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Role of soil nitrogen amendments in management of ozone stress in plants: a study of the mechanistic approach

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Abstract

In view of the increasing concentration of tropospheric ozone (O_3), it becomes important to develop mitigative measures which help in sustaining plant yield. On the basis of a few experiments, soil nitrogen amendments have given some promising results and can be used in partial amelioration of O_3 stress in plants. The aim of the present experiment was to evaluate the interactive effects of ambient O_3 and nitrogen treatments on selected physiological, biochemical and morphological characteristics of palak (*Beta vulgaris* L. var Allgreen) plants. The experimental design consisted of three nitrogen treatments i.e, control (no supplement nitrogen), recommended dose (N_1) and 1.5 x recommended dose (N_2). The results of the experiment showed that the mechanisms through which nitrogen amendments assisted the plants to cope with O_3 stress were dominated by the physiological features like stomatal conductance at low nitrogen dose treatment, while at higher nitrogen dose treatment, biochemical characteristics are more prominent. Alteration in the enzymatic and non-enzymatic antioxidants in nitrogen treated plants as compared to control, clearly suggest a reduction in O_3 stress upon nitrogen amendments. Biomass allocation pattern is also modified by nitrogen treatments to sustain higher yield. The positive effect of biomass allocation was more prominent in N_2 plants where yield increments were higher. The yield increments, as compared to control were higher in N_2 as compared to N_1, indicating that higher nitrogen dose was more effective in partial amelioration of O_3 stress. However, more experiments are required to be planned to determine the exact dose of nitrogen amendments needed for different plants.

Keywords: *Ozone stress, nitrogen amendments, stomatal conductance, antioxidants, biomass allocation*.

Introduction

Tropospheric ozone (O_3) is now established as the most phytotoxic air pollutant responsible for damaging vegetation, crop productivity and human health due to its oxidative nature and high concentration over agricultural areas (Emberson et al., 2018). High ambient O_3 levels have detrimental effects upon crop plants by inducing reduction in photosynthesis, variation in carbon allocation, biomass loss and yield reduction (Mills et al., 2018; Ainsworth et al., 2017; Gao et al., 2017). Tropospheric ozone (O_3) is no longer considered as a regional problem, but has gained a valid global significance. Monitoring studies indicate that the surface-level O_3 concentration has increased more in the northern hemisphere, especially across the tropics than in the southern hemisphere (Ziemke et al., 2011). The spike in emissions of O_3 precursors in South-East Asia is not only accountable for rise in surface O_3 levels in Asia, but also over North America (Parrish et al., 2012; Lin et al., 2012) due to prominent inter-continental long range transport of O_3 from East Asia to North America through the prevailing winds (Doherty., 2015). Significant yield curtailments is a well-recognized outcome of increasing global O_3 concentration (Yi et al., 2016; Cotrozzi et al., 2016; Ainsworth et al., 2012; Avnery et al., 2011a). Lal et al., (2017) estimated the losses of wheat and rice crop yields using surface O_3 data from 17 sites across India and estimated an annual loss of 4.2-15% for wheat and 0.3-6.3% for rice.

India being a developing nation, has been an important source of O_3 precursors due to accelerated industrialization and urbanization processes and weak legislations (Tiwari and Agrawal, 2018, Agathokleous et al., 2016; Pandey et al., 2018). India also provides suitable conditions for O_3 formation due to its prevailing tropical conditions (Tiwari et al., 2008). The 8-hourly mean average of tropospheric O_3 concentration in Varanasi varied between 24 and 62.35 ppb monitored from 2002 to 2006 (Tiwari et al., 2008). Detailed and continuous monitoring studies done at a suburban site in Varanasi from 2002 to 2012 not only showed gradual hike in ground level O_3 concentration, but also temporal and spatial variations of the same (Tiwari and Agrawal, 2018). The wintertime monthly average of O_3 concentration at the same experimental site varied between 32.33 to 53.2 ppb (Mishra et al., 2013), whereas in summer the concentration gradually increased from 55.21 to 62.2 ppb (Singh et al., 2014; Sarkar et al., 2015). The surface level O_3 concentration monitored by Dey et al., (2014) from February 2013 to May 2013 was found to be as high as 66.8 ppb in Durgapur, West Bengal.

Nutrient amendments have been frequently used to sustain a consistent growth in plants and to help repair the damage incurred by the various stresses. Nitrogen (N) is fourth most abundant element in living beings, but apparently the most important constituent supporting plant growth. N application, on one hand, ensures the maintenance of higher protein levels which can be utilized for the repair processes, and, on the other hand allows the remobilization of nutrients to reproductive parts thus sustaining higher yield (Zeng et al., 2017; Diaz-Mendoza et al., 2016; Distelfeld et al., 2014). Yendrek et al. (2013) reported that N application may partially mitigate the negative effects of O_3 on a few morphological parameters of plants. Nitrogen is an essential part of RuBisCO, an enzyme that takes part in initial reactions of photosynthesis (LeBauer and Treseder., 2008), and as hike in photosynthesis is proportionate to increase in stomatal conductance (Zhang et al., 2018b). These studies, although prove the partial ameliorative nature of N amendments towards O_3 stress in plants, the mechanism affecting the plant's performance is still debated. Plants can either increase their antioxidative defense against O_3 induced ROS burst, can fix more C to be utilized for O_3 induced damage, or can divert more biomass towards the reproductive structures, thus sustaining higher yield upon N amendments (Podda et al., 2019). The aim of the present experiment is to evaluate the mechanism adopted by plants for mitigation of ambient O_3 stress upon N amendments analyzing the variations in biochemical, physiological and morphological parameters of *Beta vulgaris L. var. Allgreen* grown under ambient O_3 concentrations at a suburban site in Varanasi. The hypotheses we present in this study are (i) Plants treated with lower N doses focus on stomatal adjustments while higher nitrogen application strengthens the detoxification mechanism in plants, and (ii) Plants with higher N application are more responsive in altering biomass allocation, thus sustaining higher yield, even under O_3 stress.

