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ORIGINAL ARTICLE

Changes in growth pattern and rhizospheric soil biochemical properties of a leguminous tree species *Leucaena leucocephala* under long-term exposure to elevated ozone

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Abstract
Increasing concentrations of ground-level ozone (O_3) exert significant impacts on the plants, but there is limited data for belowground processes. We studied the effects of long-term exposure of elevated O_3 (EO_3) on plant growth parameters (plant height and biomass) and biochemical parameters (nutrients, microbial biomass and enzymatic activities) of rhizospheric soil of leguminous tree species *Leucaena leucocephala*. *L. leucocephala* seedlings were grown under ambient O_3 (AO_3) and EO_3 (+20 ppb above ambient) under Free Air Ozone Concentration Enrichment (O_3-FACE) facility and changes in plant growth and their rhizospheric soil properties were studied during 6, 12, 18 and 24 months of EO_3 exposure. *L. leucocephala* showed significant reductions in shoot length, root biomass, shoot biomass, leaf biomass and total biomass during 12, 18 and 24 months of exposure to EO_3. Total nutrients in rhizospheric soil like carbon and phosphorus were significantly reduced after 24 months of EO_3 exposure. Most of the available nutrients showed significant reduction after 6, 12 and 24 months of EO_3 exposure. A significant decrease was apparent in microbial biomass carbon, nitrogen and phosphorus after 6, 12, 18 and 24 months of EO_3 treatment. Significant reductions were observed in extracellular enzymatic activities (dehydrogenase, alkaline phosphatase, β-glycosidase, fluorescein diacetate, arylsulfatase, cellulase and protease) of soil after 6, 12 and 24 months of EO_3 exposure. These results suggest that increasing O_3 concentrations will directly impact *L. leucocephala* growth as well as have indirect impact on the nutrient contents (C, N, and P), microbial biomass and extracellular enzymatic activities of rhizospheric soil of *L. leucocephala*. Our results suggest that continuous increase in O_3 concentrations will have serious implications for aboveground plant growth and belowground soil fertility in this region considered as O_3 hotspot.

Keywords Elevated ozone · Biomass · Rhizosphere soil parameters · Leucaena · Extracellular enzyme · O_3-FACE

Introduction

Tropospheric ozone (hereafter referred to as O_3) is the most important secondary air pollutant of high concern for vegetation and ecosystems all over the world (IPCC 2014). Ozone is formed from the reaction of precursor gases such as oxides of nitrogen (NOx), carbon monoxide (CO) and volatile organic compounds (VOCs) in the presence of sunlight (Bytnerowicz et al. 2007; Fowler et al. 2008; Yamaji et al. 2008). Due to continued dependence on fossil fuels, the concentration of O_3 will continue to rise in the future, especially in developing countries (Stocker et al. 2013; IPCC 2014). According to the IPCC scenario, the Representative Concentration Pathway (RCP) 8.5 corresponds to a highest greenhouse gas emissions trajectory for future climate (IPCC 2013). At RCP 8.5, the risk of O_3 injury in global vegetation is projected to rise by 70% from 2000 to 2100 (Sicard et al. 2017). The largest O_3 increase is expected in the tropic and sub tropic countries, e.g., India and other Southeast Asian countries (Wild et al. 2012; Cooper et al. 2014; Guarin et al. 2019). In India, high O_3 concentration has been reported in Indo-Gangetic Plains (IGP) region (Oksanen et al. 2013; Dolker and Agrawal 2019).

Tropospheric O_3 causes phytotoxicity to fast-growing woody species, which has been well documented in the

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North American and European tree species, e.g. *Betula pendula* (Kontunen-Soppela et al. 2007, 2010), Hybrid Poplar clone *Populus maximowizii* × *P. trichocarpa* (Orendovici-Best et al. 2008), *Populus alba* × *P. glandulosa* (Hu et al. 2015) and *Quercus ilex* (Gerosa et al. 2015). O_3 causes a number of physiological and biochemical changes in the leaves of a tree, including a decline in photosynthesis, stomatal closure, loss of chlorophyll content, early senescence and reduced productivity (Matyssek et al. 2007; Tausz et al. 2007; Pina and Moraes 2010) resulting reductions in total biomass (Gerosa et al. 2015; Hu et al. 2015). However, few studies have been conducted on Asian trees under O_3 stress (Yamaguchi et al. 2007; Li et al. 2016). In India, the impact of elevated O_3 on a fast-growing leguminous tree species *Leucaena Leucocephala* (Lam.) de Wit under O_3-FACE condition was first time reported by us (Singh et al. 2021). However, the effects of O_3 on belowground processes have been less examined, especially in forested ecosystems (Kasurinen et al. 2005; Matyssek et al. 2012).

Tropospheric O_3 can impact nutrient cycling by two mechanisms, i.e. direct and indirect, namely by (a) changing the chemical property of plant tissue, (b) changing the quantity/quality of litter fall, (c) impact on belowground biomass and root exudates of plant, (d) indirectly altering microbial structure and functioning, and (f) indirectly influencing soil chemical properties and their processes (Mills et al. 2013). While the diffusion of O_3 into the soil is relatively less (Toet et al. 2009), EO_3 exposure causes an indirect effect on belowground microbial populations mainly due to the reduction in belowground carbon (C) allocation (Andersen 2003). Similarly, O_3 affects the plant roots and various soil processes (Pregitzer et al. 2008; Nikolova et al. 2010) and indirectly affects the absorption and distribution of macro-nutrients, e.g. phosphorus (P) and nitrogen (N) (Inclan et al. 2005; Piikki et al. 2007; Weigt et al. 2012; Zheng et al. 2013).

In soil, microbes are important biological indicator for soil enzymatic activities. Various soil enzyme activities have been used to measure soil process, soil fertility and establish their biological index (Beck 1984; Stefanic et al. 1984; Pascual et al. 2000; Pal et al. 2013; Satapute et al. 2019). Several plant growth-promoting fungi (PGPFs) isolates like *Trichoderma*, *Penicillium*, *Fusarium*, and *Phoma* in rhizosphere soils are known for solubilization of phosphates, minerals, and micro-nutrients (Singh et al. 2011; Jogaiah et al. 2013). There are number of soil enzymes which are involved in the degradation and hydrolysis of major litter (fallen leaves) components which are associated with C (β-glucosidase), P (phosphatase), N (urease), and S (aryl sulfatase) cycling (Karaca et al. 2010; Adetunji et al. 2017). Some other soil enzymes that can be used as biological soil quality indicators include dehydrogenase, protease and cellulase (Karaca et al. 2010; Adetunji et al. 2017). Soil enzymes are closely linked with soil organic matter (SOM), soil macro- and micro-nutrients, and microorganism/microbial biomass activity. Soil enzyme activities are extremely responsive to environmental changes and can provide clues for a range of changes in the soil–plant structure (Saiya-Cork et al. 2002; Burns et al. 2013). In soil, the rhizospheric zone of the root system is reported to be more sensitive to O_3 than the non-rhizospheric (bulk) zone of soil (Chen et al. 2009; Cao et al. 2018).

