Journal of Applied Biology & Biotechnology Vol. 9(04), pp. 137-150, July, 2021
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2021.9419

Micro-morphological and anatomical response of groundnut (*Arachis hypogaea* L.) cultivars to ground-level ozone

Indra Jeet Chaudhary, Dheeraj Rathore*
School of Environment and Sustainable Development, Central University of Gujarat, Gandhinagar, India

ARTICLE INFO

Article history:
Received on: January 04, 2021
Accepted on: February 16, 2021
Available online: July 10, 2021

Key words:
Arachis hypogaea, tropospheric ozone, OTC, visible injury, stomata

ABSTRACT

Tropospheric ozone is a phytotoxic gaseous pollutant with global warming potential that disrupts the plants growth and development directly or through climate change. Ozone enters into a plant's body through stomatal pores and develops oxidative stress, which results in injury to foliage and modifies leaf micro-morphology and anatomy. A field study was conducted to assess the morphological, micro-morphological, and anatomical response of groundnut cultivars (*Arachis hypogaea* L.) to enhance the level of ozone. This study observed ozone-like visible injury symptoms on all groundnut cultivars. Visible injury was maximum in cultivar Dh-86 and minimum in cultivar TPG-41. Micro-morphological characteristics, such as increase in stomata, epidermal cells number, and its index, were also increased under enhanced ozone-exposed plants. The highest stomatal index was found in cultivar TPG-41 and lowest were noted in cultivar GG-20. Cultivars TAG-24 > TG-37A > and Dh-86 show moderate modification in the morphological and micro-morphological characteristics of plants. Elevated ozone also affected the stomatal movement and leaf internal tissue. Most of the stomata of all the groundnut cultivars were observed as closed during the enhanced ozone exposure, suggesting a protective mechanism from ozone stress. The study concluded that the micro-morphological and anatomical characteristics are important aspects to determine the effect of ozone on plants and to influence plants sensitivity to ozone. On the basis of these characteristics, cultivar TPG-41 was found to be less sensitivity, while cultivar Dh-86 was found to be highly sensitive to ozone pollution.

1. INTRODUCTION

Rapid industrialization, growing cities, and increasing vehicular load have caused serious environmental pollution and have affected plant life [1–3]. Ozone is a secondary air pollutant in the tropospheric atmosphere, formed by the reaction of sunlight and originator gases, including SO_x, NO_x, and volatile organic carbon, generated through anthropogenic activities [4,5]. The rapid increase in ground-level ozone concentration has come to be a global concern due to its direct phytotoxic effect or indirect effect through global warming [6–8]. Researches from the past four to five decades acknowledge the detrimental effects of ozone on plants. A higher ozone concentration could constrain photosynthesis [9], reduce yields and biomass [10], and also change the allocation of photosynthesis in plant organs. Ozone is also reported to cause foliar injury [11,12] and changes to stomatal conductance [13].

Ozone primarily enters the plant through stomatal pores [14] and generates oxidative stress [7,15]. To counteract this stress, the plant develops a series of defense response. However, most of the studies describing plants defense response to ozone are based on antioxidative efficiency and/or stomatal conductance. However, previously it was shown that balancing the density of stomata and cell division in developing leaves is directly connected with the plant's response to environmental stress [16–18]. The study of Navea et al. [19] reveals a specific defense mechanism in case of drought stress in which plants turn down the number of stomata and refer to it as 'stomata abortion'. Chaudhary and Rathore [3], Paakkönen et al. [20], and Aasamaa et al. [21] found decreased stomatal pores or aperture size due to the abiotic stress. Studies on plant response to air pollution, including ozone, have observed an increased stomatal density of plants [22,23]. Ferdinand et al.

**Corresponding Author*
Dheeraj Rathore, School of Environment and Sustainable Development, Central University of Gujarat, Gandhinagar, India.
E-mail: dheeraj.rathore@cug.ac.in

[23] observed an ozone-sensitive clone *Prunus serotina*, which has larger stomatal density and a lesser ratio of palisade per spongy parenchyma. The authors also asserts that resistance can be described by separate gas permeability, which is based on extending the structure of the leaf, as gas resistance of palisade tissue is larger than that of spongy tissue.

Alteration to stomata and abundance as a response to stress is also expressed as a major pollutant absorption controlling mechanism [24]. Stengleina et al. [25] stated that the density of stomata is basically affected by both the beginning of stomatal development and the build-up of epidermal cells (EC). The build-up of EC turns into a function of various environmental and developmental variables; the altitude of the experimental site can even influence stomatal index (SI) and its density. The redox state of plant cells gets unbalanced due to ozone, which leads to modifications in metabolic processes and gene expression, which affects cell growth and development. Cell division on the whole is sensible to the redox state of plant cells whereby oxidative stress hinders the cell cycle and hence proliferation [26]. Ozone sensitivity was dissimilar among plant species. However, a lot of unanswered questions come forward regarding the possible mainspring of ozone sensitivity or resistance. Furthermore, the effects of ozone on internal tissues of plants are also missing.

Therefore, the present experiment was conducted to assess the effects of ozone on plant development, micromorphology, and anatomy of groundnut (*Arachis hypogaea* L.) cultivars and their possible role in plant defense. This study hypothesized that the stomatal distribution and movement influence plant sensitivity to ozone.

2. MATERIALS AND METHODS

2.1. Site Description and Ozone Treatment

Five cultivars of groundnut (*Arachis hypogea* L), viz. TG-37A, TPG-41, TAG-24, GG-20, and Dh-86, were selected for the present experiment. Crop and cultivars were selected on the basis of popularity among the formers in the region. The experiment was carried out in open top chambers (OTCs), sized 4 × 4 × 3.5 m, established at the research field of the Central University of Gujarat (23.2156°N, 72.6369°E) during February to May 2017. OTCs were made up of multi-layered clear polycarbonate sheet (3 mm thick) to provide maximum available sunlight. Plants were grown in plots sized 1 m^2 using a regular agronomical practice for groundnuts. The soil of the field was measured with the help of a pH meter and was found to be slightly alkaline (pH 7.4). The soil texture was determined based on sand, silt, and clay percentages and was found to be sandy loam of medium fertility. Each plot was mixed with 250 g vermicompost during field preparation and was applied with the recommended dose of NPK in the ratio of 40:40:20 kg/ha. The plots were regularly irrigated ensuring sufficient water supply and weeds were managed manually. A randomized block design was opted with two treatments, i.e., enhanced ozone (provided −75.91 ± 11.42 ppb of mean ozone concentration for 4 hours) and ambient ozone (ozone concentration varied between 13.6 and 40 ppb) (Fig. 1). Ambient ozone concentration and temperature were checked with the help of an ozone analyzer [Genesis (LEDM)] and temperature sensor (open top chamber with temperature sensor (HK Tempsensor), data logger (Ambetronic, TC800D), ozone generator, Genesis Technologies, India) throughout the study period. Ozone fumigations were conducted from 11.00 am to 03.00 pm every day from seed germination till harvesting.

2.2. Plant Morphology and Ozone Visible Injury

The morphological characteristics of plants are illustrated by the image and number of roots, leaves and branches that were counts at 20, 40, and 60 days after sowing (DAS). Ozone visible injury was also identified at 20, 40, and 60 DAS of plants. Various parameters were measured for visible injury, such as the number of injured plants per plot, the number of injured leaves per plant, and the number of chlorosis and necrosis spots per plant.

2.3. Micro-Morphological and Anatomical Characteristics

2.3.1. Microscopic observation

Micro-morphological characteristics of the leaf were measured using a digital microscope (Milton Instruments, Mumbai,

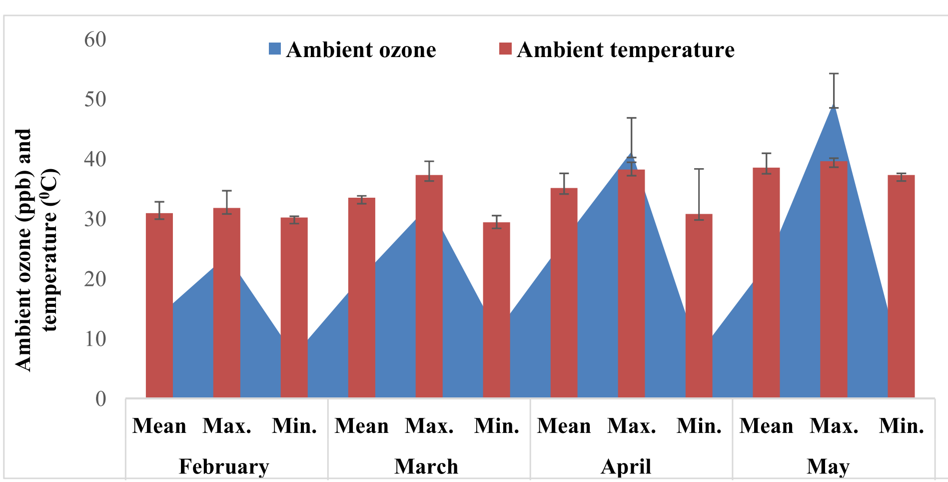

Figure 1: Monthly average ambient ozone (ppb) and temperature (°C) during the study periods of groundnut cultivars. (Mean ± standard deviation of three replicates presented by thin vertical bars.)