Materials and Methods

Experimental site

The study was conducted at botanical garden, Banaras Hindu University, a suburban site of Varanasi located in Eastern Gangetic plains of Indian subcontinent at 25º14' N latitude, 82º03' E longitude and 76.19 m above sea level. The study was conducted under natural ambient conditions between the winter months of January 2017 and March 2017. The region experiences a humid subtropical climate with distinct summer, rainy and winter seasons. Variations in the climatological data are depicted in Table 1.

Plant Material

The test plant was Palak (*Beta vulgaris* L. var. Allgreen), a popular leafy vegetable preferred mainly for the iron content in the diet. This high yielding variety was developed by Indian Agricultural Research Institute (IARI), New Delhi and can be grown in all seasons. The yield is about 12.5 tonnes green per hectare in 6-7 cuttings made at 15-20 days interval. Due to its short self-life, the local market of palak largely depends upon its local production.

Experimental design

The pots were prepared using agronomic practices. Farmyard manure was added uniformly during soil preparation. The soil is sandy loam in texture (sand 45%, silt 28%, clay 27%) having organic carbon content 0.67%, pH 7.4, nitrogen content 0.12% and phosphorus content 0.065%. Seeds of Palak were hand sown in the third week of January. After germination, subsequent thinning was done manually such that the density of the plants was reduced to five plants per pot. Pots were watered every alternate day and identical water regime was maintained for each pot. Three types of nitrogen profiles (urea as nitrogen supplement) were given, keeping one as control (C) with no nitrogen supplement, the second as low nitrogen (N_1) in which recommended dose was provided (20 kg ha^{-1}), and the last as high nitrogen (N_2) in which 1.5 x recommended dose was given (50 kg ha^{-1}). The nitrogen amendments were given twice during the lifetime of the plants, once after 25 days after germination (DAG) and another after 40 DAG. Five replicates of each treatment, were maintained.

O_3 monitoring

Eight hourly monitoring (8:00 to 16:00 hours) of O_3 was done daily from seed germination to seed maturation. Ambient O_3 concentration was monitored daily for eight hours (8.00- 16.00 h), from germination to harvesting of the plants, with the help of Ambient O_3 Analyzer, Model-APOA 370, Horiba, Japan. The ambient O_3 concentration was measured by drawing air through a Teflon tube kept above the canopy height. Calibration of the monitoring instrument was conducted weekly by known concentration O_3.

Plant sampling and analysis

Two samplings were done at 30 and 45 DAG and the different parameters were analyzed.

Physiological characteristics

Photosynthetic rate (Ps), stomatal conductance (g_s), transpiration rate (t) and internal CO_2 (Ci) were analyzed using portable photosynthetic system (LI-COR Model LI-6400XT, Version 6.2.5). The system was calibrated using a CO_2 source (509 ppm concentration). Five plants per treatment were selected and the measurements were made on cloud free dates between 9:00 and 10:00 h. The sampling was done only at 45 DAG and fully expanded 4^{th} leaf from the top was selected for taking the measurements.

Photosynthetic pigments

Two fully expanded leaves were randomly picked from three plants per treatment at 30 and 45 DAG, and the contents of photosynthetic pigments, chlorophyll (Chl a, Chl b, TChl) and carotenoids were assessed. 100 mg of fresh leaf sample were taken and homogenized with 10ml of 80% acetone. It was centrifuged and the optical densities were taken at 480 and 510 nm for carotenoids and 645 and 663 nm for chlorophyll using a UV-Vis spectrophotometer (Systronics-2203). Chlorophyll and carotenoids were estimated using methodology given by Maclachlan and Zalik (1963) and Duxbury and Yentsch (1956) respectively.

Biochemical parameters

For the quantification of biochemical parameters, fully grown leaves were randomly picked from five plants per treatment at 30 and 45 DAG. For ascorbic acid analysis, leaf samples were homogenized in oxalic acid and Na-EDTA extraction solution. 2, 6- Dichlorophenol- indophenol (DCPIP) dye was used to develop color and the absorbance was taken at 520 nm. Ascorbic acid was quantified using the method of Keller and Schwager (1977). Total phenolic content was estimated by homogenizing the leaf sample in acetone and then using the Folin- Ciocalteau reagent and Na_2CO_3 through the method of Bray and

Thorpe (1954). For protein extraction, fresh leaves were homogenized in Tris buffer (0.1 M) followed by mixing of Trichloroacetic acid (10%) and then dissolved in 0.1 N NaOH. Protein content was estimated using the method of Lowry et al. (1951). Lipid peroxidation in the leaf tissue was determined in terms of malonedialdehyde (MDA) content by thiobarbituric acid as described by Heath and Packer (1960). For estimation of SOD, fresh leaf was homogenized in 10 ml cold phosphate buffer and activity was determined by inhibition of photochemical reduction of nitro blue tetrazolium at 560 nm (Fridovich, 1974).

