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OPTIMIZING *VIGNA RADIATA* L. HEALTH: ROLE OF NITROGEN AMENDMENTS IN COMBATING OZONE STRESS

Gereraj Sen Gupta, Umesh Kumar and Supriya Tiwari*
Department of Botany, Institute of Science, Center of Advanced Studies, Banaras Hindu University, Varanasi-221005, India
*Corresponding author E-mail: supriyabhu@gmail.com
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ABSTRACT

The current experiment involved two distinct *Vigna radiata* L. (Moong bean) cultivars, namely HUM-12 and HUM-16. It was conducted under ambient ozone (O_3) conditions and included three different levels of inorganic nitrogen application: N_1(recommended dosage), N_2 (1.5 times the recommended amount), N_3 (2 times the recommended amount), as well as a control group. The primary aim of this study was to assess the effectiveness of soil nitrogen amendments in mitigating the impact of ambient ozone stress on the two *Vigna radiata* cultivars. Our findings indicate that nitrogen amendments can serve as an efficient strategy for alleviating O_3induced damage in plants. Significant outcome of these amendments is the enhancement of antioxidant enzyme activities, which plays a vital role in helping plants manage the stress caused by ambient O_3. Notably, in the more tolerant HUM-16 cultivar, nitrogen amendments proved to be particularly effective in fortifying the plant's antioxidant defences. This study underscores, applying 1.5 times the recommended dose of soil nitrogen amendments was sufficient to partially mitigate O_3 induced injury. Interestingly, increasing the nitrogen dose to twice the recommended level (as was done in our case) did not confer any additional advantages to the plant's metabolism when compared to plants treated with the lower nitrogen dosage (1.5 times the recommended amount).
Keywords : *Vigna radiata,* O_3 stress, ROS metabolism, antioxidative defence, nitrogen amendments.

Introduction

Ozone (O_3) ranks among the prominent air pollutants, exerting detrimental effects on plant life, and thus the agricultural output (Gupta and Tiwari, 2020; Kittipornkul *et al*., 2021, Nigar *et al*., 2023; Madheshiya *et al*., 2023). O_3, previously regarded as a harmful gas in the lower atmosphere, is now on the rise globally in the current era marked by extensive urbanization and industrialization. Pronounced impacts of the rising O_3 concentrations in developing countries are primarily due to unregulated emissions of ozone precursor substances such as carbon monoxide (CO), volatile organic compounds (VOCs), nitrogen oxides (NOx), and methane (CH4) from industrial and transportation activities, as evidenced by research conducted by Dentener *et al*. (2005) and Akimoto *et al*. (2015).O_3 formation and its entry in the apoplastic leads to the generation of Reactive Oxygen Species (ROS), which is a natural component of plant metabolism and additional doses of exposure to O_3 stress amplifies its production, with the apoplastic region serving as the primary site for ROS generation, as documented in studies by Nauchi *et al*. (2019) and Mignolet-Sprut *et al*. (2016). The insidious impacts manifest in the form of necrosis, lesions, and chlorosis that beset plants, ultimately culminating in a distressing reduction in crop yields, thereby posing a formidable challenge to agricultural production (Oksanen *et al*., 2013; Kittipornkul *et al*., 2021; Ansari *et al*., 2022; Shahzadi *et al*., 2022). Proietti *et al*. (2016) showed its adverse impact on forests, while Gilliland *et al*. in 2016 observed similar effects on grasslands. The toxic effects of this ozone extend to native crops and vegetation, resulting in potential losses in productivity and the occurrence of injurious physiological and biochemical effects, as observed by Calatayud *et al*. (2004) and Fuhrer (2009). Studies conducted by Yadav *et al*. (2020), Ansari *et al*. (2023) and Chaudhary and Agrawal, (2015), indicated that chronic exposure to O_3 significantly decreases biomass production and total foliar protein (Mishra and Agrawal, 2015), reflecting its impact on the photosynthetic capacity of plants. Ozone's influence on plants extends to various physiological processes, including a decrease in quantum yield, alterations in membrane permeability, disruption of electron transport in photosystem II, and interference with CO_2 fixation, photosynthesis, and stomatal conductance, as noted by Chen *et al*. (2015) and Yadav *et al*. (2020). Additionally, Emberson *et al*. (2018) revealed that elevated concentrations of O_3 have reducedthe chlorophyll contents followed by premature onset of leaf senescence, ultimately leading to diminished photosynthesis. Many researches have been conducted since last few years, in search for the ways to reduce the impact of tropospheric O_3 on plants. In view of the above, the present study was

conducted to ameliorate (partially or fully) the adverse effects of tropospheric O_3 on two different cultivars of *Vigna radiata*sp. (moong bean), a leguminous plant.

