

Article

Greater Application of Nitrogen to Soil and Short-Term Fumigation with Elevated Carbon Dioxide Alters the Rhizospheric Microbial Community of *xTriticocereale* (Triticale): A Study of a Projected Climate Change Scenario

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Abstract

An attempt was made to understand the interactive consequences of subjecting a rhizospheric microbial community of *xTriticocereale* (Triticale) to higher CO₂ levels and soil nitrogen addition in the short term in a tropical agro-ecosystem. Open-top chambers (OTCs) were used to grow the test crops for a single season under ambient CO₂ (AC) and elevated CO₂ (EC) along with two variable N dosages: recommended (N₀: 0.053 g N/kg of soil) and high (N₂: 0.107 g of N/kg of soil) levels. Variations in the composition of microbial communities and abundances were investigated using phospholipid fatty acid analysis (PLFA). A significantly ($p < 0.001$) increased microbial biomass content (MB) was observed under EC compared to AC, while the addition of N had a minor effect. A decreased fungi/bacteria (F/B) ratio (~38%) was observed with high N application in the CO₂ enrichment treatment. Bacteria were more abundant, while fungal abundance decreased under N₂ and EC. Gram (+ve) bacteria used these conditions to thrive under N₂ and EC, while Gram (–ve) bacteria declined. No significant effects on actinomycetes were noticed in any of the treatments. However, eukaryotes acquired more benefits and flourished in response to EC. Varied responses were noted for the Shannon diversity index (H') under EC. Overall, (i) bacteria (Gram-positive) and eukaryotes dominated under EC and high N addition, while fungi decreased, and (ii) EC and high levels of N addition did not affect actinomycetes. Short-term exposure under the given conditions was found to alter the rhizospheric microbial community. However, multiple season studies are needed to elucidate whether these short-term responses are transient or continuous.

Keywords: elevated CO₂; nitrogen addition; soil microbial diversity; phospholipid fatty acid analysis; *xTriticocereale* (Triticale)



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1. Introduction

Cumulative net CO₂ emissions from 1850 to 2019 were 2400 ± 240 GtCO₂. More than half (58%) of these emissions occurred between 1850 and 1989 [1400 ± 195 GtCO₂], and

about 42% of emissions occurred between 1990 and 2019 [$1000 \pm 90 \text{ GtCO}_2$]. Global net anthropogenic GHG emissions were estimated at $59 \pm 6.6 \text{ GtCO}_2\text{-eq}$ in 2019, which is about 12% ($6.5 \text{ GtCO}_2\text{-eq}$) higher than in 2010 and 54% ($21 \text{ GtCO}_2\text{-eq}$) higher than the levels in 1990 [1]. The climate is clearly changing, with 40% increases in carbon dioxide (CO_2) concentrations reported; compared to concentrations of 280 ppm during the pre-industrial era in 1850, this increased up to 422 ppm by December 2023 [2]. It is challenging to predict the responses of the biosphere against future CO_2 levels, as there are several gaps regarding our knowledge of this subject. Although sufficient work has been performed on aboveground responses, detailed information about soil belowground in terms of microbiome composition, function, and soil C and N bio-geochemical processes is still sparse [3].

Many studies have validated the fact that EC has a stimulating effect on a plant's growth and photosynthesis [4]. As the CO_2 concentration in soil is much higher than in the atmosphere, the direct consequences of EC on soil-dwelling microbes are trivial compared to the indirect effects attenuated by plants [5]. There are two different views regarding the indirect effect of EC on soil microbial communities. Some researchers [6] claim that EC can effectuate soil carbon loss, which is considered positive feedback, while others [7] maintain that it can sequester C in soil, which is considered negative feedback.