Morphological and biomass allocation parameters

For the determination of morphological parameters and biomass, five plants per treatment were taken. Morphological parameters like root (RL) and shoot length (SL), number of leaves per plant (NL), leaf area (LA), and root (RB) and shoot (SB) dry weights were analyzed at 30 and 45 DAG. Leaf area was measured using a Leaf Area Meter (Model LI-3000, LICOR, Inc., USA). For biomass analysis, monoliths containing single plant with intact roots were carefully dug out thoroughly washed by placing a sieve of 1mm under running tap water. Their parts were separated and oven dried at 80^0C till a constant weight was obtained. Dry weight of the plants was taken and expressed in gm plant^{-1}. For better understanding of the biomass allocation patterns different growth indices like, root shoot ratio (RSR), specific leaf area (SLA), specific leaf weight (SLW), leaf area ratio (LAR), and leaf weight ratio (LWR) were calculated from dry weight data using the formulae modified by Hunt (1982). All the growth indices were calculated on the basis of dry weight data.

Yield

Yield of the test plants was determined dry weight of the edible part (shoot) (10 replicates per treatment) of the plant. The edible parts were harvested at 60 DAG and the fresh weight was taken.

Statistical analysis

All statistical analysis were done using SPSS/PC$^+$ program for microcomputer (version 16). Mean and standard error were calculated. Level of significance at different treatments was calculated by two-way analysis of variance (ANOVA). Duncan's multiple range test was performed as post hoc on parameters subjected to one way ANOVA test.

Results

Ambient Ozone Concentrations

During the experimental period, eight hourly mean of O_3 concentration varied from 45.16 ppb in January to 47.74 ppb in March, 2017. The mean ambient O_3 concentration during growth period was found to be 42.60 ppb (Fig 1).

Physiological Parameters

Photosynthetic rate increased significantly by 29.77% and 54.71% for N_1 and N_2 treatments respectively as compared to control, but it did not vary significantly among the treatments (Table 1). Stomatal conductance (g_s) decreased by 9.1% for N_1 treatment but increased by 29.16% for N_2 treatment as compared to control and both the variations were insignificant (Table 1). Internal CO_2 concentration (Ci) showed insignificant reductions for both treatments as compared to control. In case of transpiration rate, both the treatments showed increased value as compared to control but both were insignificant (Table 1).

Photosynthetic Pigments

Chl a, Chl b, TChl and carotenoids content were found to be higher in N_1 than N_2, as compared to control (Fig 2). The above trend followed at both 30 and 45 DAG. Two-way ANOVA tests showed that the Chl a content varied significantly due to age and treatment only, however, Chl b content varied significantly due to treatment only (Table 2). Total chlorophyll and carotenoids also varied significantly due to age and treatment only (Table 2).

Biochemical Parameters

Ascorbic acid content increased only for N_1 treatment at 30 DAG by 48.9% (Fig 3) and its content did not show significant variations due to individual factors like age and treatment but the variations were significant due to age x treatment interactions (Table 2). Ascorbic acid decreased significantly only for N_2 treatment at 45 DAG by 38.17% as compared to control (Fig 3). Similarly phenol level only increased for N_1 treatment at 30 DAG (Fig 3), but two-way ANOVA test showed that it varied significantly for all individual factors like age and treatment along with age x treatment interactions (Table 2). Phenol level decreased significantly at 45 DAG for both N_1 and N_2 treatments by 6.4% and 30.86% respectively as compared to control (Fig 3). Lipid peroxidation (LPO) measured in terms of malonedialdehyde (MDA) contents decreased significantly by 14.66% only for N_2 treatment at 30 DAG (Fig 3). For 45 DAG, LPO decreased as compared

to control, but the reduction was significant only for only the N_2 treatment (Fig 3). MDA content did not vary significantly due to age but varied significantly at both treatment and age x treatment interactions according to the two-way ANOVA test (Table 2). The protein content increased for all the treatments at both DAG but the increments were insignificant (Fig 3). According to two-way ANOVA tests, the protein content varied significantly due to age, treatment and age x treatment interactions (Table 2). SOD activity increased significantly by 43.7% and 79.16% for N_1 and N_2 treatments at 30 DAG, but the increments were insignificant at 45 DAG (Fig 3). Two-way ANOVA tests showed that the variations were significant for the individual factors i.e; age and treatment but insignificant due to age x treatment interactions (Table 2). Proteins contents did not show any significant variations at any N treatment at 30 DAG, whereas it increased significantly at both the treatments at 45 DAG (Fig 3).

Morphological Parameters

At 30 DAG, RL increased significantly by 10.85% and 21.7% for N_1 and N_2 treatments respectively, as compared to control. Root length decreased by 16.2% for N_1 but increased by 5.6% for N_2 as compared to control at 45 DAG (Fig 4). SL was found to be 74.55 and 93.66 % significantly higher for N_1 and N_2 treatments, respectively, as compared to control at 30 DAG and 21.9 and 53.7% significantly higher for N_1 and N_2 treatments as compared to control at 45 DAG, respectively (Fig 4). According to two-way ANOVA tests both RL and SL varied significantly for individual factors like age and treatment but varied insignificantly for age x treatment interactions (Table 2). Similarly, number of leaves showed a significant hike of 82.18 and 94.0 % for N_1 and N_2 treatments, respectively, as compared to control at 30 DAG and increments of 57.6 and 46.1% for N_1 and N_2 treatments, respectively, as compared to control at 45 DAG (Fig 4). With supplementation of nutrients the leaf area showed an exponential increase for N_1 and N_2 treatments as compared to control at 30 DAG but showed a slight increase at 45 DAG (Fig 4). Two-way ANOVA tests showed significant variations for age and treatment factors for both number of leaves and leaf area but varied insignificantly for age x treatment interaction (Table 2). Root biomass showed a hike of 35.0 and 100.0% for N_1 and N_2 treatments respectively at 30 DAG but at 45 DAG it increased by 10.3 and 17.9% for N_1 and N_2 treatments respectively (Fig 4). According to the two-way ANOVA tests, RB showed significant variations only for age factor (Table 2). At 30 DAG, the shoot biomass increased significantly at both N_1 and N_2 treatments respectively but at 40 DAG the treatments varied insignificantly for both the treatments, as compared to control (Fig 4). Two-way ANOVA tests showed that SB varied significantly only for individual factors like age and treatments but showed insignificant variations for age x treatment interaction (Table 2).