Legumes are often referred to as an economical source of nutrition for individuals with limited means. According to Turan *et al*. (2021) legumesare an excellent source of protein, boasting a content of 240 grams per kilogram. They contribute significant amounts of crucial minerals like iron (0.03–0.06 grams per kilogram) and zinc (0.02–0.04 grams per kilogram), which are pivotal for human growth and development, as emphasized by Akcura *et al*. (2019). According to Swaminathan, (1974), legumes are recognized as the most affordable source of supplementary proteins. Furthermore, legumes offer a spectrum of essential nutrients, including minerals and vitamins. Studies have indicated that legumes are notably rich in vitamin C, and in some varieties, increased levels of riboflavin and niacin levels were also observed (Swaminathan, 1988). Considering the legumes to be one of the most important crops, various strategies has been utilized by the researchers to minimize the adverse effects of O_3 on plants. According to Nigar *et al*., 2023, O_3 exposed *Vigna* plants when treated with $Mg(NO_3)_2$ and Ethylenediurea (EDU)showed reduced stress, in terms of reduced leaf injury and higher chlorophyll and biomass content. Similar observations were obtained by Agrawal *et al*., 2005 and Perera *et al*., 2022, where application of EDU lead to increased levels of photosynthetic pigments, protein contents and ascorbic acid contents in the *Vigna radiata* L. var. Malviya Jyoti, plants. Gupta and Tiwari, 2020 also revealed the positive effects of nitrogen amendment in two different cultivars of *Cymopsis tetragonoloba* L. var. PUSA-N and S-151, in terms of increased enzymatic antioxidant activity, higher photosynthetic efficiency thus increased yield. Further studies conducted on twelve different cultivars of Vigna varieties revealed that the application of ascorbic acid and silicic acid to the leaves helped counteract the adverse impacts of O_3(120 ppb) by reorganizing metabolic processes resulting in a reduction in visible damage, the preservation of growth, and an improvement in antioxidant levels (Shahzadi *et al*., 2022 & 2023). The present study focuses on the efficiency of nitrogen amendmentsin minimising the adverse effects of O_3 on *Vigna* cultivars. The research extended its efforts to delve into the intrinsic adaptability of Vigna plants when confronted with elevated concentrations of O_3. It sought to understand whether these plants would respond by either bolstering their inherent antioxidative mechanisms to counteract the detrimental effects of O_3 or by demonstrating resilience in the face of such stressful environmental conditions. In essence, the study aimed to uncover how Vigna plants navigate the challenges posed by heightened O_3 levels, whether through enhanced defence mechanisms or by displaying a capacity to endure the adverse conditions.

Material and Methods

Experimental Site and Design

The research was conducted in the Botanical Garden of Banaras Hindu University, situated in Varanasi, Uttar Pradesh, India, within the eastern Gangetic plains. The study carried out in the months of January to March. The geographical coordinates of the studied location were 25.018°N latitude and 82.001°E longitude, with an elevation of about 76.19 meters above sea level. The experimental site's soil had a pH ranging from 7.2 to 7.6. The soil at the site is characterized as sandy-loam, composed of 45% sand, 28% silt, and 27% clay.

In this study, a complete randomized split-plot design was adopted. The cultivars were kept as the main plots and treatments as subplots. The treatment plots measured 1.5 x 1.5 meters and were separated by 20 cm wide and 15 cm high soil bunds. Four treatment levels were implemented, including control plots, each replicated three times. For both cultivars, 24 subplots were prepared, ensuring the soil was finely tilled. Enriched all plots with recommended doses of inorganic fertilizers (phosphorous, nitrogen, and potassium) as a basal dressing (50 kg P, 25 kg N, and 25 kg K per hectare of land in the form of super-potash, urea, and muriate of potash, respectively). Seeds, treated with Rhizobium, were manually sown in rows with a 30 cm separation between rows and 15 cm between seeds. Maintained four different treatment levels: control (no nitrogen doses, C), recommended nitrogen dose (N_1), 1.5 times recommended (N_2), and 2 times the recommended (N_3).