Maharashtra) and scanning electron microscopy (SEM) (Model: EVO 18, Make: Carl ZEISS). The sample for micro-morphological studies was collected and shifted to the laboratory in desiccators to avoid the effect of humidity. For stomatal study through a compound microscope, leaf abaxial surfaces were peeled off using dissecting needles and forceps and were finally washed with clean water. After that, each specimen was stained with safranin (1% aqueous) for 3–10 minutes and excess stains were washed using deionized water and then the stained cuticle was mounted on glycerine jelly and detected under a microscope. The number of stomata and EC and its index was calculated as per the equation of Salisbury [27], i.e., ($SI = S \times 100/S + E$) using a compound microscope.

where SI = stomatal index, S = No. of stomata/unit leaf area, and E = number of EC/unit leaf area.

2.3.2. SEM analysis

For SEM [Model: EVO 18, Make: Carl ZEISS] analysis, the specimen of the collected leaf sample was cut in 2–4 mm pieces and fixed (Primary fixation) to 2.5% Glutaraldehyde/Karnovsky's fixative for 6 hours at 4°C. After that, the samples were washed in 0.1 M phosphate buffer, for three changes each of 15 minutes at 4°C. After primary fixation, the same sample was ready for post-fixation.

For post-fixation, 1% osmium tetroxide was used for 2 hours at 4°C.

With regard to washing and dehydration, the sample was washed in 0.1 M phosphate buffer for three changes each of 15 minutes at 4°C to remove the unreacted fixative. After that, the specimen was dehydrated using increasing concentrations of acetone to remove water by the following procedure:

For dehydration, increasing concentrations of acetone, such as 30%, 50%, 70%, and 90%, were used for 30 minutes of each step, and finally 100% (dry acetone) was used.

After that, the specimen was dried by air and critical point drying (critical point, i.e. 31.5°C at 1,100 p.s.i.) and kept in desiccators.

With regard to specimen mounting and coating, the specimen was mounted on aluminum stubs with a carbon tape. The sample was coated using a sputter coater to make the sample conductive.

2.4. Leaf Anatomy

The anatomy of the selected groundnut leaf sample was analyzed by a compound microscope. A fresh leaf of groundnut sample was taken for the observation of anatomical characteristics. Fine transverse sections of the leaf were taken and stained with safrannin and mounted on glycerine. The specimens were covered with a cover slip after mounting to be observed in a compound microscope.

2.5. Statistical Analysis

Data were analyzed with three replicates (mean ± standard deviation). MS Excel 2010 was used for standard deviation. Significant correlation and variance of treatment, cultivars, and parameters were calculated with the help of regression. All data, such as morphology, visible injury, and micromorphology, were correlated with injured plants per plot of selected cultivars. Data were also analyzed through three-way analysis of variance (ANOVA) test using Statistical Package for the Social Sciences (SPSS) (SPSS Inc., version 17.0) for assessing the significance of quantitative changes in different parameters of groundnuts' response to ozone treatments at different sampling intervals.

3. RESULTS

3.1. Visible Injury

Foliar injuries on the plants are the first visible symptoms of the ozone pollution. Interveinal chlorosis and necrotic stippling were observed on the adaxial surface of the leaves of enhanced ozone-treated groundnut plants (Table 1 and Fig. 2). Chlorotic spots

Table 1: Ozone-like injury such as total number of plants injured, number of leave injured, chlorosis per plant, and necrosis of groundnut cultivars.

Cultivars	DAS	Total no. of plant plots^{-1}	Total No. of plant injured plots^{-1}	No. of leaf injured plant^{-1}	Chlorosis plant^{-1}	NECRO plant^{-1}
	20 DAS	50	2.33 ± 0.45	7.66 ± 0.67	5.66 ± 0.87	2.33 ± 0.33
TG-37A	40 DAS	20	13.33 ± 0.73	13.66 ± 0.86	11.66 ± 0.66	2.66 ± 0.67
	60 DAS	15	7.66 ± 1.67	18.33 ± 1.33	15.33 ± 0.78	3.00 ± 1.33
	20 DAS	50	2.33 ± 1.33	5.33 ± 0.93	3.66 ± 0.12	2.00 ± 1.20
TPG-41	40 DAS	20	12.33 ± 1.33	13.33 ± 0.83	11.33 ± 1.50	3.00 ± 1.50
	60 DAS	15	6.66 ± 0.67	15.66 ± 1.67	12.33 ± 2.12	3.33 ± 0.93
	20 DAS	50	1.33 ± 1.33	7.33 ± 0.63	4.00 ± 1.1	2.00 ± 1.10
TAG-24	40 DAS	20	8.33 ± 0.83	11.00 ± 1.80	8.33 ± 1.33	2.66 ± 0.67
	60 DAS	15	8.00 ± 0.1	14.33 ± 0.33	10.00 ± 1.50	4.00 ± 1.10
	20 DAS	50	3.66 ± 1.7	13.00 ± 1.56	8.33 ± 1.03	3.33 ± 0.73
GG-20	40 DAS	20	15.33 ± 0.63	18.33 ± 1.33	16.00 ± 1.02	3.66 ± 0.55
	60 DAS	15	9.66 ± 0.57	21.00 ± 1.52	17.33 ± 1.23	4.33 ± 0.80
	20 DAS	50	2.66 ± 1.67	7.33 ± 1.30	8.66 ± 1.57	3.66 ± 0.66
Dh-86	40 DAS	20	16.66 ± 1.87	19.00 ± 1.11	16.33 ± 1.33	4.33 ± 0.50
	60 DAS	15	12.66 ± 1.97	22.00 ± 1.28	19.00 ± 1.38	5.33 ± 0.28

Figure 2: Ozone-like visible injury on selected groundnut cultivars. (A) ambient ozone, (B) chlorosis and necrotic spots, and (C) visible injury on TG-37A, TPG-41, TAG-24, GG-20, and Dh-86.

were found to be higher than the necrotic spots in all the selected cultivars. Injury was identified as ozone-like because it was either absent or very less in ambient ozone (data not mentioned). Ozone visible injury was higher in older leaves than in younger leaves. The number of injured leaves was increased with the duration of treatment and was found to be maximum at 60 DAS in all the cultivars tested. The maximum number of injured plants was noted in cultivar Dh-86 (12.66 m^{-2}) and the minimum injury was noted in cultivar TAG-24 (6.66 m^{-2}). The number of injured leaves was also higher in cultivar Dh-86 (22 $plant^{-1}$) at 60 DAS and the minimum number of injured leaf was found in cultivar TPG-41 (5.33 $plant^{-1}$) at 20 DAS. The trends of injury among the selected cultivars were GG-20 > Dh-86 > TG-37A > TPG-41 > TAG-24 under the acute level of ozone (Fig. 2) at all the sampling durations.

3.2. Plant Morphology

An acute level of ozone changed the morphology of tested groundnut cultivars. At the initial growth stage, the number of leaves of all the cultivars remained similar for enhanced ozone and control plants; however, it was highly affected by an enhanced level of ozone at latter growth stages. Maximum variation in the total number of leaves per plant was noticed in cultivar Dh-86 (−40%) at 40 DAS, while minimum reduction was found in cultivar TPG-41 (−6.52%) at 60 DAS due to the enhanced level of ozone (Table 2).

Similar to the number of leaves, shoots and root branching of selected groundnut cultivars were also affected by enhanced ozone exposure (Table 2). The effect of ozone on branching groundnut cultivars was higher during the early growth stage. Branching of the shoot was highly affected in cultivar TAG-24 (−66.66%) at 40 DAS and cultivar TPG-41 showed minimum reduction in the number of shoot branches (−16.75%) at 60 DAS. A higher reduction in root branching was found in cultivar TG-37A (−36.60%) at 60 DAS, while minimum root branching reduction was found in cultivars GG-20 (−2.30%) at 40 DAS.

