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Response of tropical trees to elevated Ozone: a Free Air Ozone Enrichment study

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Abstract Tropospheric ozone (O_3) has become one of the main urban air pollutants. In the present study, we assessed impact of ambient and future ground-level O_3 on nine commonly growing urban tree species under Free Air Ozone Enrichment (FAOE) condition. During the study period, mean ambient and elevated ozone (EO_3) concentrations were 48.59 and 69.62 ppb, respectively. Under EO_3 treatment, stomatal density (SD) significantly decreased and guard cell length (GCL) increased in *Azadirachta indica*, *Bougainvillea spectabilis*, *Plumeria rubra*, *Saraca asoca* and *Tabernaemontana divaricata*, while SD increased and GCL decreased in *Ficus benghalensis* and *Terminalia arjuna.* Proline levels increased in all the nine plant species under EO_3 condition. EO_3 significantly reduced photosynthetic rate, stomatal conductance (gs), and transpiration rates (E). Only *A. indica* and *N. indicum* showed higher gs and E under EO_3 treatment. Water use efficiency (WUE) significantly increased in *F. benghalensis* and decreased in *A. indica* and *T. divaricata*. Air Pollution Tolerance Index (APTI) significantly increased in *Ficus religiosa* and *S. asoca* whereas it decreased in *B. spectabilis* and *A. indica*. Of all the plant species *B. spectabilis* and *A. indica* were the most sensitive to EO_3 (high g_s and less ascorbic acid content) while *S. asoca* and *F. religiosa* were the most tolerant (low g_s and more ascorbic acid content). The sensitivity of urban tree species to EO_3 is a cause of concern and should be considered for future urban forestry programmes. Our study should guide more such studies to identify tolerant trees for urban air pollution abatement.

Keywords Ozone · Urban trees · Stomata · Photosynthesis · APTI

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Introduction

Rapid pace of industrialization and urbanization has resulted in high levels of air pollution and this problem is more common and serious in fast developing countries like China and India than the rest of the world (Health Effects Institute, 2019). Air pollution is generally caused by industries, power plants, automobiles using fossil fuel, and other agricultural practices including crop residue burning. Urban air pollution is

mainly caused by automobiles and roadside dust. Main pollutants include oxides of nitrogen (NOx), carbon monoxide (CO), sulfur dioxide (SO_2), ozone (O_3), and suspended particulate matter ($PM_{2.5}$ and PM_{10}). In most of the Indian cities including Lucknow (capital city of Uttar Pradesh), two main urban pollutants of concern are O_3 and particulate matter. Both of these pollutants have caused one of the highest mortality in India (Health Effects Institute, 2019). In India, there are reports of decrease in productivity of rice, wheat, and maize crops due to rising O_3 in Indo-Gangetic Plains (IGP) (Oksanen et al., 2013). Using GEOS-Chem model, Lu et al. (2018) predicted that in future O_3 pollution problem in India may become much severe.

Ambient O_3 is mainly produced by the photochemical reactions of air pollutants such as volatile organic compounds, carbon monoxide, and oxides of nitrogen in the presence of sunlight (Ainsworth, 2017). In humans, exposure to high level of O_3 may result in respiratory and cardiovascular diseases, and premature mortality (Levy et al., 2005). In plants, EO_3 causes reduced photosynthesis and accelerated senescence (Ainsworth, 2017; Wittig et al., 2009). Stomata play crucial role in gas exchange between plants and atmosphere. Plants control the amount of O_3 entering into the leaves through stomatal responses (Hoshika et al., 2015). Various leaf morphological characteristics (e.g., stomatal density and pore size, leaf weights and areas) are related to sensitivity/tolerance of plants to O_3.

Plants play a vital role in air pollution remediation (Nowak et al., 2014). Plants growing in urban environments mitigate significant amount of air pollution through filtering, intercepting and absorbing pollutants (Nowak et al., 2014). Manes et al. (2016) reported a monetary value of USD 47 and 297 million for PM_{10} and ozone removal, respectively by urban and peri-urban trees in 10 cities of Italy. Nowak et al. (2014) reported that trees and forests removed 14.4 million tons of air pollution in 2010 in the USA, with human health effects valued at 6.8 billion USD. Generally native/introduced plants are grown around roads and dividers depending upon their height, growth architecture, functional group (deciduous/evergreen), leaf structure etc. But plants respond differently to ambient air pollution. There have been reports of responses of tree species to air pollutants in terms of their relative tolerance or sensitivity (Gao et al., 2016; Wen et al., 2004). However, information on the impacts of current and future levels of O_3 on trees is scarce in India. We recently studied impact of long term (2 years) exposure of elevated ozone (+20 ppb above the ambient) on photosynthetic traits and anti-oxidative defense system of *Leucaena leucocephala*, a tree of great economic importance (Singh et al., 2021). It was found that there were several negative long lasting physiological and biochemical impacts on *Leucaena*. The results revealed that *Leucaena* exhibited greater sensitivity to O_3 during initial stage of exposure, i.e., up to 12 months than the later stage (Singh et al., 2021).

Air Pollution Tolerance Index (APTI) is used to evaluate relative sensitivity/tolerance levels of plants to air pollution. APTI uses four parameters, viz., leaf extract pH, relative water content (RWC), total chlorophyll, and ascorbic acid content. These parameters are estimated and computed to obtain a numerical APTI of a plant (Singh et al., 1991). Large numbers of studies have been carried out to evaluate APTI of different plant species (Kaur et al., 2020). It is to be noted, however, that all the APTI related studies have been done on plant species that were growing in urban environment with current levels of pollution. However, it is necessary to explore and understand the responses of common plants to future levels of pollution to identify pollution tolerant plants, which in turn would provide useful information/suggestions to policy makers and urban planners.

Therefore, the present study evaluated the impact of EO_3 on APTI, and physiological and stomatal characteristics of nine common tropical plants, grown in Free Air Ozone Enrichment (FAOE) facility.

Materials and methods

The experimental site was located in the garden of CSIR-National Botanical Research Institute (CSIR-NBRI), Lucknow along the southern bank of river Gomati at 26°55′ N latitude, 80°59′ E longitude and 113 m asl altitude.

FAOE setup

CSIR-NBRI FAOE setup consists of 4 rings each of 10 m diameter; two rings each for ambient and EO_3. The O_3 exposure system in FAOE ring consists of horizontal and vertical pipes and nozzle systems, with a maximum height of 5 m. Ozone was produced from

pure oxygen using an ozone generator, and dispensed from the vertical and horizontal pipes through nozzles. O_3 concentrations were constantly monitored from the centre of each ring using UV photometric ozone analyzers (Model O_3 LEDM, Automatikprodukter, AP). More details of FAOE are given in Singh et al. (2021). The O_3 exposure was done for 8 h day^{-1} (from 09:00 to 17:00 h) during study period. In the EO_3 ring, O_3 concentration was kept + 20 ppb above the ambient levels. Hourly recorded readings were averaged for each day. Ozone exposure started on 1 January 2018 and ended on 31 March 2018. AOT40 (Accumulated Ozone exposure over a Threshold of 40 ppb (=80 µg/m³)) values were calculated (Fig. 1).

Plant material and growing condition

Nine dominant tropical plant species which commonly grow in urban landscapes outside the forest were selected for the study. They were *Azadirachta indica*, *Bouganvillia spectibilis*, *Ficus benghalensis*, *Ficus religiosa*, *Nerium indicum*, *Plumeria rubra*, *Saraca asoca*, *Tabernaemontana divaricata* and *Terminalia arjuna* (Table 1). Approximately 1-year-old seedlings were planted in plastic pots of 12″ diameter (filled with garden soil) and grown under ambient conditions. Fifteen days before start of ozone exposure, seedlings with similar height and basal diameter were transferred to FAOE rings. In every ring, there were 5 replicate plants of each of the nine plant species. So for one particular plant species, there were total 20 seedlings in four rings. Inside the ring, pots were kept at a distance of 60 cm from one another. All plants were watered as and when required to avoid water stress. Triplicate samples were collected for each plant species from each treatment for determining different parameters in the last week of the experiment. For leaf gas measurements and stomatal characteristics, replicate numbers are given is respective figure legends.

Measurement of pH of leaf extract

0.5 g of fresh leaf was crushed in 50 ml of distilled water and pH of the extract was determined after calibrating pH meter (digital) with buffer solutions of pH values 4, 7, and 10 (Singh & Rao, 1983).

Relative water content estimation

Fresh leaves were plucked and fresh weight was taken. The leaf petioles were then immersed in water wrapping the leaf lamina with an aluminum foil for 4 h. The leaf was dried using a blotting paper and then weighed again to obtain the turgid weight. The leaves were then dried in an oven at 70 °C for 72 h and the dry weight was recorded (Barrs & Weatherley, 1962).

