Phylogenetic Conservation of Microbial Responses to Elevated Ozone

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Abstract

Background: Global tropospheric ozone has doubled due to emissions from fossil fuel and biomass burning, and thus reduces plant primary productivity and crop yields. In contrast, little is known about how elevated tropospheric ozone affects soil microbial communities in the cropland ecosystem and whether such effects are sensitive to nitrogen (N) supply. Here, we examined the responses of bacterial and fungal communities in maize soils to elevated ozone (+60 ppb ozone) across different levels of N fertilization (+60, +120, and +240 kg N $ha^{-1}yr^{-1}$).

Results: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Elusimicrobia*, and *Planctomycetes* were altered by N fertilization or elevated ozone ($P < 0.05$). No interactive effects between N fertilization and elevated ozone were observed for bacterial and fungal communities ($P > 0.1$). While bacterial responses to N fertilization were phylogenetically conserved, bacterial and fungal responses to elevated ozone were phylogenetically conserved, showing universal homogeneous selection (homogeneous environmental conditions leading to more similar community structures). The ozone-responsive phyla were generally correlated ($P < 0.05$) with plant biomass, plant carbon (C) uptake, and soil dissolved organic C.

Conclusions: Our study highlighted that microbial response to elevated ozone displayed a phylogenetic clustering pattern, suggesting that response strategies to elevated ozone stress may be a phylogenetically conserved ecological trait.

Background

Although nitrogen (N) fertilization improves crop production [1], its overuse has resulted in excessive N accumulation in soil, leading to higher N emissions from soil to the atmosphere [2]. To date, tropospheric deposition of N in global ecosystems has increased and is projected to increase continuously within the first half of the 21st century [3]. Nitrogen fertilization in agricultural soils also contributes to nitrous oxide emissions, representing a significant greenhouse gas [4]. Therefore, reducing N fertilization is necessary to mitigate global climate changes. Meanwhile, tropospheric ozone has increased since the industrial revolution because of the increased levels of reactive N oxide radicals and reduced volatile organic compounds [5, 6]. As an essential component of air pollution and greenhouse gas, many countries face elevated tropospheric ozone problem [7], with the maximum daily average ozone concentration at up to 70 ppb across China in 2018 [8] and increasing 1-2% annually throughout the 21^{st} century [9, 10]. Tropospheric ozone can inhibit crop growth, photosynthesis, and flowering [11], reducing crop production [12]. In accordance, the elevated tropospheric ozone has decreased approximately 10% maize yields in the United States from 1980 to 2011 based on historical observations [12] and reduced 6-8% annual crop yields in China [13]. The N use efficiency of crops decreased by elevated tropospheric ozone through decreasing N uptake, which is one of the main reasons that drop crop yields [8].

Both N fertilization and elevated tropospheric ozone might affect the soil microbial community, which plays critical roles in mediating geochemical cycles of C, N, phosphorus, and sulphur [14] that support the plant growth [15]. Long-term N fertilization decreased soil microbial biomass and diversity [16], and increased the ratio of Gram-positive bacteria to Gram-negative bacteria [3]. In contrast, elevated ozone can either decrease [11] or increase [17] soil microbial diversity. The relative abundance of certain soil bacterial populations was altered by elevated ozone. For example, nitrifying bacteria and N-fixing bacteria affiliated to *Sphingomonadaceae*, *Rhizobiaceae*, and *Nitrospiraceae* were increased by elevated ozone in maize soils [10]. In addition, soil nutrient availability and resource distribution were altered under elevated ozone due to alterations in the ratio of fungi to bacteria [18]. Five-year elevated ozone treatment reduced soil organic C (5.6-17%) and N contents (8.2-27.8%), which reduced the N fertilization efficiency of crops [19]. However, our previous studies found no interactive effects of N fertilization and elevated ozone on maize biomass and production [8]. Whether there are interactive effects of N fertilization and elevated ozone on soil microbial communities remains obscure.

A meta-analysis study suggests that microbial response to N fertilization is phylogenetically conserved [20], i.e., closely related microbial taxa respond more similarly to N fertilization than those that are distantly related. Microbial responses to other environmental changes, such as drought, extreme desiccation, and rewetting, are also phylogenetically conserved [21-23]. In contrast, it is unclear whether microbial responses to elevated ozone are also phylogenetically conserved. . In this study, three N fertilization levels (60, 120, and 240 kg N $ha^{-1}yr^{-1}$) and two ozone levels (ambient and ambient + 60 ppb ozone) were employed to investigate the effect of N fertilization levels and elevated ozone on soil microbial community during the whole growth cycle of maize. We aim to address the following questions: (i) whether and how interactions of N fertilization and elevated ozone alter soil microbial community; (ii) whether both soil microbial responses to N fertilization and elevated ozone are phylogenetically conserved in maize agro-ecosystem; (iii) which microbial populations are involved in response to N fertilization and elevated ozone, and whether there are similarity or difference between microbial populations that responded to N fertilization and elevated ozone.