Table 2: Number of roots, number of leaves, and number of branches of groundnut cultivars under enhanced ozone and ambient ozone.

Cultivars	Treatments	No. of root 20 DAS	40 DAS	60 DAS
TG-37A	Enhanced ozone	4.00 ± 0.06	13.66 ± 0.67	15.00 ± 0.53
	Ambient ozone	5.00 ± 0.05	16.00 ± 0.25	23.66 ± 0.66
	No. of leaf			
	Enhanced ozone	4.00	8.00 ± 0.3	16 ± 0.57
	Ambient ozone	4.00	10.00 ± 0.5	19.66 ± 0.67
	No. of branch			
	Enhanced ozone	0.00	3.00 ± 0.36	3.33 ± 0.33
	Ambient ozone	0.00	3.00 ± 0.54	4.66 ± 0.66
TPG-41	No. of root			
	Enhanced ozone	4.33 ± 0.33	10.33 ± 0.33	13.33 ± 0.33
	Ambient ozone	5.00 ± 0.33	13.66 ± 0.67	16.33 ± 0.33
	No. of leaf			
	Enhanced ozone	4.00	7.00 ± 0.50	14.33 ± 0.33
	Ambient ozone	4.00	11.00 ± 0.50	15.33 ± 0.33
	No. of branch			
	Enhanced ozone	0.00	2.66 ± 0.67	3.33 ± 0.33
	Ambient ozone	0.00	3.66 ± 0.67	4.00 ± 0.68
TAG-24	No. of root			
	Enhanced ozone	4.66 ± 0.66	10.33 ± 0.33	12.00 ± 0.65
	Ambient ozone	5.66 ± 0.70	14.66 ± 0.66	16.00 ± 0.33
	No. of leaf			
	Enhanced ozone	4.00	6.00 ± 0.30	10.00 ± 0.65
	Ambient ozone	4.00	8.00 ± 0.01	14.33 ± 0.33
	No. of branch			
	Enhanced ozone	0.00	1.00 ± 0.28	1.66 ± 0.67
	Ambient ozone	0.00	3.00 ± 0.24	4.60 ± 0.67
GG-20	No. of root			
	Enhanced ozone	5.33 ± 0.33	14.00 ± 0.35	15.00 ± 0.08
	Ambient ozone	6.33 ± 0.33	14.33 ± 0.33	15.66 ± 0.66
	No. of leaf			
	Enhanced ozone	4.00	7.00 ± 0.2	18.00 ± 0.25
	Ambient ozone	4.00	9.00 ± 0.25	22.00 ± 0.22
	No. of branch			
	Enhanced ozone	0.00	2.00 ± 0.25	2.33 ± 0.33
	Ambient ozone	0.00	3.00 ± 0.34	3.66 ± 0.67
Dh-86	No. of root			
	Enhanced ozone	4.66 ± 0.7	12.00 ± 0.36	13.00 ± 0.54
	Ambient ozone	6.00 ± 0.75	12.66 ± 0.67	14.00 ± 0.58
	No. of leaf			
	Enhanced ozone	4.00	6.00 ± 0.6	13.00 ± 0.35
	Ambient ozone	4.00	10.00 ± 0.8	15.00 ± 0.22
	No. of branch			
	Enhanced ozone	0.00	2.66 ± 0.67	3.00 ± 0.35
	Ambient ozone	0.00	3.66 ± 0.67	5.00 ± 0.57

3.3. Micro-Morphological Characteristics

The result of the study found that the enhanced level of ozone modified the micro-morphological characteristics of groundnut cultivars. Enhanced level of ozone increased the number of stomata and EC in all selected groundnut cultivars (Fig. 3). Among the tested crop cultivars, maximum increase in stomata was recorded in cultivar TPG-41 (62.5%) at 20 DAS, while a minimum increase in cultivar GG-20 (10%) at 40 DAS was noted. The trend of increase in stomata due to enhanced ozone was TPG-41 > TAG-24 > Dh-86 > TG-37A > GG-20. While the highest increase in EC was noted in cultivar TPG-41 (33.33%) at 60 DAS, the lowest number of EC count was noted in cultivar TG-37A (5.45%) at 20 DAS. The trends of increase were TPG-41 > GG-20 > TAG-24 > Dh-86 > TG-37A. Higher SI was also recorded among the plants grown under an enhanced level of ozone (Fig. 3). Maximum SI was found in cultivar TG-37A (27.06) at 60 DAS and minimum in cultivar Dh-86 (14.63) at 20 DAS. The trends of increase in SI under enhanced ozone exposure is TG-37A > TPG-41 > TAG-24 > Dh-86 > GG-20.

Enhanced ozone also interferes with the stomatal opening of plant leaves. The present study shows that exposure of groundnut plants to enhanced ozone deduced stomata openings and maximum stomatal pores were closed in enhanced ozone-exposed plants than ambient ozone (Figs. 4 and 5).

3.4. Anatomical Modification

Elevated ozone also influences the internal structure of the plant leaf (Fig. 6). An enhanced ozone-treated plant showed thin epidermis than ambient ozone-treated plants. Xylem and phloem of the foliage were also affected under enhanced ozone exposure. Vessel elements of xylem reduced in size, despite the increase in the number. In contrast to xylem, the phloem tissue was expanded but became significantly disorganized and collenchyma was reduced (Fig. 6). An enhanced level of ozone also affected the palisade cells and the damage can be seen in Figure 6B. Mesophyll cells were also reduced in size than ambient ozone plants. Xylem and phloem of elevated ozone-treated plants become compact with minimum distancing and size (Fig. 6C and D).

3.5. Regression and Three-Way ANOVA Test

Data were analyzed by regression and ANOVA (three factors) test for significant variation between treatments, age, and cultivars of selected parameters. Pearson's correlation analysis with R^2 values nearest to one shows a strong relationship with the number of injured plants per plot (Fig. 7). The number of stomata and EC shows a strong relationship when compared to other parameters, while the number of injured leaves per plant was highly correlated with chlorosis of leaves per plant in cultivar TG-37A. The highest R^2 (37%) value was found in the number of injured leaves per plant. In cultivar TPG-41, the highest R^2 (75%) value was noted in necrotic spots per plant. Chlorosis (CHLO) and necrotic showed a strong relationship with each other, while the number of injured leaves slightly correlated with these parameters. EC, chlorosis, and the number of stomata highly correlated in cultivar TAG-24 with R^2 (95%) values are same in all three parameters, while the number of injured leaves is slightly correlated (Fig. 7). For cultivar GG-20, the plant necrotic spots showed higher R^2 values (99%) and chlorosis of the plant was slightly correlated with R^2 value (73%). The higher R^2 value of cultivar Dh-86 was found in SI (92%) and