Biomass Allocation Pattern

RSR reduced significantly for all the treatments at both ages of sampling, except the N_1 treatment of 30 DAG which was insignificant (Table 3). SLA increased by 5.6% for N_1 treatment but decreased by 11.35% for N_2 treatment at 30 DAG and both were significant but at 45 DAG both the values had insignificant reductions (Table 3). SLW decreased significantly by 9.3% for N_1 treatment and increased significantly by 12.5% for N_2 treatment at 30 DAG, but at 45 DAG both the values increased insignificantly (Table 3). LAR did not show any significant variations for all the treatments at both sampling stages, except N_2 treatment at 30 DAG, where LAR showed an decrement of 3.6% as compared to control (Table 3). LWR increased significantly by 9.3% and 6.6% for N_1 and 8.6% and 11.2% for N_2 at 30 and 45 DAG respectively, as compared to control (Table 3).

Yield

The yield showed significant increase by 58.12% for N_1 and 71.2% for N_2 treatments as compared to control.

Discussion

O_3 monitoring data of the present study clearly indicate that O_3 concentration were high enough to produce significant negative effects on plants. The present experimental site was characterized by high temperature and high sunlight intensity during the experimental period, which provides favorable conditions for O_3 formation (Monks et al., 2015). The dependency of O_3 concentration on meteorological factors is clearly reflected by the high O_3 episodes during the later part of the experiment which is marked by the soaring temperatures. The global mean 6-months summer time O_3 concentration (M12) from 2010 – 2014 is reported to be 40.1 ppb with average O_3 concentration in South-Asia being 41 ppb (Mills et al., 2018). Monitoring studies done at the present experimental site during the period 2002 – 2012 clearly depicts and shows increasing trend of O_3 concentration (Tiwari et al., 2018). Earlier studies done at the present experimental site

have also reported high O_3 concentration (Singh et al., 2018; Yadav et al., 2020). Certain modelling studies have also predicted significantly high impacts of O_3 on crops and vegetation in Indo-gangetic plains (Burney and Ramanathan, 2014; Ghude et al., 2014).

The negative effects of O_3 are well cited in literature (Hu et al., 2020; Shao et al., 2020, Ghosh et al., 2020, Feng et al., 2019) and soil nitrogen amendments are established to be an effective way of managing O_3 stress in plants (Podda et al., 2019; Zhang et al., 2018 a & b). The role of soil nitrogen amendments in minimizing O_3 stress in plants is determined by the ability of the extrinsically applied nitrogen to annihilate the unbalance between the physiological (gaseous exchange) and biochemical (antioxidative response) characteristic of plants under O_3 stress (Podda et al., 2018). The present study showed that N addition significantly increased the rate of photosynthesis (Ps) at both the treatments, however the increment was higher at N_1 as compared to N_2. This observation is consistent with other studies (Singh et al., 2009; Yamaguchi et al., 2007; Fusaro et al., 2017; Shang et al., 2018; Zhang et al., 2018 a&b). However, Pandey et al. (2018) reported reductions in Ps in two cultivars of wheat HD2967 and Sonalika upon O_3 stress and nutrient fertilization. Increased Ps under nitrogen treatment indicates more amount of C being fixed. As such, enhanced photosynthetic rate upon N application can be attributed to the increased RuBP activity (Maheshwari et al., 1993). However, positive effects of N treatment on process related to photochemistry have also been reported (Fusaro et al., 2017). Palmorth et al. (2014) reported that N treatment enhances the capacity to channel the flow of energy through photosystems, thus sustaining the photosystem functionality and photosynthetic yield, even at lower g_s. Stomatal conductance (g_s) plays a significant role in determining Ps, however, ample of studies have reported their uncoupling under O_3 stress (Singh et al., 2009; Agathokleous et al., 2011; Feng et al., 2016). In the present study, g_s reduced significantly by 9.1% at N_1 treatment, while increased significantly at N_2 treatment. Reduction in g_s is an adaptive mechanism in plants to minimize the entry of O_3 (Biswas et al., 2008). In the present study, plants under N_1 treatment are capable of utilizing the stomatal closure as a mechanism to minimize O_3 injury. Zhang et al. (2018) however, have reported that N enrichment increased O_3 uptake by 5-10% in Oxford Poplar clone. This aberrant behavior of g_s in two N treatments disturbs the Ci/Ca (rates of internal and ambient CO_2) which is responsible for their differential behavior under different nitrogen treatment regimes. Ci/Ca increased at N_2 treatment, indicating a relative decrease in stomatal limitation to photosynthesis and a relative increase in non-stomatal limitations such as biochemical factors (Watanabe et al., 2014). On the basis of the physiological data of the present experiment, Ps of O_3 exposed plant is sustained by reducing g_s at low nitrogen treatments (N_1) whereas at high N treatment (N_2), a clear decoupling of Ps and g_s is observed which suggests that management of O_3 injury is governed by the biochemical parameters. Previous studies have also confirmed increased gs in plants upon high N application (Pandey et al., 2018; Urairi et al., 2016). The N treated plants showed significantly high chlorophyll and carotenoid contents as compared to untreated plants which may play an important role in compensating against O_3 stress (Kitao et al., 2015).

