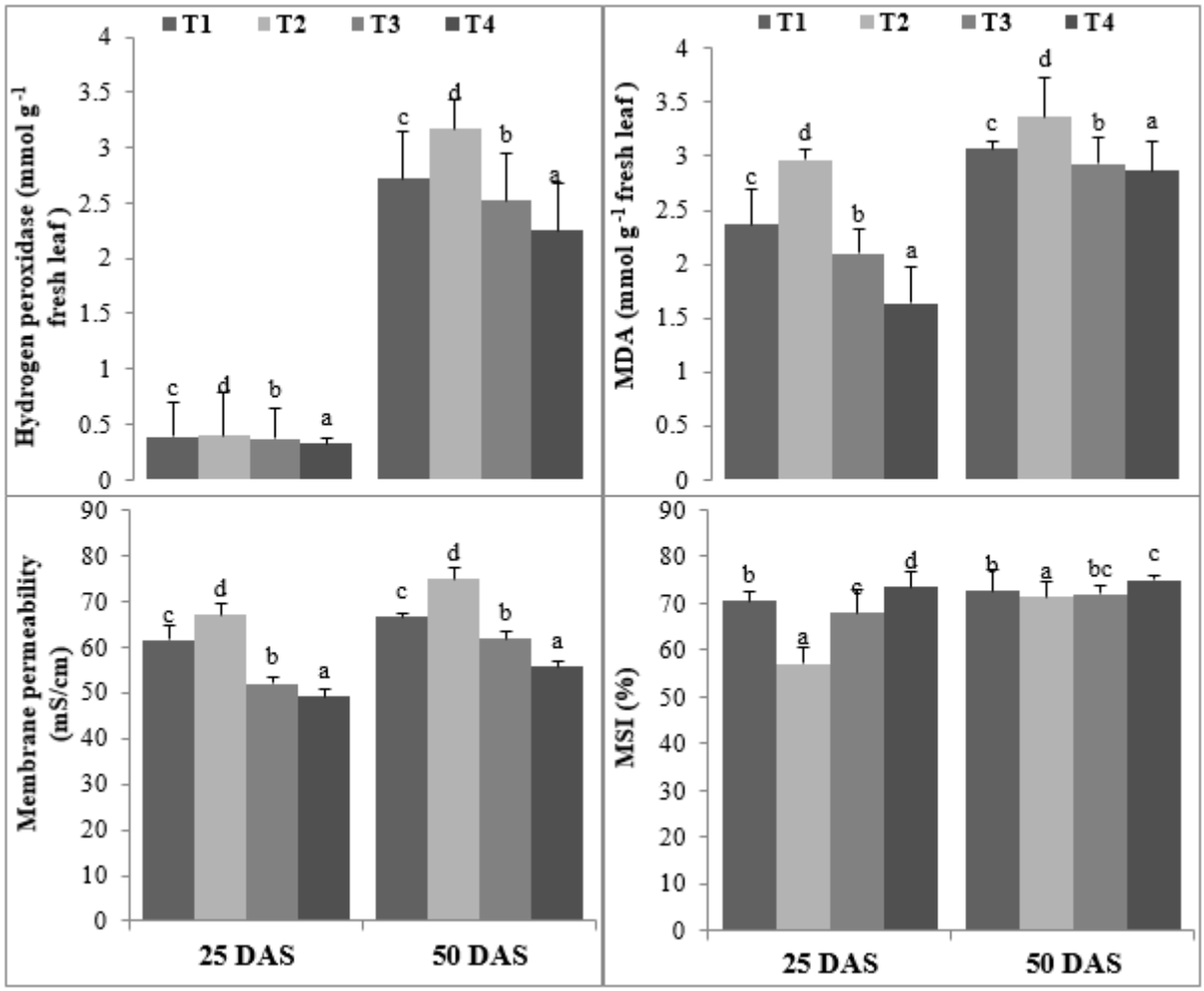

Figure 2

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) 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).

