Plant Archives Vol. 22, No. 1, 2022 pp. 375-385 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2022.v22.no1.058

ROLE OF BIOCHEMICAL AND BIOPHYSICAL CHARACTERISTICS IN MANAGEMENT OF O_3 INJURY IN PLANTS THROUGH SOIL NUTRIENT AMENDMENTS

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(Date of Receiving : 16-10-2021; Date of Acceptance : 31-01-2022)

ABSTRACT

The present study focuses on mitigative measurements adopted to manage ozone (O_3) stress by application of different doses of nutrient (N, P, K) treatments. Two cultivars of maize (*Zea mays* L. var HHM-1 and Malviya hybrid-2) growing under ambient O_3 stress conditions were treated with three doses of nutrients (NPK); recommended (N_1), 1.5x recommended (N_2) and 2x recommended (N_3) in near natural condition. The antioxidant pool, secondary metabolites, photosynthetic efficiency and gas conductance parameters were assessed. Results of our experiment suggest that the plants treated with nutrients responded better than the plants without nutrient, which served as control. Increased antioxidant activities in both the maize cultivars upon nutrient treatment resulted in decline of O_3generated superoxide radicals and H_2O_2 contents, indicating a reduction in O_3 stress. It was observed that the antioxidant response upon treatment was more prominent in HHM-1, as compared to MH-2, in which major proportions of antioxidant stimulations were observed during the vegetative period only. SOD and CAT played an important role in defining the plant's defense and regulating the SOR and H_2O_2 contents in both the maize cultivars at both vegetative and reproductive stages. The results of the present experiment clearly suggest that nutrient amendments can be effectively used in partially mitigating ambient ozone stress, however more experimentation with different crop varieties is required to prove the expediency of nutrient amendments.

Keywords: Tropospheric ozone, oxidative stress, antioxidants, mitigating.

Introduction

Tropospheric ozone (O_3), a well-established secondary oxidative pollutant is one of the most prominent air pollutants, greatly affecting the crop productivity (Li *et al*., 2021; Yadav *et al*., 2020; Peng *et al*., 2019; Pleijel *et al*., 2019; Embersen *et al.,* 2018; Mills *et al*., 2018). As per the modeling studies. South Asia is predicted to emerge as an important O_3 hotspot in the coming times (Cooper *et al*., 2014; Dentener *et al*., 2006). Projections related to emission of O_3 precursors also indicate a substantial increase in the O_3 concentration in South Asia (Chang *et al*., 2017). In addition, the climate change scenario is also expected to augment the process of O_3 formation in the atmosphere (Demuzere *et al.,* 2019).

Enhanced production of pernicious reactive oxygen species (ROS) and elicitation of the cellular antioxidant pools are two well acknowledged initial consequences of O_3 injury in plants (Peng *et al*., 2019; Yadav *et al*., 2019; Zhang *et al*., 2019). A number of biochemical and metabolic adjustments are observed in O_3 stressed plants which include membrane lipid peroxidation, degradation of nucleic acid, protein oxidation, pigment rearrangement etc, which result in collapse of the cellular machinery (Tiwari and Agrawal, 2018; Chaudhary *et al*., 2017). Although the reduction in O_3 induced plant productivity can be attributed to quite a few factors like modifications in biomass utilization pattern, reduced phloem translocation efficiency, dominance of cellular repair processes, stimulation of secondary metabolite pathways etc, however, decline in the carboxylation efficiency is considered to be the most influential of all (Wilkinson *et al*., 2012).

In view of the perilous outcomes of O_3 on plant metabolism; it is of absolute urgency that some ameliorative measures are implemented. Soil nutrient amendments have shown very promising results in partial mitigation of O_3 stress in plants (Sengupta and Tiwari, 2020; Podda *et al*., 2019; Tatsumi *et al*., 2019; Pandey *et al*., 2018; Harmens *et al*., 2017; Kinose *et al*., 2017). Although the mechanisms underlying the alterations in defense processes are not very clear, Sengupta and Tiwari (2020) have suggested

stimulation of antioxidant enzymes, especially those of Halliwell–Asada pathway strengthened the defense system of the plants enabling them to minimize the ozone injury. It has been further affirmed that nutrient application positively affected the photosynthetic potential (Zhang *et al*., 2018), along with adjustments in stomatal conductance (Marzuoli *et al*., 2018; Zhang *et al*., 2018; Harmens *et al*., 2017).

The aim of the present study was to assess the role of soil nutrient amendments in management of O_3 stress, by evaluating the response of biochemical and physiological attributes of two maize (*Zea mays* L.) cultivars with varying O_3 sensitivities. We propose the following hypotheses:

(i) Antioxidant pool responded more positively in O_3 tolerant cultivar as compared to O_3 sensitive cultivar and the invigoration was more prominent at the vegetative stage than in the reproductive stage, upon similar nutrient treatment regimes.

(ii) O_3 induced uncoupling of rate of photosynthesis and stomatal conductance was not modified upon nutrient application and increased rate of photosynthesis of nutrient treated plants was attributed to increased N availability.

Material and Methods

Experimental area

The botanical garden of Institute of science, Banaras Hindu University, Varanasi, has been chosen as the experimental study site. Area with the geographical dimensions (25◦ 16′ 14.1" N, 82◦ 59′ 20.9″ E) was found to have alluvial and sandy loam type of soil, with 45%, 28% and 27% of sand, silt and clay respectively. The color of soil varied from pale brown to dark brown with pH being slightly alkaline ranged from 7.2-7.4.

Experimental framework, plant material and sampling

The experimental plot was designed such that the larger plot contained two different cultivars. The larger plot was divided into two halves and each half-contained twelve subplots (triplicate of four different treatments viz. C, N_1, N_2, N_3) of 1.5x1.5 dimension each. The framework followed the randomized split plot arrangement where different levels of nutrient treatment were in subplots and the cultivars were taken as the whole plot. The field preparation was done employing standard agronomic practices and drip irrigation technique was used to water each plot. Controlled ploughing of the field was done with fine tilth and given a basal treatment of recommended doses of inorganic fertilizers, i.e. nitrogen in form of urea (25 kg), phosphorous (50 kg) in form of super-phosphate and potassium (25 kg) in form of muriate of potash, per hectare of land. The moisture in the soil was checked and optimized and then the seeds were sown manually in each sub-plots during mid-June. After germination, the plants were thinned and the distance between the plants in each row was maintained at 15cm. Three different doses of nutrients (NPK) were applied viz. recommended (N_1), 1.5x recommended (N_2) and 2x recommended (N_3) where N, P, K for N_1, N_2, and N_3, were 80, 40, 40; 120, 60, 60 and for 160, 120, 120 kg ha^{-1} respectively. Control plots were also maintained in triplicate for each cultivar, where no supplemental nutrients were added. Nutrient treatment was given at two stages, vegetative (40 DAG) and reproductive (60 DAG) stages.

Two maize cultivars, Malviya Hybrid-2 (MH-2) and HHM-1 were selected as experimental plant. HHM-1 was developed in 2000 by CCS Haryana Agricultural University, Karnal by the cross of HKI 536 with HKI 295. Maturity time for this cultivar is about 100-105 days with an average yield of 60 q ha^{-1}. MH-2 was developed in 2007 by the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi by the cross of HUZ M185 with HKI 1105 strain. This cultivar matures in about 90-95 days and has an average yield of 54q ha^{-1}.