Experimental Material

Two distinct varieties of *Vigna radiata*, leguminous plants, namely HUM-12 and HUM-16 were chosen for the present experiment. The legume, commonly known as moong bean, is typically grown as an annual crop in the North-East plain zone, including the states of Uttar Pradesh, Bihar, Jharkhand etc. Between these two cultivars, HUM-12 exhibits a larger whose maturation period ranges from 60 to 65 days, while HUM-16 matures bit early, typically between 55 to 60 days. The expected yield per hectare for HUM-12 and HUM-16 falls within the range of 15-20 quintals and 12-14 quintals respectively. Both the seeds were procured from the Indian Institute of Agricultural Sciences, Banaras Hindu University.

Ozone (O_3) Monitoring

Continuous daily monitoring of ambient O_3 levels were carried out between 8:00 AM to 4:00 PM at the experimental site from January to March. An ambient O_3 Analyzer (Model-APOA 370, Horiba, Japan) was employed at the canopy level of the plants, commencing from germination, and continued until the plants were harvested. The O_3 analyser underwent weekly calibration using known O_3 concentrations. Hourly O_3 concentrations were documented, and the AOT 40 (8 hrs) for each month during the study was calculated using the formula by Mauzerall and Wang (2001):

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

Here, 'Co_3' signifies the average O_3 values per hour in parts per billion (ppb), 'n' denotes the number of hours where O_3 values exceeded 40 ppb, and 'i' represents the index.

ROS Metabolism

(i) 2.4.1 Superoxide radicals:

Superoxide radicals ($O_2^{o\,-}$) were quantified using a method based on Elstner and Heupel (1976), with certain adaptations. Initially, leaf samples were homogenized in a 65 millimolar (mM) phosphate buffer at pH 7.8 and then subjected to cold centrifugation at 2800g for a duration of 15 minutes at 4°C. Following centrifugation, the supernatant was meticulously collected and introduced into an assay mixture comprised of 10 mM hydroxylamine hydrochloride

and 65 mM phosphate buffer (pH 7.8). This mixture was allowed to incubate for a period of 30 minutes at 25°C. Subsequently, a sequence of additions was carried out, including 17 mM sulfanilamide and 7 mM α-naphthylamine. After each addition, the mixture was incubated once again, this time for 20 minutes at 25°C. The absorbance of the resulting solution was then measured spectrophotometrically at 530 nm, utilizing a reagent blank for reference. Finally, the obtained result was compared to a standard curve derived from nitrite salt to determine the total quantity of $O_2^{\circ -}$ generated within the sample.

(ii) 2.4.2 Hydrogen peroxide contents:

The H_2O_2 content was estimated following the method outlined by Alexieva *et al*. (2001). Initially, 0.5 grams of fresh leaves were taken and meticulously homogenized in 5 milliliters of 0.1% trichloroacetic acid (TCA) under cold conditions. After homogenization, the mixture underwent centrifugation at 12,000 rpm for 15 minutes, resulting in the extraction of the supernatant. Subsequently, the extract was subjected to the sequential addition of 10 millimolar (mM) potassium phosphate buffer and 1 millimolar (mM) potassium iodide solution. The entire reaction mixture was left undisturbed in the dark for a duration of 1 hour, during which it developed a pale-yellow color. Finally, the absorbance of the mixture was measured at 390 nm using a double-beam spectrophotometer (Model 2203, Systronics, India).

Enzymatic Antioxidants

Homogenized 0.2 grams of fresh leaf tissue in liquid nitrogen to extract the enzymes. Five mililiters of extraction buffer (consisting of 1 M phosphate buffer with a pH of 7.0, along with Poly vinyl pyrrolidone (PVP), Ethylene Diamine Tetra Acetic-acid (EDTA), phenyl methane sulfonyl fluoride (PMSF), and Triton-X-100, all maintained at 4°C) were used to extract the enzymes from the homogenized leaf tissues. Subsequently, the resulting mixture were centrifugated at 12,000 rpm, andutilized the supernatant in various assay mixtures. Evaluated enzymatic activities, including superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX), and catalase (CAT), by following the procedures outlined by Fridovich (1974), Anderson (1996), Nakano and Asada (1987), and Aebi (1984), respectively.