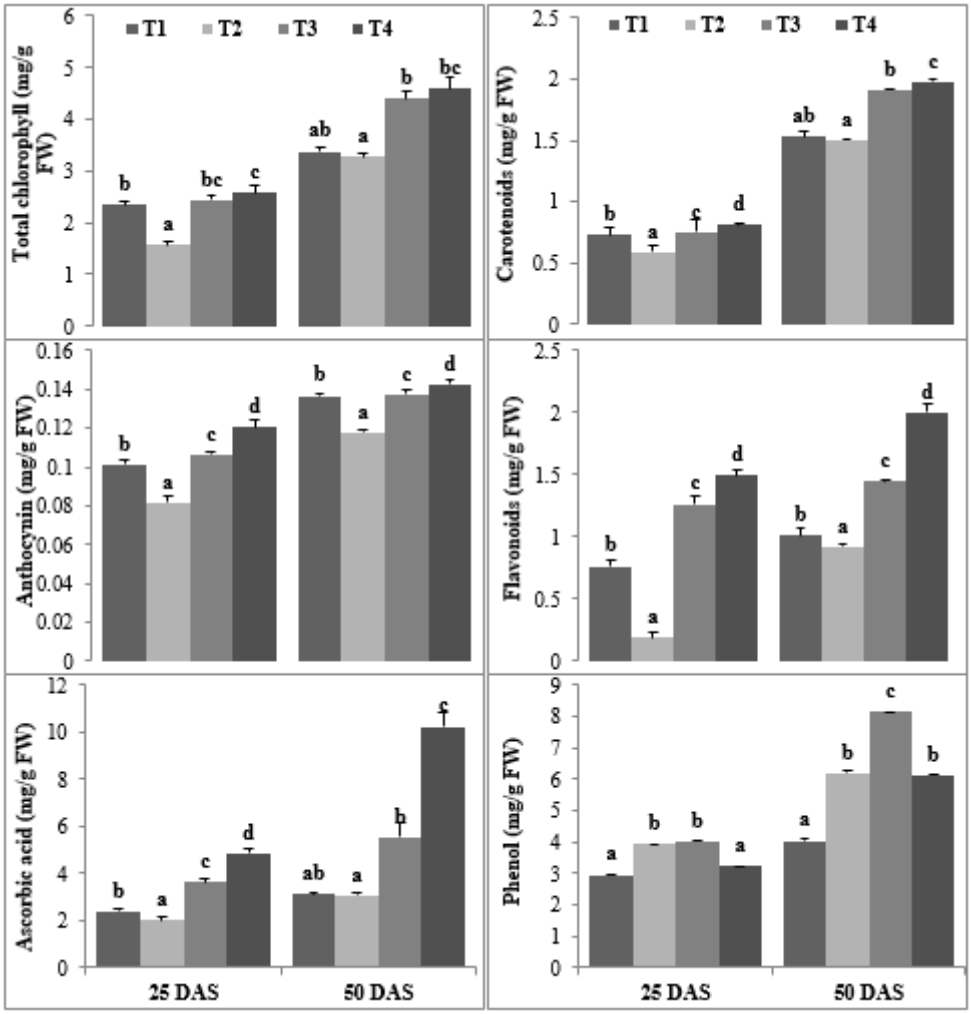

Figure 3

Role of natural and synthetic ascorbic acid on total chlorophyll (mg/g fresh wt.), carotenoids (mg/g fresh wt.), anthocyanin (mg/g fresh wt.), flavonoids (mg/g fresh wt.), ascorbic acid (mg/g fresh wt.) and phenol (mg/g 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).