Relative water content (RWC) was calculated as per the following equation (Evans & Ting, 1974):

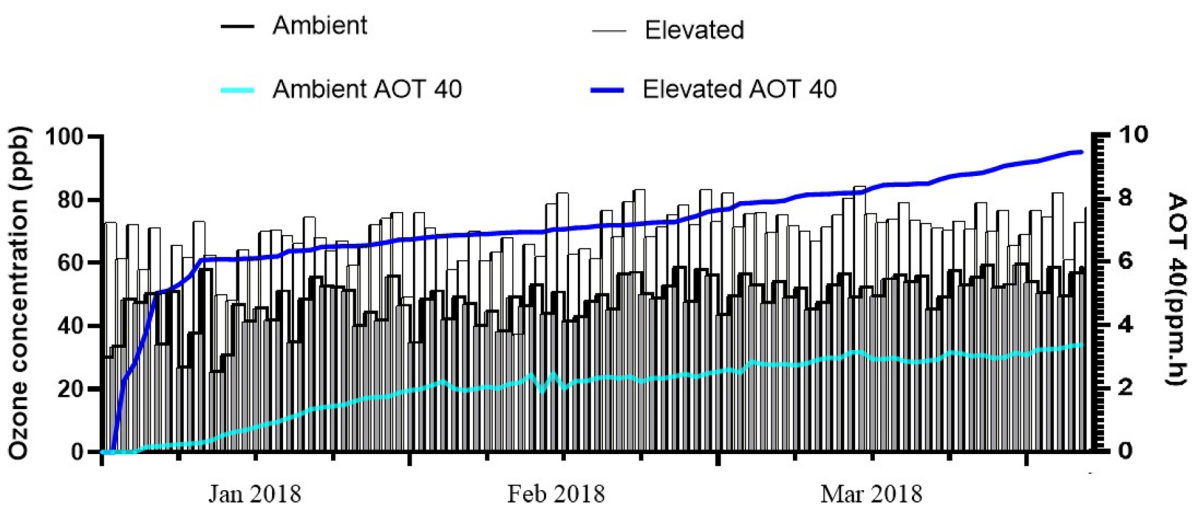

Fig. 1 Average daily O3 concentrations (ppb) and AOT40 (ppm h) values for ambient and elevated O3 rings

Table 1 Plant species and their basic characteristics

S. no	Botanical name	Common name	Family	Habit growth form	Plant functional type (leaf habit)	Leaf type
1	*Azadirachta indica* A. Juss	Neem	Meliaceae	Tree	Semi-evergreen	compound
2	*Bougainvillea spectabilis* Willd	Bougainvillea	Nyctaginaceae	Shrub	Evergreen	simple
3	*Ficus benghalensis* L	Bargad	Moraceae	Tree	Evergreen	simple
4	*Ficus religiosa* L	Peepal	Moraceae	Tree	Deciduous	simple
5	*Nerium indicum* Mill	Kaner	Apocynaceae	Shrub	Evergreen	simple
6	*Plumeria rubra* L	Plumeria	Apocynaceae	Tree	deciduous	simple
7	*Saraca asoca* (Roxb.) Willd	Ashok	Annonaceae	Tree	Evergreen	simple
8	*Tabernaemontana divaricata* R.Br. ex Roem. & Schult	Chandni	Apocynaceae	Shrub	Evergreen	simple
9	*Terminalia arjuna* (Roxb.) Wight & Arn	Arjun	Combretaceae	Tree	Evergreen	simple

$$RWC(\%) = \frac{FW - DW}{TW - DW} \times 100$$

where

FW = Fresh weight (g)
DW = Dry weight (g)
TW = Turgid weight (g)

Total chlorophyll content estimation

For estimation of total chlorophyll, 0.5 g fresh leaves, from each plant representing ambient and O_3 treated, were homogenized in chilled 80% acetone and homogenates were centrifuged at 10,000 g for 10 min. The absorbance of the clear supernatant was taken at 663 and 646 nm on a UV–VIS spectrophotometer (Spectra Max Plus, Molecular devices, USA). The concentration of total chlorophyll was calculated and expressed as mg g^{-1} fresh weight of leaves following Lichtenthaler (1987):

$$\text{Total Chl} = \frac{20.2 \times A_{646} + 8.02 \times A_{663} \times V}{1000 \times w \times d}$$

where A_{663} and A_{646} represent optical density at specific wavelengths

v = final volume (ml) of chl extract
w = fresh weight (g) of leaves
d = distance of light path (cm)

Ascorbic acid content estimation

Ascorbic acid (AA) was determined following Omayl et al. (1979). The leaf sample of 0.5 g was crushed in 2.5 ml of 10% TCA followed by centrifugation at 3500 g for 20 min. Re-extracted and the volume was made up to 5 ml. Of this 0.5 ml aliquot was taken and 1 ml DTC reagent (prepared by 3 g DNPH + 0.4 g thiourea and 0.05 g $CuSO_4$ in 100 ml of 9 N H_2SO_4) was added. Subsequently it was incubated for 3 h at 37 °C in water bath. After that 0.750 ml of ice cold 65% H_2SO_4 was added and left for 30 min. The spectrophotometer reading at 520 nm OD was recorded. Ascorbic acid content was calculated using standard curve of ascorbic acid and expressed as mg g^{-1} fresh weight.

Calculation of air pollution tolerance index

The air pollution tolerance index (APTI) for various plant species from ambient and EO_3 rings was calculated as per the equation given by Singh and Rao (1983). It uses four parameters namely ascorbic acid content, total chlorophyll content, relative water content, and pH of leaf extract.

$$\text{APTI} = \frac{[A(T + P) + R]}{10}$$

where A = Ascorbic acid content (mg g^{-1} fresh weight)

T = Total chlorophyll (mg g^{-1} fresh weight)
P = pH of leaf extract
R = Relative water content of leaf (%)

Determination of leaf gas exchange parameters

Gas exchange parameters viz., the rate of net photosynthesis (P_N), stomatal conductance (g_s), and transpiration (E) were measured in 3rd or 4th fully

expanded leaves with a portable photosynthesis system (Li-6800, LI-COR, Lincoln, NE, Nebraska, USA). The photosynthetically active radiation (PAR) was maintained at 1000 µmol (photons) m^{-2} s^{-1}, the level of CO_2 in the leaf chamber was maintained at 400 µmol CO_2 mol^{-1}. The vapor pressure deficit level was less than 3 kPa, the leaf temperature was 30 °C and the relative humidity was 60——65%. All the gas exchange parameters were measured between 8:00 and 11:00 h on four plants per treatment.

Measurement of stomatal density and guard cell length

Stomatal density (SD) and guard cell length (GCL) were measured on the same leaves which were used for gas exchange analysis. Canada balsam was used to create impressions on the abaxial leaf surface. Later, cello tape was applied to take negative impressions of leaves and the impressions were placed on glass slides. Images were captured using a Leica DM2500 microscope attached to a Leica DFC450C camera (Leica Microsystems, Wetzlar, Germany). Twelve images (3 images per leaf from 4 independent plants) were analyzed for stomatal density and guard cell length. SD was expressed as number of stomata/mm^2.

Estimation of proline content

Proline content was estimated following a modified protocol of Bates et al. (1973). 500 mg of leaf tissue was homogenized in 1 ml of 3% (w/v) aqueous sulfosalicylic acid. Then 96% acetic acid and 3% sulfosalicylic acid (2:1) were added, followed by ninhydrin reagent. The mixture was set aside in a water bath at 100 °C for 1 h and then it was allowed to cool at room temperature. Toluene was added and the absorbance of a fraction with toluene aspired from the liquid phase was measured at a wavelength of 520 nm. Proline concentration was estimated using a standard curve and expressed as µmol proline g^{-1} fresh weight of leaves.

Statistical analysis

All the bar graphs were prepared using Sigma Plot 11.0 (Systat Software, Inc., Richmond, CA, USA). A pairwise comparison was done using Student's *t*-test to examine the level of significance between the ambient and elevated ozone (EO_3) exposed plants. All data are means ± standard deviation (SD) from independent measurements made on four individual plants. The significance levels are shown as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

Results and discussion

Ozone concentration and AOT 40 values

The mean ozone concentrations during the experimental period were 48.59 ppb in ambient ozone (AO_3) ring and 69.62 ppb in EO_3 ring, indicating about 1.4 times enhancement in O_3 levels due to ozone treatment (Fig. 1). Calculated AOT40 (accumulated exposure over a threshold 40 ppb) values for ambient and EO_3 rings were 2.47 ppm h and 8.53 ppm h, respectively (Fig. 1).