Yield Attributes

At the time of the final harvest, yield attributes were evaluated by harvesting 10 plants per treatment, and the weight of seeds per plant was recorded.

Statistical Analysis

One-way ANOVA, was performed to assess treatment differences in measured parameters. Data normality and homoscedasticity was confirmed and a2x2 factorial multivariate ANOVA examined the individual and interactive effects of age, treatment, and cultivar. SPSS software (Version 21.0, IBM Corp, Armonk, NY) was used for statistical analysis.

Result

O_3 Monitoring

The average ambient concentration of O_3 at the experimental site exceeded the phytotoxic threshold, measuring at 52.6 parts per billion (ppb) during the experimental period. The highest recorded O_3 concentration reached 68 ppb, while the lowest was 37 ppb. By the end of the experiment, the accumulated ozone exposure over 40 ppb (AOT40) had reached 9885 ppb per hour, with the highest monthly value of 3,578 ppb per hour occurring in March (see Figure 1).

ROS Metabolism

H_2O_2 content was found to decrease by 14.28% in N_1 treated plants of HUM-12, while 26.53 and 26.93% depreciation was observed in N_2 and N_3 treatments, respectively, at 45 DAG. Higher depreciation was observed in HUM-16 cultivar given by 17.94, 30.76 and 31.79% in N_1, N_2 and N_3 treated plants, respectively (Fig. 2).

SOR showed similar depreciation in nitrogen treated plants. It showed 19.35, 27.41 and 27.9% decrease in N_1, N_2 and N_3 treated plants in HUM-12 cultivar while 6.97, 20.93 and 20.94% depreciation were observed in HUM-16 at N_1, N_2 and N_3 treatments, respectively, at 45 DAG (Fig 2).

Enzymatic Antioxidants

The nitrogen treated plants of HUM-12, revealed 9.85, 57.74 and 60.56% increments in APX activity for N_1, N_2 and N_3 treatments while greater increments were observed in HUM-16 given by 9.87, 53.08 and 55.55% in N_1, N_2 and N_3 treatments, respectively (Fig. 3). GR activity in HUM-12 increased by 31.81% in N_2 treated plants while in N_2 and N_3 treatments, 36.36 and 36.82% increments were observed. In HUM-16, the GR activity was found to increase by 5, 20 and 20% in N_1, N_2 and N_3 treated plants (Fig. 3). The catalase and superoxide dismutase activity were found to increase in a very similar in response to the increased doses of nitrogen. The HUM-12 cultivar showed 6.3, 24.45 and 24.04% increments in CAT activity while 3.96, 17.84 and 18.01% increments were observed in case of HUM-16 cultivar in N_1, N_2 and N_3 treated plants, at 45 DAG (Fig. 3).The SOD activity increased by 1.86% in N_1 dose while 8.69 and 8.7% increments were observed in N_2 and N_3 nitrogen doses, respectively in HUM-12 cultivar. In HUM-16, greater increments in SOD activity were observed, viz. 0.54, 15.04 and 15% in N_1, N_2 and N_3 treated plants, respectively (Fig. 3).

Yield Attributes

Yield of plants in terms of weight of seeds per plant showed greater increments in HUM-16 upon higher nitrogen doses, as compared to HUM-12 cultivar. HUM-16 showed 28.14, 84.73 and 83.23% increments in weight of seeds per plant at N_1, N_2 and N_3 treatments respectively while 11.92, 75.17 and 75.25% increments were observed in HUM-12 cultivar, respectively (Fig. 4).

Discussion

The current research site exhibits elevated levels of O_3, a fact substantiated by earlier monitoring investigations (Yadav *et al*., 2019; Gupta *et al*., 2020). The monitoring conducted during the ongoing experiment revealed an average seasonal O_3 concentration of 52.6 ppb. The concentration surpasses the values documented in prior research, signifying a rising trend in tropospheric O_3 levels at this location. South Asian nations, with India as a focal point, are already recognized as future O_3 hotspots, as discussed in the studies Emberson *et al*. (2018).In the current research, O_3