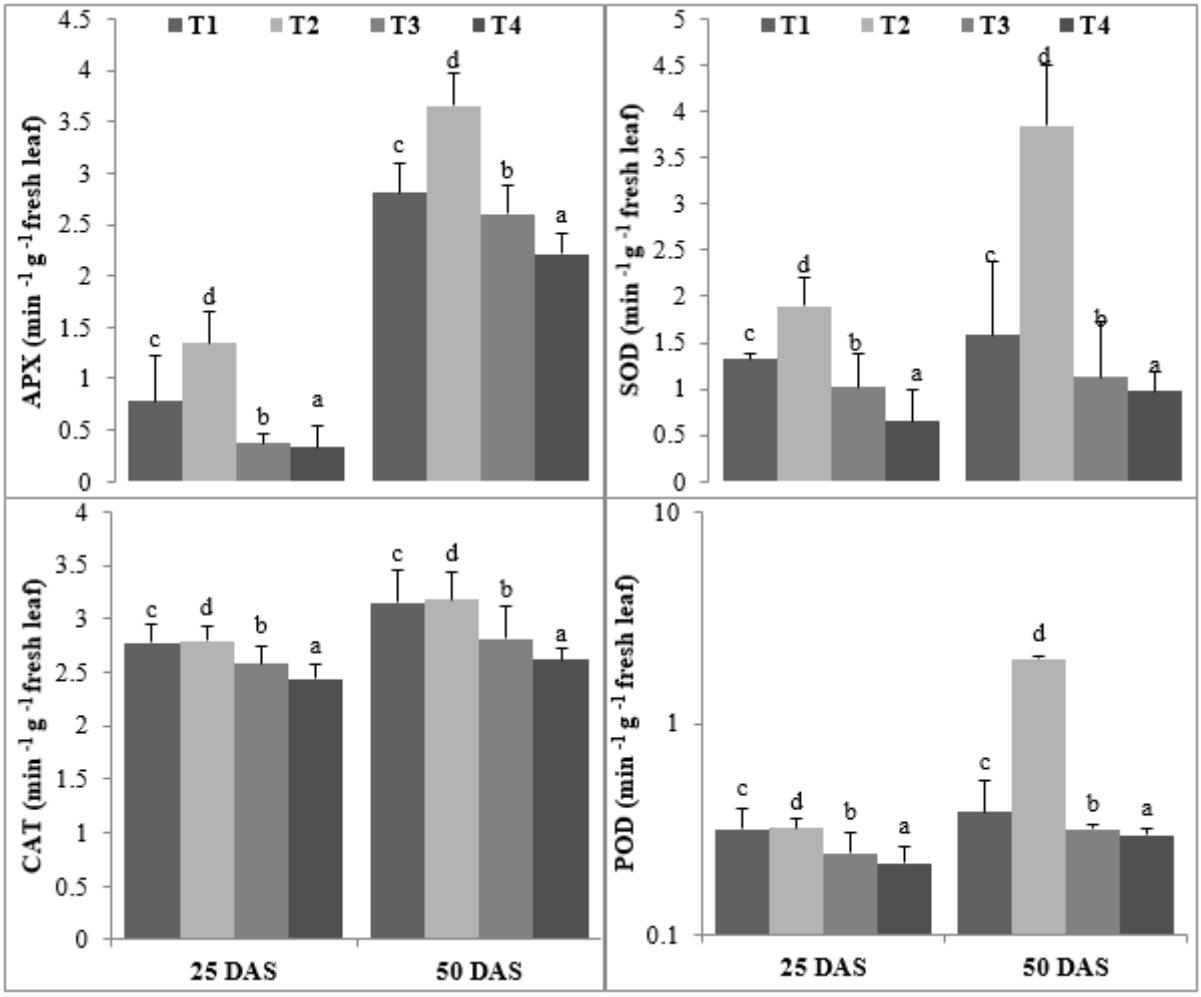

Figure 4

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).