During the period under study, maximum ambient O_3 levels were recorded in the month of March, followed by February and January (Fig. 1). High ambient O_3 concentrations in Indo-gangetic region of India were also reported by Tiwari et al. (2008) and Singh et al. (2010). This high concentration has been attributed to longer sun-shine hours, hot weather and lower relative humidity (Sharma et al., 2016). We have earlier reported high ambient O_3 and AOT40 values for Lucknow city (Maurya et al., 2020; Singh et al., 2021).

Impact of EO_3 on pH of leaf extract

The pH of the cell is an important factor for intracellular trafficking of vesicles and proteins, and the transport of small molecules like hormones apart from maintaining optimum environment for various biochemical reactions (Verweij et al., 2008). The pH of leaf extract of *F. religiosa* was 7.32 at ambient condition while that of *T. arjuna* and *S. asoca* were below 6.0 at ambient condition. Other plant species had pH between 6.0 and 7.0 under both the treatments. Most plant species showed decreasing pH values under EO_3 as compared to ambient O_3 (Table 2). Only in *Ficus religiosa*, pH significantly ($p<0.05$) increased from 7.32 (ambient O_3) to 8.72 (EO_3). In fact, it was an important contributor to high APTI

value of *F. religiosa* under EO_3 condition. Zhang et al. (2016) reported that the greater is the value of leaf extract pH, the stronger is the ability of plants to absorb SO_2 and NOx. The pH can also affect the reactivities of ascorbic acid towards ozone. Chang et al. (2021) have shown that higher acidity in airway-lining fluids could cause the lower reactivity of ascorbic acid to ozone. This implies that antioxidant ability of ascorbic acid against O_3 may be significantly suppressed at low pH of leaf extract.

Impact of EO_3 on Relative Water Content (RWC)

RWC is the water present in leaves relative to the full turgidity. Decreased RWC is associated with protoplasmic permeability, loss of water and dissolved nutrients, and early senescence of leaves (Agrawal & Tiwari, 1997). RWC in a plant body helps maintaining its physiological balance under stress conditions such as exposure to air pollution (Zhang et al., 2016). RWC was significantly greater ($p < 0.05$) in ambient conditions in comparison to EO_3 condition in *F. benghalensis*, *S. asoca*, *P. rubra*, *F. religiosa* and *N. indicum*, where it ranged between 90 and 97%. It ranged between 80 and 90% in *T. arjuna*, *T. divaricata*, and *B. spectabilis* (Table 2). RWC was minimum in *A. indica*. Exposure to EO_3 resulted in significant decline in RWC in *B. spectabilis* (15%), and *A. indica* (14%) in comparison to their respective controls. Previous studies have shown reduced leaf RWC due to drought (Deeba et al., 2012), high temperature (Duan et al., 2017), and air pollution (Zhang et al., 2016). Thus, in an urban environment, high leaf RWC helps plants maintain their physiological balance and enhance their tolerance ability to stress conditions.

Impact of EO_3 on total chlorophyll content

EO_3 caused significant decrease in chlorophyll content in most plant species (Table 2). In comparison to their respective control plants, maximum decrease was recorded in *F. benghalensis* (54.5%) followed by *A. indica* (47.35%), *T. arjuna* (42.24%), *T. divaricata* (29.15%), *P. rubra* (28.16%), *B. spectabilis* (13.55%) and *F. religiosa* (9.86%). *N. indicum* did not show any significant difference. Degradation of chlorophyll pigment has been widely used as an indicator of air pollution (Ninave et al., 2001). Reduced chlorophyll content due to EO_3 is due to destruction of chlorophyll

Table 2 Air pollution tolerance index (APTI) values of the studied plants in ambient ozone (AO_3) and elevated ozone (EO_3) conditions. Values are means + S.D., $n = 3$. The significance levels are shown as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ according to Student's *t*–test

Species	O_3 conditions	Ascorbic Acid (mg g^{-1})	Total Chl (mg^{-1} Fw)	Leaf extract pH	Relative Water Content (%)	APTI values
Azadirachta indica	Ambient	5.33 ± 0.15	9.34 ± 0.07	6.09 ± 0.07	76.23 ± 1.94	15.84 ± 0.22
	Elevated	6.85 ± 0.4**	4.92 ± 0.43**	6.30 ± 0.19 ns	71.58 ± 1.73*	14.84 ± 0.12**
Bouganvillea spectabilis	Ambient	4.63 ± 0.68	10.35 ± 0.095	7.11 ± 0.02	80.46 ± 8.4	16.0 ± 2.00
	Elevated	3.24 ± 0.04 ns	8.95 ± 0.88 ns	6.85 ± 0.09 ns	68.12 ± 3.9*	11.9 ± 0.48*
Ficus benghalensis	Ambient	10.76 ± 0.39	8.95 ± 0.30	6.60 ± 0.64	96.53 ± 2.60	26.40 ± 1.11
	Elevated	13.61 ± 1.73*	4.07 ± 0.29***	7.78 ± 0.68 ns	95.92 ± 1.58 ns	24.78 ± 1.26 ns
Ficus religiosa	Ambient	13.37 ± 0.18	5.28 ± 0.16	7.33 ± 0.02	94.57 ± 1.33	26.32 ± 0.41
	Elevated	14.84 ± 0.24*	4.76 ± 0.09*	8.72 ± 0.03***	93.38 ± 0.48 ns	29.34 ± 0.24*
Nerium indicum	Ambient	3.86 ± 0.39	9.98 ± 1.22	6.51 ± 0.03	90.86 ± 1.7	15.4 ± 1.15
	Elevated	5.27 ± 0.25**	9.73 ± 0.10 ns	6.44 ± 0.06 ns	93.10 ± 0.6 ns	17.8 ± 0.48 ns
Plumeria rubra	Ambient	10.67 ± 0.20	6.95 ± 0.47	6.86 ± 0.04	94.63 ± 4.1	24.1 ± 0.88
	Elevated	13.68 ± 0.03**	4.99 ± 0.06*	6.78 ± 0.08 ns	93.89 ± 2.0 ns	25.4 ± 0.18 ns
Saraca asoca	Ambient	4.86 ± 0.02	6.09 ± 0.02	5.86 ± 0.03	96.33 ± 0.92	15.44 ± 0.08
	Elevated	9.75 ± 0.05***	7.05 ± 0.33*	5.90 ± 0.01 ns	93.60 ± 0.54 ns	21.99 ± 0.31***
Tabernaemontana divaricata	Ambient	6.57 ± 0.37	10.20 ± 0.06	6.52 ± 0.03	83.16 ± 4.86	19.30 ± 0.79
	Elevated	9.26 ± 0.10**	7.23 ± 0.14***	6.45 ± 0.12 ns	82.23 ± 5.08 ns	20.88 ± 0.43 ns
Terminalia arjuna	Ambient	4.51 ± 0.12	8.67 ± 0.08	5.79 ± 0.06	87.43 ± 7.47	15.26 ± 0.77
	Elevated	6.32 ± 0.12***	5.01 ± 0.03***	5.54 ± 0.10 ns	86.24 ± 2.35 ns	15.29 ± 0.25 ns

(Saitanis et al., 2001), or damage to thylakoid membranes (Kivimäenpää et al., 2005). Moreover, O3 indirectly affects chlorophyll content by accelerating leaf senescence (Donnelly et al., 2000). *S. asoca* was the only plant which showed enhanced chlorophyll content (15.78%) in response to EO_3 treatment. Higher chlorophyll content provides tolerance to plants against air pollutants (Prajapati & Tripathi, 2008).

Impact of EO_3 on ascorbic acid content

Ascorbic acid (AA) is a natural antioxidant and a strong reducing agent. It plays an important role in pollution tolerance and protects the plant against oxidative damage (Castagna & Ranieri, 2009).

In response to EO_3 treatment, all the plants had greater levels of AA content compared to their respective controls except *B. spectabilis* (Table 2). The increase was more than 100% in *S. asoca*, 41% in *T. divaricata*, 40% in *T. arjuna*, 36% in *N. indicum*, 28% in *A.indica* and *P. rubra*, 26% in *F. benghalensis*, and 11% in *F. religiosa*. In contrast, in *B. spectabilis*, AA content was reduced by 30% in comparison to the control (Table 2).