The results of the present experiment suggest that the palak plants under N_2 treatment have invested more of their resources in stimulating the anti-oxidative defense system which was responsible for their sustained yield under O_3 stress. The results are synchronized with the results of Podda et al. (2019), which states that the antioxidant compounds were stimulated in Oxford poplar clone upon N application. Ascorbic acid, an important component of the Halliwell-Asada pathway and considered to be the first line of plant's defense system against O_3 stress (Noctor & Foyer, 1998) declined significantly in N_2 treatment at both the sampling ages, indicating its active utilization in scavenging the excess ROS produced due to oxidative stress (Pellegrini et al., 2016). Plants with N_1 treatment, however, showed an increment in ascorbic acid contents at 30 DAG which indicate an accumulation of ascorbic acid due to reduced ROS accumulation. It is to be noted that reduced gs in N_1 plants leads to lesser accumulation of ROS, as compared to N_2 plants. SOD, an important enzyme of the plant's defense machinery also showed significant increments at both the treatments and at both the sampling ages which again signifies a reduction in O_3 stress under nitrogen treatments. SOD is a scavenger of superoxide radicals (O_2^{o-}) generated in the apoplast and results in the formation of hydrogen peroxide (H_2O_2). SOD showed significant increase for both N_1 and N_2 at 30 DAG as compared to control, which suggests that the plant in its early stage is increasing its defense potential. More increments in SOD under high nitrogen treatment (N_2) as compared to low nitrogen treated (N_1) plants indicate more production of SOD in the former in order to quench the excessive ROS generated. Further, significant reductions in MDA

content in N_2 plants reflect higher membrane stability as compared to untreated control plants. Malonedialdehyde (MDA) is the end product of ROS- induced oxidation of polyunsaturated fatty acids (PUFA) and is generally used as a biomarker of lipid peroxidation (Bhatacharjee., 2015). Reduction in LPO in N_2 plants suggests that N nitrogen enrichments helped the plant in scavenging the ROS generated through increased antioxidant defense system. These results clearly suggest the prominent role of biochemical parameters in managing O_3 stress in N_2 treated plants in spite of high g_s. Two way ANOVA studies have also shown that most of the biochemical parameters varied with treatment indicating their significant and differential role at different nitrogen dose treatments. Earlier Podda et al. (2018) also reported the prominent role of antioxidant pool in nitrogen treated plants against O_3 stress. The results of the present study clearly verify our hypothesis that low nitrogen treatment (N_1) helped in managing O_3 stress in plants through physiological modifications while high nitrogen treatment (N_2) strengthened the repair mechanisms by stimulating the antioxidant pool.

The biochemical/physiological response of the plants growing in nitrogen amended soil demonstrated positive effects on growth and yield characteristics as well. The positive response of nitrogen amendments was more prominent in the above ground parts as compared to below ground parts in terms of their length and biomass. Nitrogen addition stimulated the formation of new leaves as reflected by increased number of leaves in N_1 and N_2 plants as compared to control. New leaves had more potential for photosynthesis and may be capable of compensating for the decreased photosynthesis induced by O_3 in damaged older leaves (Maurer and Matyssek, 1997; Zhang et al., 2018). However, the increments in number of leaves per plant were higher in N_1 than N_2 as compared to control, at 45 DAG, which elucidates the larger increase of Ps in former. Significant variations with respect to treatment as indicated by Two way ANOVA studies strengthens the differential response of palak plants towards the two doses of nitrogen amendments.

An interesting feature which is observed in the present study is that the yield of N_2 is higher than that of N_1 plants as compared to control, despite of the fact that Ps was higher in N_1 as compared to control. This unusual response is clearly attributed to the differential biomass allocation strategy adopted by palak plants at different nitrogen amendments doses. O_3 stress is well known to disturb the normal biomass allocation pattern of plants which is an important factor responsible for causing yield reduction in plants (Black et al., 2012). Nitrogen amendments positively modify the resource allocation, which assist the plant metabolite processes to sustain yield even under O_3 stress. In the present experiment the different biomass allocation indices like SLA, SLR, LWR etc clearly reflect the nitrogen amendments have modified the resource allocation in such a manner that more photosynthates are retained in the above ground portion and are utilized in the development of leaves. The increment in LWR was of higher magnitude in N_2 than N_1 plants as compared to control, which suggests that the tendency of retaining more biomass in leaves was more prominent in N_2 as compared to N_1. Further, higher reduction in SLA and increment in SLW in N_2 as compared to N_1 plants as compared to control signifies the tendency of N_2 plants to favor leaf expansion which is evident from the higher increments in leaf area in N_2 plants than in N_1, as compared to control.

Yield, increased significantly for both N_1 and N_2 treated plants, as compared to control. However, the mechanism of sustaining yield through nitrogen amendments varied between the two nitrogen doses. N_1 plants sustained their yield by minimizing their O_3 intake which is evident through the reduced stomatal conductance. N_2 plants, however depended more upon the non-stomatal factors such as stimulation of antioxidant pool to maintain their yield even under O_3 stress conditions. Biomass allocation pattern, although was beneficial for both N_1 and N_2 plants but its possible effects were more prominent in N_2 plants. Although carbon fixation was less in N_2 plants, its yield was higher than in N_1 plants; as compared to control, which can be attributed to the biomass allocation strategy of N_2 plants.

Conclusion

The present study clearly demonstrated that soil nitrogen amendments can be effectively used in management of O_3 injury in plants. However, the strategy adopted by the plants to cope with O_3 stress may vary depending upon the dose of nitrogen amendment. While the plants grown at low nitrogen dose used the strategy of avoidance (i.e, reduced stomatal conductance to exclude from leaf extracellular space), the plants grown at higher nitrogen dose focused mainly on repair processes (i.e, activation of detoxification systems).