Leucaena leucocephala (Fabaceae) is a perennial and economically important fast-growing leguminous tree widely distributed across the world. It has root nodules that contain nitrogen-fixing bacteria and arbuscular mycorrhizal fungi (AFM) that fix N and P, thereby improving soil quality (Cubillos-Hinojosa et al. 2019). Previous studies have reported that fast-growing trees such as *Populus nigra* are more sensitive to O_3 than slow-growing tree species such as *Fagus sylvatica* (Bortier et al. 2000; Hayes et al. 2015). Thus, we hypothesized that the continuous open-air exposure to EO_3 will adversely affect the growth as well as soil biochemical properties of *L. leucocephala* rhizospheric soil. Our specific objectives were (i) to observe the growth response of *L. leucocephala* to EO_3 (ii) to determine the impact of EO_3 on rhizospheric soil nutrients (iii) to measure the impact of EO_3 on extracellular enzymes and soil microbial biomass.

Materials and methods

Experimental site and set-up

The Free Air Ozone Concentration Enrichment (O_3-FACE) experiment was set up at CSIR-NBRI, Lucknow city (26.55°N 80.59°E), India (Fig. 1). The study site has a humid subtropical climate and annual precipitation is about 700 mm and the highest precipitation is recorded from July to mid-September month. The O_3-FACE ring consists of four hexagonal shaped rings (10 m diameter and 5 m height) with horizontal and vertical galvanized iron (GI) pipes having nozzles for the release of O_3. Two rings are used for elevated ozone (EO_3) and two for ambient ozone (AO_3) exposure.

Seeds from 10-year-old *L. leucocephala* tree, growing in CSIR-NBRI, campus, were used. For seed germination, healthy seeds were sterilized with 3% hydrogen peroxide solution for 15 min and rinsed thoroughly with double distilled water. Sterilized seeds (about 100) were sown in vermin-compost: sand (2:1) filled trays in the controlled poly house conditions (temperature 29 ± 1 °C and humidity 65 ± 5%). After germination of new leaves, 100 seedlings were transplanted in 30 L polypropylene pots. After 30 days of transplantation, 48 *L. leucocephala* seedlings (having

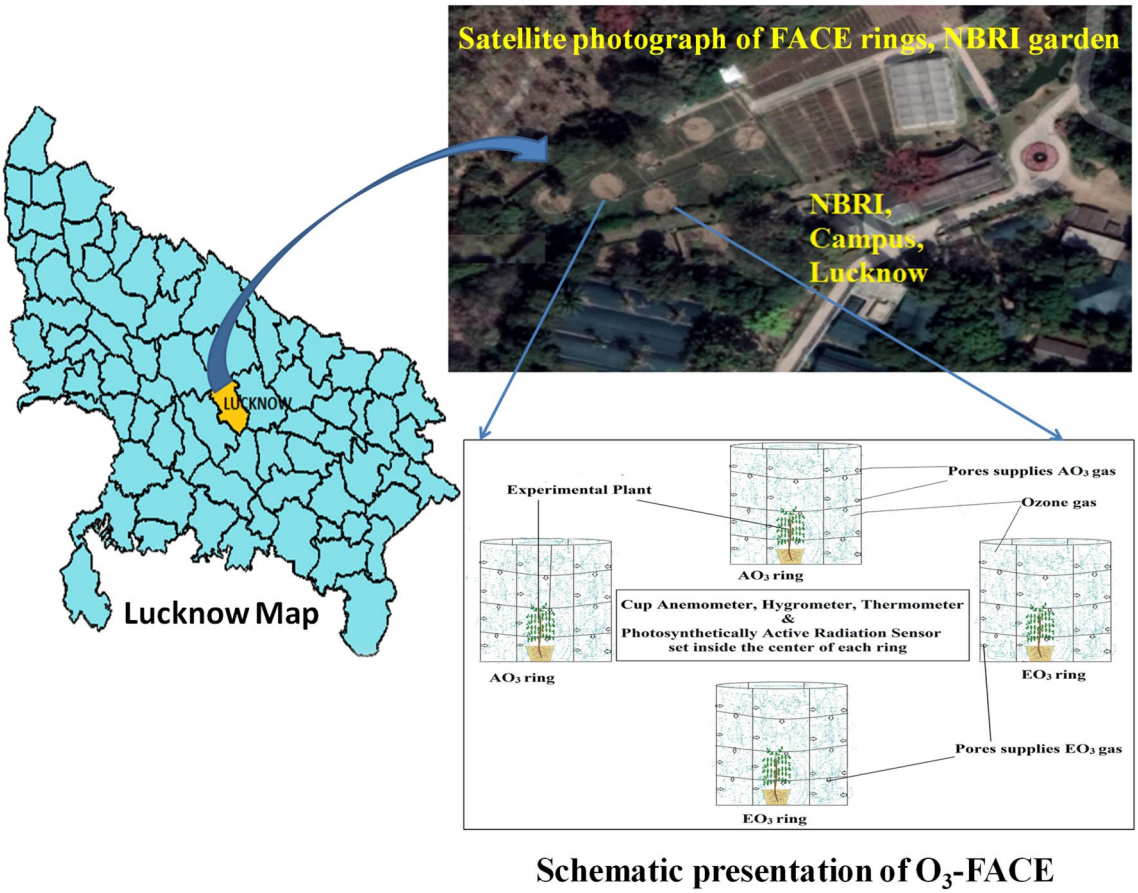

Fig. 1 Experimental site showing O_3-FACE facility

similar height and basal diameter) were placed in O_3-FACE rings for the O_3 exposure (each ring having 12 pots each).

Ozone exposure

In the present study, O_3 fumigation started from 10th Jan 2018 and after 6 months of exposure, the first sampling was done on 14th Jun 2018, 12 months sampling was done on 29th Dec 2018, 18 months sampling was done on 28th Jun 2019 and final harvesting was done on 31st Dec 2019. O3 exposure was provided daily 9 h from 09:30 to 17:30 h. O_3 generator (model no. S3-300, AM Ozonics Pvt. Ltd. Mumbai, Maharashtra, India) produces ozone from ambient air pure oxygen source and directly dispensed through nozzles on *L. leucocephala* seedling from the upwind vertical and horizontal pipes. O_3 concentrations were continuously monitored from the middle of each AO_3 and EO_3 ring using UV photometric O_3 analyzers (Model O_3 LEDM) made by Automatikprodukter. The concentration of EO_3 was maintained at around plus 20 ppb above AO_3 concentration as per the projections reported by Sicard et al. (2017). All the meteorological data (temperature and relative humidity), were transferred by Supervisory Control and Data Acquisition (SCADA) software. During the experiment, the accumulation over a threshold hourly O_3 concentration of 40 ppb in daylight hours (AOT40) were calculated in ppm.h for EO_3 and AO_3 treatments for different time intervals (Supplementary Table S1). The O_3 supply was stopped in some environmental conditions such as under the higher wind speed (>10 ms^{-1}), drizzling, and low photosynthetically active radiation (PAR) (<100 mmol m^{-2}).