Plant samplings were done at 55 DAG (vegetative stage) and at 75 DAG (reproductive stage). Five plants were selected randomly from each replicate, i.e. fifteen plants were selected for each cultivar. Fully developed leaves (third from the top) were taken for evaluation of enzymatic antioxidants, non-enzymatic antioxidants and physiological characteristics, in both the cultivars.

Ambient Ozone monitoring

Eight hourly (8h) ambient O_3 monitoring was done every day between 8.00-16.00 hrs with the help of O_3analyzer, Model- APOA 370, Horiba, Japan. Monitoring was done above the canopy height from the day of seed sowing and was continued till the harvesting of the crop. Fortnightly calibration of the instrument was done to ensure its data validity. AOT 40 (8h) was calculated once a month employing a formula given by Mauzerall and Wang (2001).

$$AOT40 = \sum_{i=1}^{n} [CO_3 - 40]$$

where, 'i' is the index, 'n' designates the hours (in number), 'Co_3' indicates the mean O_3 values per hour ppb.

Biochemical Assays:

Fourth fully matured, healthy leaves were tagged and plucked manually from replicated sub-plots at 55 and 75 DAG from each treatment for estimation of non-enzymatic and enzymatic parameters:

(i) Pigment contents

Pigments such as chlorophyll and carotenoids were determined employing the formula given by Maclachlan and Zalik (1963) and Duxbury and Yentsch (1956), respectively. For determination of total chlorophyll, 0.5 g of fresh leaves were homogenized in 20 ml of 80% acetone and centrifuged at 4032 rpm for 15 minutes. The supernatant was gently transferred in a test-tube and their concentrations were estimated. The optical densities were taken at 663 and 645 nm wavelength for calculation chlorophyll a and b contents and at 480 and 510 nm for calculation of carotenoid contents, using double beam spectrophotometer (Model 2203, Systronics, India).

(ii) Enzymatic antioxidants

Enzymatic activities such as glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) enzymes were evaluated as described by Anderson (1996), Nakano and Asada (1987), Fridovich (1974) and Aebi (1984), respectively. To

determine the activity of enzymes, 0.2 g of freshly plucked leaf tissues were taken and were homogenized in liquid nitrogen. The homogenized leaves were subjected to 5 ml of extraction buffer made from 1M phosphate buffer of pH 7.0, having Poly vinyl pyrrolidone (PVP), Phenyl Methane Sulfonyl Fluoride (PMSF), Ethylene Diamine Tetra Acetic-acid (EDTA) and Triton-X-100 at 4°C and were centrifuged at 12,000 rpm. The supernatants were separated from the sediments and were used to measure the activities of enzymes.

(iii) Non-enzymatic antioxidants

For ascorbic acid contents (AsA), total phenolic content and lipid peroxidation (LPO) were estimated using the method of Keller and Schwager (1977), (Bray and Thorpe, 1954) and Heath and Packer (1968) respectively. For AsA, the leaf samples were homogenized in a solution containing NaEDTA and oxalic acid. Then DCPIP was used as a dye to develop color whose absorbance was measured at 520 nm. For total phenolic content, the leaf samples were homogenized in acetone and then centrifuged at 6000 rpm for 15 mins. Supernatant was allowed to react with Folin-ciocalteu reagent and Na_2CO_3 and optical density was measured at 650 nm. Lipid peroxidation (LPO) was determined in terms of malondialdehyde (MDA) content by using thio barbituric acid (TBA) and finally optical density was measured at 532 and 600 nm.

(iv) Hydrogen peroxide and superoxide radical contents

H_2O_2 and O_2^{o-} contents were measured using the method of Alexieva *et al*., (2001) and Elstner and Heupel (1976) respectively. For estimation of H_2O_2, leaf samples were homogenized in 5ml of 0.1% trichloroacetic acid (TCA). The homogenized sample is then centrifuged at 12,000 rpm for 15 minutes and taken the supernatant carefully. It is then mixed with 10mM of potassium phosphate buffer and 1mM of Potassium iodide solution, which were sequentially added and the solution was incubated in dark for 1hr. The solution was observed until it turns to pale-yellow, then absorbance was taken at 390 nm using double-beam spectrophotometer (Model2203, Systronics, India). For O_2^{o-} contents, the fresh leaves were homogenized in 65mM phosphate buffer (pH 7.8) and centrifuged at 4°C for 15 minutes at 2800g. Decant the supernatant and mixed with the assay mixture consisting of 10 mM, 7.8 pH of hydroxyl amine hydrochloride and 65mM phosphate buffer and was kept at 25°C for 30 minutes. Then added 17mM Sulfanilamide and 7mM α-naphthylamine sequentially and was kept at 25°C for 20 minutes. Following the incubation, the absorbance was taken at 530 nm using double-beam spectrophotometer (Model2203, Systronics, India) against a reagent blank. The optical densities were matched against the standard curve of nitrite salt and calculated O_2^{o-} generated in the sample.

Physiological parameters

The physiological parameters such as rate of photosynthesis (Ps) and stomatal conductance (gs) were estimated using portable photosynthesis system (Model LI-6200, LI-COR, USA). The measurement was done on third fully expanded mature leaf from the top of three randomly selected plants per plot. On field physiological experiments were performed between 8:00 am and 11:00 am on cloud free days at 55 and 75 DAG. During the measurement, the instrument was calibrated with the help of a known source of CO_2 of 510 ppm and the PAR photosynthetically active radiation was set at 1200 mmol $m^{-2}s^{-1}$. The measurements were done using Infra-Red Analyzer of LICOR photosynthetic system.

Statistical analysis

For analyzing principal and the interactive effects of treatment, age and mean O_3 concentration, a three-way multivariate analysis of variance, (ANOVA) was successfully executed. The Levene's F test was done to check the assumption of homoscedasticity followed by each ANOVA. All the above statistical analysis was performed using IBM SPSS/PC$^+$ statistics.

Results

Ozone monitoring

The mean ambient O_3 concentration during the entire experimental period was found to be 50.5 ppb, however the minimum and maximum ambient O_3 concentration varied between 40.17 and 55.4 respectively. The AOT 40 value thus calculated was found to vary from 5254 (minimum) to 7246 ppb (maximum) which was recorded in the month of July and September respectively (Figure 1).

Photosynthetic pigments

Total chlorophyll content in leaves of HHM-1 and MH-2 were found to have increased at all level of treatments in reproductive phase. In HHM-1, the total chlorophyll contents were found to increase by 23.46, 31.99 and 32.55% in N_1, N_2 and N_3 treatments respectively in reproductive stage while in vegetative stage, the increments were18.94, 26.48 and 28.28% in N_1, N_2 and N_3 treatments respectively (Table 1). In MH-2, similar trend of increments was observed in reproductive stage but it did not follow in vegetative stage (Table 2). In case of total carotenoid content, all the treatment levels were not found to increase significantly however N_2 and N_3 treatments showed similar fashion of increase.

Rate of photosynthesis and stomatal conductance

Significant increase in the Ps and gs were observed in both the cultivars. Ps raised by 19.7, 21.99 and 22.16% in N_1, N_2 and N_3 treatments, respectively, in HHM-1 cultivar and 18.6, 24.9 and 25.02% increments were seen in vegetative stage of MH-2(Figure 2). Homogenous trend was followed in HHM-1 and MH-2 cultivar at reproductive stage as well. In case of gs, 2.4, 3.2, 3.6% and 9.6, 11.71,12.01% increase was found in N_1, N_2 and N_3 treatments of HHM-1 and MH-2 cultivar respectively, at vegetative stage, however, higher but similar trend was followed at the reproductive stage also.