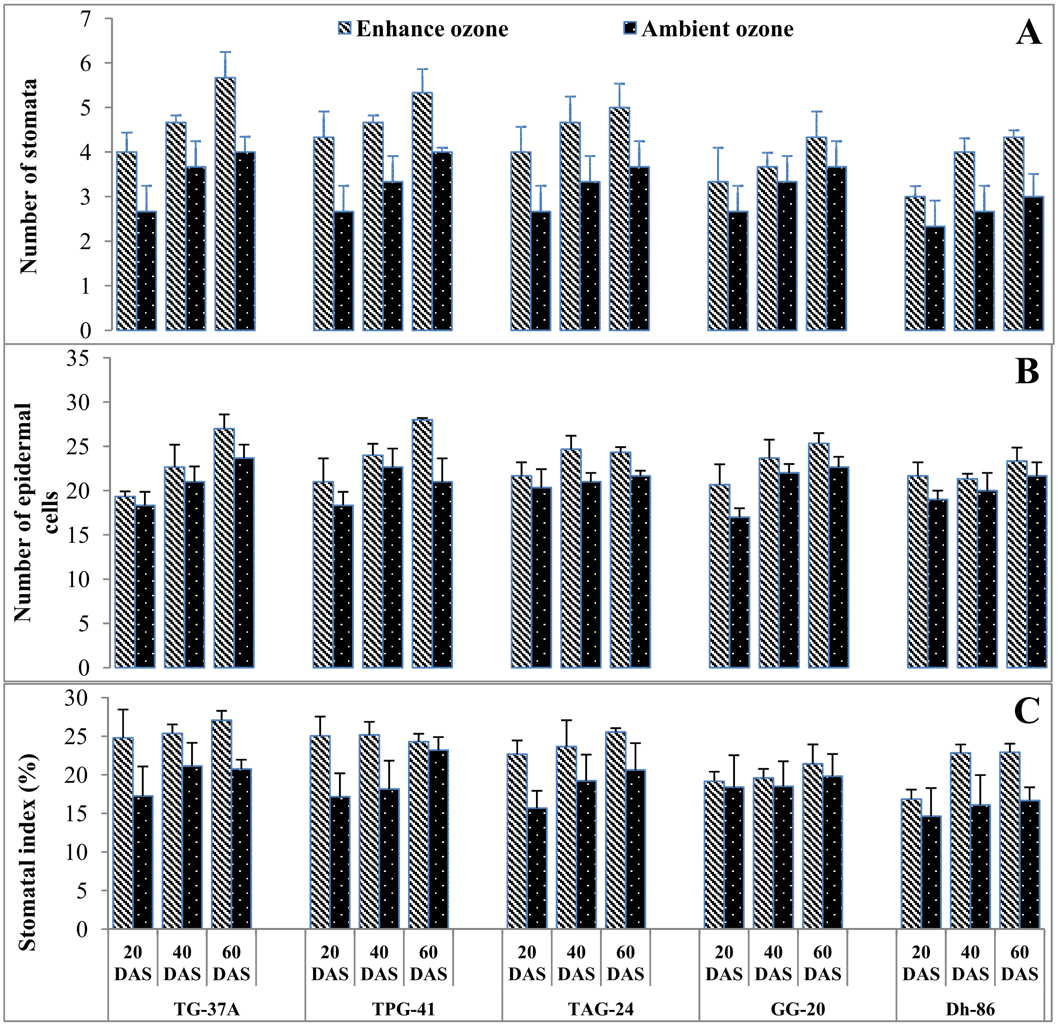

Figure 3: Microscopic observation. (A) Number of stomata, (B) number of EC, and (C) SI (%) of groundnut cultivars under enhance and ambient ozone-treated plants. (Mean ± standard deviation of three replicates presented by thin vertical bars.)

the lowest R^2 value was for the number of EC (10%). The number of injured leaves, stomata, and chlorosis was slightly correlated with SI. Overall, sensitive cultivars showed a strong relationship with the number of injured plants per plot. In sensitive cultivars, the number of injured plants per plot was higher; therefore, the number of injured leaves per plant and chlorosis necrosis was also higher (Fig. 7).

Three-way ANOVA test (three-factor ANOVA) confirmed the significant levels of treatment, DAS, and cultivars (Table 3). Cultivars wise the number of stomata significantly varied at $p < 0.01$ level and the level of significance of SI was $p < 0.05$, while the number of EC showed no significant levels. Selected parameters, such as the number of stomata, EC, and SI of groundnut, were highly significant with treatments ($p < 0.001$). The level of significance of the number of stomata and EC showed the same values ($p < 0.01$), while the significant level of SI was $p < 0.05$. Selected parameters' relationship with Cult.*Treat. Cult.*DAS, Treat.* DAS, and Cult.*Treat.*DAS showed no significant level, except the number of EC; the significant level of the number of EC was $p < 0.05$.

4. DISCUSSION

The present study shows that the enhanced level of ozone caused a negative effect on groundnut cultivars. The micro-morphological and anatomical modifications observed in selected cultivars show higher variability in the injuries of plants. On the basis of morphological, micro-morphological, and anatomical modification, ozone-sensitive cultivars were highly affected than ozone-tolerant cultivars. Besides having global warming potential, ozone is one of the major gaseous pollutants that directly affected plant growth and productivity [7,9,10,28]. However, its effect depends on the genotype and prevailing environmental condition [29,30].

Foliage is the primary plant organ that is exposed to ozone and shows visible symptoms. Foliar injury is generally the first visible sign of injury to plants from ozone exposure and indicates lessened physiological processes in the leaves [31]. However, these symptoms vary with the genotypes and are restricted to sensitive species and the plant site exposed to the ozone [32]. All the groundnut cultivars tested in the present experiment showed injury under enhanced ozone exposure. However, the total number

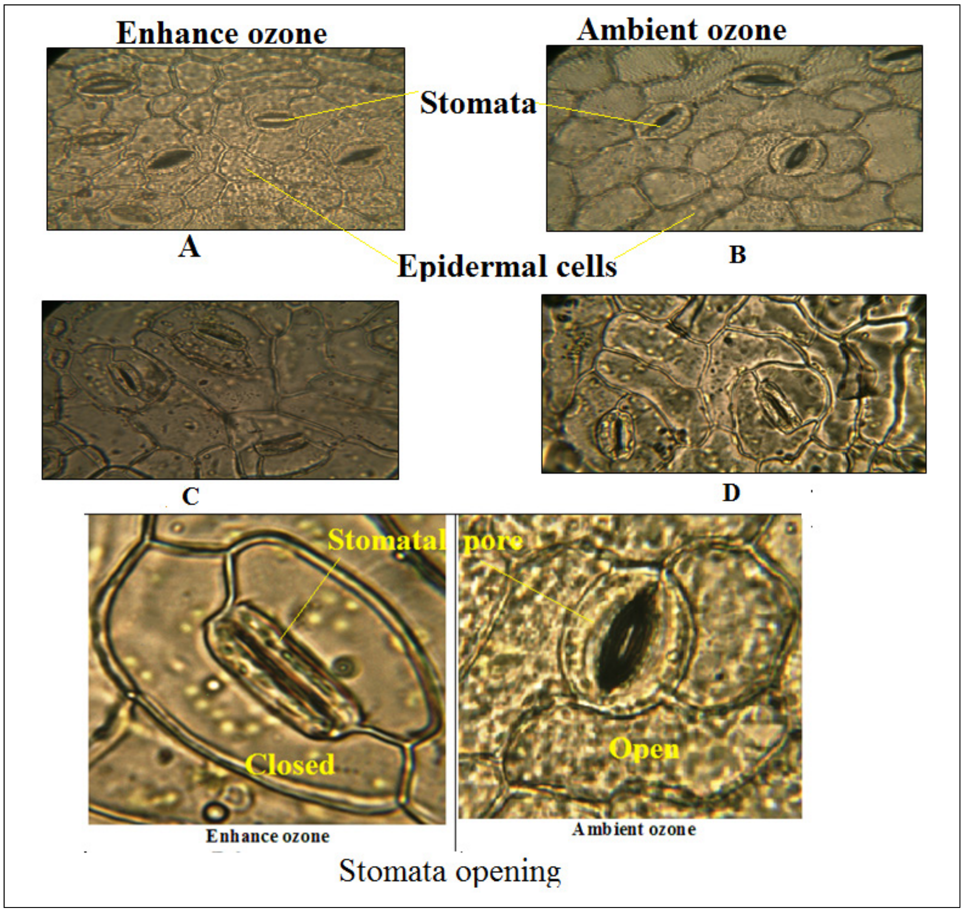

Figure 4: Microscopic observation of the number of stomata, number of EC, and stomatal opening of groundnut cultivar TPG-41. (A) Enhanced ozone, (B) ambient ozone and cultivar Dh-86, (C) enhanced ozone, and (D) ambient ozone.

of plant affected and the number of leave affected in each plant was varied. Leung et al. [28] explained that the sensitive species had a higher injury than resistant species. A higher number of chlorosis and necrotic spots were found in cultivar Dh-86, suggesting its higher sensitivity to ozone, while cultivar TPG-41 showed reduced sensitivity to ozone with the least number of chlorosis and necrotic spots. Similar to this study, Basahi et al. [33] and Islam et al. [34] also reported visible ozone injury under ambient ozone to olive and mung bean plants, respectively. Hayes et al. [35] reported increased ozone-induced leaf injury in *Phaseolus vulgaris* with increased ozone exposure. In the present experiment, we also found that the ozone-induced injury was higher in mature leaves. This may be due to the longer duration to ozone exposure to older leaves than younger ones.

The reduction in the number of leaves per plant is an indicator of an unhealthy and stressed condition. The reduction in leaves per plant reduced total carbon assimilation and NPP of the ecosystem. The reduction in leave was seen in all the groundnut cultivars, despite their sensitivity to ozone. However, a higher sensitive cultivar Dh-86 had a higher reduction and tolerant cultivar TPG-41 had the least reduction in leaves. Consistent production of new leaves can be a reason for lesser leaf reduction in tolerant cultivars. Leung et al. [28] reported the production of new leaves in tolerant cultivar of *Phaseolus vulgaris* which reduces the percentage of ozone-affected leaves. Furthermore, the least affected branching of tolerant species also maintained the number of leaves. Similar to our study, Cotrozzi et al. [36] also reported 25%–60% phylloptosis in *Quercus* cultivars under ozone exposure. The minimum reduction in the number of branches per plant in cultivar TPG-41 is also an indication of tolerance of this cultivar than other cultivars. Tsukahara et al. [37] reported ozone-induced reduction in rachis branches in two cultivars of rice and explained this as the effect of ozone on genes near RM3430 markers. However, a surprising reduction in branching of root under enhanced ozone cannot be explained as ozone effect as the effect of ozone on underground plant part is not direct [38].