levels significantly exceeded the allowable threshold set by the European Union, which is 40 ppb. This excessive O_3 concentration had a detrimental impact on the growth and performance of the experimental plants. Utilizing soil nitrogen supplements can serve as an effective approach for mitigating O_3induced damage in plants. In the current study, the application of soil nitrogen amendments played a role in supporting the metabolic processes of two V*igna radiata* cultivars, HUM-12, and HUM-16. This support was achieved by boosting their defence mechanisms, which led to increased enzymatic antioxidant activity when compared to untreated plants exposed to O_3. The reduction in O_3induced stress in plants due to nitrogen treatment is evident from the substantial decrease in the levels of both O_2^{o-} and H_2O_2 in both cultivars of *Vigna radiata*. Elevated levels of reactive oxygen species (ROS) were noted in two types of tropical legumes, namely PUSA-N and S-151, when exposed to increased concentrations of O_3 (Gupta and Tiwari, 2020), simultaneously, the addition of nitrogen was found to reduce the adverse effects of higher O_3 concentration. Gupta and Tiwari (2020) also documented the beneficial impacts of nitrogen treatment on plants exposed to O_3. Podda *et al*., 2019, also observed a noteworthy decrease in O_2^{o-} and H_2O_2 levels, as well as improved membrane stability when applying 80 kg of nitrogen per hectare at both normal and elevated O_3 concentrations (14.4, 43.8, and 71.1 ppm/h). In our present study, significant declines in H_2O_2 levels were observed in the HUM-16 cultivar when compared to the HUM-12, across all nitrogen doses. This observation implies that HUM-16 demonstrated a higher degree of effectiveness in removing the surplus reactive oxygen species (ROS) produced as a result of oxidative stress and in preserving the integrity and stability of the cell membranes through nitrogen treatment, even when subjected to similar O_3 conditions. These findings indicate that HUM-16 displays greater resistance to stress induced by O_3. Our finding was further supported by the ANOVA results (Table 1), that reveals significant variation in ROS in different cultivars due to variable doses of nitrogen.

The activities of antioxidant enzymes (namely APX, GR, CAT, and SOD) exhibited significant increases in both varieties of *Vigna radiata* when exposed to nitrogen treatment, as compared to control ones. These elevated enzyme activities indicate a bolstered defence mechanism in plants and a reduction in O_3 induced stress as a result of nitrogen treatment. Within our study, the enhancements in enzyme activities were more pronounced in the HUM-16 cultivar than in the HUM-12 across all doses of nitrogen. This suggests that HUM-16 responded more favourably to nitrogen treatments and could activate its defence mechanisms more effectively when confronting oxidative stress. This reflects the greater resilience of HUM-16 compared to HUM-12. Similar findings of heightened enzyme activities in tolerant wheat cultivars were also reported by Gupta and Tiwari (2020). The findings of the present study were further supported by the ANOVA results, that shows significant effect of nitrogen treatment in *Vigna* cultivars particularly CAT and SOD. Increased catalase activity suggests better scavenging of H_2O_2 radical generated in the apoplastic region of the plant cell while higher SOD activity reveals efficient scavenging of O_2^{o-}. Similar increments in the activity of APX and GR were observed suggesting better performance of HUM-16 cultivar to cope up with the adverse situation due to the O_3 stress. Higher activities of HUM-16 cultivars subsequently revealed greater yield in terms of weight of seeds per plant as compared to the HUM-12 cultivar (Fig. 4).

To conclude, soil nitrogen amendments can be adopted as an efficient measure to manage O3 injury in plants. Stimulation of antioxidant enzyme activities under nitrogen amendments is an important feature of plants to cope with ambient O3 stress. The present study depicts that 1.5-times recommended dose of soil nitrogen amendments was sufficient in partial mitigation of O_3 injury and that higher nitrogen doses (2-times recommended as in this study) may not be more effective in mitigating O_3 injury as the higher nitrogen treatment did not provide any extra advantage to the plant's metabolism. Findings from this study can be used in breeding programs for developing O_3 tolerant cultivars. However, more experiments are required on O_3–N sensitivity interactions that focus on fertilisation management practices such as nitrogen dose and timing of application.

In conclusion, it is observed that O_3 injury in plants can be effectively managed through the application of soil nitrogen amendments, which result in the stimulation of antioxidant enzyme activities crucial for coping with O_3induced stress. It is further noted in our study that the partial mitigation of O_3 injury is achievable with a nitrogen dose 1.5 times the recommended level, while increasing the nitrogen dosage to twice the recommended amount does not yield additional advantages in terms of plant metabolism. These findings suggest potential implications for breeding programs aimed at the development of O_3tolerant cultivars. Nevertheless, further experimentation is deemed necessary to explore interactions related to O_3 and nitrogen sensitivity, particularly regarding nitrogen dosage and the timing of its application.