Methods

The study site and experimental manipulations

The soils used in the present study were collected from an agricultural station located in Tangjiapu (40° 48′ N, 115° 99′ E), China [8]. The most commonly used maize cultivar (Zhengdan 958) in China was planted in a 6.1 L pot. Three N fertilization treatments were randomly distributed in the open-top chambers (OTCs): N60, 60 kg N $ha^{-1}yr^{-1}$; N120, 120 kg N $ha^{-1}yr^{-1}$; and N240, 240 kg N $ha^{-1}yr^{-1}$. After the growth status of plants reached the 4-leave stage (4 maize leaves were grown), elevated ozone treatments were performed on June 30, 2019 to elevate the tropospheric ozone concentration by 60 ppb. Elevated ozone treatments were applied with an electrical discharge ozone generator (HY003, Chuangchen Co., Jinan, China), whose concentrations were monitored by an ultraviolet absorption ozone analyzer (Model 49i; Thermo Scientific, Franklin, Massachusetts, USA). The OTCs supplied with ambient air were used as control. Six OTCs made by the toughened glass were used in this study; thus, there were three OTCs for elevated ozone treatments. In addition, three replicates for each N fertilization treatment were distributed in each OTC. In total, six treatments and nine replicates for each treatment in this study were conducted until the mature stage (September 24, 2019).

Sample collection

Soil samples were collected at the end of September 2019. The soil samples were sieved (2 mm) to remove litter, roots, and stones. They were then put into airtight polypropylene bags, and placed in a cool box at 4°C during transportation to the laboratory. Soil samples were then divided into two subsamples. Subsamples for soil geochemical analyses were stored at 4 °C. Subsamples for microbial community composition analysis were stored at -80 °C.

Determination of plant properties and soil geochemical properties

Soil pH was measured with a pH meter (Model PHS-3C, Shanghai Precision and Scientific Instrument Co. Ltd., Shanghai, China) after shaking the soil in deionized water (1:2.5 w/v) suspensions for 30 min. Soil total organic carbon (TOC) was determined by the potassium dichromate-volumetric method. Dissolved organic carbon (DOC) was extracted by adding 120 ml of deionized water to 40 g soil samples (1:3 w/v) as described [24]. After being centrifuged at 4000 rpm for 10 min and passed through a 0.45 μm membrane, the filtered extracts were used for DOC analysis. Total N (TN) was determined by Kjeldahl digestion. Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted by 0.01 M potassium chloride (1:10 w/v) for 30 min and then detected by auto-analyzer (Alpkem, Perstorp Analytical Company, Wilsonville, OR, USA). Total phosphorus (TP) was measured using the molybdenum-antimony colorimetric method after being treated by melted sodium hydroxide. Available phosphorus (AP) was measured after being treated by hydrochloric acid-ammonium fluoride extraction. Total potassium (TK) was determined by atomic absorption spectrophotometer (Z-2300, Hitachi, Japan) after being treated by melted sodium hydroxide, and available potassium (AK) was extracted by ammonium acetate. Plant biomass, plant carbon uptake, and plant N uptake were measured by an elemental analyzer (Vario EL III, Elementar, Germany) as described before [8].

Illumina sequencing of 16S rRNA genes and ITS amplicons

Following the instructions, the whole soil DNA was extracted from 0.5 g soil samples by PowerSoil Kit (MOBIO, Carlsbad, CA, USA). Bacterial 16S rRNA gene V4-V5 hypervariable regions were amplified using primers 515F (5'-GTGCCA GCM GCC GCG GTA A-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') combined with adapter sequences and barcode sequences. The ITS2 regions were amplified using primers ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). Purified amplicons were sequenced by Magigene Inc., Guangzhou, China, on a HiSeq2500 platform (Illumina Inc., San Diego, CA, USA). Chimera detection and removal were accomplished using the Gold Chimera-Free reference database via the USEARCH option in the UCHIME algorithm. Quality-filtered reads were truncated to an equal length. Unosie3 were applied to generate ASV table, and ASVs were calculated using the usearch -unoise3 command. The representative sequence of each ASV was assigned to a taxonomic lineage by classified against the SILVA database (version 132) for 16S rRNA gene and UNITE database (version 7.2) for ITS sequences.

Statistical analyses

ANOVA was applied to test the effects of N fertilization and elevated ozone on plant and soil geochemical properties, microbial diversity. FDR correction was performed to adjust the *P*-values obtained for multiple comparisons.

The alpha diversity indices were calculated using R functions 'alpha.g' in the 'vegan' and 'ieggr' packages. The nonmetric multidimensional scaling (NMDS) analysis based on the abundance weighted Bray-Curtis distance was used for comparing the microbial communities in different treatments. Permutational multivariate analysis of variance (Adonis) based on Bray–Curtis distance was used to determine the microbial differences between treatments [25]. Canonical correlations analysis (CCA) was conducted to detect the interactions of plant properties, soil geochemical properties, and microbial communities [25]. The function 'envfit' in the 'vegan' package evaluated the association of microbial community variation and each environmental variable in CCA. The function 'permutest' in the 'vegan' package was used to test the significance of the CCA model.

The significance of ASVs changed by elevated ozone or N fertilization was determined by the Student *t*-test. For N fertilization treatments, the significantly changed ASVs were firstly selected by $P < 0.05$ between N240 and N60. Then, ASVs that increased with N fertilization levels and ASVs that decreased with N fertilization levels were selected, respectively. For ozone treatments, the significantly changed ASVs were selected by $P < 0.05$ between ambient and elevated ozone treatments. The ASVs increased by N fertilization or elevated ozone treatments were defined as positive responses. The ASVs decreased by N fertilization, or elevated ozone treatments were defined as negative responses. Pearson correlation analysis was conducted to evaluate correlations between the significantly changed microbial populations with plant and soil geochemical properties. FDR correction was performed to adjust the *P*-values.