Stomata development during cell differentiation is confirmed to be regulating by genes that are simultaneously regulating physiological parameters, such as stomatal conductance. The present study found an increase in stomata as well as SI under enhanced ozone exposure in all the groundnut cultivars, suggesting a reduced size of EC under ozone pollution. Islam et al. [34] reported that the ozone pollution interferes with stomatal functioning causing increases in conductance, sluggish stomatal response to environmental factors, or stomatal closure, depending on the species and ozone exposure. Wang et al. [39] and Taiz and Zeiger [26] suggested that between sensitive leaves, young mature ones that developed after the plant had been exposed to significant

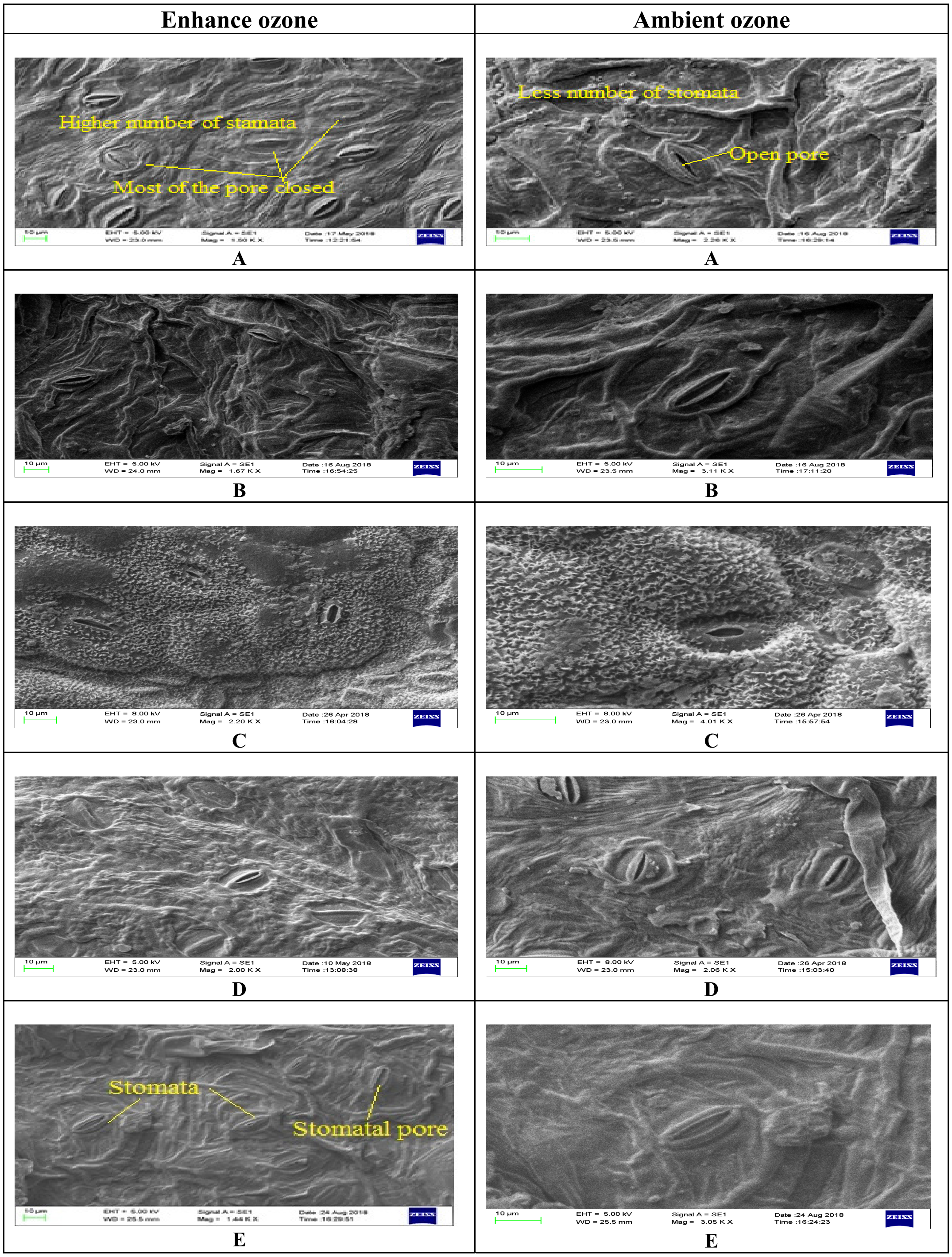

Figure 5: Micro-morphological modification identified by SEM of selected groundnut cultivars (A) TG-37A, (B) TPG-41, (C) TAG-24, (D) GG-20, and (E) Dh-86 under enhanced and ambient ozone.

cumulative doses of ozone stopped cell division of EC earlier than the old ones did. From another aspect, leaves which developed later maintained generous cell growth for a longer duration. This phenomenon may be due to ozone-convinced oxidative stress, which changes the redox state of cells and hence proliferation. The expansive growth of cell decreased the stomata number per mm^2 of epidermis in sensitive strain and increased EC size. Guard cells development changed similarly after the plants had been exposed to the climate of the summer season and high cumulative ozone doses; the recently developed leaves of sensitive plants had higher-sized guard cells and thus found the larger stomatal apparatus. Cultivar TPG-41 showed maximum increase in EC suggesting its adaptability to enhanced ozone by reducing epidermal cell size and stomatal pores to reduce conductance. The idea of SI normalizes the epidermal cell expansion effect on the density of stomata [25]. SI (Fig. 2) of sensitive strain was mainly lower, but

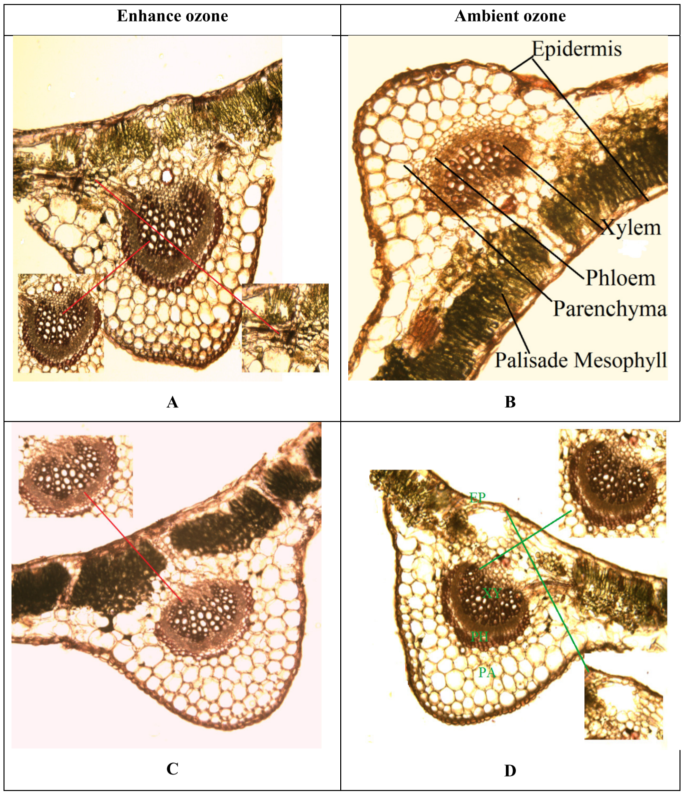

Figure 6: Cross-section of groundnut ozone-tolerant cultivar TPG-41. (A) Enhanced ozone, (B) ambient ozone and ozone-sensitive cultivar Dh-86, (C) enhanced ozone, and (D) ambient ozone.