The experimental evidence proves that EC conditions alter the chemical properties of litter, which may be the reason behind the decelerated rate of carbon and nitrogen cycling [8,9]. It is also the cause behind the slow rate of decomposition of organic matter [10] or another process, i.e., the immobilization of nutrients by microbes. In opposition to this, Hungate et al. (1997) reported on the enhanced rates of C and N transformations under EC that provide evidence for a +ve feedback reaction in the C and N dynamics in soil and ultimately lead to enhanced plant growth under EC [11].

Nitrogen, which is a key player in regulating many biological and chemical responses, causes both direct and indirect repercussions on the microbial community. Globally, terrestrial ecosystems have been experiencing unprecedented reactive nitrogen (N) depositions for many decades, which is expected to further increase by ~2.5 times in the present century [12]. Additionally, agricultural practices add large amounts of N into the soil with the intention of achieving more production. Any alterations in N availability in soil regulate the quantity and quality of carbon inputs, which are ultimately associated with an alteration in microbial physiology and compositional aspects [13]. Broadly, these indirect drivers can affect microbial activities. Furthermore, the progressive N limitation (PNL) effect under EC can be alleviated by adding N to the soil [14].

Limited information exists on the bilateral consequences of climate change drivers and other components of the environment on soil microbiomes, especially in sub-tropical agro-ecosystems. Also, our present knowledge is limited to how N undergoes rhizodeposition through plant roots to the soil under EC and its ensuing metabolism by soil-dwelling microbes.

Plant/crop type is another dimension that determines microbial community structures. Many scientific reports are available that explain that plants encourage some specific communities in their rhizospheric zones [15]. "*xTriticocereale* (Triticale)" is the first successful crossover between wheat (*Triticum aestivum* L.) and rye (*Secale cereal* L.), resulting in the birth of a hybrid called *xTriticocereale* (Triticale). It was developed at the end of the 19th century, and the goal behind this was to gather the positive points of wheat (with the highest yield potential amongst all cereals and good nutritional value) and rye (with strong resistance) to make them expressive in a single plant. As a food crop, *xTriticocereale* (Triticale) has already been considered a hardy and fit crop, and it is expected to play a significant role in the food of impoverished populations. Thus, *xTriticocereale* (Triticale)

was chosen as the perfect candidate to carry out this study, since it has advantages in both listed crops.

In the present investigation, we assessed the consequences of the short-term subsection of experimental EC and additional N inputs to hypothesized changes that may occur at the compositional level of existing microbial communities of the rhizosphere of *xTriticocereale* (Triticale). Our hypothesis behind this experiment was as follows: (i) EC alone and in interaction with additional N change the composition of microbial communities in the rhizosphere of the test crop. We also attempted to test, during short-term exposure to both EC and high N, which factor is most dominant in governing microbial compositions.

2. Materials and Methods

2.1. Experimental Site

A pot experiment was conducted growing *xTriticocereale* (Triticale) as the test crop inside open-top chambers (OTCs) for the experimental elevation of CO₂ levels. The crop was grown around mid-November, and the experiment was conducted at the research farm of the Indian Agricultural Research Institute, New Delhi (Latitude: 28°35' N; Longitude: 77°12' E; and Altitude: 228.16 m above mean sea level). The climate of the experimental site is semi-arid and sub-tropical. The annual average rainfall is 710 mm, and the mean daily maximum and minimum air temperatures during the wheat growing season (mid-November to end of March) vary between 10 and 29 °C and 2–12 °C. The soil is sandy loam in texture and belongs to the Typic Haplustept soil subgroup of the upper Indo-Gangetic Plains. The sand, silt, and clay contents were 68.8%, 19.0%, and 12.2%, respectively. The initial physico-chemical properties of the soils that were used to fill the pots are presented in Table 1.

Table 1. General physico-chemical properties of the experimental soil.