Plant sampling and biomass analysis

Randomly, six replicates of plant samples were selected from AO_3 and EO_3 ring for the measurement of shoot length (SL) and after SL, plant were harvested completely for measurement of dry biomass, aboveground (stems and leaves) and belowground (root) biomass at different time intervals (after 6, 12, 18, and 24 months of O_3 exposure) in year 2018–2019. The material of the replicate plant sample was broken into different parts, and the different parts of the plant were weighed separately. To obtain dry biomass, plant samples were oven-dried at 60 °C to the constant weight. *L.*

leucocephala tree biomass was measured in per gram dry weight basis. Leaf biomass (LB), root biomass (RB), shoot biomass (SB), total biomass (TB) and root to shoot (R/S) ratio were measured in the samples.

Soil sampling and physico-chemical analysis

During four exposure periods (after 6, 12, 18, and 24 months) from 2018 to 2019, the rhizospheric soil particles loosely attaching to the roots of harvested *L. leucocephala* were removed by lightly hand shaken method for 10 min. The remaining moist soil layer (1–3 mm) adhering to the roots surface was considered as rhizosphere soil (Nazih et al. 2001), which was collected for testing by shaking and gentle brushing of the roots (Massaccesi et al. 2015). The six replicates of EO_3 and AO_3 treated rhizospheric soil samples were homogenized separately and passed through a 2 mm sieve to remove roots, stones, macrofauna and litter fractions in the laboratory. These rhizospheric soil samples were further separated into three sub-samples. The 1st sub-sample was desiccated at 25 ± 1 °C room temperature till soil achieved stable weight—prior to examination of soil physico-chemical parameters and 2nd sub-sample was kept at 4 °C for the estimation of microbial biomass C (MBC), P (MBP), and N (MBN). A 3rd sub-sample was directly frozen at − 20 °C for the assessment of soil enzymatic activities.

Prior to the experiment, the physico-chemical and biological parameters were measured in garden soil (Table 1) where soil texture was measured by the hydrometric method. Soil bulk density, particle density and porosity were determined by the method of Black et al. (1965). In 1st sub-sample of soil, pH was determined in 1:2.5 ratio of soil:water extract using a pH meter (Model EA940, Orion, U.S.A). Soil total organic carbon (TOC) was assessed by the method of Walkley and Black (1934). Available nitrogen (AN) and total nitrogen (TN), respectively, were estimated by Stanford and Smith (1978) method and Kjeldahl nitrogen method (Jackson 1958). Available phosphorous (AP) content was determined by Olsen (1954) method. Total phosphorous (TP) was estimated by Sparling et al. (1985) method, followed by hot plate acid (nitric acid/perchloric acid) digestion in ratio 3:1 at high temperature (180 °C) for 6 h. Available sulfur (AS) was estimated using the Turbidity method of Chesnin and Yien (1950).

Table 1 Physico-chemical properties of the garden soil before start of O_3 treatment (Jan 2018) at the O_3-FACE site in NBRI, Lucknow

	O_3-FACE soil
Physical factors	
pH	8.7 ± 0.02
Bulk density (mg m^{-3})	1.4 ± 0.02
Particle density (mg m^{-3})	3.3 ± 0.5
Porosity (%)	59.9 ± 0.7
Soil texture (%)	50, 28, 23
Chemical factors	
Available phosphorus (mg kg^{-1})	130 ± 12
Available nitrogen (mg kg^{-1})	360 ± 20
Available sulfur (mg kg^{-1})	14.9 ± 4
Total organic carbon (mg kg^{-1})	8600 ± 112
Total nitrogen (mg kg^{-1})	742 ± 10
Total phosphorus (mg kg^{-1})	1401 ± 54
Biological factors	
Microbial biomass carbon (mg kg^{-1})	240 ± 9
Microbial biomass phosphorus (mg kg^{-1})	84 ± 5
Microbial biomass nitrogen (mg kg^{-1})	48 ± 3
Dehydrogenase (µg TPF g^{-1} 24 h^{-1})	3.2 ± 0.02
β-glucosidase (µg *p*-nitrophenol g^{-1} h^{-1})	9.1 ± 1
Alkaline phosphatase (µg *p*-nitrophenol g^{-1} h^{-1})	105 ± 3
Fluorescein diacetate hydrolysis (µg fluorescein g^{-1} 2 h^{-1})	8.6 ± 0.3
Aryl sulfatase (µg *p*-nitrophenol g^{-1})	280 ± 30
Protease (µg *p*-tyrosine g^{-1} 2 h^{-1})	28.2 ± 3
Cellulase (µg glucose g^{-1} 24 h^{-1})	1.4 ± 0.01

Mean value ± standard error (SE) ($n = 6$)

Soil microbial biomass C, P, and N analyses

Soil MBC, MBP, and MBN were estimated using the chloroform fumigation–extraction method as specified by Vance et al. (1987), Brookes et al. (1982) and Brookes et al. (1985), respectively, in 2nd sub-sample of soil. For estimation of MBC and MBN, 0.5 M of potassium sulfate (K_2SO_4) extraction solution was used, while 0.5 M of sodium bicarbonate ($NaHCO_3$) extraction solution was used estimate of MBP.

For MBC characterization, the extracted solution was filtered with Whatman No. 42 filter paper and the filtrate solution was examined by potassium dichromate ($K_2Cr_2O_7$) method as described by Vance et al. (1987). In this method, the filtrate was titrated with 0.4 N ammonium ferrous sulfate solutions till the solution color turned green. The C concentration in the extract solution was examined by the formula given below:

$$\text{Microbial biomass carbon}\left(\text{mg kg}^{-1}\right) = \frac{\text{Fumigated} - \text{Non Fumigated}}{\text{Kc Value}} \times 10^4 \quad (1)$$

where Kc value is 0.33 (Sparling and West 1988).

The percentage of organic C present as microbial C is expressed as the ratio of MBC to TOC (MBC/TOC) and is known as the microbial quotient. For MBP characterization, the filtrate solution was measured by Olsen and Sommers

1982) method. In this, 5 mL of the filtrate was taken and 1 mL of composite reagent was added for developing the blue color after 5 min. After this, sample absorbance was taken at 885 nm using a Helios UV-β spectrophotometer (Thermo Electron Corporation, England). The concentration of MBP in the extract solution was determined by the formula given below:

$$\text{Microbial biomass phophorus}\left(\text{mg kg}^{-1}\right) = \frac{\text{Fumigated} - \text{Non Fumigated}}{\text{Kp Value}} \times 10^4 \quad (2)$$

where Kp value is 0.40 (Sparling and West 1988).