To assess whether the microbial responses to N fertilization or elevated ozone are phylogenetically conserved, representative sequences of these significantly changed ASVs were aligned using the DECIPHER package [26]. An ML tree with 100 bootstrap replications was constructed with RAxML v8.0, using the GTR + Gamma distribution model [27]. We then applied consenTRAIT analysis to test whether an ASV's response to N fertilization or elevated ozone was related to the microbial phylogeny [28]. The tree was traversed from the root to the tips, recording the deepest nodes where >90% of the descending tips (ASVs) shared the same directional response (a 'consensus' clade). The genetic depth (average distance of the node to its descending tips) and each consensus clade's size (total number of the descending tips) were calculated. The genetic depth of clades with a single descending tip (i.e., ASV) was calculated as half the branch length to the nearest neighbor as previously recommended [28]. Finally, the mean genetic depth, τD, of the consensus clades sharing positive or negative responses was calculated. Simulated τD values were calculated by randomizing the responses among the tips 1000 times, to assess the statistical significance of phylogenetic conservation of N fertilization levels or elevated ozone. The probability of phylogenetic conservation (non-randomness) of the traits was calculated as the fraction of simulated τD values that were greater than or equal to the observed τD [20].

The taxonomy of clades whose response to N fertilization levels or elevated ozone was significantly more positive or negative than expected by chance was calculated based on the number of ASVs that had a positive or negative response at each taxonomic level. We performed a two-tailed fisher's exact test against each taxonomic level's equal distribution of positive and negative responses.

Results

Plant and soil geochemical properties

Both N fertilization and elevated ozone exhibited significant effects on most plant and soil geochemical properties (Table S1). Specifically, N fertilization increased plant biomass, plant N uptake, plant C uptake, and soil ammonium (NH_4^+) concentration, whereas decreased soil available phosphorus (AP) ($P < 0.05$, Table S2). In contrast, elevated ozone decreased plant biomass, plant C uptake, soil pH, dissolved organic carbon (DOC), and AP but increased soil NH_4^+ and nitrate (NO_3^-). No interactive effects of N fertilization and elevated ozone were observed on plant and soil geochemical properties except for soil total potassium (TK) and available potassium (AK) ($P < 0.05$, Table S1).

Microbial community composition and diversity

After resampling at 45 000 reads per sample, a total of 2 430 000 amplicon sequences for 16S rRNA gene representing bacterial communities were generated, resulting in 18 260 amplicon sequence variants (ASVs). Most of these sequences were affiliated to *Proteobacteria* (24.8-26.3%), *Acidobacteria* (15.6-17.7%), and *Chloroflexi* (12.7-14.6%) (Fig. S1a). The most abundant genera included *Anaerolineaceae* UTCFX1 (3.1-3.8%), *Nitrosomonadaceae* MND1 (1.8-2.5%), and *Pyrinomonadaceae* RB41 (1.9-2.3%) (Fig. S1b). Bacterial alpha diversity (Shannon index) was decreased ($P < 0.05$) by N fertilization (Fig. 1a) but was not affected by elevated ozone (Fig. 1b). No interactive effect between N fertilization and elevated ozone was observed on bacterial alpha diversity (Table S3).

Similarly, a total of 1 684 152 ITS sequences were generated after resampling at 31 188 reads per sample, resulting in 4 036 ASVs representing fungal communities. Most sequences were affiliated to *Ascomycota* (42.3-48.8%), *Glomeromycota* (9.6-16.8%), and *Basidiomycota* (3.6-6.4%) (Fig. S1c). The most abundant genera included *Gibberella* (6.0-9.4%), *Claroideoglomus* (4.1-6.4%), and *Fusarium* (4.0-5.0%) (Fig. S1d). Contrary to bacteria, fungal alpha diversity was not affected by N fertilization (Fig. 1a) but was decreased ($P < 0.05$) by elevated ozone (Fig. 1b). No interactive effect between N fertilization and elevated ozone was observed on fungal alpha diversity (Table S3).

There were significant (Adonis, $P < 0.05$) main effects of N fertilization and elevated ozone on both bacterial and fungal communities (Table S4). The effects of N fertilization ($F = 1.58$) and elevated ozone ($F = 1.66$) on the bacterial community were generally equal, but the effect of elevated ozone on the fungal community ($F = 2.09$) was much larger than that of N fertilization ($F = 1.36$) (Fig. 1c). There was no interaction between N fertilization and elevated ozone on microbial communities ($P > 0.1$, Table S4).

Phylogenetic conservation of microbial responses to N fertilization

A total of 1 593 bacterial ASVs were changed in relative abundance by N fertilization ($P < 0.05$, Fig. 2a). These ASVs were mainly affiliated to *Proteobacteria* (31.3%), *Planctomycetes* (10.5%), *Chloroflexi* (10.4%), and *Bacteroidetes* (10.2%). 26.0% of the significantly changed ASVs were increased relative abundances by at least two-fold by N fertilization, and 45.3% of the significantly changed ASVs were decreased by at least 50% by N fertilization (Fig. S2a). 86.2% of the ASVs affiliated with *Actinobacteria* was increased by N fertilization (two-tailed exact test, $P < 0.05$, Fig. 2c). On the contrary, most of the ASVs affiliated to *Gammaproteobacteria* (69.2%), *Deltaproteobacteria* (75.5%), *Bacteroidetes* (69.3%), *Elusimicrobia* (91.3%), and *Planctomycetes* (65.5%) were decreased by N fertilization, suggesting that bacterial response to N fertilization was largely at the phylum level. We also examined other taxonomic levels and found a consistent response to N fertilization (Fig. 2c). The mean genetic depth (τD) of ASVs with both positive and negative responses ranged from 0.041 to 0.049 (average τD = 0.045, permutation test, $P < 0.05$, Table 1), demonstrating around 9% of average sequence dissimilarity in the 16S rRNA gene amplicon showed consistent response to N fertilization.