AA is the most abundant water-soluble antioxidant in plants (Gallie, 2013) and is considered as an important molecule for plants' tolerance to various abiotic stresses, e.g., metal stress (Dixit et al., 2001), heat stress (Kumar et al., 2013), and drought stress (Dolatabadian et al., 2009). In our study, a direct relationship was obtained between AA content of plants and ozone concentration, suggesting that greater Reactive Oxygen Species (ROS) production due to O_3 induces higher production of AA (Bellini & De Tullio, 2019). Potential mechanisms include direct detoxification of O_3-generated ROS by AA or alteration of redox signaling pathways that regulate O_3-induced responses (Bailey et al., 2019). Plants use several strategies to combat O_3 stress, including O_3 avoidance (stomatal control) or O_3 tolerance (detoxification/repair system). The protective role of AA, a ROS-scavenger is also supported by the enhanced O_3-sensitivity shown by mutants deficient in AA synthesis (Conklin et al., 1996) and by transgenic plants overexpressing ascorbate oxidase (Sanmartin et al., 2003).

Air Pollution Tolerance Index (APTI) in response to EO_3

APTI of *F. religiosa*, *T. arjuna*, and *T. divaricata* were greater in EO_3 condition as compared to the ambient condition. Highest APTI values of 29.34 ± 0.24 (ambient) and 26.32 ± 0.41 (elevated) were in *F. religiosa*, and were lowest in *T. arjuna* (15.29 ± 0.25 and 15.26 ± 0.77). APTI values of *T. divaricata* were 20.88 ± 0.43 and 19.30 ± 0.79 in elevated and ambient O_3, respectively (Table 2). APTI values for *F. benghalensis* were 27.07 and 25.05, for *A. indica* 20.88 and 16.70, for *S. asoca* 18.97 and 21.52, respectively in elevated and ambient O_3. The APTI values in *Nerium indicum* were16 ± 1.15 and 18 ± 0.48, and the least were in *Bougainvillea spectabilis* i.e. 16 ± 1.15 and 11 ± 0.48 in ambient and EO_3, respectively. Thus, the most significant change in APTI was in *Bougainvillea spectabilis* whereas, the least change was in *Nerium indicum*.

It is evident that APTI values of plants changed under EO_3 condition. Some plants showed better APTI values under EO_3 and some showed lower APTI values under EO_3 treatment. Higher Greater APTI values are associated with higher tolerance of plants to air pollutants (Jyothi & Jaya, 2010; Pandey et al., 2016). According to a classification by Singh et al. (1991), plants having APTI values less than 12 are regarded as sensitive, between 13–16 are intermediate tolerants, between 17–20 are considered moderately tolerants and those with APTI value of more than 20 are tolerant to air pollutants. The variation in APTI values can be attributed to the variation in any of the four parameters used for computation of the index. In the present study, *Bougainvillea* APTI values reduced maximally in EO_3 treatment, which may be attributed to less ascorbate, less chlorophyll content and low RWC. *S. asoca* APTI values increased maximally under EO_3, which could be due to increased ascorbate content, more chlorophyll, and constant RWC. Das et al. (2010) categorized *A. indica*, growing around a steel manufacturing factory, as sensitive due to its low APTI value. Similar response was also observed in *A. indica* in the present study. Mukherjee and Agrawal (2018) reported *A. indica* to be sensitive to air pollutants (SO_2, NO_2 and O_3). Although this plant maintained its antioxidant level but failed to maintain its water balance and resource utilization. Similarly in the present study, *A. indica* showed increase in antioxidants but had reduced RWC and total chlorophyll content which probably affected its photosynthetic performance under EO_3 treatment.

The linear regression plots of individual variables with APTI in both ambient and EO_3 condition showed a high positive correlation between APTI score and ascorbic acid content (Fig. 2a, b). However, total chlorophyll content was weakly and negatively correlated with APTI score. A positive correlation between RWC and APTI score was also obtained (Fig. 2a, b).

Impact of EO_3 on leaf stomatal density (SD) and guard cell length (GCL)

Stomata play a crucial role in determining the O_3 influx to leaves, because the majority of the O_3 enters the leaf via stomatal pores (Vainonen & Kangasjärvi, 2015). Since O_3 enters leaves of the plant through stomata, O_3 influx are highly dependent on stomatal density and conductance (Shang et al., 2021). Stomatal density decreased significantly in *A. indica*, *P. rubra*, *S. asoca*, and T. *divaricata* by 35%, 37%, 33%, and 23%, respectively, while it significantly increased in *F. benghalensis* and. *T. arjuna* by 14% and 19%, respectively (Fig. 3a). Neufeld et al. (2019) reported that 100 ppb of O_3 tended to reduce stomatal density, suggesting structural changes in plants. On the other hand, some studies have reported increased stomatal densities in trees due to high O_3 (Baek et al., 2018; Frey et al., 1996).

GCL significantly increased in *A. indica*, *B. spectabilis*, *P. rubra*, *S. asoca* and *T. divaricata* by 18.5%, 9%, 19%, 22%, and 13.7%, respectively, while it significantly decreased in *F. benghalensis, F. religiosa* and in *T. arjuna* by 13.3%, 11.6%, and 11.4%, respectively under EO_3 treatment as compared to the ambient conditions (Fig. 3b). Light micrographs (Fig. 4) and scanning electron

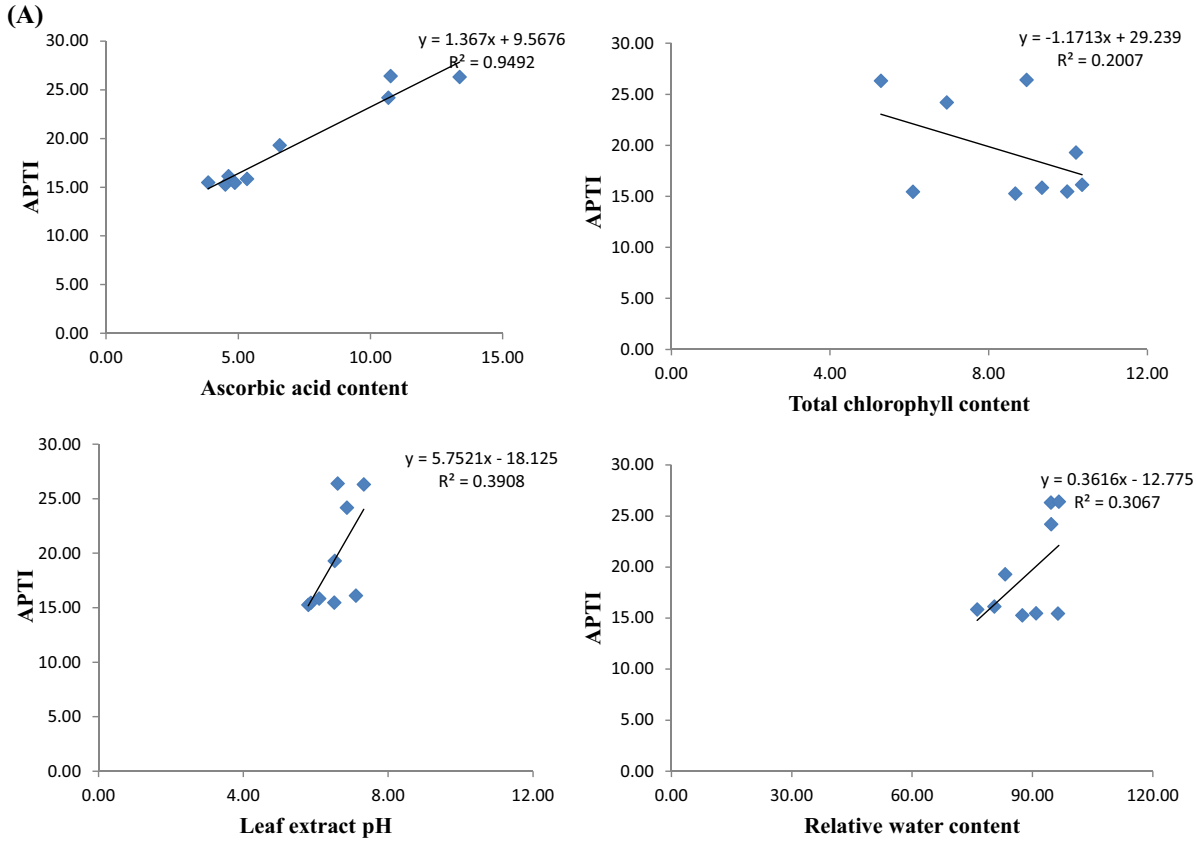

Fig. 2 **A** Linear regression plots of APTI of plants growing in ambient ring with individual variables. **B** Linear regression plots of APTI of plants exposed to elevated ozone with individual variables