Similarly, for MBN characterization, the filtrate solution was analyzed using the Kjeldahl method. The MBN concentration in the extract was determined by the formula given below:

$$\text{Microbial biomass nitrogen}\left(\text{mg kg}^{-1}\right) = \frac{\text{Fumigated} - \text{Non Fumigated}}{\text{Kn Value}} \times 10^4 \quad (3)$$

where Kn value is 0.54 (Brookes et al. 1985).

Soil enzymatic assays

In third sub-sample of soil, dehydrogenase (DHA) activity was analyzed by the method of Casida et al. (1964) at 485 nm and expressed as µg triphenyl formazan (TPF) g^{-1} 24 h^{-1}. β-glycosidase (βG) activity was analyzed as per the method of Eivazi and Tabatabai (1988) at 420 nm. The activity of soil alkaline phosphatase (AlkP) was determined by Tabatabai and Bremner (1969) method at 400 nm. AlkP and βG enzyme activities were expressed as µg *p*-nitrophenol g^{-1} h^{-1}. Fluorescein diacetate hydrolase (FDA) activity was determined by the method of Adam and Duncan (2001) at 490 nm. FDA hydrolase enzyme activity was expressed as µg fluorescein g^{-1} 2 h^{-1}. Aryl sulfatase (ASA) and protease (PA) activities were measured by using the standard methods of Dick (2011)and Srivastava et al. (2012), respectively. ASA and PA were expressed as µg *p*-nitrophenol g^{-1} h^{-1} and µg *p*-tyrosine g^{-1} 2 h^{-1}, respectively. Cellulase (CeA) activity was measured by the method of Tabatabai (1994) and expressed as µg glucose g^{-1} 24 h^{-1}.

Statistical analysis

Statistical analyses of data (EO_3 Vs AO_3 treatment) were done for all parameters and summarized as mean ± standard error (SE). Under EO_3 and AO_3 treatments, mean values of tree biomass and soil parameters were differentiated using a paired Student's t test. Primarily, the normality (Shapiro–Wilk W test) and homogeneity (Levene's test) of data were tested by SPSS 16.0 software. Univariate General Linear model (repeated measures one-way analysis of variance i.e. RM-ANOVA) was performed between subject factor (EO_3 and AO_3) and within subjects' variable (time intervals) to find the effect of time on biomass and soil biochemical responses in O_3-FACE using SPSS 16.0 software. The outcomes were considered statistically significant at $p \leq 0.05$.

Results

The O_3-FACE system

The *L. leucocephala* was exposed to O_3 under field conditions (O_3-FACE) from Jan. 2018 through to Dec. 2019. During the exposure period, AO_3 concentration varied from 40.0 to 64.4 ppb, 40.1 to 51.6 ppb, 40.4 to 69.3 ppb and 40.1 to 52.9 ppb at 6, 12, 18 and 24 months, respectively (Table S.1). The concentration of EO_3 ranged between 56.7 and 79.9 ppb, 51 and 71.5 ppb, 62.6 and 82.2 ppb and 59.7 and 79.3 ppb at 6, 12, 18 and 24 months, respectively. The average concentration of AO_3 was 50.8, 43.3, 52.6, and 46.0 ppb at 6, 12, 18, and 24 months exposure period, respectively. During the experimental period, the mean values of EO_3 were 70.9, 62.0, 72.8 and 65.3 ppb at 6, 12, 18 and 24 months exposure period, respectively. During the exposure period, cumulative AOT40 values for AO_3 were 18.5, 24.3, 46.0 and 56.4 ppm.h, whereas cumulative AOT40 values for EO_3 exposure were 53.3, 91.4, 148.1 and 191.9 ppm.h at 6, 12, 18 and 24 months exposure period, respectively (Table S.1).

Effects of long-term ozone exposure on plant growth

Elevated O_3 exposure negatively impacted the biomass of *L. leucocephala* at different time intervals (Table 2). A significant reduction was observed in SL after 12 (21%) and 24 (15%) whereas reduction in RB was observed after 12 (14%), 18 (14%) and 24 (12%) months of exposure to EO_3, respectively. SB reduced significantly after 12 (16%) and 24 (11%) months of EO_3 exposure.

Similarly, a significant reduction was observed in LB after 18 (20%) and 24 (37%) months of EO_3 exposure. TB of *L. leucocephala* declined significantly by 15, 12, and 15% after 12, 18, and 24 months of EO_3 exposure, respectively, in comparison to AO_3. Elevated O_3 had no significant impact on the R/S ratio of the *L. leucocephala*. According to one-way RM-ANOVA, the interaction between O_3 and time was significant for SB, LB, TB and R/S ratio, whereas SL and RB did not show any significant interaction (Table 2).

Table 2 Effect of ozone on *L. leucocephala* biomass at different exposure periods

Response variables	6 month		12 month		18 month		24 month		RM-ANOVA		
Growth factors	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	Time	Ozone	Time x Ozone
Shoot length (m)	2.58 ± 0.11	2.36 ± 0.12	3.28 ± 0.09	2.60 ± 0.11**	4.49 ± 0.10	4.21 ± 0.12	5.44 ± 0.17	4.61 ± 0.18*	***	**	ns
Root biomass (g)	345 ± 20	289 ± 23	961 ± 17	831 ± 28*	1438 ± 26	1233 ± 33*	1512 ± 43	1330 ± 37*	***	***	ns
Shoot biomass (g)	363 ± 13	332 ± 9	1768 ± 68	1481 ± 59*	2148 ± 81	1935 ± 83	2998 ± 81	2659 ± 56*	***	**	*
Leaf biomass (g)	126 ± 3	109 ± 6	251 ± 11	220 ± 7	410 ± 8	330 ± 10**	607 ± 40	383 ± 20**	***	***	***
Total biomass (g)	830 ± 30	729 ± 35	2980 ± 59	2537 ± 46***	3996 ± 89	3497 ± 108*	5116 ± 99	4372 ± 53***	***	***	***
Root: Shoot ratio	0.95 ± 0.05	0.86 ± 0.05	0.55 ± 0.03	0.57 ± 0.04	0.67 ± 0.02	0.64 ± 0.02	0.51 ± 0.02	0.50 ± 0.02	***	ns	*

The student's *t* test shows individual differences between AO_3 and EO_3 and the repeated measurement one-way ANOVA shows interactions between ozone and time interval. Data are mean ± standard error (SE) ($n = 6$). Asterisks indicate the level of significance, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

Effects of long-term ozone exposure on soil chemical properties

Continuous exposure to EO_3 resulted in a significant alteration in the rhizospheric soil chemical properties of *L. leucocephala* except for pH, whereas time had a significant influence on all soil chemical parameters (Table 3). The soil pH ranged between 8.2 and 8.9 and showed no significant change under EO_3 compared to AO_3 during the experimental period. Under overall exposure periods, the values of available nutrients in the soil ranged between 101–252 mg kg^{-1} for AP, 224–448 mg kg^{-1} for AN and 10.1–21.5 mg kg^{-1} for AS (Table 3). Almost all the available form of nutrients showed a declining trend, though differently, under EO_3 exposure in comparison to AO_3. Soil AS and AP contents were significantly ($p \leq 0.05$) lower after 6, 12, and 24 months of EO_3 exposure except 18 months of exposure period. Soil AN was found decreased after 6 and 12 months of exposure period but significant reductions were observed after 18 and 24 months of EO_3 exposure period.