A total of 96 fungal ASVs were changed in relative abundance by N fertilization, which was mainly affiliated to *Ascomycota* (26.0%) and *Glomeromycota* (20.8%) (Fig. 2b). Even though 64% (16 out of 25 ASVs) of the ASVs affiliated to *Ascomycota* and 80% (16 out of 20 ASVs) of the ASVs affiliated to *Glomeromycota* were decreased by N fertilization (Fig. S2a), the changes were not significant (two-tailed exact test, $P > 0.05$, Fig. 2c). Besides, The τD of ASVs with both positive and negative responses were not significant (permutation test, $P > 0.05$, Table 1), indicating that fungal responses to N fertilization were not phylogenetic conserved.

Phylogenetic conservation of microbial responses to elevated ozone

A total of 1 387 bacterial ASVs were changed by elevated ozone (Fig. 3a). 25.6% (355 out of 1 387 ASVs) of these ASVs were increased in relative abundances by at least two folds by elevated ozone, and 29.0% (402 out of 1 387 ASVs) of these ASVs were decreased at least 50% by elevated ozone. Most of the ASVs affiliated to *Alphaproteobacteria* (71.6%), *Actinobacteria* (69.4%), and *Chloroflexi* (81.6%) were increased, whereas most of the ASVs affiliated to *Gammaproteobacteria* (68.1%), *Bacteroidetes* (90.1%), and *Elusimicrobia* (88.9%) were decreased by elevated ozone (two-tailed exact test, $P < 0.05$, Fig. S2b). , These suggested that bacterial responses to elevated ozone occurred at the phylum level. We also examined other taxonomic levels and found a consistent response to elevated ozone (Fig. 3c). The mean τD of ASVs with positive and negative responses ranged from 0.050 to 0.052 (average τD = 0.051, permutation test, $P < 0.05$, Table 1), demonstrating around 10.2% of average sequence dissimilarity in the 16S rRNA gene amplicon showed consistent response to elevated ozone.

A total of 220 fungal ASVs were significantly changed by elevated ozone (Fig. 3b). Among them, 33.6% of these ASVs were increased in relative abundances by at least two folds by elevated ozone, and 45.9% of these ASVs were decreased by at least 50% (Fig. S2b). These 220 fungal ASVs were mainly affiliated to *Ascomycota* (27.3%) and *Glomeromycota* (25.9%). 71.7% (43 out of 60 ASVs) of the ASVs affiliated to *Ascomycota* were increased, whereas 93.0% (53 out of 57 ASVs) of the ASVs affiliated to *Glomeromycota* were decreased by elevated ozone (two-tailed exact test, $P < 0.05$, Fig. S2b). All taxonomic levels showed consistent responses to elevated ozone (Fig. 3c). The mean τD of ASVs with positive and negative responses ranged from 0.18 to 0.26 (average τD = 0.22, permutation test, $P < 0.05$, Table 1).

Comparison of microbial responses to N fertilization and elevated ozone

Phylum *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were significantly changed by both N fertilization and elevated ozone (Figs. 2 and 3). However, only 3% of bacterial ASVs and 2% of fungal ASVs were overlapped in response to N fertilization and elevated ozone (Fig. S3), suggesting that N fertilization and elevated ozone changed different microbial populations. The average τD of bacterial response to N fertilization was 0.045, suggesting that bacterial response to N fertilization was between family and genus levels (Fig. S4). However, the average τD of bacterial response to elevated ozone was 0.051, suggesting a bacterial response to N fertilization was between family and order levels. Hence, bacterial response to elevated ozone was slightly more deeply conserved than to N fertilization.

Relationships of the significantly changed microbial populations with plant and soil geochemical properties

Plant biomass, plant N uptake, and plant C uptake were positively correlated with *Actinobacteria*, which were increased by N fertilization ($P < 0.05$, Table 2). In contrast, those properties were negatively correlated with *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Elusimicrobia*, and *Planctomycetes*, all of which were decreased by N fertilization.

Plant biomass and plant C uptake were also negatively correlated with the phylum *Alphaproteobacteria* and *Chloroflexi* ($P < 0.05$, Table 3), both of which were increased by elevated ozone. However, they were positively correlated with all phyla that showed negative responses to elevated ozone. Soil DOC was positively correlated with bacterial phyla that showed positive responses to elevated ozone but was negatively correlated with *Alphaproteobacteria*, which was increased by elevated ozone. For fungal communities, plant biomass and plant C uptake were marginally and positively correlated ($P < 0.1$) with *Glomeromycota*, showing a negative response to elevated ozone. Plant biomass and plant C uptake were negatively correlated ($P < 0.05$) with Ascomycota, positively responding to elevated ozone.

Discussion

Long-term N fertilization usually decreased both bacterial and fungal alpha diversity [29, 30], especially under high N fertilization levels [29]. In this study, N fertilization also decreased ($P < 0.05$) bacterial alpha diversity (Fig. 1). In contrast, no significant effect of N fertilization on fungal alpha diversity was observed, despite a decreasing trend of fungal alpha diversity with increasing N fertilization levels. This may be due to the shorter duration of N fertilization than other studies [30]. Elevated ozone decreased ($P < 0.05$) fungal alpha diversity, which was also found in soils of two endemic trees in subtropical China [31]. No interactive effects of N fertilization and elevated ozone were observed on plants and most soil geochemical properties closely correlated with microbial community [32], indicating that N fertilization could not alleviate the adverse effect of elevated ozone on plant and microbial communities. Together, our findings revealed no interactive effect of N fertilization and elevated ozone at the tested levels on soil microbial community.