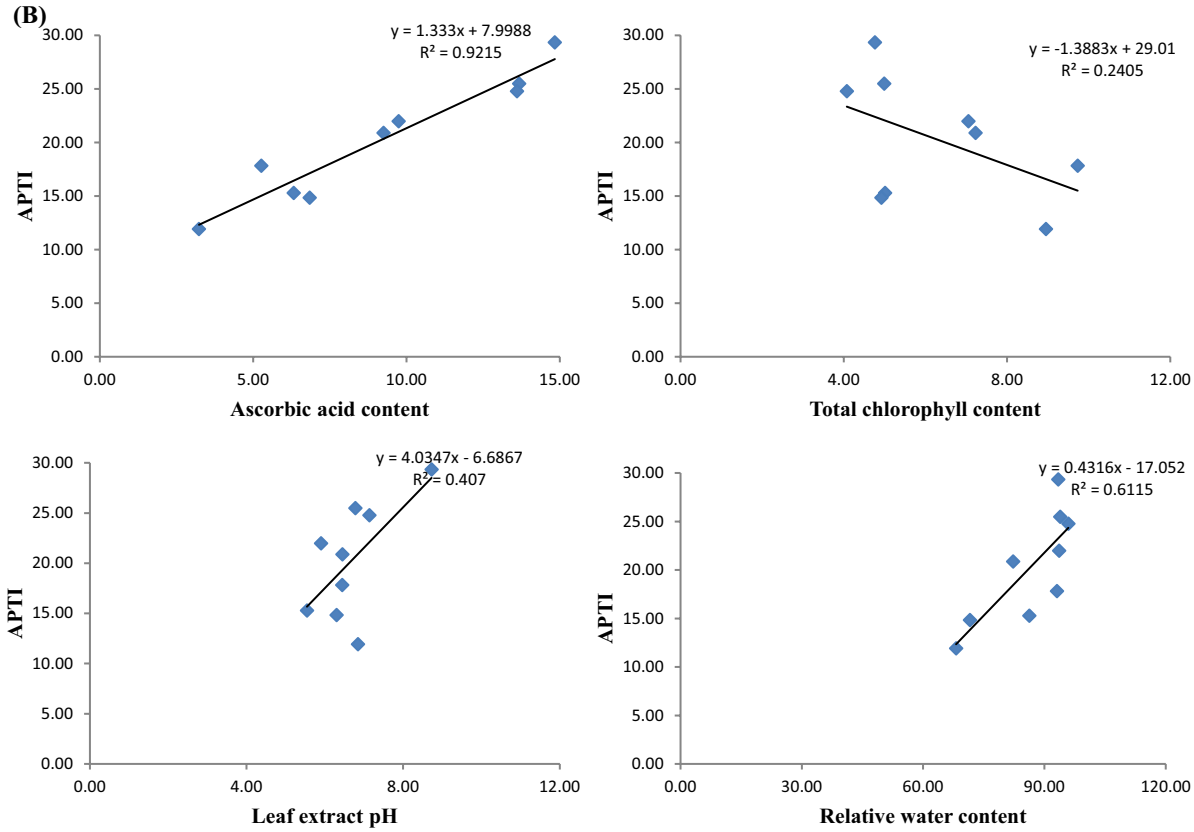

Fig. 2 (continued)

micrographs (Fig. 5) also show the changes as mentioned above. Guard cells control the rates of CO_2/O_3 entry by regulating the apertures of the stomatal pores in the leaf epidermis. Reduced stomatal conductance is commonly observed after O_3 exposure (Pearson & Mansfield, 1993), and it is possible that these reductions reflect a primary response of the guard cells to O_3 (Maier-Maercker, 1999). Torsethaugen et al. (1999) showed in *Vicia faba* that O_3 directly affects guard cells, targeting inward K^+ channels, and inhibits stomatal opening. One possible mechanism for the observed O_3 effect on guard cells, was direct oxidation of the channel proteins. Alternatively, elevation of cytosolic Ca^{2+} levels after O_3 exposure is another plausible mechanism to explain these responses.

The effects of air pollutants on stomatal responses have been reported extensively, and the responses are very complex among the plants that vary with the environmental stressors. Ambient O_3 enters into the leaves of the plant through stomata and its influx is greatly dependent on the stomatal density and stomatal conductance (Bertolino et al., 2019; Lee et al., 2021). Decrease in SD and GCL limits g_s and transpiration (E), showing a shift towards minimum use of water and avoidance of O_3 influx (Hoshika et al., 2015). Elagoz et al. (2006) reported significantly higher O_3 induced g_s rates in O_3 sensitive bush bean than tolerant ones. This indicates that stomatal movement plays an important role in plants' response to O_3.

Higher stomatal densities and larger aperture size were found in O_3 sensitive bush bean cultivars exposed to 60 ppb O_3 (Elagoz et al., 2006).

Impact of EO_3 on proline content

Proline level in all the species increased more in EO_3 than the ambient condition. Proline increased in leaves of *A. indica*, *B. spectabilis*, *F. religiosa*, *N. indicum*, *P. rubra*, *S. asoca* and *T. arjuna* by 3.7 2.05,

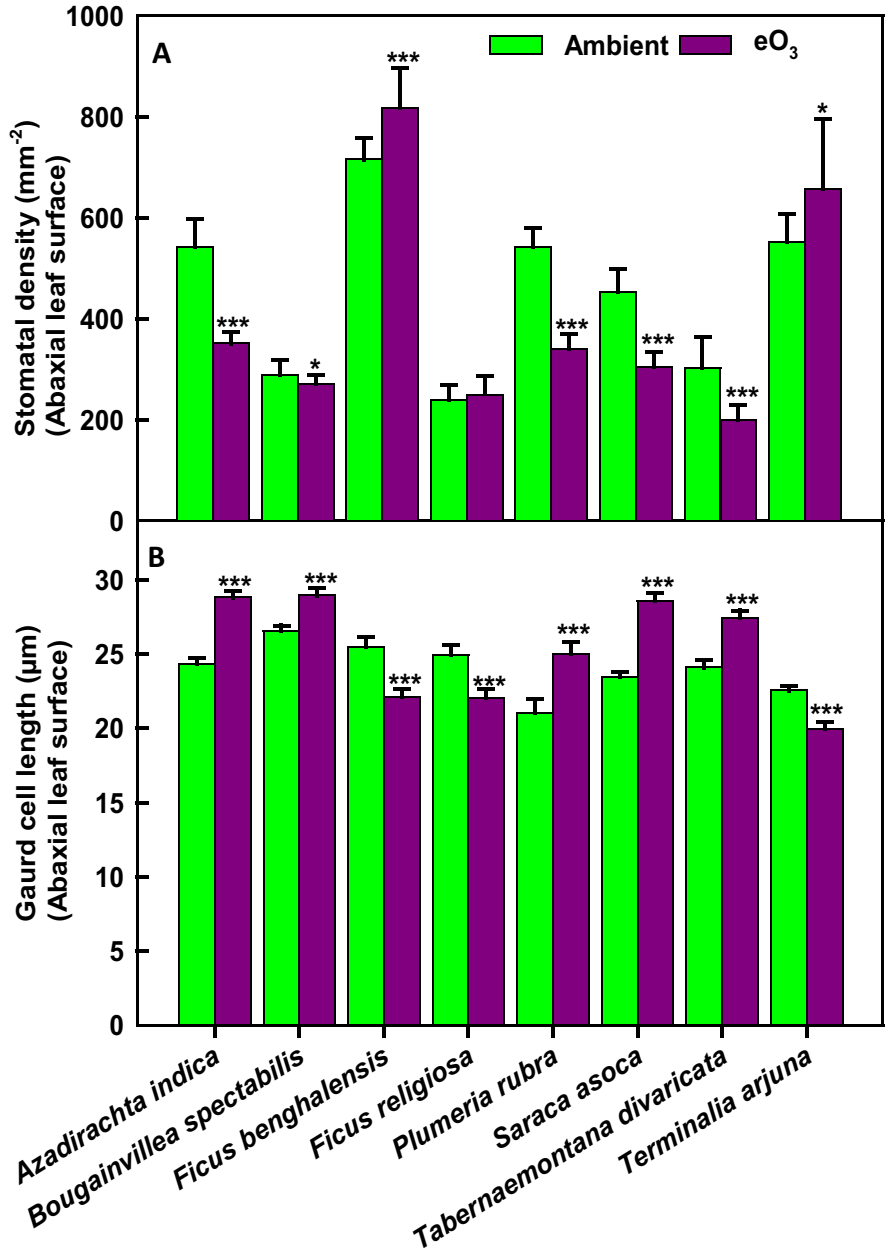

Fig. 3 Stomatal density, **A** (mm^2) and guard cell length, **B** (µm) of different plant species grown under ambient and elevated O_3 (+20 ppb above ambient) conditions. Values are mean + S.D., $n = 12$. The significance levels are shown as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ = according to Student's t – test

3.7, 1.81, 2.3, 2.19 and 2.98 fold, respectively. The increase was not significant in *F. benghalensis* and *T. divaricata* under EO_3 as compared to ambient condition (Fig. 6).