The values of soils TOC, TN, and TP under overall exposure periods, ranged between 6460–9960 mg kg^{-1},715–830 mg kg^{-1}, and 1010–1790 mg kg^{-1}, respectively (Table 3). The ratio of carbon to nitrogen (C/N) ranged between 7.8 and 12.6 under the entire O_3-FACE experiment. The total forms of nutrients as well as the C/N ratio did not show any significant change after 6, 12, and 18 months of EO_3 exposure, however, soil TOC, TP and C/N ratio declined significantly by 17, 15, and 15% after 24 months of EO_3 exposure, respectively. One-way RM-ANOVA showed interaction between O_3 and time which was significant for AP, TOC, and C/N ratio whereas pH, AS, AN and TP did not show any significant interaction (Table 3).

Effects of long-term ozone exposure on soil microbial biomass C, P and N

Continuous exposure to EO_3 resulted in reduction in soil MBC, MBP, MBN and their ratio along with soil nutrients except for MBC/MBN ratio. Whereas, time had a significant influence on MBC, MBP, MBN and their ratio with nutrients of *L. leucocephala* rhizospheric soil (Table 4). Soil MBC, MBP and MBN values ranged between 270 and 430 mg kg^{-1}, 49.3 and 91.7 mg kg^{-1} and 40.6 and 65.1 mg kg^{-1}, respectively, during the whole exposure (AO_3 and EO_3) periods (Table 4). Significant reductions in MBC were observed after 12 (23%), 18 (15%) and 24 (22%) months of EO_3 exposure, while significant reductions in MBP were observed after 6 (22%), 12 (22%), 18 (17%) and 24 (36%) months of EO_3 exposure. Similarly, soil MBN was reduced significantly by 18, 13, 19 and 14% after 6, 12, 18 and 24 months of EO_3 exposure, respectively, under O_3-FACE experiment (Table 4).

Table 3 Soil nutrient parameters of *L. leucocephala* rhizospheric soil at different exposure periods

Soil nutrient factors	6 month		12 month		18 month		24 month		RM-ANOVA		
	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	Time	Ozone	Time x Ozone
pH	8.5 ± 0.06	8.3 ± 0.04	8.6 ± 0.04	8.6 ± 0.02	8.6 ± 0.04	8.4 ± 0.04	8.8 ± 0.04	8.5 ± 0.07	*	ns	ns
Available phosphorus (mg kg^{-1})	138 ± 2.0	114 ± 3.7***	174 ± 2.5	147 ± 4.3***	126 ± 1.1	123 ± 1.3	225 ± 8.0	192 ± 4.4**	***	***	ns
Available nitrogen (mg kg^{-1})	374 ± 24	299 ± 12	286 ± 26	243 ± 12	340 ± 23	250 ± 12*	355 ± 12	280 ± 20***	***	***	**
Available sulfur (mg kg^{-1})	15.3 ± 0.01	12.9 ± 0.05***	20.0 ± 0.38	17.1 ± 0.56*	14.0 ± 0.39	12.1 ± 0.52	17.8 ± 0.18	15.3 ± 0.18***	**	***	ns
Total organic carbon (mg kg^{-1})	8623 ± 84	8491 ± 84	8867 ± 235	8667 ± 208	8623 ± 84	8491 ± 85	8975 ± 220	7426 ± 288*	***	**	*
Total nitrogen (mg kg^{-1})	747 ± 8	743 ± 10	780 ± 6	769 ± 5	747 ± 8	755 ± 5	822 ± 3	803 ± 11	***	***	***
Total phosphorus (mg kg^{-1})	1418 ± 26	1353 ± 23	1454 ± 78	1224 ± 59	1498 ± 62	1463 ± 56	1732 ± 29	1479 ± 44*	***	**	ns
C:N ratio	11.6 ± 0.23	11.4 ± 0.19	11.4 ± 0.31	11.3 ± 0.29	11.6 ± 0.16	11.2 ± 0.15	10.9 ± 0.30	9.3 ± 0.39*	***	*	*

The student's *t* test shows individual differences between AO_3 and EO_3 and the repeated measurement one-way ANOVA shows interactions between ozone and time interval. Data are mean ± standard error (SE) ($n = 6$). Asterisks indicate the level of significance, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

Microbial biomass ratio, i.e. MBC/MBP was significantly increased after 6 (22%) and 24 (23%) months of EO_3 exposure, however, MBC/MBN ratio was reduced significantly only after 12 (11%) months of EO_3 exposure. Soil MBC/TOC ratios were significantly lower after 12 (21%) and 18 (14%) months of EO_3 exposure, while MBP/TP ratios were reduced significantly after 6 (18%), 18 (16%) and 24 (25%) months of EO_3 exposure as compared to AO_3. Soil MBN/TN ratios were decreased significantly after 12 (22%), 18 (16%) and 24 (20%) months of EO_3 exposure. RM-ANOVA results showed significant interactions between O_3 and time on MBC, MBP, MBC/MBN, MPC/MBP and MBC/TOC (Table 4).

Effects of long-term ozone exposure on soil enzymatic activity

Elevated O_3 and time had individual impact on soil enzymatic activities. RM-ANOVA result showed significant interaction between O_3 and time for soil enzyme parameters which are summarized in Fig. 2. Soil enzyme activities varied during the whole exposure (AO_3 and EO_3) period and ranged between 2.1 and 5.3 µg TPF g^{-1} 24 h^{-1} for DHA, 7.7 and 17.8 µg *p*-nitrophenol g^{-1} h^{-1} for βG, 104 and 190 µg *p*-nitrophenol g^{-1} h^{-1} for AlkP, 8.02 and 16.6 µg fluorescein g^{-1} 2 h^{-1} for FDA, 32.0 and 60.4 µg *p*-tyrosine g^{-1} 2 h^{-1} for PA, 225 and 480 µg *p*-nitrophenol g^{-1} h^{-1} for ASA and 1.14 and 3.63 µg glucose g^{-1} 24 h^{-1} for CeA.

The AlkP activities in rhizospheric soil of *L. leucocephala* showed a significant reduction of 15, 14, 18 and 20% after 6, 12, 18 and 24 months of EO_3 exposure, respectively (Fig. 2). Similarly, reduction in soil FDA enzymes activities were observed after 6 (13%), 12 (23%), 18 (12%) and 24 (22%) months of EO_3 exposure. After 6, 12 and 24 months of EO_3 exposure, a significant reduction was observed in DHA, βG, PA and CeA activities. The ASA activity showed declining trend after 6 and 24 months of EO_3 exposure.