Metabolites and antioxidants

Significant decrease in the AsA contents was observed in both the stages of MH-2 and HHM-1 cultivars, upon nutrient treatment (Figure 3). In MH-2, significant decline in AsA contents were seen in all the treatments however greater depreciation were observed in N_2 treatment (30.13%) of vegetative stages. In case of HHM-1, significant reductions

in AsA were observed at both the stages in N_2 and N_3 treatments but a noticeable decrease was observed in N_2 (39.9%) and N_3 (49.2%) treatments of reproductive stage. In total phenolics, significant decline was observed in N_2 (35.76%) and N_3 (36.23%) treatments of vegetative phase while 16.81, 27.51 and 29.31% decline were observed in N_1,N_2 and N_3 respectively, at reproductive phase (Figure 4).

Enzymatic antioxidants such as SOD, GR, APX and CAT showed significant increments in their activities in all the stages of both the cultivars at N_1, N_2 and N_3 treatments. In case of HHM-1, SOD activity showed higher increments at reproductive stage of N_1 (22.03%), N_2 (53.81%) and N_3 (53.81%) treatments while in vegetative stage, significant increase were observed in N_2 (54.21%) and N_3 (55.42%) treatments only (Figure 6). In MH-2, the reproductive stage showed significant increments in N_2 (6.47%) and N_3 (7.11%), however, vegetative stage did not show significant increments with higher doses (Figure 6). Similar trend of increments was observed in CAT as well as APX activities, wherein HHM-1 showed greater efficiency as compared to MH-2. In case of CAT, the reproductive stage of HHM-1 showed 59.44, 68.21 and 68.94% increments in N_1, N_2, and N_3 respectively, however, 5.46, 14.61 and 15.01% increments were observed in MH-2 cultivar (Figure 6). Similar trend was followed in vegetative phase also. In APX, the reproductive stage of HHM-1 showed 25.8, 48.38 and 49.22% increment in N_1, N_2, and N_3 respectively (Figure 5).However, 12.52, 24.82 and 25.07% increase in were observed in MH-2 cultivar and similar trend of increments were observed in vegetative phase also (Figure 5).In case of GR, vegetative as well as reproductive stage revealed significant increments in all the treatments.TheN_1 (21.05%), N_2 (47.36%), and N_3 (47.36%)treatments in reproductive stage of HHM-1 were higher as compared to MH-2 and same trend was followed in vegetative stage (Figure 5).

Reactive oxygen species and lipid peroxidation

H_2O_2 contents followed a significant depreciation in HHM-1 as well as in MH-2 cultivars, but higher reductions were observed at vegetative stage of HHM-1, at N_1(10.37%), N_2(29.62%) and N_3(29.62%) treatments as compared to N_1(6.78%), N_2(28.50%)and N_3(32.01%) in MH-2 cultivar (Figure 7).The reproductive stage also followed similar trend in both the cultivars, but the reductions were 7.68, 21.47, 21.90% and 6.43, 10.48, 11.21% in N_1, N_2 and N_3 treatments respectively. The O_2^{0-} contents of HHM-1 and MH-2 in vegetative stage followed N_1(16.43%), N_2(23.26%), N_3(25.74%) and N_1 (12.42%), N_2 (18.32%), N_3 (18.92%) reductions respectively, and similar regime was recorded in reproductive stage also (Figure 7). In case of lipid peroxidation (LPO), greater reductions were seen in HHM-1 cultivar as compared to MH-2. In HHM-1, 20.93, 33.40 and 33.39% and 29.50, 39.82 and 40.26% reductions were seen in N_1, N_2 and N_3 treatments of vegetative and reproductive stage respectively and the results of MH-2 was homogenous with vegetative as well as reproductive stages(Figure 8).

Discussion

The recorded high concentration of O_3 during the experimental period is concurrent with monitoring data of the previous studies done at the same site (Sengupta and Tiwari, 2020; Ghosh *et al*., 2020; Yadav *et al*., 2020,2021; Fatima *et al*., 2019). O_3 concentration during the study period was high enough to cause significant yield reduction in crop plants, and as such a planned approach to minimize O_3 stress was of paramount importance. Soil nutrient amendment have shown some encouraging results as far as the response of biochemical and biophysical qualities of plants are concerned (Sengupta and Tiwari, 2020; Podda *et al*., 2019). In this study the abatement of O_3 induced oxidative stress, upon nutrient amendments is well evident through significant reductions in contents of superoxide radicals and H_2O_2 in nutrient treated plants as compared to control. The mitigative effect of nutrient amendments was further demonstrated by reduction in membrane lipid peroxidation in nutrient treated plants as compared to control, in both the cultivars. Increased production of ROS and high degree of membrane lipid peroxidation are important indicators of O_3 induced oxidative stress (Podda *et al*., 2019; Gill and Tuneja, 2010). Several studies have documented enhancement in ROS production in plants upon O_3 exposure (Yadav *et al*., 2019; Wang *et al*., 2014; Tiwari and Agrawal 2011). The positive effects of nutrient amendments on the cultivar and biochemical feedback were also reported by Podda *et al*. (2019), on O_3 sensitive oxford poplar clone and Pandey *et al*. (2018) on two cultivars of wheat. It was observed that the two maize cultivars responded differentially to the nutrient amendments. Reduction in ROS contents and lowering of membrane lipid peroxidation was higher in O_3 exposed cultivar HHM-1 as compared to MH-2 under similar nutrient treatment regimes. This indicates that HHM-1 is more responsive to nutrient treatment as compared to MH-2.

The enhanced ROS scavenging potential of a plant upon nutrient treatment is directly correlated to the modification of the cellular antioxidant pool. In our study, the activity and content of enzymatic and non-enzymatic antioxidants increased upon nutrient treatment with most significant increase at N_2. It has been confirmed that nutrient application brings about modifications in the cellular redox state confirming their role in scavenging ROS (Podda *et al*., 2019). The results of the present study clearly indicate that HHM-1 showed higher increments in enzyme activity upon nutrient treatment as compared to MH-2. These results can be compared with those reported by Sengupta and Tiwari (2020) they have observed that the antioxidant pool of the O_3 tolerant cultivar of *C. tetragonoloba*, PUSA-N, responded more positively to nitrogen amendments as compared to O_3 sensitive cultivar S-151. In the present study, it was observed that response of antioxidants upon nutrient treatment was more prominent at the vegetative stage in both the cultivars. However, in HHM-1, the antioxidative response was further maintained in the reproductive stage as well, under similar nutrient treatments. The differential response of antioxidant pool upon nutrient treatment perhaps defines the sensitive or resistant nature of the two cultivars towards oxidative stress. SOD and ascorbic acid are important antioxidants which are directly involved in ROS detoxification and are considered as plant's first line of defense (Manry *et al*., 2020). A number of studies have linked the increased SOD activity with reduced O_3 stress in plants (Fatima *et al*., 2018; Singh *et al*., 2018; Rai *et al*., 2015; Kashyap *et al*., 2020). In present study, an increment of SOD activity was recorded, coupled with a