Soil Properties	Values/Inferences
pH (soil/water1/2.5)	7.9
Electrical conductivity (EC) (dSm ^{−1})	0.64
Textual class	Sandy loam
Soil type	Typic Haplustept
Bulk density (Mg m ^{−3})	1.61
Water-holding capacity (%)	32.6
Available N (g kg ^{−1})	0.30
Available P (kg ha ^{−1})	11.7
Available K (kg ha ^{−1})	225
Oxidizable soil organic carbon (g kg ^{−1})	3.7

2.2. Open-Top Chamber Experiment Details

The OTCs were fabricated using an aluminum frame and lined with a PVC plastic sheet of 1.2 mm thickness, which transmits 90% of sunlight. It is permeable to all three ranges of UV light. The dimension of each OTC was as follows: a diameter of 3 m and a height of 2.5 m. The upper part of each OTC has a truncated and open cone of 0.5 m, set at a height of 2.5 m to alleviate any possible dilution of CO₂ via an air current, and the top was kept open to maintain a near-natural microenvironment inside the chamber. The schematic sketch of OTC is presented in Figure 1. The solenoid valve and PV tubing surrounding the OTCs were fabricated to release pure CO₂ gas (99.7% (v/v) CO₂) from a commercial-grade cylinder fitted with a regulator (DURA; make: ESAB, India). In two OTCs, a level of 580 ± 20 µmol mol^{−1} CO₂ (between 09:00 h and 17:00 h) was maintained for elevated CO₂ treatment (EC). As OTC is also expected to vary in micro-environmental parameters a bit, we used two other chambers as chamber controls, but without any external supply of CO₂.

(this was considered an ambient treatment and the average level of CO₂ was recorded at $384 \pm 13 \mu\text{mol mol}^{-1}$ during the experiment), and two OTCs with CO₂ elevation. Inside each OTC, pot replication was 25. At a one-hour interval, the CO₂ levels were monitored using sensors fitted inside the OTCs.