Table 1: F-ratio and level of significance of selected enzymatic and non-enzymatic characteristics of *Vigna radiata* L.

	Cultivar	**Treatment**	**CxT**
APX	116.83^{***}	483.29^{***}	0.21^{ns}
GR	676.17^{***}	35.48^{***}	0.00^{ns}
CAT	2295.20^{***}	7647.45^{***}	80.03^{***}
SOD	1771.50^{***}	958.85^{***}	101.46^{***}
H_2O_2	149.42^{***}	61.19^{***}	0.14^{ns}
O_2^{o-}	202.70^{***}	51.24^{***}	6.16^{*}

C, cultivar; T, treatment; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant

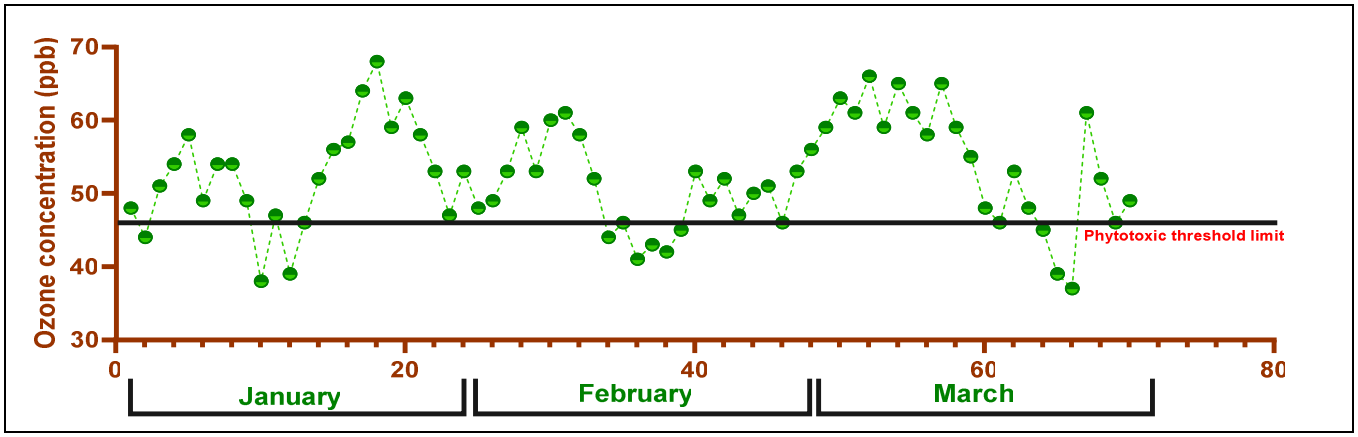

Fig. 1 : Daily ambient ozone (O_3) concentration, measured hourly between 0800 hours and 1600 hours, at the experimental site (Botanical-garden of Banaras Hindu University, Varanasi, Uttar Pradesh, India. January-March, 2020)