reduction in AsA content, which indicates an efficient scavenging of ROS. HHM-1 showed significant increments in SOD as well as AsA, which was higher than MH-2 during both the sampling stages, at N_2 treatment as compared to control. However, in MH-2, increments in SOD activity were insignificant at both the stages, and reduction in ascorbic acid content was of higher magnitude during the vegetative stage (30.13% at vegetative stage and 17.82 at reproductive stage) upon N_2 nutrient treatment. These results clearly suggest that the stimulation of the antioxidant pool upon nutrient amendments was confined till the vegetative stage only in case of MH-2, whereas for HHM-1, the enhanced response of antioxidant pool was evident at both vegetative and reproductive stages. This observation clearly indicates that nutrient amendments were more efficient in case of HHM-2 as compared to MH-2 cultivar. The response of SOD and AsA pool well coincided with the variations in superoxide radical contents, which showed higher magnitude of reduction in HHM-1 as compared to MH-2 upon nutrient treatment. Further reductions in SOR contents were of higher magnitude at vegetative stage than at reproductive stage under similar nutrient treatments in both the cultivars. It is to be noted that the antioxidants and SOR showed maximum significant variations at N_2 treatment as compared to control. Presence of high H_2O_2 contents is an important indicator which indicates the stressed condition of plants (Anjum *et al*., 2016; Del Rw, 2015). Yadav *et al*., 2019 have reported enhanced H_2O_2 accumulation in wheat cultivars exposed to elevated O_3. In the present study, reduced H_2O_2 contents in both the cultivars, O_3 exposed maize plants upon nutrient treatments provide a concrete evidence of reductions of O_3 induced oxidative stress. Reduction in H_2O_2 contents can be attributed to increased levels of CAT and APX activities. CAT and APX are the primary enzymes for metabolizing stress induced H_2O_2 in plants (Anjum *et al*., 2016). In the present study, enhancement in CAT and APX activity was more pronounced in O_3 tolerant HHM-1 cultivar which corresponds to more reductions in H_2O_2 contents, more prominently at vegetative stage. In MH-2 also, the ROS scavenging activity of enzymes in nutrient treated plants was more distinguishable during the vegetative stage. Their observations clearly validate our first hypothesis.

Increased chlorophyll content is a well observable fact reported in some earlier studies and can be attributed to the increased availability of nitrogen (Holder *et al*., 2020). In the present study, higher increments in total chlorophyll contents were recorded in HHM-1 in plants at N_2 treatment at both vegetative and reproductive phases. In MH-2, higher increments in chlorophyll contents were recorded at the vegetative phase upon nutrient treatments, which did not show any additional increments at reproductive phase. Enhanced senescence is a well- known feature of plants experiencing O_3 stress. On the basis of this observation, it was hypothesized by Holder *et al*. (2020) that increased chlorophyll contents can be attributed to the increased resource allocation to the newest leaves as the older ones withered away due to premature senescence. The Ps and gs, both increased upon nutrient amendments in both the cultivars. However, it was observed that the magnitude of variations in both the parameters was asynchronized. Observations recorded at the end of the growing season showed increments of 30.53 and 14.52% in Ps and gs, respectively, in MH-2 at N_2 treatments, whereas, no significant increment was observed in gs, at N_2 treatment. These observations suggest a clear uncoupling of Ps and gs in both the maize cultivars treated with nutrient doses. Studies have shown that O_3 exposure causes a decoupling of photosynthesis and conductance (Lombardozzi *et al*., 2012; Singh *et al*., 2009). The present study indicates that although nutrient amendments were able to partially alleviate the well-marked O_3 induced stomatal sluggishness (Paoletti and Grulke, 2010; Hoshika *et al*., 2014), yet the decoupling phenomenon could not compensation. The result of the present study suggests that increment in the photosynthesis yield was attributed to non –stomatal factors, increased chlorophyll contents and higher carboxylation efficiency being a few of them.

Conclusion

The result of the present study provides evidence for the positive response of nutrient amendments in managing O_3 stress, in the two cultivars of maize. Increased antioxidant activities in both the maize cultivars upon nutrient treatment reduced the O_3 generated superoxide radical and H_2O_2 contents indicate a reduction in O_3 stress. It was observed that the antioxidant response upon treatment was more prominent in HHM-1, as compared to MH-2, in which major proportions of antioxidant stimulations were confined to the vegetative phase only. However, in HHM-1, significant stimulation of antioxidant pool was also recorded during the reproductive stage, which explains the more positive O_3 ameliorative effect of nutrient amendments, as compared to MH-2. SOD and CAT played an important role in defining the plant's defense and regulating the SOR and H_2O_2 contents in both the maize cultivars at both vegetative and reproductive stages.Positive response of the biophysical factors also suggests the efficient O_3 amelioration of nutrient amendments. However, the nutrient amendments were not able to alleviate the O_3 induced uncoupling of rate of photosynthesis and stomatal conductance and the increased rate of photosynthesis is attributed to the non-stomatal factors like increase in the concentration of photosynthetic pigments and hence the carboxylation efficiency.

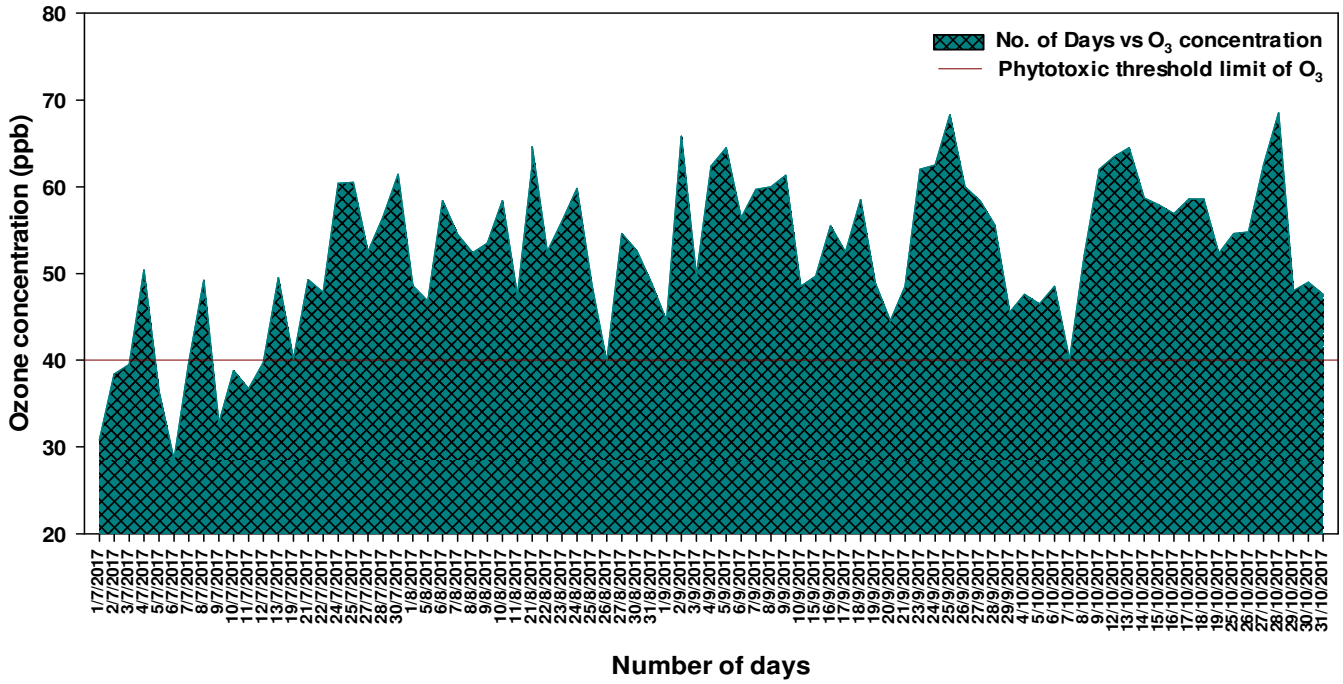

Fig. 1 : Eight hourly (0800 hours- 1600 hours) daily average O_3 concentration during the period July, 2017 to October, 2017.