Microbial responses to environmental changes can be phylogenetic conserved, including drought, specific carbon resources utilization, precipitation, and N fertilization [20, 22, 23, 28]. In this study, bacteria with positive and negative responses to N fertilization were phylogenetically conserved at a genetic depth (τD) of 0.041 and 0.049 (Table 1). A previous study showed that the positive response of bacteria to over three years' N addition was conserved at a genetic depth of 0.020 [23]. A meta-analysis of soil bacterial communities from 13 field experiments across five continents was conducted. Results showed that bacterial responses to N addition were phylogenetically conserved within (τD = 0.018) and across sites (τD = 0.017) [20], which are comparable but smaller than our observation (τD = 0.045), probably owing to the larger more significant amount of N used in this study. Bacterial responses to elevated ozone were also phylogenetically conserved. Bacterial positive and negative responses to elevated ozone were phylogenetically conserved at a genetic depth (τD) of 0.051 and 0.052, which are slightly higher than the conserved genetic depths in response to N fertilization. Similarly, bacterial responses to drought and nitrogen fertilization were also conserved in similar phylogenetic depth based on field manipulation of precipitation and nitrogen fertilization for over three years [23].

The fungal community was more influenced by elevated ozone than N fertilization (Fig. 1), with more prominent τD value in response to elevated ozone. These results indicated that the fungal responses to elevated ozone are more phylogenetically conserved than N fertilization. Similarly, the response of fungal community to drought was more conserved than that to N fertilization [23]. Hence, microbial stress response might be more conserved than resource utilization [20], as reactions to stress may involve the interactions of many parts of the genome [33], more than what is required for resource utilization.

Nitrogen fertilization increased the relative abundance of *Actinobacteria*, but decreased the relative abundance of *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Elusimicrobia*, and *Planctomycetes* (Fig. 2). The increase of *Actinobacteria* by N fertilization was consistent with a previous study showing that *Actinobacteria* was increased by N addition [20]. The rise of *Actinobacteria* by N fertilization was highly correlated with the increase of plant biomass and soil NH_4^+. This is consistent with a long-term N fertilization study showing that N fertilization promoted the growth of *Actinobacteria* in agro-ecosystems, coinciding with increased soil available N by N fertilization [16]. The phylogenetically conserved phyla showing negative responses to N fertilization were *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Elusimicrobia*, and *Planctomycetes*, also broadly consistent with a previous study [20]. Some bacterial populations affiliated with *Proteobacteria* and *Planctomycetes* are N-fixing populations [34], dispensable upon N fertilization.

Elevated ozone increased the relative abundance of *Alphaproteobacteria*, *Actinobacteria*, and *Chloroflexi*, but decreased *Gammaproteobacteria*, *Bacteroidetes*, and *Elusimicrobia* (Fig. 3). *Chloroflexi*, increased by elevated ozone in this study, was also found as one of the dominant bacterial phyla after treatment with ozone in the bioreactor [35], suggesting strong resistance of *Chloroflexi* to ozone. The increase of soil NO_3^- by elevated ozone can be attributed to the increase of nitrite-oxidizing *Chloroflexi* [36], thus favoring nitrate-reducing *Chloroflexi* [37]. *Actinobacteria* was increased by elevated ozone. The increase of *Actinobacteria* was mainly correlated with the increase of soil NH_4^+ and NO_3^-, which provide more available N for the growth of *Actinobacteria* [16]. *Bacteroidetes* was decreased by elevated ozone in this study. *Bacteroidetes* utilizes complex algal and plant-derived polysaccharides as carbon and energy resources [38]. Hence, the decrease of plant biomass and soil DOC by elevated ozone might induce the decrease of *Bacteroidetes*, consistent with our

observations of positive correlation ($P < 0.05$) between plant biomass, soil DOC and *Bacteroidetes* (Table 3). The relative abundance of *Actinobacteria* was increased, while those of *Gammaproteobacteria*, *Bacteroidetes*, and *Elusimicrobia* were decreased by both N fertilization and elevated ozone. However, only 3% of bacterial ASVs were changed by N fertilization and elevated ozone, indicating that N fertilization and elevated ozone influenced different bacterial populations at lower taxonomic levels.

Only fungal response to elevated ozone was phylogenetically conserved (Table 1). Elevated ozone increased the relative abundance of *Ascomycota*, but decreased the relative abundance of *Glomeromycota* (Fig. 3). Plant properties and soil DOC showed significant correlations with fungal populations changed by elevated ozone (Table 3). *Ascomycota*, one of the major components of plant pathogenic fungi [39], presented a negative correlation ($P < 0.001$) with plant properties, suggesting a potential threat to plant health under increasing ozone. *Glomeromycota*, dominated by arbuscular mycorrhizal fungi (AMF), showed a negative response to elevated ozone. Recent study also found that elevated ozone changed AMF community composition and decreased AMF colonization [40]. The growth of *Glomeromycota* depends on soil DOC, also mainly derived from root exudation of plant [41]. A recent analysis including 239 studies exploring dry root mass of woody plants found that elevated ozone generally decreased root biomass [42]. Hence, elevated ozone reduces typically the allocation of plant C resources to the soil, consistent with the reduced soil DOC by elevated ozone in this study. *Glomeromycota* forms symbioses with the roots and contribute to plant growth and production [43, 44], and enhance environmental adaption of the plant to complicated environment [45]. The inoculation of AMF increased 68% of shoot biomass and 131% of root biomass when the ozone concentrations were over 80 ppb, and then increased crop production under elevated ozone stress based on a meta-analysis including 20 studies [46]. Therefore, the increase of *Ascomycota* and the decrease of *Glomeromycota* under elevated ozone might result in stunted growth and plant disease and further decrease plant biomass and production.