The activation of antioxidant systems at N_2 treatment was able to protect the photosynthetic machinery even when the O_3 uptake was high. The more prominent efficiency of biomass allocation pattern in N_2 treated plants was able to retain more biomass in the above ground parts of the plants, thus sustaining higher yield even though the carbon fixation was low. However, further studies are required to establish the application, concentration and timings of different nitrogen fertilization regimes under ambient and elevated O_3, for different economically as well as ecologically important plants.

Table.1- Physiological parameters of palak plants at 45 DAG at different dose treatments (Mean ± SE). Value not followed by same letters between rows are significantly different at $p<0.05$.

	Photosynthetic rate (µmol CO_2 m^{-2} S^{-1})	**Stomatal conductance (mol H_2O m^{-2} S^{-1})**	**Internal CO_2 concentration (µmol mol^{-1})**	**Transpiration rate (mmol H_2O m^{-2} S^{-1})**
C	12.83 ± 0.85^b	0.48 ± 0.20^a	304.49 ± 22.17^a	5.13 ± 0.102^a
N_1	16.65 ± 1.11^a	0.43 ± 0.09^a	283.66 ± 9.59^a	4.59 ± 0.069^a
N_2	15.85 ± 0.96^a	0.62 ± 0.17^a	317.13 ± 16.06^a	5.73 ± 0.21^a

Table.2- F ratio and level of significance of selected morphological and biochemical parameters of palak plants. ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

Parameters	Age	Treatment	Age X Treatment
Root length (cm plant^{-1})	58.79***	3.63*	2.38
Shoot length (cm plant^{-1})	54.88***	27.59***	1.35
Number of leaves (plant^{-1})	5.38***	7.59**	0.19
Leaf Area (cm^{2} plant^{-1})	19.41*	7.13**	0.40
Root biomass (g plant^{-1})	43.37***	0.227	0.48
Shoot biomass (g plant^{-1})	26.41***	5.23*	1.22
Chlorophyll a (mg g-^{1} FW)	27.236***	74.358***	1.281
Chlorophyll b (mg g^{-1} FW)	0.984	83.903***	2.686
Total chlorophyll (mg g-^{1} FW)	8.40*	95.325***	0.574
Carotenoids (mg g^{-1} FW)	18.914	119.836***	2.337
Ascorbic acid contents (µmol g^{-1} FW)	0.484	0.341	7.159***
Phenol content (µmol g^{-1} FW)	20.976***	23.613***	8.129**
MDA content (nmol g^{-1} FW)	4.23	252.95***	33.12***
Protein content (mg g^{-1})	0.391	1.76	2.04

Table.3- Variations in selected growth indices of palak plants at 30 DAG and 45 DAG at different nitrogen dose treatments (Mean ± SE). Values not followed by same letters between rows are significantly different at $p<0.05$.

	RSR		SLA		SLW		LAR		LWR	
	30 DAG	45 DAG	30 DAG	45 DAG	30 DAG	45 DAG	30 DAG	45 DAG	30 DAG	45 DAG
C	0.17 ±0.008^{b}	0.262 ±0.027^{b}	305.02 ±6.35^{c}	163.02 ±23.47^{a}	0.0033 ±0.00007^{c}	0.0062 ±0.0009^{a}	260.70 ±5.17^{b}	150.77 ±8.71^{a}	0.85 ±0.006^{b}	0.78 ±0.006^{b}
N_1	0.069 ±0.005^{b}	0.20 ±0.006^{ab}	332.26 ±10.77^{b}	150.97 ±12.52^{a}	0.003 ±0.00009^{b}	0.0067 ±0.0005^{a}	310.45 ±8.61^{b}	125.84 ±11.09^{a}	0.93 ±0.004^{a}	0.83 ±0.004^{a}
N_2	0.076 ±0.009^{a}	0.151 ±0.024^{a}	270.39 ±2.90^{a}	138.69 ±16.69^{a}	0.0037 ±0.00003^{a}	0.0074 ±0.0007^{a}	251.07 ±4.08^{a}	121.16 ±17.33^{a}	0.92 ±0.008^{a}	0.86 ±0.018^{a}