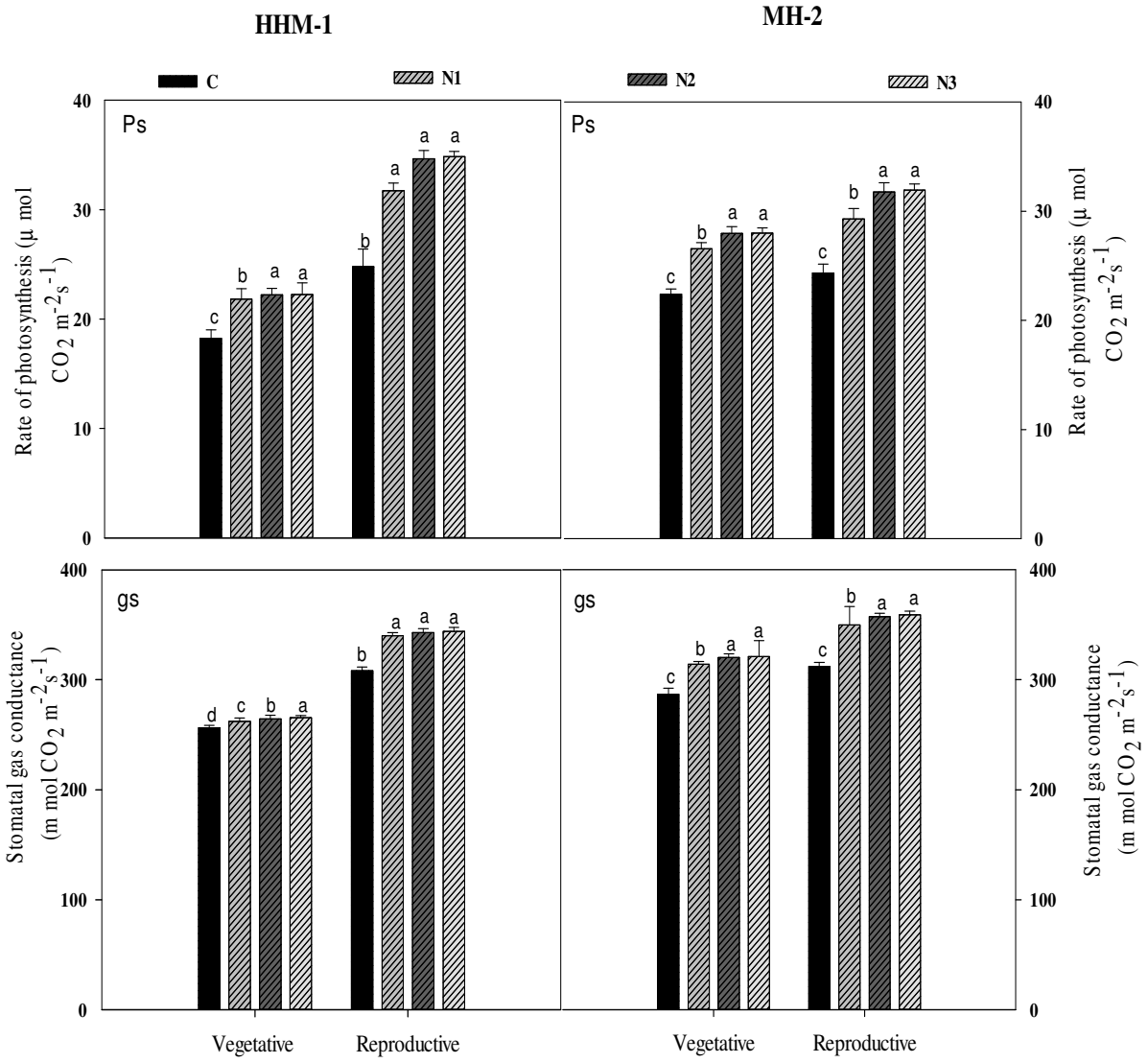

Fig. 2: Response of rate of photosynthesis (Ps) and stomatal conductance (gs) of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

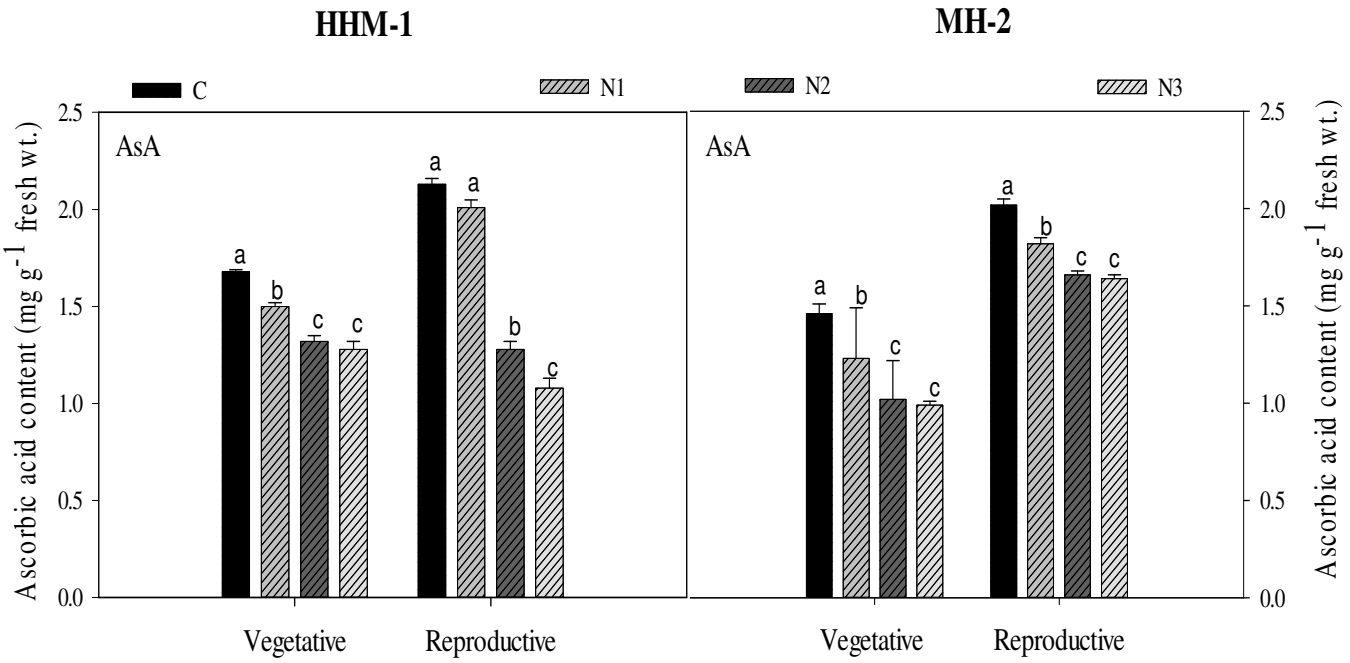

Fig. 3: Responses of ascorbic acid (AsA) contents of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