Ozone is a powerful oxidant that produces ROS when it enters into the leaves. The response of various species and cultivars to O_3 varies, and it is dependent on the evolved defense against ozone stress (Chaudhary & Rathore, 2021). Furthermore, the duration and degree of the stress affect the plant's sensitivity to O_3. One of the goals of this research was to determine whether plant's antioxidative potential confers ozone tolerance to the selected tree species. Higher concentration of proline is generally considered as an indicator of induction of defense mechanism against different stresses (Verbrugeen & Hermans, 2008). Tropospheric O_3 in the substomatal cavity forms ROS and also initiates a series of reactions which alters level of anions

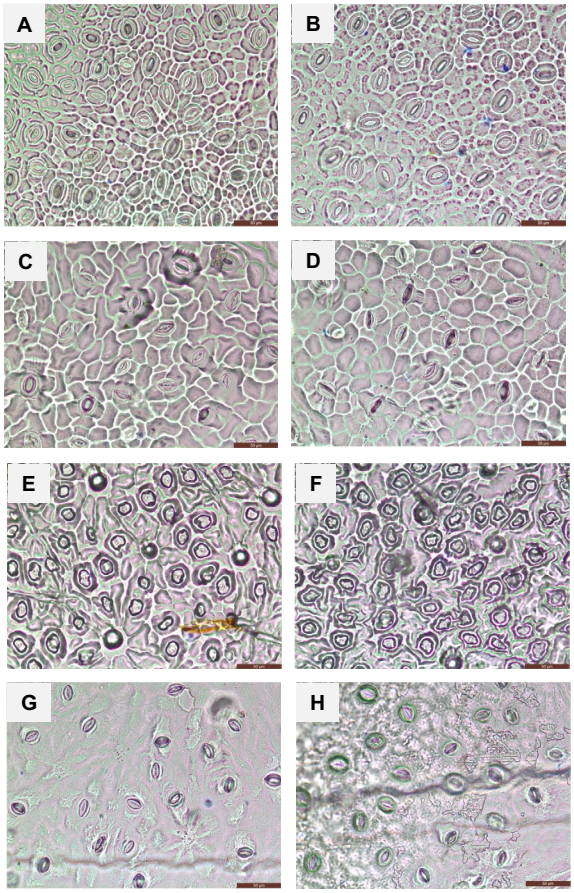

Fig. 4 Light micrographs of the abaxial surface stomata in the leaves of *Azadirachta indica* **A** ambient ozone (AO3) and **B** elevated ozone (EO3); *Bouganvillea spectabilis* **C**, AO3 and **D**, EO3; *Ficus benghalensis* **E**, AO3 and **F**, EO3; *Ficus religiosa* **G**, AO3 and **H**, EO3; *Plumeria rubra* **I**, AO3 and **J**, EO3; *Saraca asoca* **K**, AO3 and **L**, EO3; *Tabernaemontana divaricata* **M**, AO3 and **N**, EO3; *Terminalia arjuna* **O**, AO3 and **P**, EO3. Scale bar is 50 µm

and leads to increase in proline content (Mansfield & Freer-Smith, 1984). Proline protects membranes and proteins by acting as a protein-compatible hydrotope (Srinivas & Balasubramanian, 1995) and it also acts as a hydroxyl radical scavenger (Smirnoff & Cumbes, 1989). The significant increase in proline accumulation in *A. indica* and *F. religiosa* followed by *T. arjuna*, *P. rubra*, *S. asoca*, *B. spectabilis* and *N. indicum* might be important in maintaining water potential equilibrium inside cells, which in turn acted as an adaptive mechanism against EO_3 (Fig. 5) (Calzone et al., 2019).

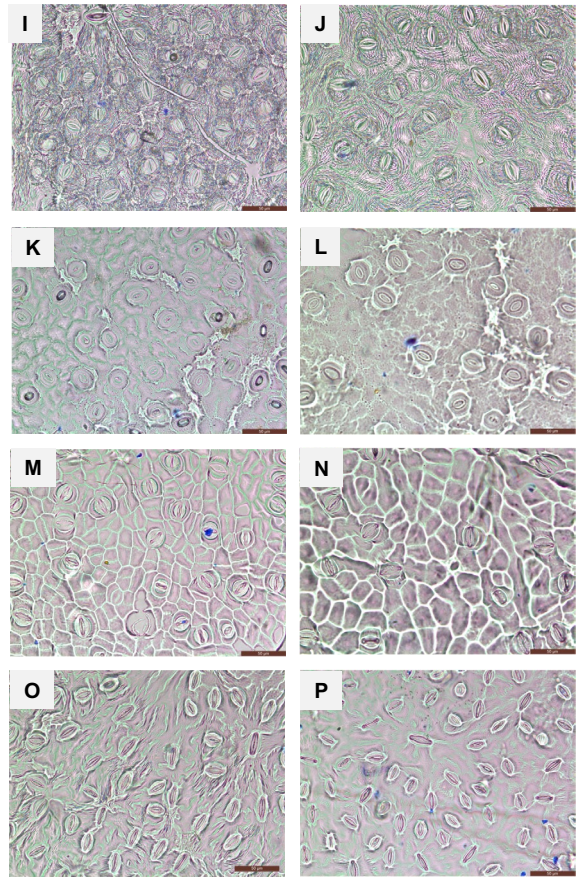

Fig. 4 (continued)

Impact of EO_3 on photosynthetic performance

The gas-exchange parameters of net photosynthesis (P_N), stomatal conductance (g_s), and transpiration (E) were monitored in ambient and elevated ozone (EO_3) conditions. P_N significantly decreased by 64.4%, 48.4%, 18.4%, 26.0%, 21.3%, 49.6%, 31.8%, and 15.4% in *A. indica*, *B. spectabilis*, *F. benghalensis*, *F. religiosa*, *P. rubra, S. asoca*, *T. divaricata* and *T. arjuna*, respectively due to EO_3 conditions (Fig. 7).

Except *A. indica*, transpiration (E) declined in all the species under EO_3 condition. *F. benghalensis* and *S. asoca* recorded the highest decline i.e. reduction by 62% and 61%, respectively (Fig. 6b). E increased only in *A. indica*. The stomatal conductance (g_s) significantly reduced in *F. benghalensis, F. religiosa*, *S. asoca*, *T. divaricata*, and *T. arjuna* by 57.6%, 52.8%, 67.1%, 25.2%, and 37.3%, respectively