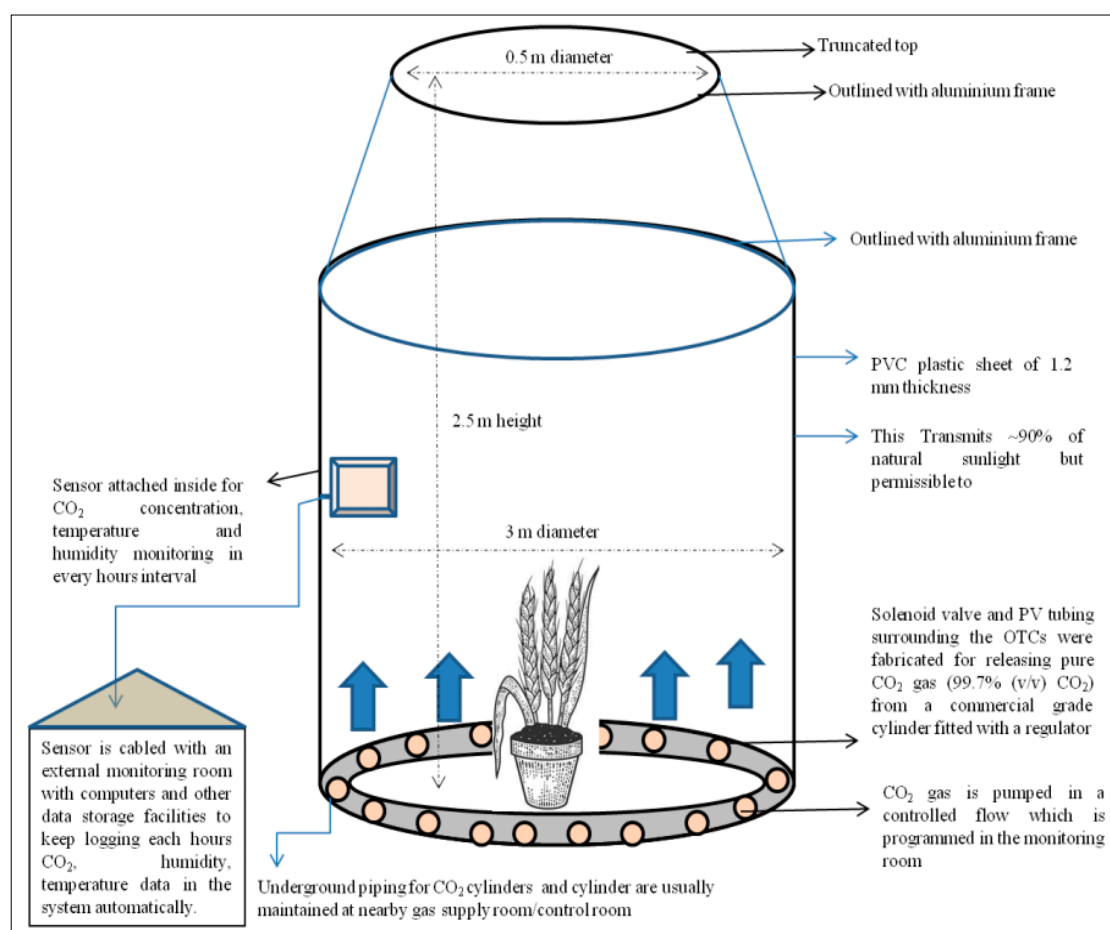


Figure 1. Schematic diagram of open-top chambers used in present study.

2.3. Crop Management and Treatments

Triticocereale (Triticale) was grown in pots inside OTCs, and each pot was filled with 5 kg of soil. Twenty-five pots were placed inside each OTC, and every treatment was allocated three replicates. The normal recommended basal dose of mineral fertilizers was mixed in the soil prior to sowing. For N, Urea was applied, and two levels of N treatment along with two different CO₂ treatments were administered (Table 2). Three split doses for N application were followed, including 50% basal, 25% at the crown root initiation (CRI) stage, and 25% at the reproductive stage. Higher N doses were applied for the latter two split doses during the crown root initiation (CRI) and flowering stage.

Table 2. Carbon dioxide (CO₂) levels and nitrogen (N) dosage maintained during the experiment.

Nitrogen (N) Doses	Carbon Dioxide	Notation Used
N ₀ (Normal recommended dose of N: 0.0533 g N/kg of soil)	Ambient CO ₂ (384 ± 13 ppm)	N ₀ AC
N ₂ Twice of N ₀ , (0.107 g N/kg soil)	Ambient CO ₂ (384 ± 13 ppm)	N ₂ AC
N ₀ (Normal recommended dose of N: 0.0533 g N/kg of soil)	Elevated CO ₂ (580 ± 20 ppm)	N ₀ EC

Table 2. Cont.

Nitrogen (N) Doses	Carbon Dioxide	Notation Used
N ₂ Twice of N ₀ , (0.107 g N/kg soil)	Elevated CO ₂ (580 ± 20 ppm)	N ₂ EC

2.4. Soil Sampling

Rhizospheric soil was sampled at the anthesis stage (85 days after sowing) from all treatments in three replicates. Root-entangled soil particles were brushed and collected separately for different treatments and replications. Furthermore, the soil samples were sieved through a 2 mm mesh sieve to ensure they were root- and plant-debris-free. A set of subsamples was stored at −20 °C until further PLFA was performed.