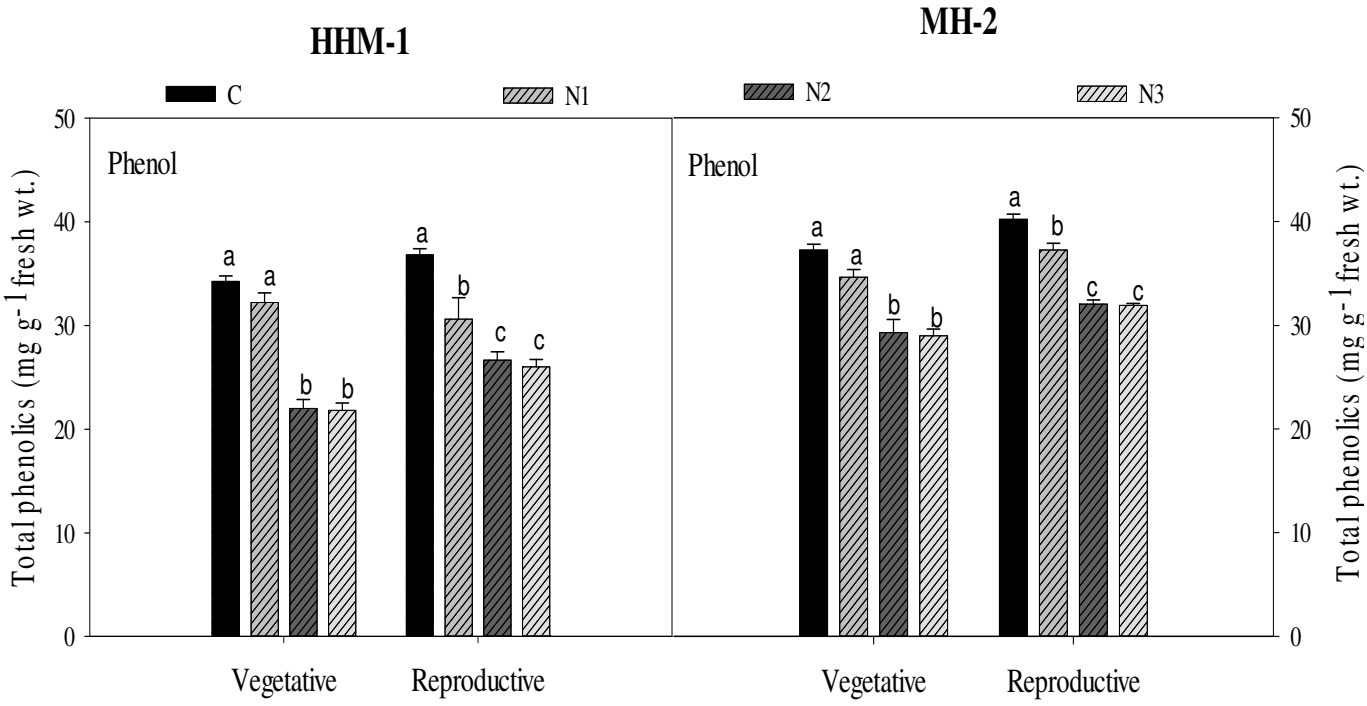

Fig. 4: Responses of phenol contents of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

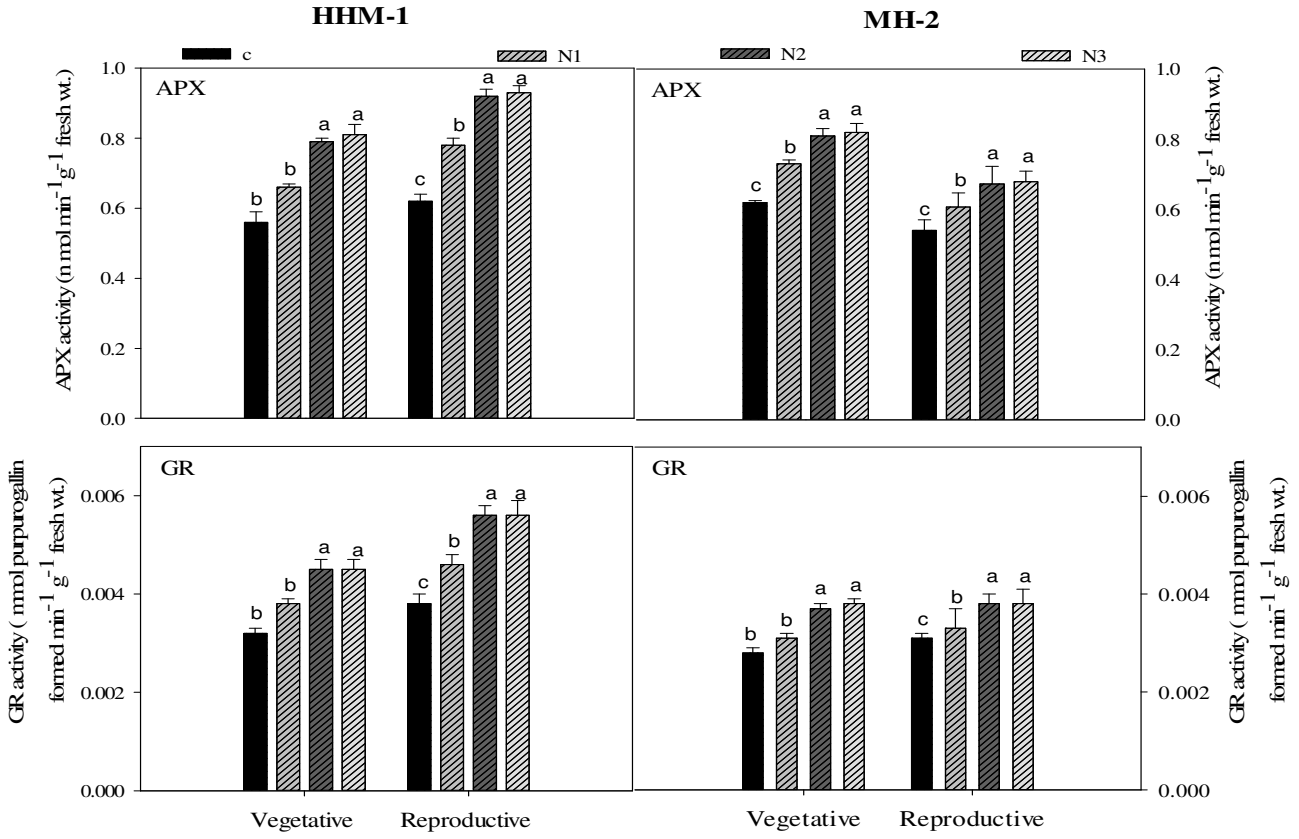

Fig. 5: Responses of ascorbate peroxidase (APX) and glutathione reductase (GR) activities of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