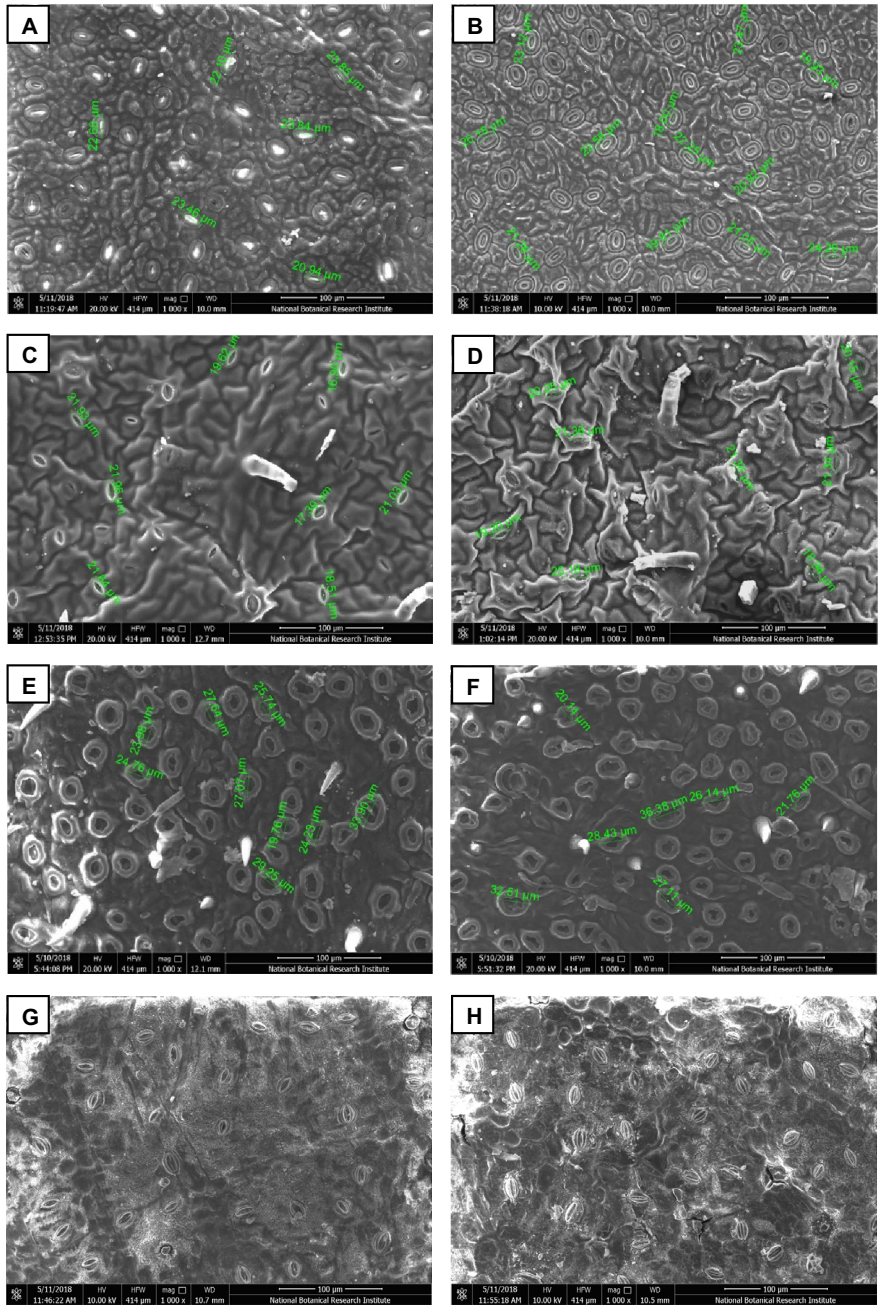

Fig. 5 Scanning electron microscopy of abaxial surface of the leaves of *Azadirachta indica* **A**, Ambient Ozone (AO3) and **B**, Elevated Ozone (EO3); *Bouganvillea spectabilis* **C**, AO3 and **D**, EO3; *Ficus benghalensis* **E**, AO3 and **F**, EO3; *Ficus religiosa* **G**, AO3 and **H**, EO3; *Plumeria rubra* **I**, AO3 and **J**, EO3; *Saraca asoca* **K**, AO3 and **L**, EO3; *Tabernaemontana divaricata* **M**, AO3 and **N**, EO3; *Terminalia arjuna* **O**, AO3 and **P**, EO3. Scale is 100 µm

under EO_3 condition compared to the ambient condition. On the other hand, g_s increased significantly in *A. indica* and *N. indicum* (Fig. 7c). Water use efficiency (WUE), which is the ratio of photosynthesis over transpiration, increased significantly under EO_3 condition only in *F. benghalensis* in comparison to the ambient condition. It significantly decreased in *A. indica* and *T. divaricata* by more than 1.5-fold (Fig. 7d). McLaughlin et al. (2007) reported that spikes in ambient O_3 significantly increased water loss of trees, suggesting that O_3-induced aberrations in the stomatal dynamics differ among the species and the environmental conditions. Hoshika et al. (2012) reported that in poplar clone, although O_3 stress resulted in loss of stomatal control over water loss, it was compensated by lower stomatal conductance and premature leaf shedding. Lower rates

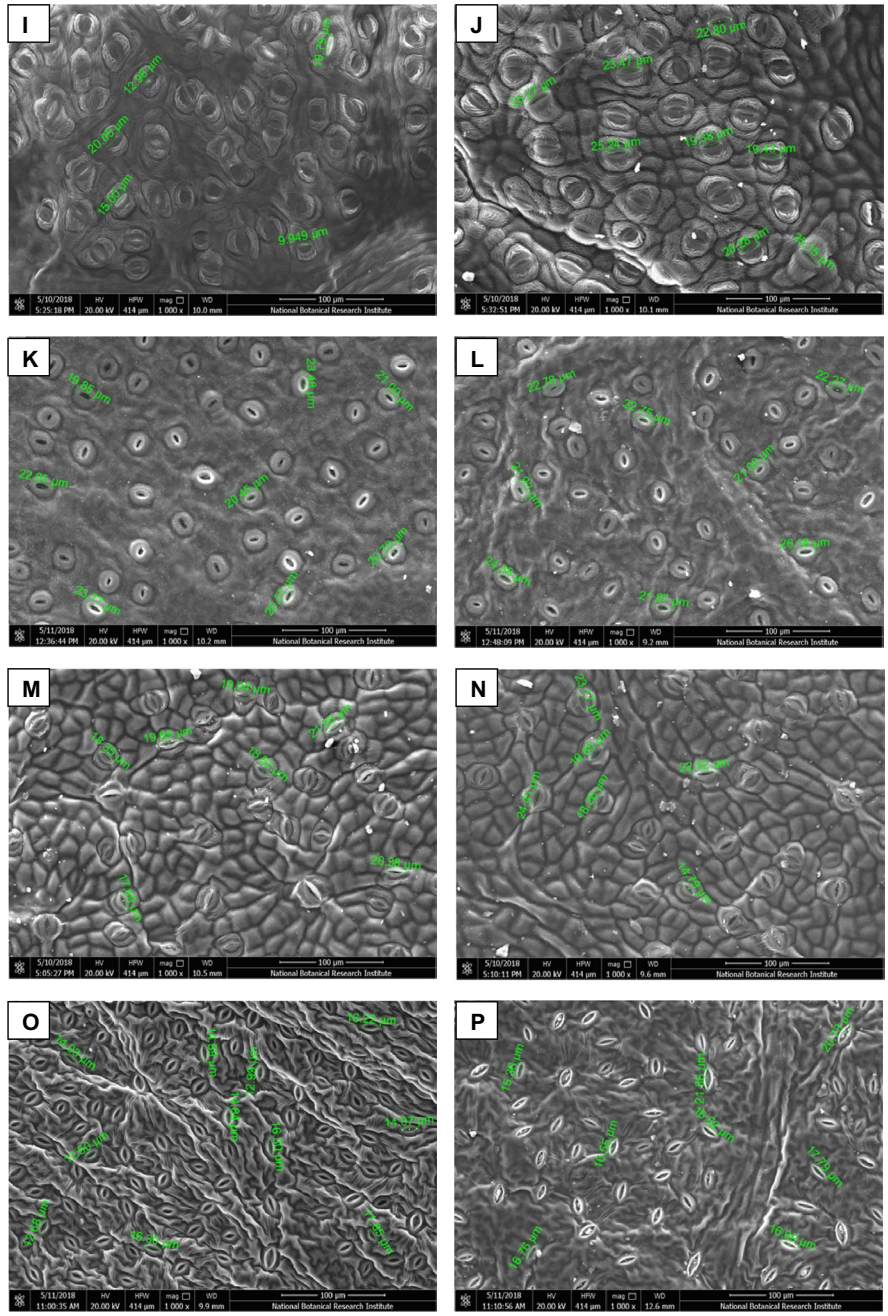

Fig. 5 (continued)

of stomatal conductance indicates a role for stomatal exclusion as a contributing factor towards reduced O_3 sensitivity as observed by Bailey et al. (2019) in contrasting soybean genotypes. Thus lower stomatal conductance seems to be an important trait associated with O_3 tolerance. This shows that stomatal exclusion is only one of the several possible tolerance mechanisms present in different plants. The lower rates of stomatal conductance along with lower transpiration rates suggest a potential link between O_3 tolerance and drought tolerance. This assumes significance as almost all avenue or roadside plants also experience drought stress in an urban environment.