2.5. Soil Microbial Composition/Structural Analysis Using Phospholipid Fatty Acid (PLFA) Composition and Content

The PLFA of rhizospheric soil samples was carried out by extracting phospholipids from processed soil samples and subsequent hydrolysis. The fatty acids were then analyzed using GC-MS following the protocol given by Buyer and Sesser (2012) [16]. The major steps, given in brief, are as follows: i. drying the sample and extracting the lipid phase using a Bligh–Dyer extractant; ii. Elution of phospholipids using solvents and further drying in a centrifugal evaporator; iii. Transesterification of phospholipids and dissolving into hexane; iv. Analysis through the Agilent 6890 gas chromatograph (GC); and v. Identification of fatty acid methyl esters (FAMES) using the MIDI PLFAD1 calibration mix and naming table. Individual PLFA values were expressed as nmoles g^{−1} soil. vi. Nomenclature of PLFAs was performed following the classification given by Zelles (1999) [17]. The bacterial-specific PLFAs were i15:0, a15:0, i16:0, 16:1ω7c, 16:1ω9c, 10Me16:0, i17:0, a17:0, cy17:0, 17:0, 18:1ω7c, and cy19:0a [18,19]. For saprophytic fungi biomarkers, 18:1ω9c and 18:2ω6 were used [20]. The amount of arbuscular mycorrhizal (AM) fungi was measured through the abundance of the 16:1ω5c fatty acid biomarker [21]. Total microbial biomass (MB) was derived by calculating the cumulative sum of all fatty acids detected in each sample.

2.6. Shannon–Weiner Diversity Index (H')

The H' index is a mathematical tool for measuring species diversity in an ecosystem for a given community. H' was calculated following the method of Kaur et al. 2005 [22].

2.7. Statistical Analysis

The influence of elevated CO₂ and microbial attributes was determined using analysis of variance (ANOVA) and a Completely Randomized Design (CRD) with two factorial analyses. Two levels of atmospheric CO₂ (ambient and elevated) and two levels of N (normally recommended and higher doses) were considered fixed effects for this model. All data were analyzed through Genstat 12th Edition for one-way ANOVA, and significance was accepted at $p \leq 0.05$.

3. Results

3.1. Microbial Biomass Content (MBC) During CO₂ Enrichment and Differential Doses of Nitrogen Application Experiment of *xTriticocereale* (Triticale)

Elevated CO₂ (EC) levels significantly ($p < 0.001$) increased the microbial biomass content under both N treatments. At N₀, an increase of ~5.9% was recorded, while for N₂, an increase of ~28.3% was observed under EC compared to AC. Among all the treatments, the highest biomass content of 112.9 nmoles·g^{−1} soil was observed under N₂ at EC, and the lowest content was found under N₀ at an AC of 96 nmoles·g^{−1} soil (Table 3; Figure 2).

Table 3. Microbial traits in the rhizosphere of *xTriticocereale* (Triticale) grown in pots at ambient CO₂, elevatedCO₂, and varying nitrogen levels.

Treatments	Microbial Biomass Content (nmoles/g of Dry Soil)	Gram- Positive Bacteria (nmoles/g of Dry Soil)	Gram -Negative Bacteria (nmoles/g of Dry Soil)	F:B Ratio	Straight- Chain Fatty Acids (nmoles/g of Dry Soil)	Branched Fatty Acids (nmoles/g of Dry Soil)	MUFA (nmoles/g of Dry Soil)	PUFA (nmoles/g of Dry Soil)	DMA (nmoles/g of Dry Soil)	18:1 ω9c (nmoles/g of Dry Soil)	18:2 ω6,9c (nmoles/g of Dry Soil)	10-Methyl (nmoles/g of Dry Soil)	Shannon– Weiner Diversity Index (<i>H'</i>)
N ₀ AC	96	0.3	0.02	0.16	0.61	0.16	0.17	0.005	0.043	0.037	0.01	0.015	0.27
N ₂ AC	88	0.3	0.02	0.18	0.49	0.24	0.18	0.13	0.032	0.044	0.013	0.017	0.4
N ₀ EC	101	0.3	0.004	0.19	0.63	0.13	0.05	0.08	0.038	0.051	0.007	0.000	0.43
N ₂ EC	112	0.26	0.006	0.17	0.51	0.18	0.03	0.05	0.021	0.034	0.01	0.008	0.49
LSD at 5%	0.0172 ***	0.00190 ***	0.00190 ***	0.0188 **	0.0014 ***	0.0013 ***	0.0014 ***	0.00135 ***	0.00135 ***	0.00136 ***	0.001 ***	0.0014 ***	0.0085 ***