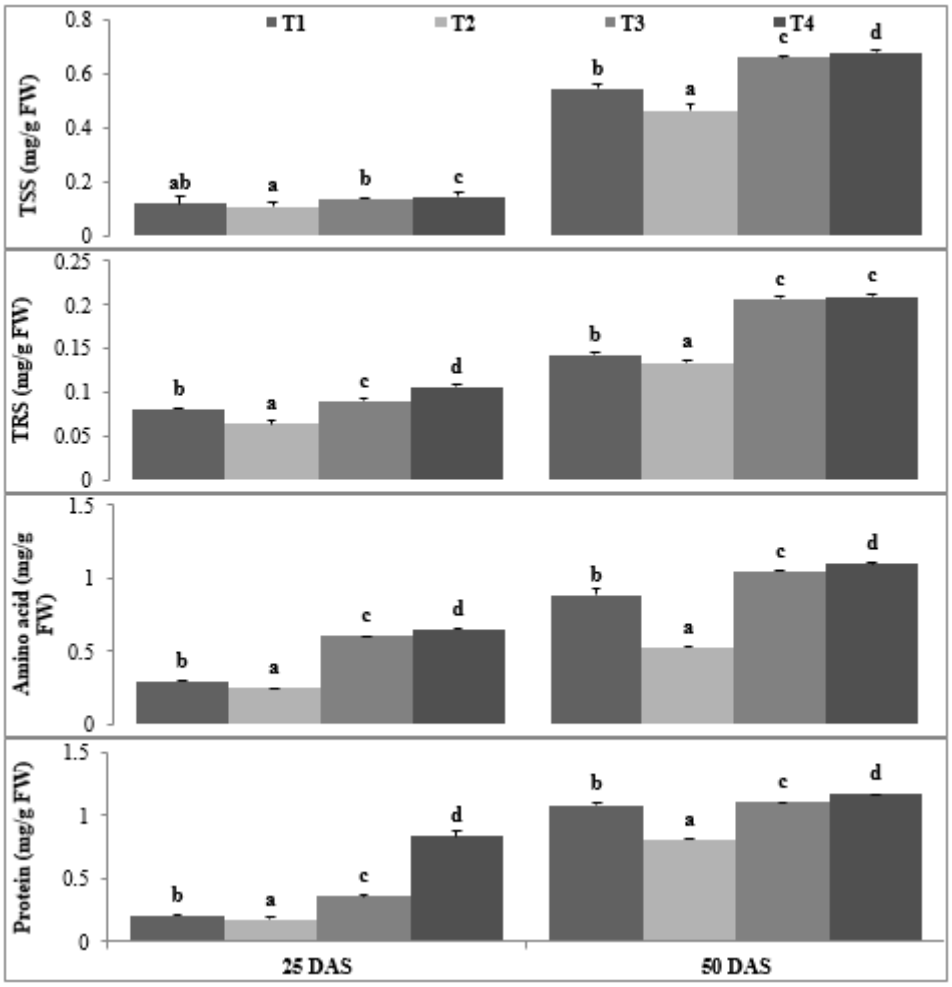

Figure 5

Role of natural and synthetic ascorbic acid on TSS (mg/g fresh wt.), TRS (mg/g fresh wt.), amino acid (mg/g fresh wt.) and protein (mg/g 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).