On exposure to EO_3, the decrease or increase in SD and GCL alters the gas exchange traits. Usually, stomatal closure is stimulated by O_3, leading to lower O_3

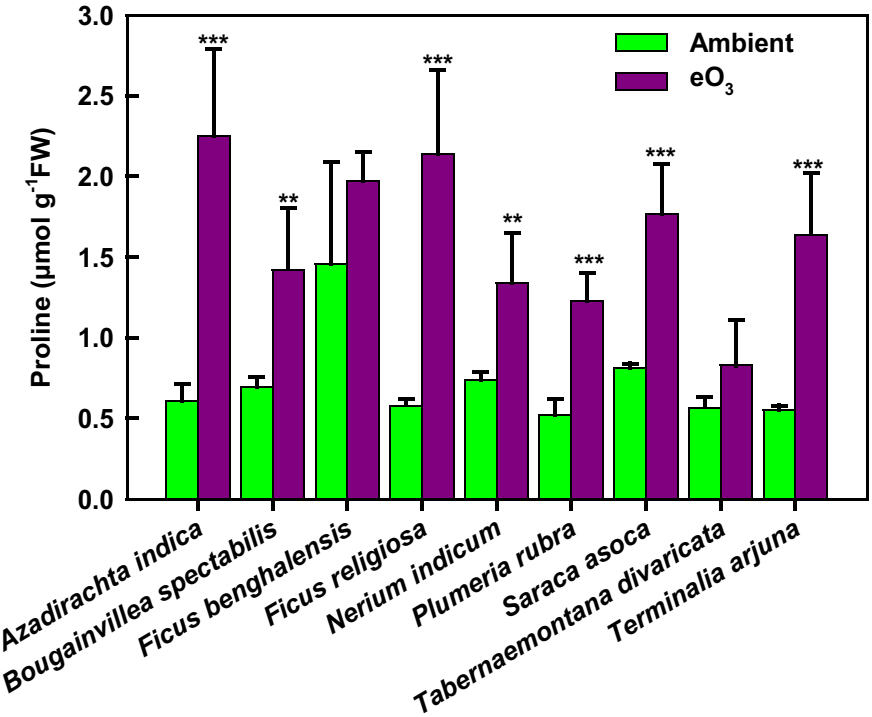

Fig. 6 Changes in level of proline in different plant species grown under ambient and elevated O3 (+20 ppb above ambient) conditions. Values are mean +/– S.D., $n = 3$. The significance levels are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ according to Student's t – test

uptake by plants and water usage due to less transpiration (Hoshika et al., 2015). In the present study, the change in stomatal conductance (g_s) in *B. spectabilis* and *P. rubra* was not significant while significant decrease was observed in *T. divaricata*, *N. indicum* and *T. arjuna* followed by *A. indica*, *F. religiosa*, *F. benghalensis* and *S. asoca* in ozone treated plants. This response may be linked to a plants' counter-measure mechanism for limiting pollutant entry by restricting its stomata. However, such a mechanism may also lower CO_2 entry and ultimately lead to reduced rate of photosynthesis and transpiration (Shang et al., 2020). In plants, photosynthesis is the most basic and important physiological process. Under stressful conditions, physiological activities of the plants usually decrease. One of the most essential metrics for monitoring plant physiological activity under stress conditions is photosynthesis. Significant decrease in photosynthesis was observed in EO_3 treated plants, viz., *T. arjuna*, *F. benghalensis*, *F. religiosa*, and *P. rubra* followed by *T. divaricata*, *S. asoca*, *B. spectabilis*, and *A. indica* compared to the ambient ozone condition. However, P_N did not vary significantly in *N. indicum* (Fig. 7). Shang et al. (2017) reported reduction in photosynthesis in Poplar clone 546 and 107 exposed to ambient+40 ppb and ambient+60 ppb compared to charcoal filtered air. Poplar clone 546 reported maximum reduction in photosynthesis with significant reduction in its stomatal conductance and total chlorophyll. In *A. indica,* reduced P_N was correlated with maximum reduction in its stomatal conductance as well as stomatal density which suggests that closure of stomata restricted uptake of O_3 which might have affected photosynthesis as well. In the present study, low stomatal conductance was correlated with low stomatal density as well as increased guard cell length in all the tree species except in *F. benghalensis* and *T. arjuna* which suggests that stomatal closure took place under EO_3 and it also affected its P_N rate. Lee et al. (2021) reported reduction in stomatal density in *Brassica juncea* exposed to EO_3 (105.6 ppb for 8 h). In *F. benghalensis* and *T. arjuna*, increase in stomatal density and reduced guard cell length along with increased WUE and reduced E could be an O_3 tolerance mechanism as it represents a shift towards maximum water utilization in CO_2 assimilation. The factors such as SD and GCL have the ability to affect stomatal mobility and the resultant g_s, and WUE (Drake et al., 2013). This flexible modulation of stomatal size and number enables plants to shift the maximum and minimum gas exchange by modifying the stomatal size in response to the environmental stress (Hetherington & Woodward, 2003).

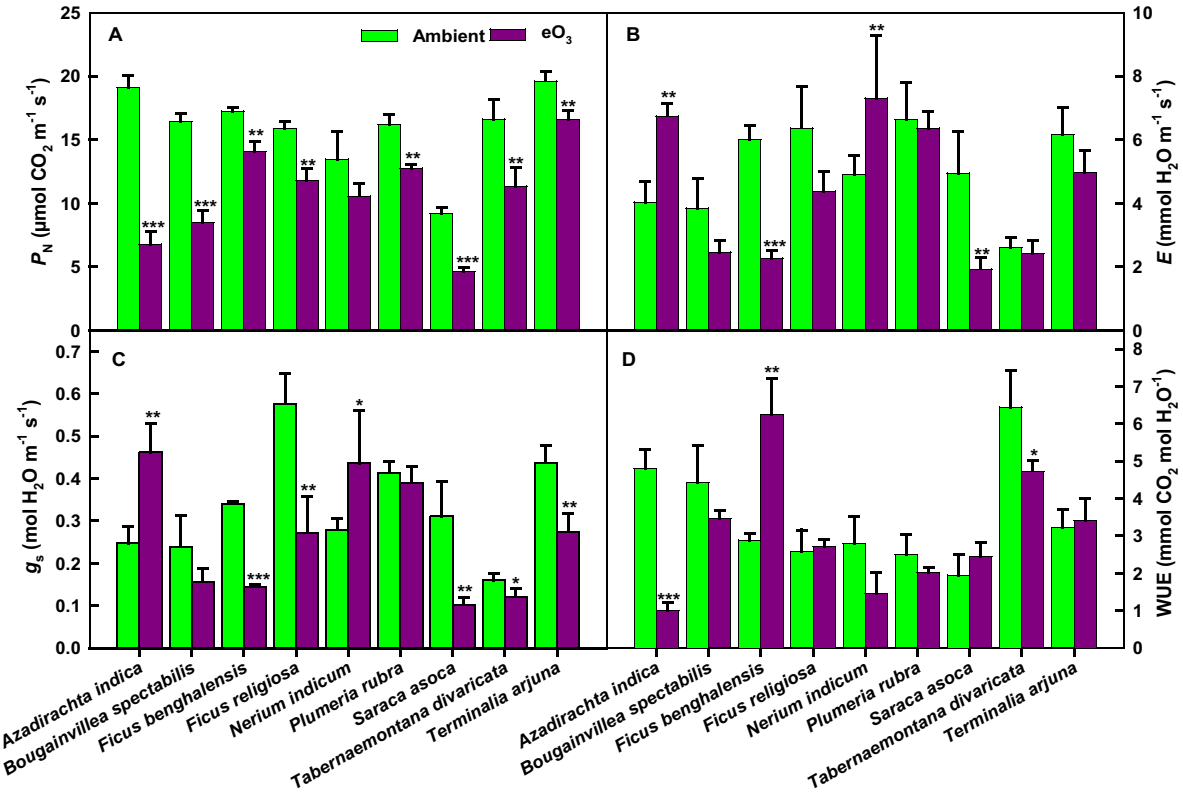

Fig. 7 Photosynthetic characteristics of different plant species grown under ambient and elevated O3 (+20 ppb above ambient) conditions. **A** Net photosynthesis, *P*N; **B** transpiration rate, *E*; **C** stomatal conductance, *gs*; **D** water use efficiency, WUE. Values are mean +/− S.D., $n = 5$. The significance levels are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ according to Student's *t* – test

Conclusion

The present study provides empirical evidence that pollution tolerant trees could be a viable strategy to improve air quality. Among the tree species studied, *S. asoca* and *F.religiosa* were the most tolerant, and *B. spectabilis* and *A. indica* were the most sensitive to EO_3. The APTI was useful, but to a limited extent in selecting tolerant plants. Therefore, it is time that a more robust and inclusive index is developed, as indicated in the present study. WUE, stomatal traits and photosynthesis rates varied among different plant species and therefore, these should be taken as O_3 responsive parameters. The species screened for O_3 tolerance in this study, though limited in number, should be useful for plantation in the areas experiencing high O_3 concentration such as many pockets of peninsular India and Indo-Gangetic plains.

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Author contribution SKB and VP conceptualized the work. RJ and RK did biochemical work. SN and RD did stomata and physiology work. Sandip Behera did scanning electron microscopy work. SKB, PAS and VP analyzed the data. RR wrote the first draft. PAS, VP, and SKB improved the first draft. All authors read and approved the final draft.

Declarations

Conflict of interest The authors declare no competing interests.

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