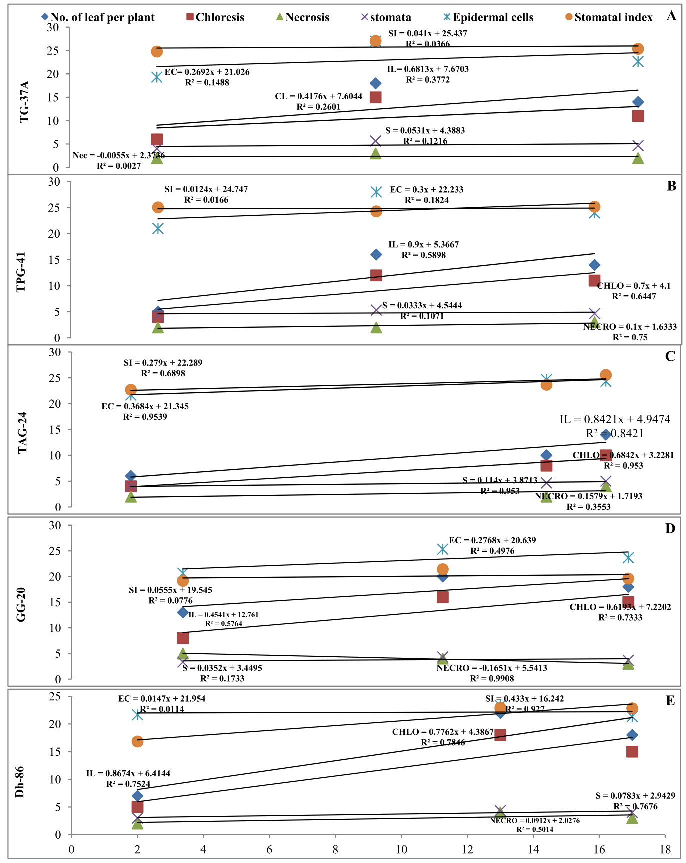

Figure 7: Pearson's correlation analysis between the total number of injured plants at 60 DAS of plant in response to number of leave injured per plant (IL), CHLO, Necrosis (NECRO), number of stomata (S), number of EC, and SI of groundnuts cultivars (A) TG-37A, (B) TPG-41, (C) TAG-24, (D) GG-20, and (E) Dh-86. Coefficient (R^2) value close to 1 expressed the strong positive correlation in between the subsets.

this difference was more significant in resistant genotypes, which developed an increase in stomata numbers, while sensitive plants had a lesser number of stomata either as an attempt to reimburse the adverse effects of ozone or as a consequence of ozone which would have caused the change in cell division and developments. If the purpose of this change had been to lower the accession of the contaminant, it should have also resulted in lower gas condition and/or transpiration rate. The reason of the different stomatal developments could have possibly been ozone-inhibited cell division and/or increased expansive growth of cells, which comparatively decreased the stomata numbers per mm^2 epidermal area. Chaudhary and Rathore [2] and Qadir et al. [40] reported higher stomata indexes due to oxidative stresses.

Moreover, the present experiment also found that the enhanced ozone leads to closure of stomata of all the experimental groundnut cultivars. This would reach a reduction in evapotranspiration and water use efficiency, including reduced capability to uptake soil water and an increase in sensible heat flux as seen in soybean by VanLoocke et al. [41] and Bou Jaoudé et al. [42]. Although stomatal closure seems to be the principal response in crops, previous studies have proposed that under chronic ozone exposure, ozone-induced eminent production of stress ethylene can lead to a reducing of the abscisic acid signal [43 ,44], which would normally lead to decreases in gas to conserve water in dry soils [45]. This could result in the crop losing control of stomatal closure, impairing water loss, and enhancing ozone uptake that would otherwise be limited by soil water stress, thus generating a feedback loop that enhances ozone damage. The present study also found that the elevated ozone caused stomatal and EC modifications and caused succulents guard cells under exposure of enhanced ozone.

Table 3: *F* ratio and level of significance of number of stomata, number of EC, and SI of groundnut cultivars obtained by ANOVA test.

Parameters	Cult.	Treat.	DAS	Cult. ×Treat.	Cult. ×DAS	Treat. × DAS	Cult. ×Treat. ×DAS
Number of stomata	3.53**	40.35***	13.00***	0.67NS	0.12NS	0.14NS	0.14NS
Number of EC	1.18NS	37.57***	33.40***	0.57NS	1.97*	1.24NS	0.94NS
SI	2.50*	19.49***	2.92*	0.70NS	0.13NS	0.08NS	0.35NS

Significant levels, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; NS = not significant.

Ozone-induced anatomical changes in groundnut cultivars were also observed in the present study. Mitu et al. [46] reported changes in spongy parenchyma, epidermis, and vascular bundles of leaves and stems of mango, mahogany, and koroi due to continuous exposure of pollutants. Reduced vessel size in leaves of groundnut suggested reduced water transport and can be correlated with stomatal closure. It would be vital to emphasize that a more detailed anatomical assessment is needed with variable species and cultivars to justify the effect of ozone on internal tissues of plants.

5. CONCLUSION

Ozone is a toxic gaseous pollutant that causes a negative effect on the micromorphology and anatomy of groundnut plants. The study observed ozone-like visible injury symptoms on all the groundnut cultivars using OTC. The results of the study showed that the visible injury was maximum in cultivar Dh-86 and minimum was in cultivar TPG-41. Micro-morphological characteristics, such as the number of stomata, EC, and SI, were increased due to elevated levels of ozone. The higher SI was found in cultivar TPG-41, while cultivars TAG-24 > TG-37A > and Dh-86 showed moderate values and the lowest SI was noted in cultivar GG-20. The enhanced level of ozone injured the leaves, micromorphology, and anatomy of all the groundnut cultivars. The results also confirmed that the stomatal closure and anatomical characteristics such as xylem, phloem, collenchyma, and mesophyll cells disorganization are important characteristics for the identification of ozone-resistant variety. On the basis of stomatal movement and distribution, anatomical changes in cultivar TPG-41 were shown as the most ozone-resistant groundnut variety among the tested cultivars. However, more studies with variable plant species and different cultivars are needed for a substantial conclusion.

Acknowledgments

The authors are grateful to the School of Environment and Sustainable Development, Central University of Gujarat, for providing necessary laboratory and field facilities, and Central Instrumentation Facility, Central University of Gujarat, for instrumentation support. The authors are also thankful to Science and Engineering Research Board (SERB), New Delhi, for financial support in the form of project (File No. YSS/2015/000724).