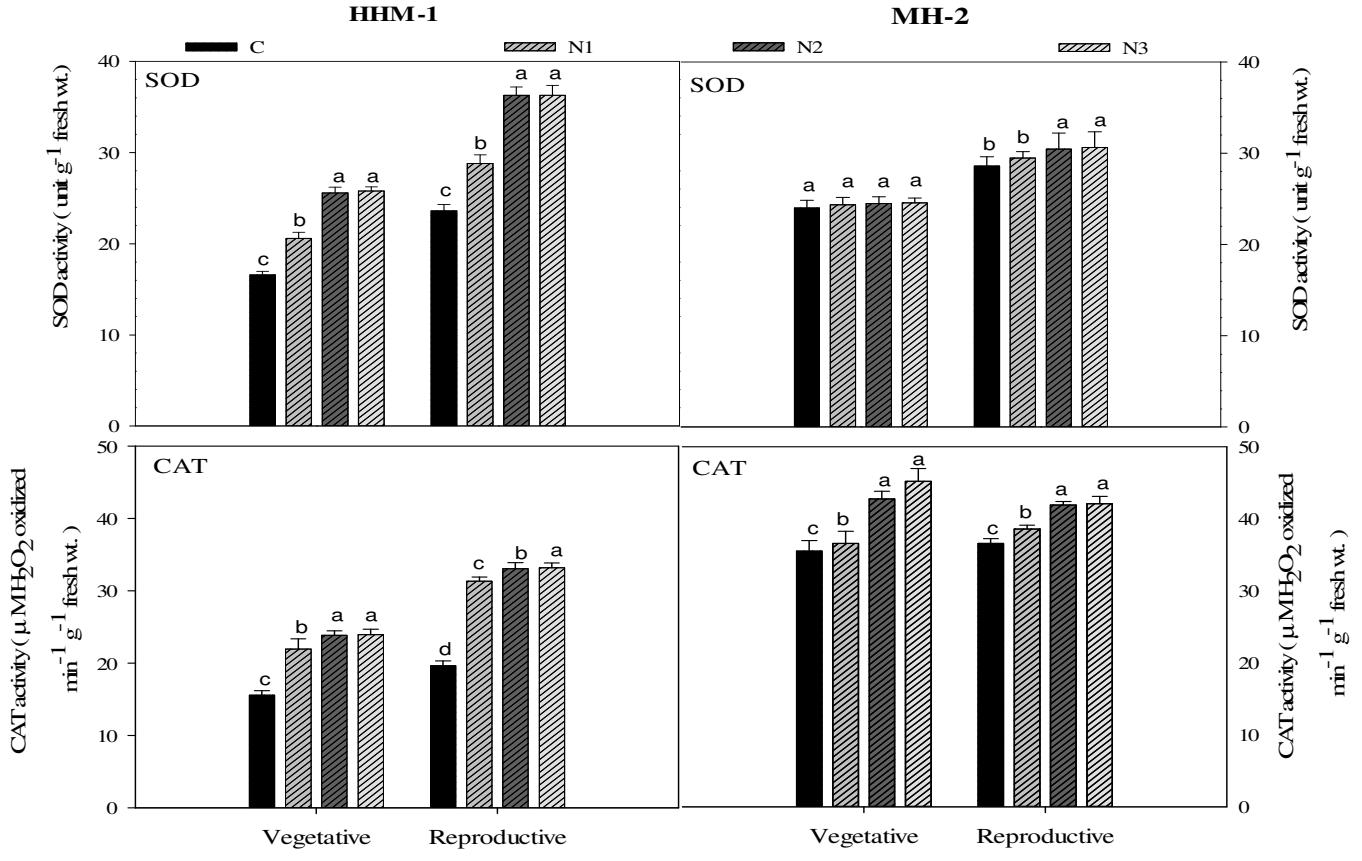

Fig. 6: Responses of superoxide dismutase (SOD) and catalase activities (CAT) of two maize cultivars exposed to O_3 stress at

vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

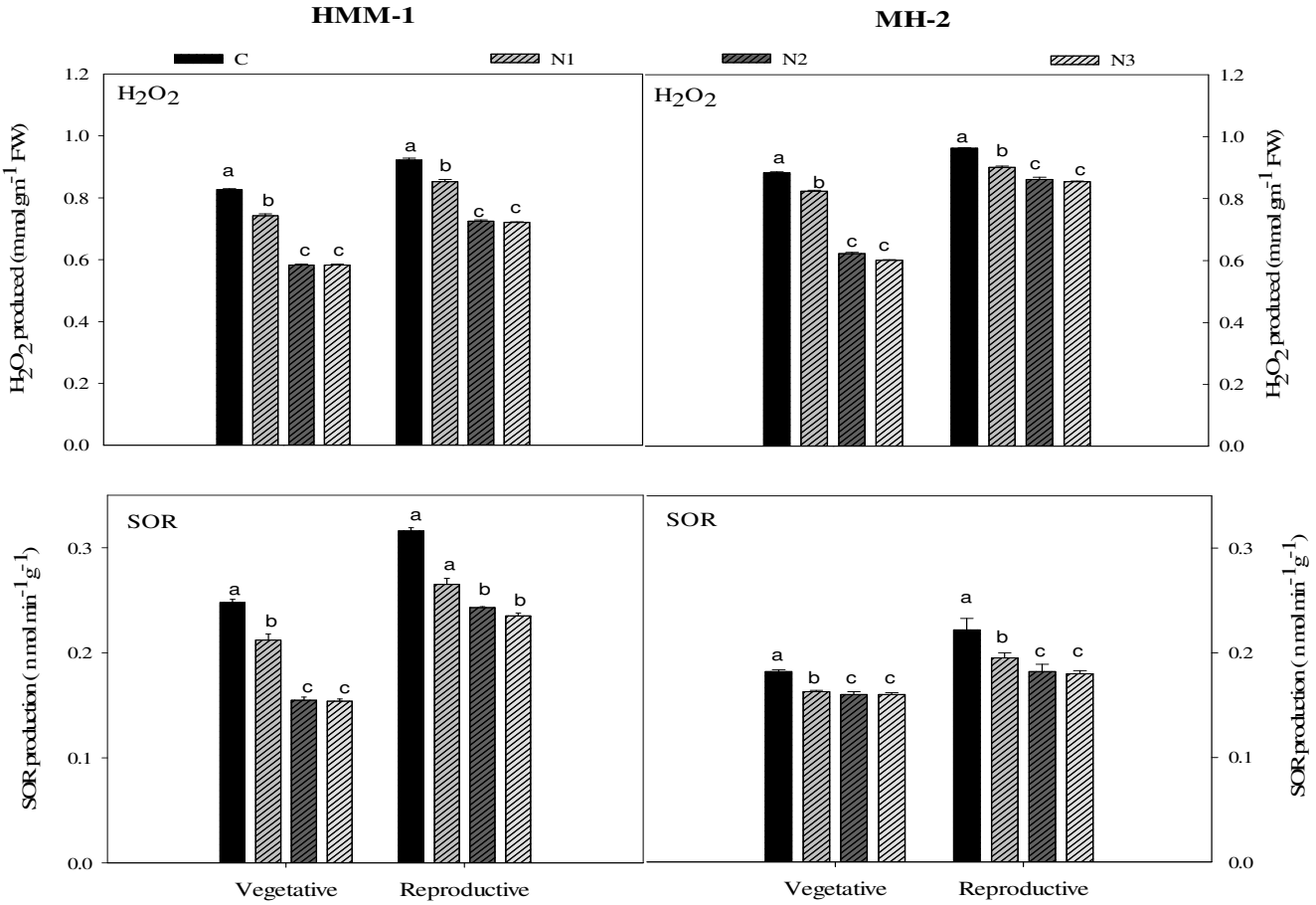

Fig. 7: Responses of superoxide radicals (O_2^{o-}) and hydrogen peroxide (H_2O_2) contents of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