Discussion

Ozone concentrations

In the present experiment, the ambient O_3 concentration was higher during the months of January to February (6 and 18 months sampling period in both years, 2018 and 2019) and from the late September to mid-November (12 and 24 months sampling period in both years, 2018 and 2019). Moreover, higher concentrations of O_3 were observed in the afternoon hours from the starting of March to June during 6 and 18 months sampling periods in both years, i.e., 2018 and 2019 because of higher temperature, longer sunshine period and less relative humidity with stable wind speed (Jain et al.

Table 4 Microbial biomass carbon, phosphorus, nitrogen, and their ratio with soil nutrients of *L. leucocephala* rhizospheric soil

Response variables	6 month		12 month		18 month		24 month		RM-ANOVA		Time x Ozone
	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	AO_3	EO_3	Time	Ozone	
Microbial biomass carbon (mg kg^{-1})	295 ± 4.22	280 ± 3.61	417 ± 4.17	322 ± 5.90***	339 ± 2.31	287 ± 2.12***	391 ± 1.18	306 ± 0.077***	***	***	***
Microbial biomass phosphorus (mg kg^{-1})	69 ± 1.31	54 ± 1.18**	77 ± 3.19	60 ± 2.64**	72 ± 1.88	60 ± 2.51*	85 ± 2.21	54 ± 0.83***	***	***	***
Microbial biomass nitrogen (mg kg^{-1})	55 ± 0.99	45 ± 1.57**	53 ± 0.69	46 ± 1.28*	61 ± 1.95	49 ± 2.56*	63 ± 0.48	55 ± 2.61*	***	***	ns
MBC:MBP ratio	4.3 ± 0.11	5.2 ± 0.14*	5.5 ± 0.24	5.4 ± 0.33	4.7 ± 0.09	4.8 ± 0.21	4.6 ± 0.10	5.7 ± 0.07***	***	**	**
MBC:MBN ratio	5.4 ± 0.14	6.3 ± 0.29	7.9 ± 0.12	7.1 ± 0.25*	5.6 ± 0.22	6.0 ± 0.35	6.2 ± 0.06	5.7 ± 0.26	***	ns	**
MBC:TOC ratio	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.04 ± 0.00**	0.04 ± 0.00	0.03 ± 0.00**	0.04 ± 0.00	0.04 ± 0.00	**	**	***
MBP:TP ratio	0.05 ± 0.00	0.04 ± 0.00*	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00*	0.05 ± 0.00	0.04 ± 0.00***	**	**	ns
MBN:TN ratio	0.40 ± 0.01	0.38 ± 0.01	0.54 ± 0.00	0.42 ± 0.01***	0.45 ± 0.01	0.38 ± 0.00***	0.48 ± 0.00	0.38 ± 0.00***	***	***	ns

The student's *t* test shows individual differences between AO_3 and EO_3 and the repeated measurement one-way ANOVA shows interactions between ozone and time interval. Data are mean ± standard error (SE) ($n = 6$). Asterisks indicate the level of significance, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

mg kg^{-1}: milligram per kilogram, *MBC:TOC ratio* microbial biomass carbon to total organic carbon ratio, *MBC:MBN ratio* microbial biomass carbon to microbial biomass nitrogen ratio, *MBN:TN ratio* microbial biomass nitrogen to total nitrogen ratio, *MBP:TP ratio* microbial biomass phosphorus to total phosphorus ratio, *MBC:MBP ratio* microbial biomass carbon to microbial biomass phosphorus ratio

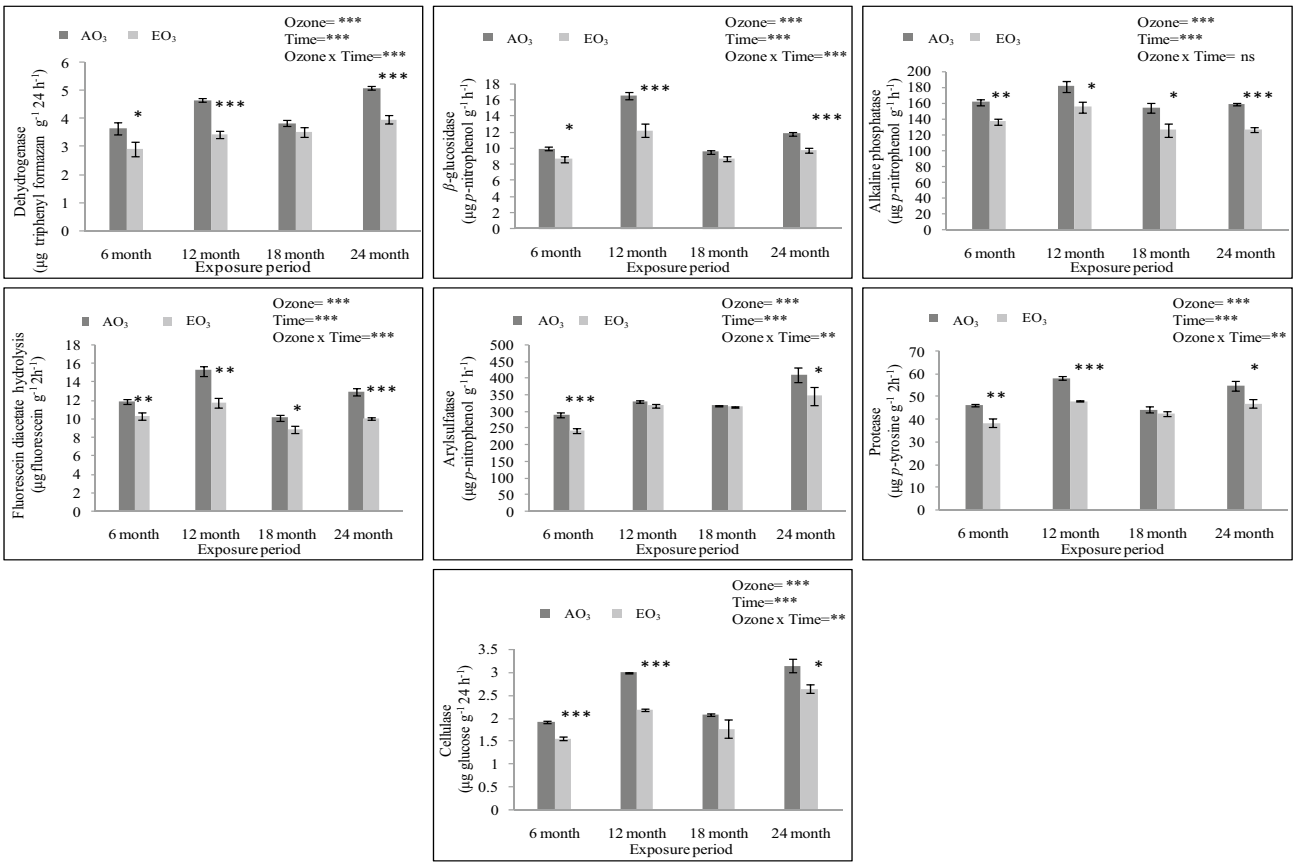

Fig.2 Soil enzymatic activities of *L. leucocephala* rhizospheric soil under elevated O_3 (EO_3) and ambient O_3 (AO_3) exposure. The student's *t* test shows individual differences between AO_3 and EO_3 and the repeated measurement one-way ANOVA shows interactions between ozone and time interval. Data are mean ± standard error (SE) ($n=6$). Asterisks indicate the level of significance, $*P<0.05$, $**P<0.01$, and $***P<0.001$

2005; Tiwari et al. 2008; Singh et al. 2010). Lower O_3 concentrations were recorded between the end of November to December and end of the July to mid-September in both years of exposure, i.e., 2018 and 2019. The low O_3 concentrations during these months were due to precipitation, which leads to washout of the O_3 precursor, and were previously discussed by Singh et al. (2021). The aim of this experimental work was to assess whether 2-year continuous exposure of EO_3 leads to changes in *L. leucocephala* growth, soil biochemical as well as soil microbial responses.