REFERENCES

1. Chaudhary IJ, Rathore D. Suspended particulate matter deposition and its impact on urban trees. Atmos Pollut Res 2018;9:1072–82; doi:10.1016/j.apr.2018.04.006
2. Chaudhary IJ, Rathore D. Dust pollution: its removal and effect on foliage physiology of urban trees. Sustain Cities Soc 2019;51:101696; doi:10.1016/j.scs.2019.101696
3. Chaudhary IJ, Rathore D. Phytomonitoring of dust load and its effect on foliar micro morphological characteristics of urban trees. J Plant Sci 2018b;2(3):170–9.
4. Wang Y, Konopka P, Liu H, Chen H, Müller R, Plöger F, et al. Tropospheric ozone trend over beijing from 2002-2020: ozonesonde measurements and modeling analysis. Atmos Chem Phys 2012;12:11175–99; doi:10.5194/acpd-12-11175-2012
5. Verstraeten WW, Neu JL, Williams JE, Bowman KW, Worden JR, Boersma KF. Rapid increases in tropospheric ozone production and export from China. Nat Geosci 2015;8:690–5; doi:10.1038/ngeo2493
6. Sitch S, Cox PM, Collins WJ, Huntingford C. Indirect radiative forcing ofclimate change through ozone effects on the land-carbon sink. Nature 2007;448:791–4; doi:10.1038/nature06059
7. Rathore D, Chaudhary IJ. Ozone risk assessment of castor (*Ricinus communis* L.) cultivars using open top chamber and ethylenediurea (EDU). Environ Pollut 2019;244:257–69.
8. Chaudhary IJ, Rathore D. Relative effectiveness of ethylene diurea, phenyl urea, ascorbic acid and urea in preventing groundnut (*Arachis hypogaea* L) crop from ground level ozone. Environ Technol Innov 2020;19:100963; doi:10.1016/j.eti.2020.100963
9. Feng ZZ, Niu JJ, Zhang WW, Wang XK, Yao FF, Tian Y. Effects of ozone exposure on sub-tropical evergreen *Cinnamomum camphora* seedlings grown in different nitrogen loads. Trees 2011;25:617–25; doi:10.1007/s00468-011-0538-x
10. Zhu X, Feng Z, Sun T, Liu X, Tang H, Zhu J, Guo W, Kobayashi K. Effects of elevated ozone concentration on yield of four Chinese cultivars of winter wheat under fully open-air field conditions. Glob Change Biol 2011;17:2697–706; doi:10.1111/j.1365-2486.2011.02400.x
11. Singh S, Agrawal SB. Use of ethylene diurea (EDU) in assessing the impact of ozone on growth and productivity of five cultivars of Indian wheat (*Triticum aestivum* L.). Environ Monit Assess 2009;159:125–41; doi:10.1007/s10661-008-0617-7
12. Agathokleous E. Perspectives for elucidating the ethylenediurea (EDU) mode of action for protection against O_3 phytotoxicity. Ecotoxicol Environ Saf, 2017;142:530–7.
13. Peng J, Shang B, Xu Y, Feng Z, Calatayud V. Effects of ozone on maize (*Zea mays* L.) photosynthetic physiology, biomass and yield components based on exposure- and flux-response relationships. Environ Pollut, 2020;256:113466.
14. Zhang L, Jia LL, Sui JX, Wen MX, Chen YJ. Ameliorating effects of three kinds of antioxidants to ozone-polluted painted nettle (*Coleus blumei* Benth.). Photosynthetica 2018;56(2):623–32; doi:10.1007/s11099-017-0693-4
15. Castagna A, Ranieri A. Detoxification and repair process of ozone injury: from O_3 uptake to gene expression adjustment. Environ Pollut, 2009;157:1461–9.
16. Nadeau JA, Sack FD. Stomatal development in arabidopsis. Arabidopsis Book 2002;1:e0066; doi:10.1199/tab.0066.
17. Miyazawa SI, Livingston NJ, Turpin DH. Stomatal development in new leaves is related to the stomatal conductance of mature leaves in poplar (*Populus trichocarpa* x P. deltoides). J Exp Bot, 2006;57:373–80.
18. Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S. Stomatal development and patterning are regulated by environmentally responsive mitogen activated protein kinases in Arabidopsis. Plant Cell 2007;19:63–73.
19. Navea C, Terrazas T, Delgado-Salinas A, Ramírez-Vallejo P. Foliar response of wild anddomesticated *Phaseolus vulgaris* L. to water stress. Genet Resour Crop Evol 2002;49:125–32.
20. Paakkönen E, Holopainen T, Kaarenlampi L. Variation in ozone sensitivity of *Betula pendula* and *Betula pubescens* clones from southern and central Finland. Environ Pollut, 2007;95:37–44.
21. Aasamaa K, Sober A, Rahi M. Leaf anatomical characteristics associated with shoot hydraulic conductance, stomatal conductance and stomatal sensitivity to changes of leaf water status intemperate deciduous trees. Aust J Phys 2000;28:765–74.
22. Paoletti E, Contran N, Manning WJ, Ferrara AM. Use of the antiozonant ethylenediurea (EDU) in Italy: verification of the effects of ambient ozone on crop plants and trees and investigation of EDU's mode of action. Environ Pollut 2009;157:1453–60.
23. Ferdinand JA, Fredericksen TS, Kouterick KB, Skelly JM. Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. Environ Pollut 2007;108:27–302.
24. Gostin IN. Air pollution effects on the leaf structure of some Fabaceae species. Not Bot Horti Agrobotanici 2009;37:57–63.
25. Stengleina SA, Arambarria AM, del Carmen M, Sevillanod M, Balattib PA. Leaf epidermal characters related with plant's passive resistance to pathogens vary among accessions of wild beans Phaseolus vulgaris var. aborigineus (Leguminosae– Phaseoleae). Flora 2005;200:285–95.

26. Taiz L, Zeiger E. Secondary metabolites and plant defense, In: Taiz L, Zeiger E (eds.). Plant physiology. 5th edition, Sinauer Associates Inc, Sunderland, MA, 2010.
27. Salisbury EJ. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. Philos Trans R Soc Lond B 1927;216:1–65.
28. Leung F, Pang JYS, Tai APK, Lam T, Tao DKC, Sharps K. Evidence of ozone-induced visible foliar injury in Hong Kong using *Phaseolus vulgaris* as a bioindicator. Atmosphere 2020;11:266; doi:10.3390/atmos11030266
29. Feng Z, Buker P, Pleijel H, Emberson L, Karlsson PE, Uddling J. A unifying explanation for variation in ozone sensitivity among woody plants. Glob Change Biol 2018;24:78–84; doi:10.1111/gcb.13824
30. Singh AA, Chaurasia M, Gupta V, Agrawal M, Agrawal SB. Responses of *Zea mays* L. cultivars 'Buland' and 'Prakash' to an antiozonant ethylene diurea grown under ambient and elevated levels of ozone. Acta Physiologiae Plant 2018;40:92; https://doi.org/10.1007/s11738-018-2666-z
31. Grulke NE. The physiological basis of ozone injury assessment attributes in Sierran conifers. In: Bytnerowicz A, Arbaugh MJ, Alonso R (eds.). Ozone air pollution in the Sierra Nevada: distribution and effects on forests. Elsevier Science, Ltd., New York, NY, pp 55–81, 2003.
32. Smith GC, Coulston JW, O'Connell BM. Ozone bioindicators and forest health: a guide to the evaluation, analysis, and interpretation of the ozone injury data in the Forest Inventory and Analysis program. Gen Tech Rep NRS34. U.S. Department of Agriculture, Forest Service, Northern Research Station, Newtown Square, PA, 2008. Available via http://www.nrs.fs.fed.us/pubs/gtr/gtr_nrs34.pdf (PDF) (Accessed 13 July 2020).
33. Basahi JM, Ismail IM, Haiba NS, Hassan IA, Lorenzini G. Assessing ambient ozone injury in olive (*Olea europaea* L.) plants by using the antioxidant ethylenediurea (EDU) in Saudi Arabia. Environ Monit Assess 2016;188(6):371.
34. Islam MT, Sattar MA, Hasmi QSI, Bueker P, Emberson L. Measurement of tropospheric ozone and effects of ethylene diurea on ozone injuries of wheat and mung bean at farming area of Bangladesh agricultural university, mymensingh Bangladesh. Bangladesh J Environ Sci 2015;28:1–8.
35. Hayes F, Sharps K, Harmens H, Roberts I, Mills G. Tropospheric ozone pollution reduces the yield of African crops. J Agron Crop Sci 2019;206(2):214–28.
36. Cotrozzi L, Remorini D, Pellegrini E, Landi M, Massai R, Nali C, et al. Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress. Physiol Plant 2016;157:69–84; doi:10.1111/ppl.12402
37. Tsukahara K, Sawada H, Kohno Y, Matsuura T, Mori IC, Terao T, et al. Ozone induced rice grain yield loss is triggered via a change in panicle morphology that is controlled by ABERRANT PANICLE ORGANIZATION 1 Gene. PLoS One 2015;10(4):e0123308; doi:10.1371/journal.pone.0123308
38. Shang B, Feng Z, Li P, Calatayud V. Elevated ozone affects C, N and P ecological stoichiometry and nutrient resorption of two poplar clones. Environ Pollut 2018;234:136–44.
39. Wang XK, Manning W, Feng ZW, Zhu YG. Ground level ozonein China: distribution and effects on crop yields. Environ Pollut 2007;147:394–400; doi:10.1016/j.envpol.2006.05.006
40. Qadir SU, Raja V, Siddiqui WA. Morphological and biochemical changes in *Azadirachta indica* from coal combustion fly ash dumping site from a thermal power plant in Delhi, India. Ecotoxicol Environ Saf 2016;129:320–8.
41. VanLoocke A, Betzelberger AM, Ainsworth EA, Bernacchi CJ. Rising ozone concentrations decrease soybean evapotranspiration and water use efficiency whilst increasing canopy temperature. New Phytol 2012;195:164–71; doi:10.1111/j.1469-8137.2012.04152.x
42. Bou Jaoudé M, Katerji N, Mastrorilli M, Rana G. Analysis of the ozone effect on soybean in the Mediterranean region. II. The consequences on growth, yield and water use efficiency. Eur J Agron 2008;28:519–25; doi:10.1016/j.eja. 2007.09.001
43. Wilkinson S, Davies WJ. Ozone suppresses soil drying- and abscisic acid (ABA)-induced stomatal closure via an ethylene-dependent mechanism. Plant Cell Environ 2009;32:949–59; doi:10.1111/j.1365-3040.2009.01970.x
44. Wilkinson S, Davies WJ. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. Plant Cell Environ 2010;33:510–25; doi:10.1111/j.1365-3040.2009.02052.x
45. Mills G, Hayes F, Wilkinson S, Davies WJ. Chronic exposure to increasing background ozone impairs stomatal functioning in grassland species. Glob Change Biol 2009;15:1522–33; doi:10.1111/j.1365-2486.2008.01798.x
46. Mitu KJ, Islam MA, Biswas P, Marzia S, Ali MA. Effects of different environmental pollutants on the anatomical features of roadside plants. Progress Agric 2019;30(4):344–51.