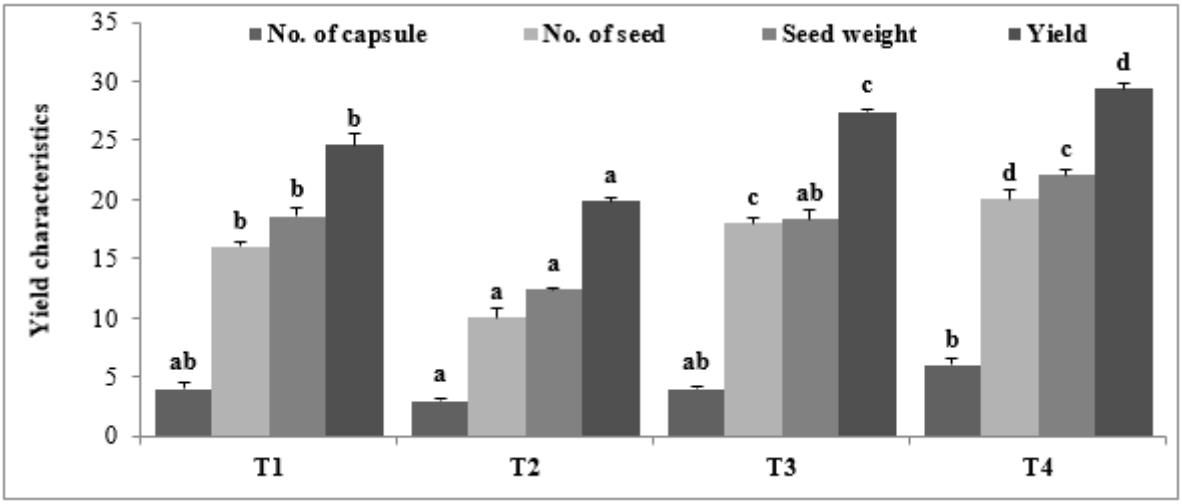

Figure 6

Role of natural and synthetic ascorbic acid on no. of capsule (plant^{-1}), number of seed (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).

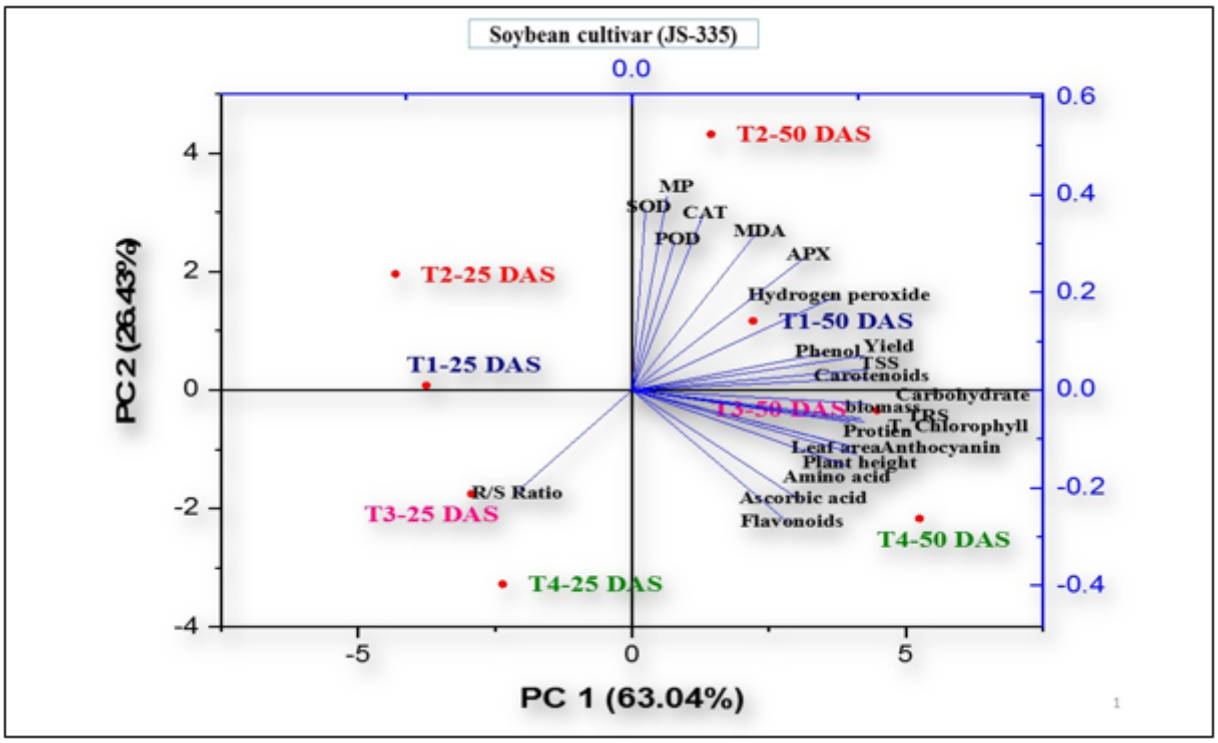

Figure 7

Principle component analysis (PCA) correlation bi-plot of growth, biomass and biochemical responses to ozone stress. Symbol represent the standardized scores on PC1 (x-axis) and PC2 (y-axis) for the ozone

stress and ascorbic acid protectants on soybean cultivar (cv. JS-335). Vector coordinates represent the correlations between standardized variables and principle components (PCs).