Response of tree growth to elevated ozone

Some studies have reported that tree species with more leaf mass per area (LMA) could be resistant to O_3 (Ribas et al. 2005; Li et al. 2017). On the other hand, long-term O_3 exposure, particularly during growing seasons is likely to result in growth reductions in fast-growing tree species (e.g., Birch) under open-field conditions (Kostiainen et al. 2006; Oksanen et al. 2007). In the present study, EO_3 treatment caused significant reductions in SL after 12 (AOT40 value 91.4 ppm h) and 24 (AOT40 value 192 ppm h) months of exposure to O_3 suggesting that *L. leucocephala* potential will be significantly reduced in capturing carbon from the atmosphere by rising O_3 in the future. A similar finding was reported in meta-analysis of various Chinese woody species (Li et al. 2017). The LB was decreased significantly at 18 (mean concentration of O_3 62.7 ppb) and 24 (mean O_3 concentration 65.3 ppb) months of EO_3 exposure. This decrease is mainly attributed to reduced photosynthesis due to EO_3 (Ainsworth et al. 2012). A previous study showed that EO_3 significantly decreased LB of *Phoebe bournei* and *Phoebe zhennan* in the subtropical region in China (Chen et al. 2018). In our experiment, significant reductions were observed in SB and RB at 12 and 24 months of EO_3 exposure. The decrease in RB could result in less productivity of terrestrial ecosystems, due to decreased soil microbial competition and may predispose trees to various stresses such as drought, nutrient deficiency, parasites, etc. (Pan et al. 2020). The plant root is highly sensitive to O_3 than the shoot (Feng et al. 2008). It has been shown that more C is retained in the aboveground parts (stem and leaves) for O_3 detoxification

and comparatively less C is allocated to belowground, leading to the larger effects of O_3 on roots (Li et al. 2019). Similar results were reported in two deciduous species, *Liriodendron chinense* and *Liquidambar formosana,* which were exposed to EO_3 in open-top chambers in China (Zhang et al. 2012). Similar findings were also reported in three meta-analyses where EO_3 concentrations reduced RB of woody species by 13% in tress of China (average concentration of O_3, 128 ppb) (Li et al. 2017), 19% in trees of European and American regions (average concentration of O_3, 101 ppb) (Wittig et al. 2009) and 23% in Poplar genus in Northern Hemisphere (average concentration of O_3, 75 ppb) (Feng et al. 2019). In the present study, reduction in TB after 12, 18 and 24 months of EO_3 exposure resulted in less resource allocation to belowground part and consequent reduction of TB which has been widely accounted worldwide (Shang et al. 2017; Li et al. 2019). Under EO_3 treatment, R/S ratio declined but it was not significant during whole exposure periods which is consistent with previous findings in Poplar clones (Gao et al. 2017; Li et al. 2019) and other meta-analyses results (Yendrek et al. 2013; Li et al. 2017). Changes in the R/S ratio result in decreased C assimilation amplifying metabolic costs and reduced phloem loading (Andersen 2003). Our results also confirmed that fast-growing tree species tend to be more sensitive to O_3 than slow-growing tree species (Bortier et al. 2000; Novak et al. 2005).

Response of soil nutrients to elevated ozone

Ozone mostly shows an indirect effect on belowground processes (Andersen 2003; reviewed in Agathokleous et al. 2016). Soil processes, e.g., organic matter decay and nutrient cycling, are determined by soil microorganisms and their relations with plants and soil abiotic (temperature, moisture, etc.) environments (Wardle et al. 2004). The labile nutrients for the plants are influenced by soil pH (Zhao et al. 2012). Variety of root exudates, mainly carbohydrates, impacts the microbial diversity and structure as well as their population (Fierer and Jackson 2006; Pietri and Brookes 2009; Cheng et al. 2013b). In our experiment, soil pH slightly decreased under EO_3 which was possibly caused by release of organic acids in root exudates. The impacts of O_3 on belowground properties (roots and soil nutrients) are mediated through leaves and photosynthesis (Kasurinen et al. 2005). We have earlier reported reduced photosynthesis in *L. leucocephala* under long-term EO_3 exposure (Singh et al. 2021). Furthermore, the exposure of O_3 results in diminished root performance, which reduces the root exudates in soil (Chen et al. 2009) and rhizospheric microbial activities (Yoshida et al. 2001). Root residues and exudates are important sources of C and N to the soil (Booker et al. 2005). In the present study, soil C was not significantly reduced under EO_3 except at 24 months of EO_3 exposure, indicating that this process is more resilient to O_3 stress. It may also be possible as C cycling is a complicated process, linking diverse groups of bacteria and fungi; some of these were enhanced while reduction was also observed in these groups under EO_3 exposure. Similar findings were observed in other studies where EO_3 diminished soil DOC (dissolved organic C) and altered the C cycle in several processes (Jones et al. 2009; Chen et al. 2015b, c; Lu et al. 2015). In our experiment, the reduction in RB under EO_3 exposure may have caused changes in the mineralization of organic form of nitrogen (TN) thus altering the soil N availability (AN) for microorganisms and disturbing soil N cycling (Booker et al. 2005; Bhatia et al. 2011). Furthermore, EO_3 caused decrease in AN from *L. leucocephala* soil after 18 and 24 months of O_3 exposure. This could have been due to either reduction in both the N substrate accessibility or reduced activities of nitrifying bacteria. Similar results were also found by Holmes et al. (2003); Kanerva et al. (2007) and Bhatia et al. (2011).

Phosphorus plays a vital role in physiological functions including photosynthesis and stomatal conductance, energy storage and transfer, cell division, development of new tissues in root (Zhang et al. 2018), nutrient allocation and growth pattern in leguminous crops (Mitran et al. 2018). The solubility of P was significantly declined after 6, 12 and 24 months of EO_3 exposure where continuous increase in AOT40 values were recorded 53.3, 91.4 and 192 ppm h, respectively. The lower P availability in soil shows negative impact of EO_3 on root nodule formation in legumes, as P is essential for energy transformation in root nodules and improved N-fixation rate in soil (Udvardi and Poole 2013; Yadav et al. 2017).