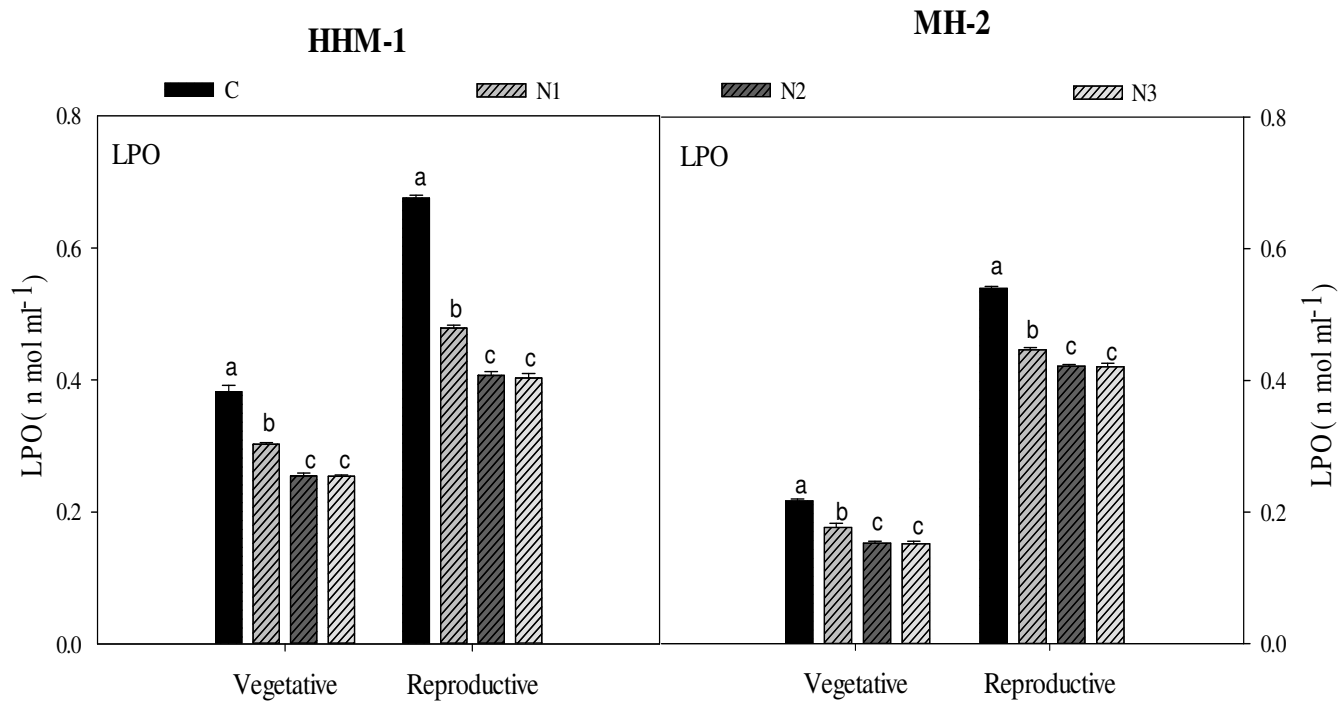

Fig. 8: Variations in MDA contents of two maize cultivars exposed to O_3 stress at vegetative and reproductive phases upon

different doses of NPK treatment (mean ± ISE). Bars with different letters in the same group show significant variation at $p<0.05$.

Table 1: Effect of NPK treatment (C, control; N_1, recommended NPK dose; N_2, 1.5-times recommended NPK dose and N_3, 2-times recommended NPK dose) on pigments of *Zea mays* L. cv. HHM-1 at 55 days after germination (DAG; vegetative stage) and 75 DAG (reproductive stage). Values are mean ± s.e

Nutrient Treatment	Chl a (mg g^1 FW)	Chl b (mg g^{-1} FW)	T Chl (mg g^{-1} FW)	Caro (mg g^{-1} FW)
		Vegetative stage		
C	28.30^{c}±0.58	17.34^{b}±1.27	45.65^{c} ±1.83	9.75^{b} ±0.62
N1	34.25^{b}±1.24	20.05^{b} ±1.18	54.30^{b} ±2.35	11.15^{b}±0.47
N2	37.40^{a}±1.24	20.34^{a} ±1.23	57.74^{a} ±2.48	11.65^{a}±0.92
N3	37.61^{a}±1.31	20.61^{a} ±1.57	58.61^{a} ±2.89	11.70^{a}±0.61
		Reproductive stage		
C	32.45^{c} ±1.10	20.53^{c} ±0.93	52.98^{c} ±2.03	11.29^{b}±0.95
N1	41.28^{b} ±0.77	24.13^{b} ±0.92	65.41^{b} ± 1.67	13.13^{b}±1.18
N2	45.23^{a} ±1.22	24.70^{a} ±0.77	69.93^{a} ±1.96	13.82^{a}±0.71
N3	45.39^{a} ±1.21	24.84^{a} ±1.45	70.23^{a} ±2.64	13.88^{a}±0.85

Table 2: Effect of NPK treatment (C, control; N_1, recommended NPK dose; N_2, 1.5-times recommended NPK dose and N_3, 2-times recommended NPK dose) on pigments of *Zea mays* L. cv. MH-2 at 55 days after germination (DAG; vegetative stage) and 75 DAG (reproductive stage). Values are mean ± s.e

Nutrient treatment	Chl a (mg g^1 FW)	Chl b (mg g^{-1} FW)	T Chl (mg g^{-1} FW)	Caro (mg g^{-1} FW)
		Vegetative Stage		
C	32.10^{b} ±0.74	18.77^{c} ±0.59	50.87^{b} ±1.34	10.29^{b} ±0.94
N1	36.99^{b} ±1.21	20.75^{bc} ±1.44	57.74^{b} ±2.37	11.52^{b} ±0.73
N2	41.40^{a} ±1.19	21.30^{ab} ±2.04	62.70^{a} ±3.21	12.10^{a} *±0.85*
N3	41.90^{a} ±1.77	21.50^{a} ±0.97	63.40^{a} *±2.71*	12.30^{a} *±1.39*
		Reproductive stage		
C	33.01^{c} *±1.03*	19.95^{c} ±0.61	52.97^{c} ±1.65	11.29^{b}±0.70
N1	39.43^{b} *±0.88*	22.34^{b} ±1.31	61.77^{b}±2.18	12.76^{b}±0.70
N2	41.46^{a} *±2.03*	23.86^{a} ±0.96	65.32^{a} ±2.98	13.70^{a}±0.47
N3	41.59^{a} *±2.02*	23.95^{a} ±1.02	65.54^{a} ±3.04	13.77^{a}±0.71

Conflict of interest: Authors declare no conflict of interest.

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