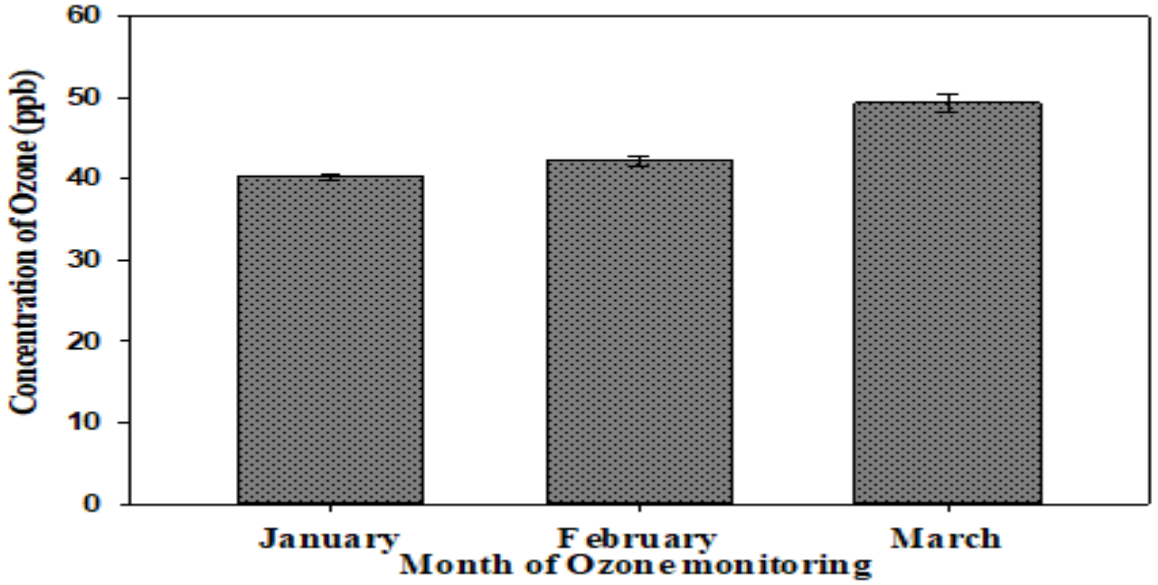

Figure 1- Eight hourly daily average and mean monthly O_3 concentration during the experimental period.

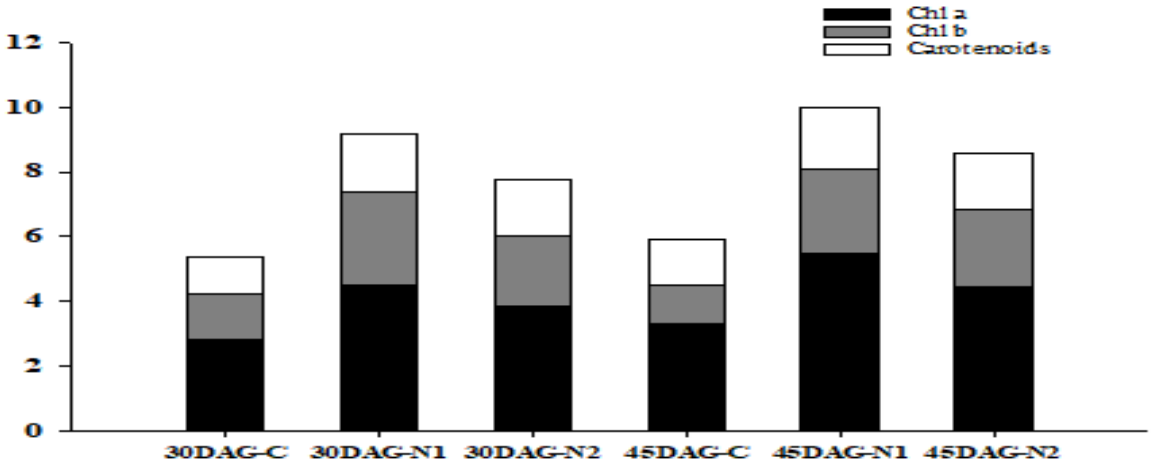

Figure 2- Response of photosynthetic pigment contents of palak plants at 30 and 45 DAG, given under different doses of nitrogen amendments (mean ± SE). Bars with different letters in the same group show significant variations at $p < 0.05$.