Conclusions

In summary, we provide evidence that microbial responses to elevated ozone are phylogenetically conserved. As no interactive effect of N fertilization and elevated ozone on microbial community and plant and soil geochemical properties, the adverse effect of elevated ozone on plant and microbial community could not be alleviated by N fertilization. Furthermore, the decrease of AMF induced by elevated ozone would aggravate the adverse effect on crops if no policy were proposed to retard the increase of tropospheric ozone. Moreover, the decrease of specific microbial species may result in the extinction of microbial species, warranting attention to protecting microbial diversity and agriculture development from the damage of increasing tropospheric ozone.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence reads of 16S rRNA gene and ITS amplicons were deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), with BioProject ID PRJNA791240. The R scripts are publicly available at https://github.com/yuzs8911/ozone_maize.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

H.L. and Z.F. conceived the study. H.L. designed the study. Z.Y. analyzed the data. J.P., Z.F., Z.Y., and M.G. contributed to preparing the materials and sequencing. The paper was written by Z.Y., Q.G., X.G., and Y.Y. with help from Q.Q, M.W, and C. M.

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Tables

Table 1 Mean genetic depth (τD) of consensus clades as calculated with the consenTRAIT algorithm.

	Bacteria		Fungi	
	Positive response	Negative response	Positive response	Negative response
N fertilization	**0.041**	**0.049**	0.101	0.135
Elevated ozone	**0.051**	**0.052**	**0.182**	**0.255**

Bold indicates that the response is significantly associated with phylogeny (permutation test; P<0.05). Consensus clades are the phylogenetic clades in which >90% of the descendant ASVs show the same direction of response.

Table 2 Pearson's correlation of plant and soil geochemical properties with ASVs responded consistently to N fertilization at phylum level.

	Actinobacteria		*Gammaproteobacteria*		*Deltaproteobacteria*		*Bacteroidetes*		*Elusimicrobia*		*Planctomycetes*	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Plant biomass	0.54	**<0.001**	-0.55	**<0.001**	-0.59	**<0.001**	-0.39	**0.016**	-0.37	**0.023**	-0.59	**<0.001**
Plant N uptake	0.67	**<0.001**	-0.66	**<0.001**	-0.70	**<0.001**	-0.58	**<0.001**	-0.57	**<0.001**	-0.72	**<0.001**
Plant C uptake	0.56	**<0.001**	-0.56	**<0.001**	-0.60	**<0.001**	-0.39	**0.016**	-0.37	**0.027**	-0.58	**<0.001**
pH	-0.05	0.832	0.13	0.590	0.02	0.922	0.09	0.780	0.14	0.538	-0.02	0.900
TOC	0.22	0.234	-0.18	0.404	-0.09	0.780	0.07	0.782	-0.03	0.900	0.00	0.975
TN	0.06	0.808	-0.03	0.900	0.03	0.900	-0.03	0.900	0.01	0.952	-0.04	0.900
TP	0.24	0.201	-0.05	0.832	0.02	0.900	0.15	0.516	0.09	0.780	0.06	0.832
TK	-0.12	0.644	0.07	0.782	0.15	0.517	0.26	0.134	0.16	0.479	0.17	0.440
DOC	0.25	0.164	0.03	0.900	0.10	0.780	0.21	0.295	0.36	**0.029**	0.08	0.782
NH_4^+	0.29	0.098	-0.31	0.071	-0.26	0.134	-0.22	0.234	-0.27	0.134	-0.29	0.098
NO_3^-	0.14	0.553	-0.09	0.782	-0.07	0.782	-0.08	0.782	-0.17	0.461	-0.06	0.832
AP	-0.27	0.134	0.28	0.114	0.33	0.054	0.32	0.065	0.41	**0.012**	0.36	**0.028**
AK	0.08	0.782	-0.05	0.833	-0.06	0.808	-0.11	0.683	-0.08	0.782	-0.11	0.685

Bold values represent $P < 0.05$ after adjusted by FDR.

Table 3 Pearson's correlation of plant and soil geochemical properties with ASVs responded consistently to elevated ozone at phylum level.

	Alphaproteobacteria		*Actinobacteria*		*Chloroflexi*		*Gammaproteobacteria*		*Bacteroidetes*		*Elusimicrobia*		*Ascomycota*		*Glom*
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r
Plant biomass	-0.44	**0.010**	-0.24	0.194	-0.48	**0.004**	0.64	**<0.001**	0.35	**0.046**	0.40	**0.017**	-0.60	**<0.001**	0.32
Plant N uptake	0.03	0.896	0.10	0.650	-0.09	0.699	0.28	0.112	-0.01	0.942	-0.08	0.714	-0.30	0.085	0.02
Plant C uptake	-0.43	**0.010**	-0.22	0.244	-0.47	**0.004**	0.63	**<0.001**	0.35	**0.046**	0.41	**0.015**	-0.59	**<0.001**	0.33
pH	-0.32	0.069	-0.35	**0.046**	-0.27	0.133	0.41	**0.017**	0.22	0.244	0.22	0.242	-0.33	0.059	0.19
TOC	0.09	0.713	0.13	0.542	-0.04	0.882	0.00	0.987	0.07	0.749	0.04	0.882	-0.03	0.896	0.08
TN	0.08	0.714	0.08	0.714	0.15	0.478	0.14	0.510	0.04	0.882	0.15	0.463	-0.05	0.821	-0.03
TP	-0.10	0.641	0.02	0.914	-0.16	0.463	0.15	0.463	0.20	0.297	0.24	0.192	-0.01	0.945	0.15
TK	-0.12	0.568	-0.09	0.705	-0.12	0.568	0.02	0.942	0.19	0.353	0.08	0.713	-0.13	0.542	0.19
DOC	-0.36	**0.042**	-0.11	0.593	-0.24	0.196	0.42	**0.014**	0.35	**0.046**	0.44	**0.010**	-0.29	0.101	0.47
NH_4^+	0.31	0.082	0.29	0.094	0.21	0.266	-0.14	0.510	-0.13	0.542	-0.12	0.584	0.26	0.140	-0.15
NO_3^-	0.44	**0.010**	0.27	0.133	0.33	0.059	-0.37	**0.040**	-0.35	**0.046**	-0.32	0.069	0.31	0.082	-0.13
AP	-0.21	0.266	-0.26	0.155	-0.18	0.367	0.16	0.435	0.27	0.136	0.37	**0.040**	-0.08	0.714	0.30
AK	-0.08	0.713	0.07	0.738	0.07	0.714	0.06	0.802	-0.01	0.942	0.18	0.366	0.17	0.424	0.02