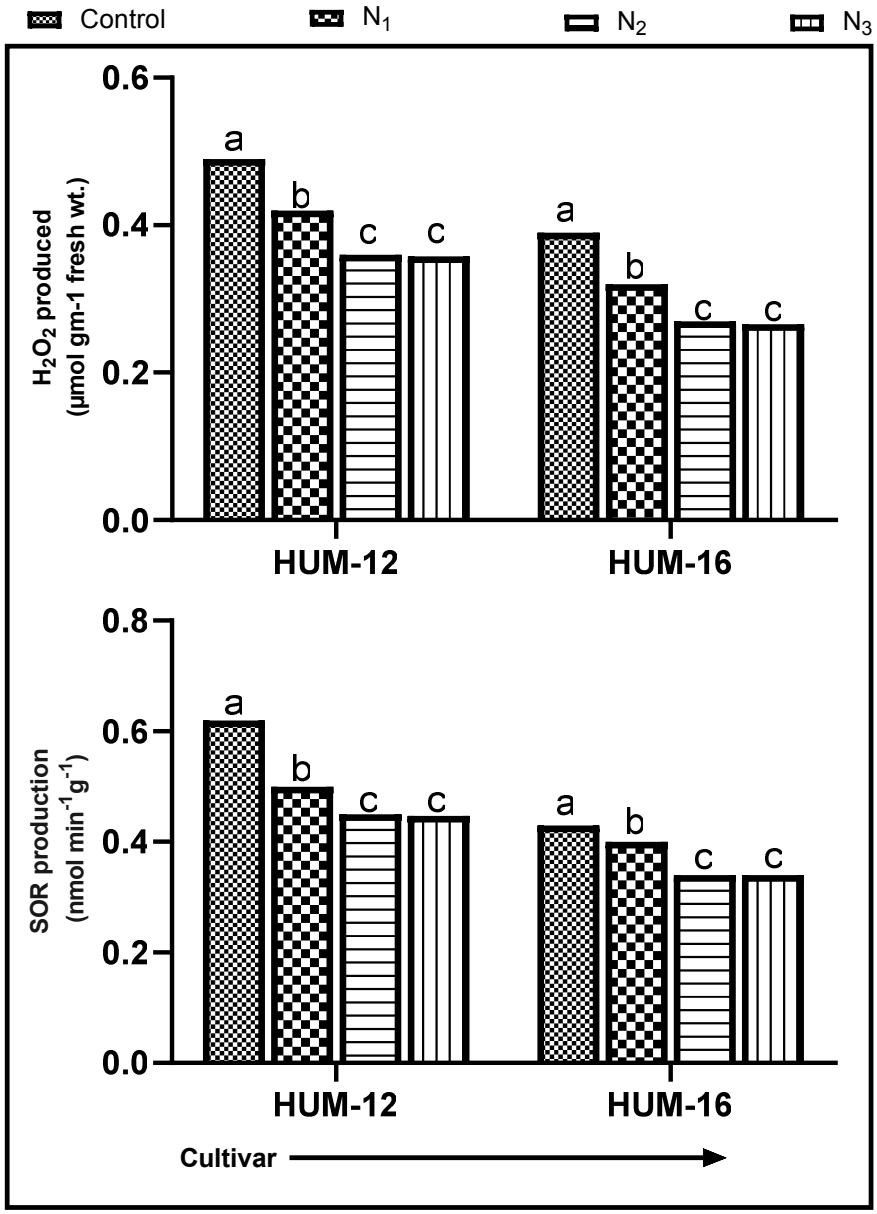

Fig. 2 : Effect of N treatment (C, control; N_1, recommended N dose; N_2, 1.5-times recommended N dose and N_3, 2-times recommended N dose) on the responses of Hydrogen peroxide (H_2O_2) and Superoxide radical (SOR) activities of Vigna radiata L. cv. HUM-12 and HUM-16 at 45 days after germination (DAG). Bars are mean± s.e. Bars with different letters in the same group show significant variation at $P < 0.05$.