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. ns—not significant. N₀: optimum nitrogen; N₂: twice the amount of optimum nitrogen; AC: ambient CO₂; EC: elevated CO₂. MUFA = Mono Unsaturated Fatty Acid: biomarker for aerobic prokaryotes and Gram-negative bacterial community; PUFA = Poly Unsaturated Fatty Acid: biomarker for eukaryotes; DMA = dimethyl acetal: biomarker for anaerobes; 18:1ω9c: fungal biomarker; 18:2ω6,9c:fungal biomarker; 10-methyl: biomarker for actinomycetes.

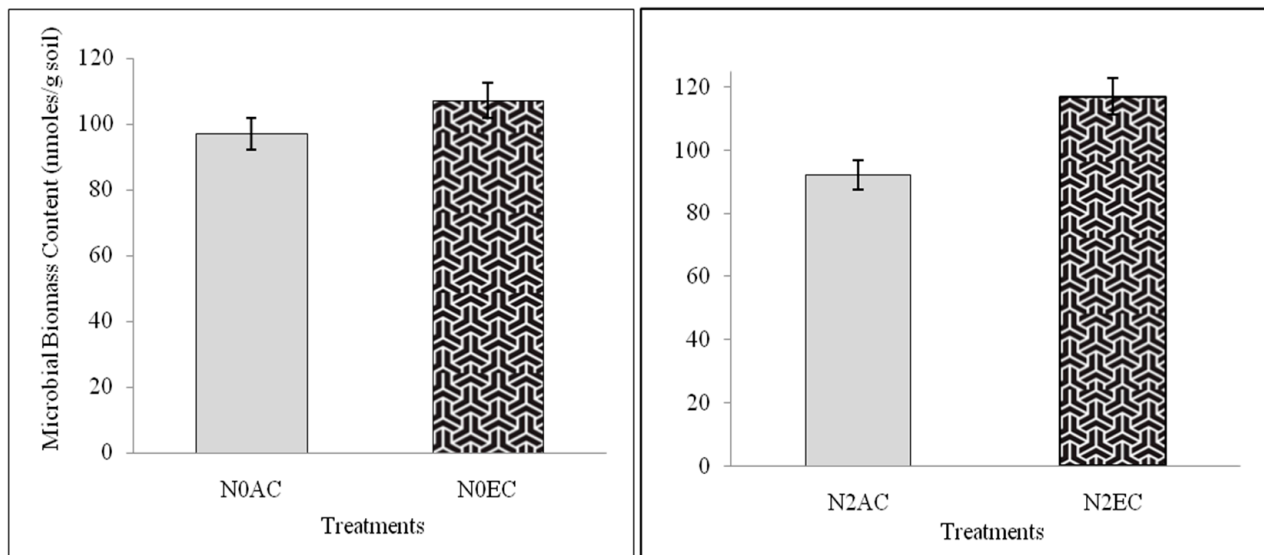


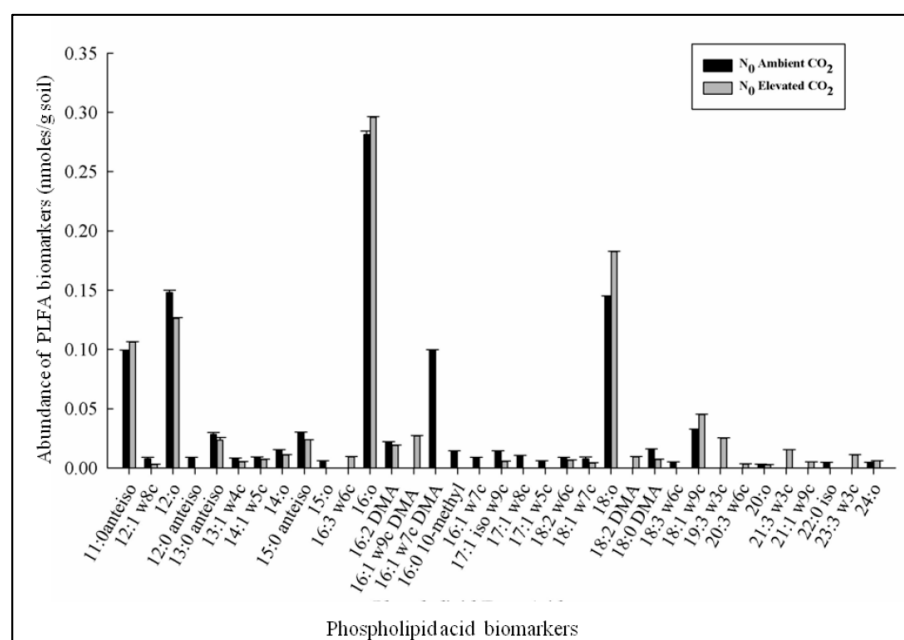
Figure 2. Total microbial biomass content in rhizosphere of *xTriticocereale* (Triticale) at ambient and elevated CO_2 and varying nitrogen