Bold values represent $P < 0.05$ after adjusted by FDR.

Figures

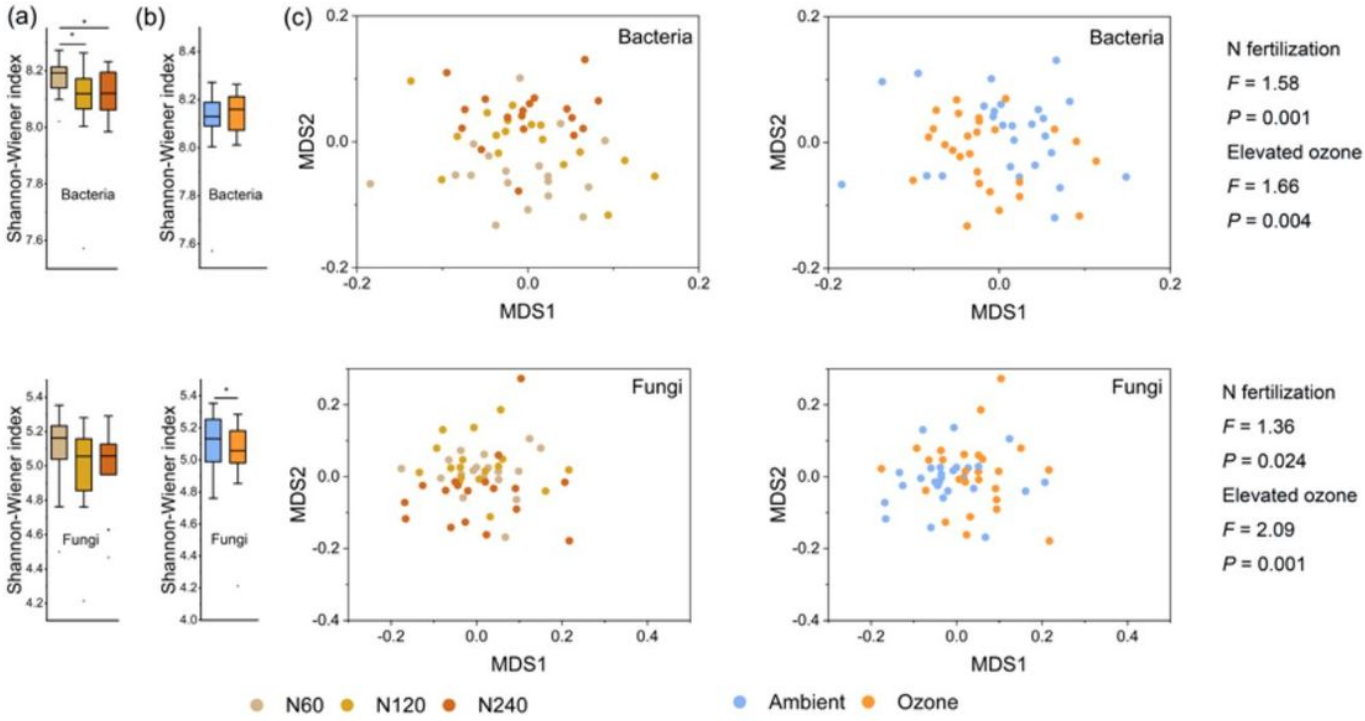

Fig. 1

Figure 1

Influence of N fertilization and elevated ozone on microbial diversity. Mean bacterial and fungal Shannon-Wiener indices under N fertilization (a) and elevated ozone treatments (b); Nonmetric multidimensional scaling analysis and Adonis based on Bray-Curtis distance (c). Significance: * P <0.05.