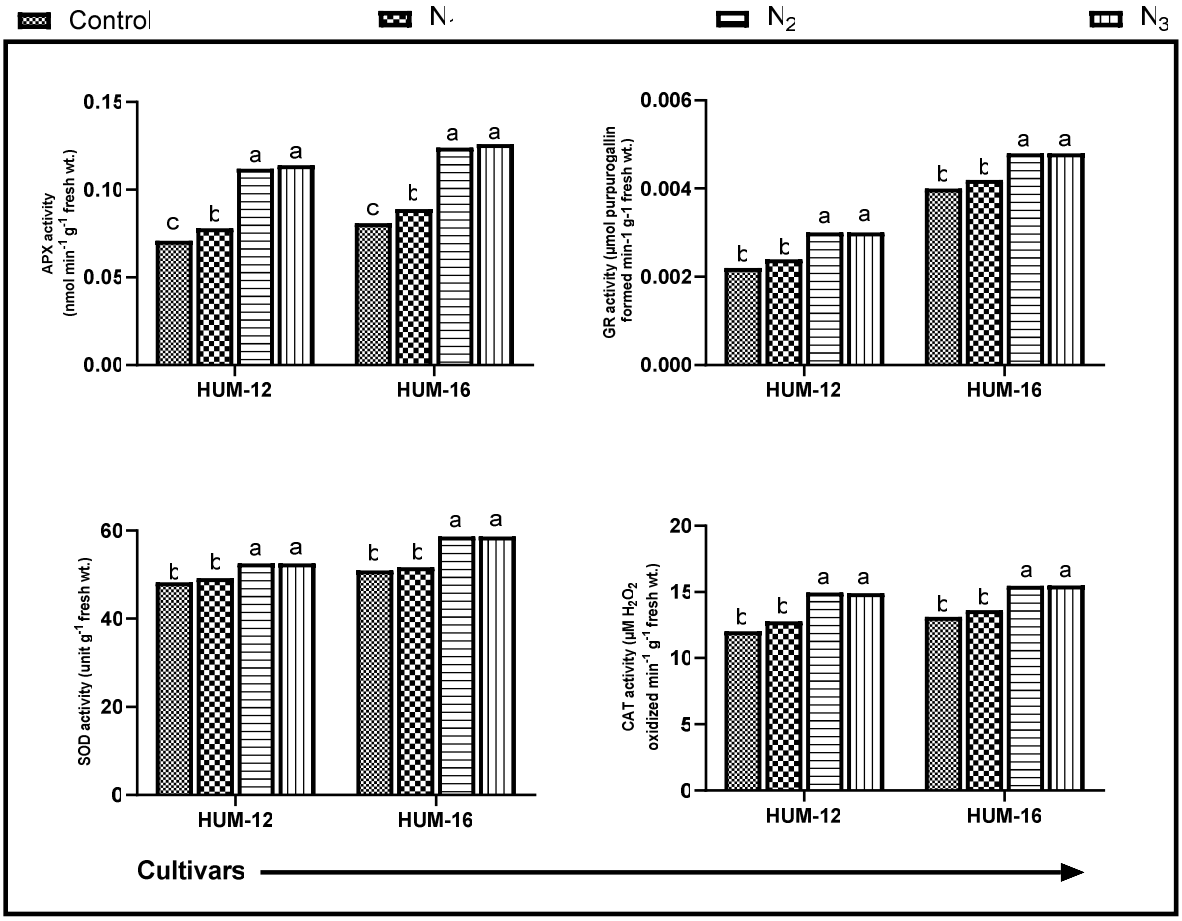

Fig. 3 : Effect of N treatment (C, control; N_1, recommended N dose; N_2, 1.5-times recommended N dose and N_3, 2-times recommended N dose) on the responses of ascorbate peroxidase (APX), glutathione reductase (GR), Superoxide dismutase (SOD) and Catalase (CAT) activities of Vigna radiata L. cv. HUM-12 and HUM-16 at 45 days after germination (DAG). Bars are mean± s.e. Bars with different letters in the same group show significant variation at $P < 0.05$.

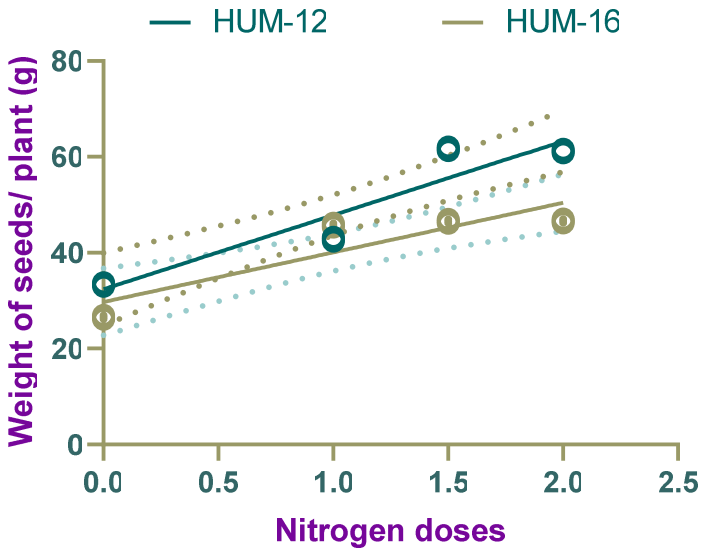

Fig. 4 : Effect of N treatment (C, control; N_1, recommended N dose; N_2, 1.5-times recommended N dose and N_3, 2-times recommended N dose) on weight of seeds/ plant (g) in HUM-12 and HUM-16 cultivars.

Conflicts of Interest:

The authors disclose that they have no conflicts of interest.

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