How to cite this article:
Chaudhary IJ, Rathore D. Micro-morphological and anatomical response of groundnut (*Arachis hypogaea* L.) cultivars to ground-level ozone. J Appl Biol Biotech 2021; 9(04):137–150.

SUPPLYMENTRY FIGURES

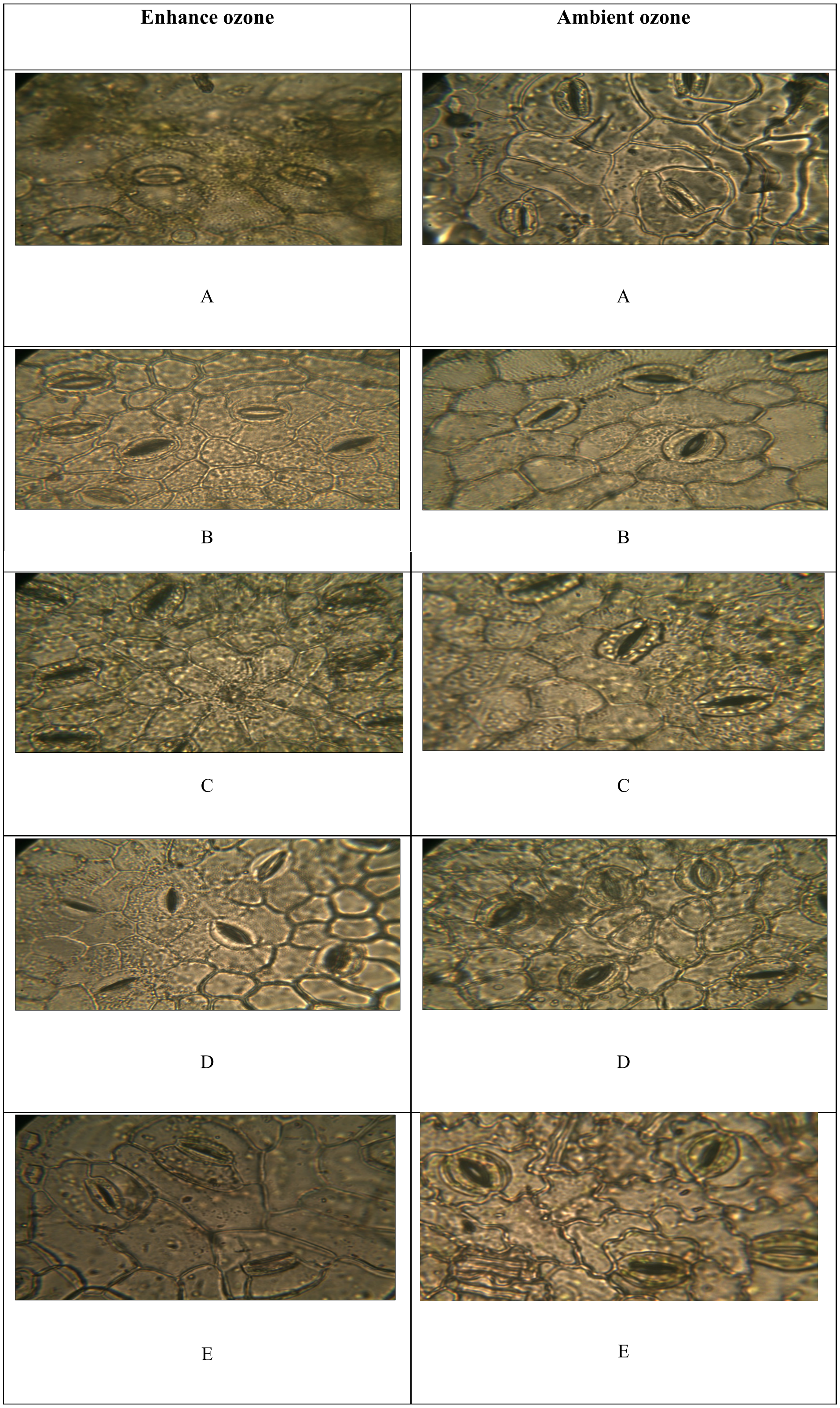

Figure S1: Modification of micro-morphological characteristics such as number of stomata, number of EC, and stomatal opening of groundnut cultivars (A) TG-37A, (B) TPG-41, (C) TAG-24, (D) GG-20 and (E) Dh-86 under enhanced ozone and ambient ozone.

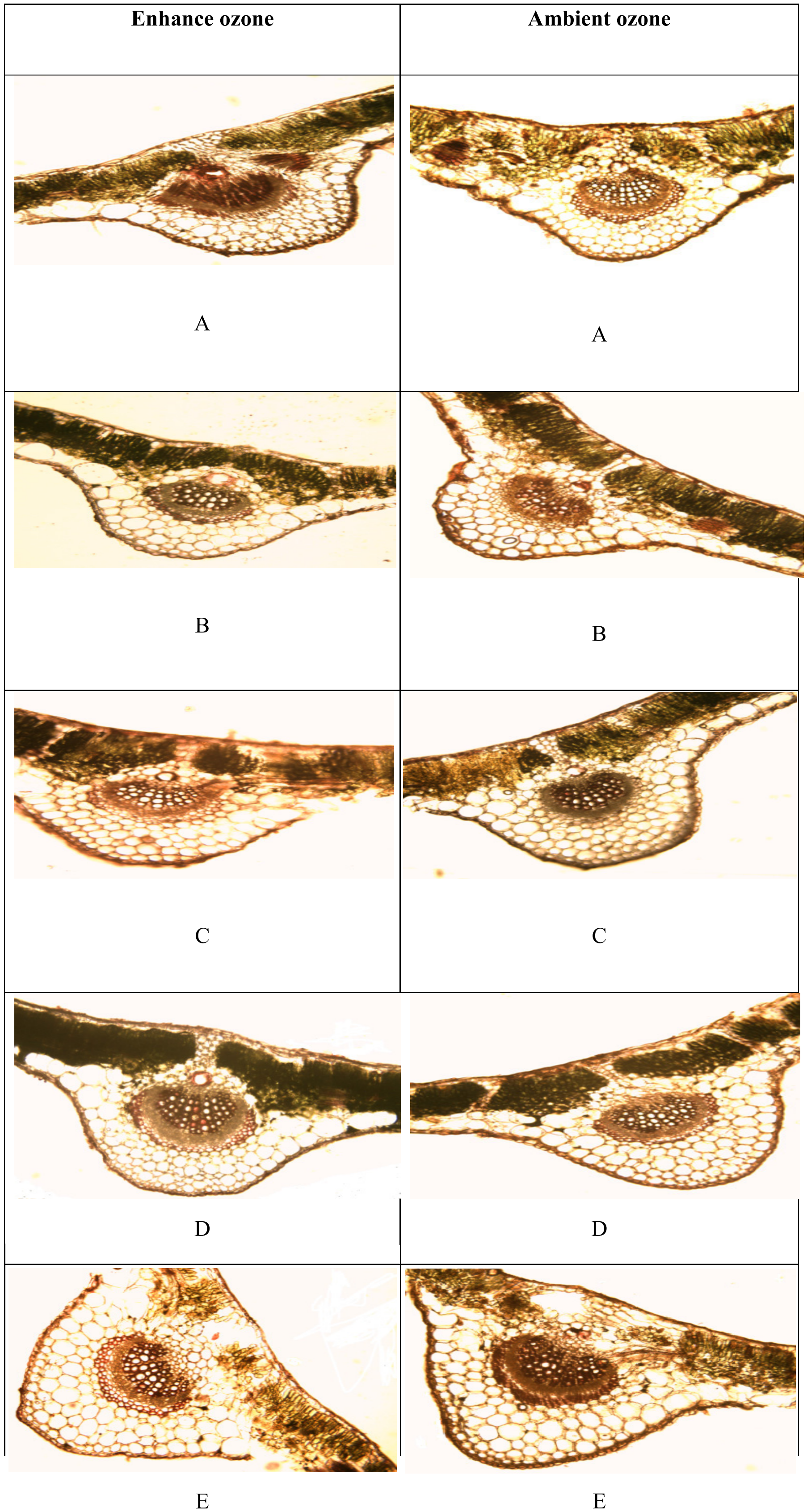

Figure S2: Cross-section of anatomical characteristic changes such as epidermis, xylem, phloem, parenchyma and palisade mesophyll cells of groundnut cultivars (A) TG-37A, (B) TPG-41, (C) TAG-24, (D) GG-20 and (E) Dh-86 under enhanced ozone and ambient ozone stress.

GRAPHICAL ABSTRACT