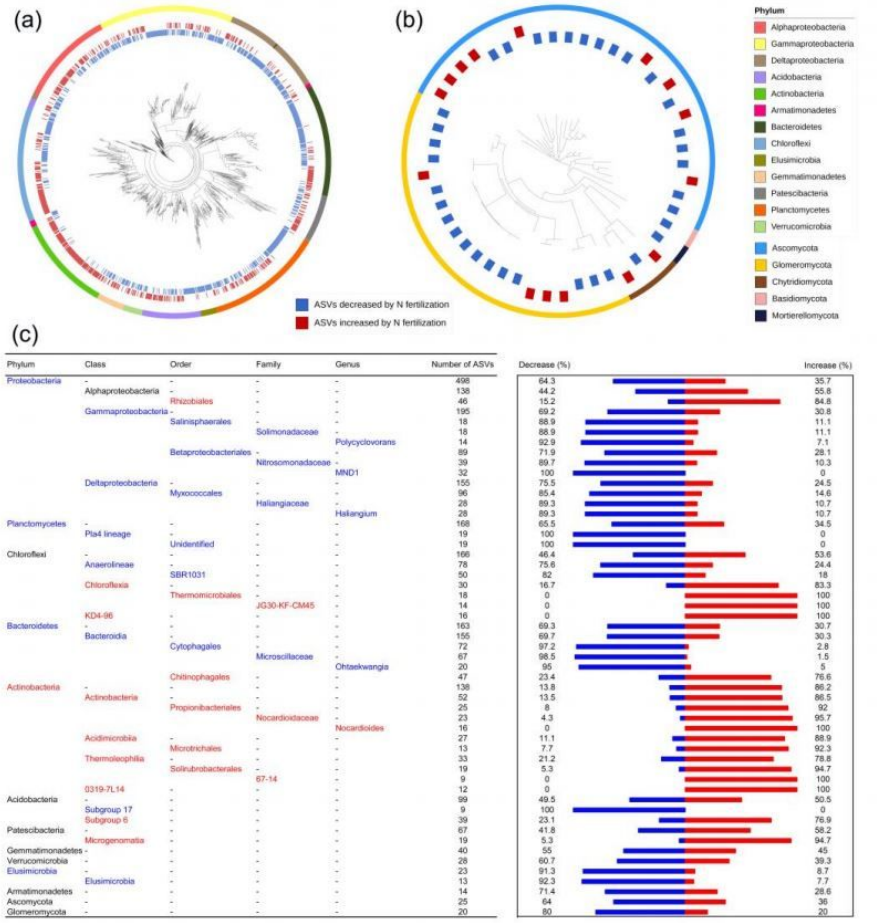

Fig. 2

Figure 2

Phylogenetic distribution of the ASVs changed by N fertilization. Phylogenetic tree of bacterial ASVs (a) and fungal ASVs (b) that changed by N fertilization. Taxonomic levels of microbial response to N fertilization (c). The response significantly more positive (red) or negative (blue) or no significance (black) than expected by chance (two-tailed exact test; $P < 0.05$) are shown. Only the phylum contains >10 bacterial ASVs are shown. The percent of ASVs that were increased (red) or decreased (blue) by N fertilization are plotted in the bar graph to the right. Higher taxonomic levels are listed in black (e.g., the class Alphaproteobacteria) when only the lower levels are significant.

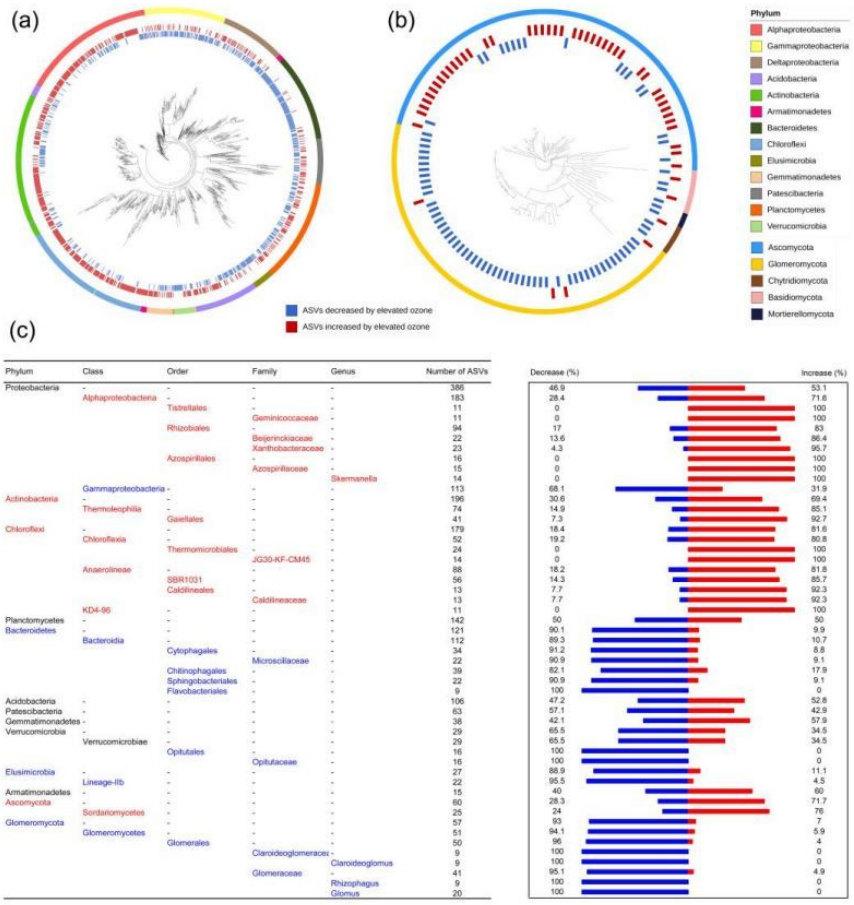

Fig. 3

Figure 3

Phylogenetic distribution of the ASVs changed by elevated ozone. Phylogenetic tree of bacterial ASVs (a) and fungal ASVs (b) that changed by elevated ozone. Taxonomic levels of microbial response to elevated ozone (c). The response significantly more positive (red) or negative (blue) or no significance (black) than expected by chance (two-tailed exact test; $P < 0.05$) are shown. Only the phylum contains >10 bacterial ASVs are shown. The percent of ASVs that were increased (red) or decreased (blue) by elevated ozone are plotted in the bar graph to the right. Higher taxonomic levels are listed in black (e.g., the class Verrucomicrobiae) when only the lower levels are significant.

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