Soil sulfur (AS) is one of the important macro-nutrient which interacts with various stress metabolites to enhance the performance of crops under different environmental conditions including drought, salinity (Gallardo et al. 2014) and O_3 (Tjoelker and Luxmoore 1991). In the present experiment, AS content was decreased after 6, 12 and 24 months of EO_3 exposure which could have also been the cause of discolouration in *L. leucocephala* leaves under EO_3. Reduced S content in soil could be due to lower activity of ASA enzyme present in the soil. Reduction in S content in soil may negatively affect the plants growth due to reduced photosynthetic activity of the plant (Kabata-Pendias and Pendias 2010).

Responses of microbial biomass C, N, and P and their ratio with nutrients to elevated ozone

Various experiments have been performed to evaluate the effect of EO_3 on soil microbial biomass, although the outcomes remain controversial. Some researchers

have observed reduction in microbial biomass (Bao et al. 2015), while others have observed increase in microbial biomass (Mörsky et al. 2008) or no difference in microbial biomass (Zhang et al. 2014) of soils under EO_3. In the present study, negative responses of soil MBC, MBN and MBP were observed due to EO_3 at different time intervals. Exposure of O_3 results in changed C flux to soil by altering rhizo-deposition and litter quality/quantity (Andersen 2003) and in our study this altered microbial biomass in rhizospheric soil of *L. leucocephala* could be due to reduction in RB and substrate availability under EO_3.

Moreover, lower availability of C may cause reduction in microbial immobilization of $NH4^+$ resulting in declined MBN in the rhizospheric zone of *L. leucocephala*. Previously, Chen et al. (2019) also reported reductions in MBC and MBN in soils of *Machilus ichangensis* and *Taxus chinensis* at AOT40 of 145 ppm.h O_3. In contrast, higher microbial biomass was observed in herbaceous flowering plant (*Eriophorum vaginatum)* after 3 year of exposure to EO_3 (Mörsky et al. 2008) and no changes were observed after 3 year of exposure to EO_3 in wheat (Zhang et al. 2014). The adverse impact of EO_3 on microbial biomass of rhizospheric soils of fast-growing tree species, e.g. *Betula papyrifera*, *Populus tremuloides*, *Betula pendula* and *Acer saccharum* have also been documented (Holmes et al. 2003; Kasurinen et al. 2005). Soil MBC/MBN ratios were found to be low in EO_3 treatment in the present study. Under various environments, the comparison between the differences in MBC/MBN is not frequently possible as the rate of mineralization differs with changing circumstances (Brookes et al. 1984; Dalal and Mayer 1987). Soil MBC/TOC ratio is also a useful parameter for the assessment of the soil inorganic C availability as well as a good indicator of alteration in organic matter because of soil condition changes (Cheng et al. 2013a). The significantly lower values of MBC/TOC after 12 and 18 months of EO_3 exposure indicated lower soil organic matter quality, resulting in low C immobilization efficiency by soil microorganisms with higher energy spent. Equally, significant reduction in MBN/TN and MBP/TP demonstrated the poor accessibility of N and P under the EO_3 condition at different time intervals.

Responses of soil enzymes to elevated ozone

Soil enzyme activities depend on various abiotic and biotic factors, e.g., temperature, water potential, pH and also on increasing concentrations of greenhouse gases like CO_2 (Rao et al. 2017) and O_3 (Wang et al. 2019). In the present study, EO_3 and time; these two factors affected quality and quantity of various soil enzymatic activities of rhizospheric soil. Soil hydrolytic extracellular enzymes play key role in degradation of labile C (βG and DHA) and labile P (AlkP) (Burns et al. 2013; Huang et al. 2013). We found that EO_3 exposure reduced soil enzymatic activities linked to C, N, P and S cycle such as βG, DHA, FDA, AlkP, PA, CeA and ASA during the course of the experiment as compared to AO_3 exposure. Although these soil enzyme activities may not be solely responsible for C, N, P and S cycling, still these soil enzyme activities catalyze biochemical reactions in the biogeochemical cycling of C, N, P and S (Kelley et al. 2011). The higher O_3 concentrations resulted in reduced activities of soil extracellular enzymes, e.g. AlkP and ASA, resulting in less availability of P and S to plant roots, thereby reducing RB (Reddy et al. 1995; Knauff et al. 2003). Soil ASA activity accelerates the hydrolysis process of organic S compound, i.e., sulfate ester or inorganic sulfate (Kertesz and Mirleau 2004).

CeA enzyme activity was reduced under the EO_3 condition which could have been reduced due to the reduction in C content in the fine roots of plants (Bottino et al. 2016). Soil CeA activity stimulates hydrolysis process and convert cellulose to D-glucose form (Hussain et al. 2009). CeA is highly available structural polysaccharide in plant cell walls with β-1, 4-glucosidic linkages and stands for about 50% of the biomass produced by CO_2 fixation in photosynthetic reactions (Eriksson et al. 1990).

Soil PA enzymatic activity was decreased significantly under EO_3 stress. In contrast to our results, higher activity of PA under EO_3 stress was attributed to higher rate of death and decay of old roots and proteins liberated through root exudation (Badri and Vivanco 2009). Under EO_3 exposure, decreased activities of these enzymes clearly showed a reduction in microbial activities of C, N and P due to altered size and properties of labile C, N and P pool which is derived from lesser litter input or root exudation. As previously stated, microbes are vital source of soil extracellular enzymatic activities (McLatchey and Reddy 1998; Jackson et al. 2013), and the decline of microbial biomass and allocation of nutrients, i.e., C and N substrate sources could affect soil enzymatic activities under EO_3 and finally diminish rates of N transformation (Shackle et al. 2000; Chen et al. 2015a).

Conclusion

The present study provides evidence about the impact of EO_3 treatments on plant-soil system through soil nutrient and extracellular enzyme activities. Our results demonstrated that EO_3 over a period of 2 years (2018 and 2019) negatively impacted *L. leucocephala* plant height and biomass. Rhizospheric soil microbial biomass and soil extracellular enzyme activities were also negatively affected by EO_3, which was mainly due to reduced belowground C allocation.

The available form of nutrients (AS and AP) was reduced during a shorter period of exposure (upto 12 months) as well as longer period of exposure (at the end of 24 months exposure period) to EO_3. However, total form of nutrient concentrations (TOC and TP) and C/N ratio in the soil were reduced significantly only at the end of 2 years exposure period. Our observations suggest that reduced plant growth induced by high concentration of O_3 can mediate changes in soil microbial communities and soil biochemistry, which in turn, have the potential to alter soil C, N and P cycling in forest ecosystems.

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Declarations

Conflict of interest Authors declare that there are no conflicts of interest.

Research involving human participants and/or animals Our research does not involve either humans or animals.

Informed consent All authors have given their consent for publication.

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