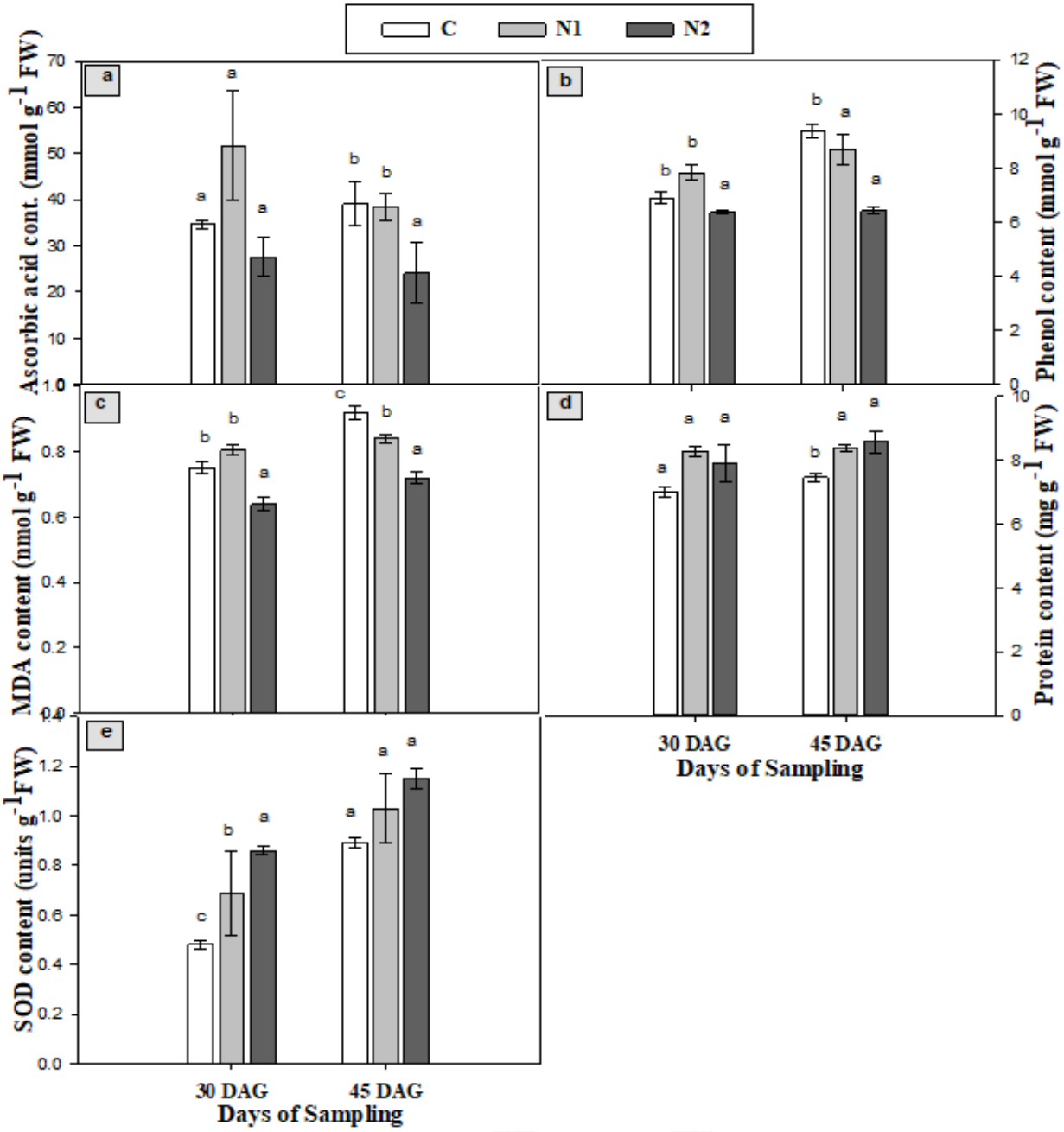

Figure 3-Response of ascorbic acid (a), phenol (b), MDA (c), protein (d) and SOD (e) contents of palak plants at 30 and 45 DAG, grown under different nitrogen amendments (mean ± SE). Bars with different letters in the same group show significant variations at $p < 0.05$.

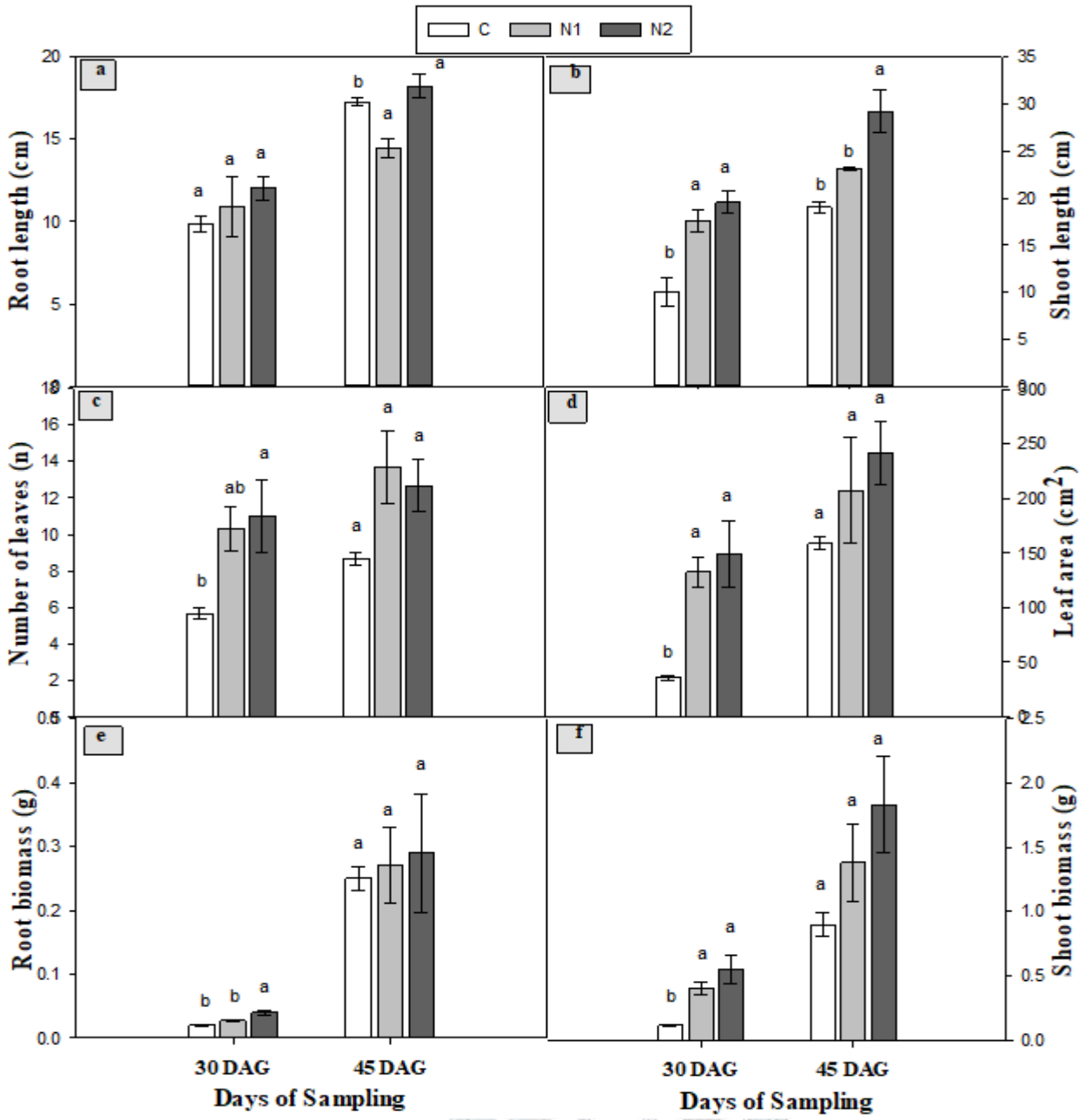

Figure 4- Root length (a), shoot length (b), number of leaves (c), leaf area (d), root biomass € and shoot biomass (f) of palak plants at 30 and 45 DAG, grown under different nitrogen amendments (mean ± SE). Bars with different letters in the same group show significant variations at $p < 0.05$.

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