levels. (Where, N_0 : normal recommended nitrogen; N_2 : Twice the normal amount of recommended nitrogen; AC: ambient CO_2 ; EC: elevated CO_2).

3.2. Phospholipid Fatty Acid (PLFA) Profiles Affected by CO_2 Enrichment and Differential Doses of Nitrogen Application in *xTriticocereale* (Triticale)

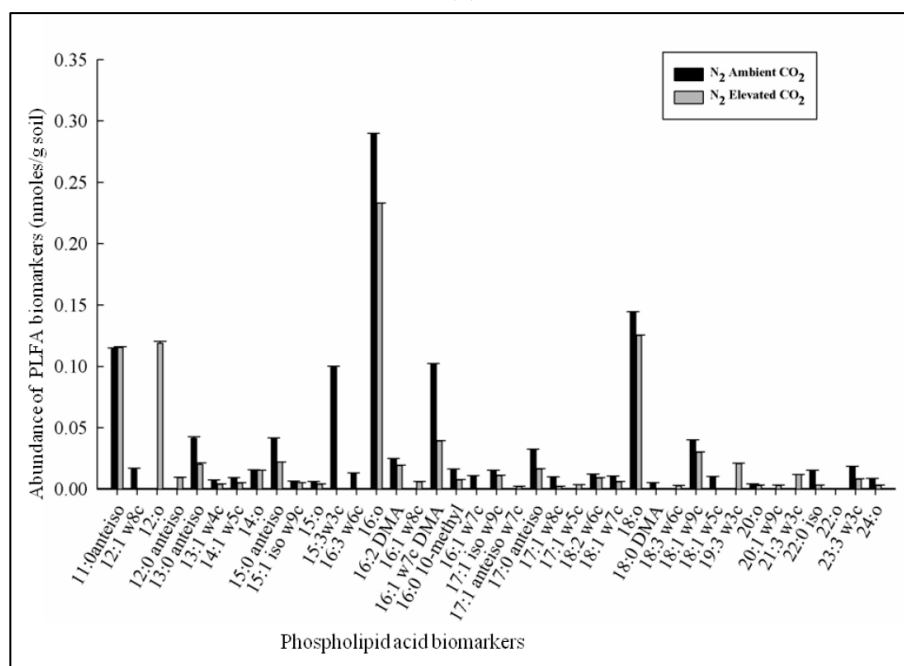
The PLFA content varied under N_0 at both AC and EC. A total of 35 PLFAs were detected, and the concentration of seven PLFAs (out of 35 PLFAs) increased significantly ($p < 0.001$) under EC; no significant effect was observed under AC (Figure 3a). Soil with higher nitrogen (N_2) depicted an increase in the total number of PLFAs at both AC and EC compared to N_0 (Figure 3b). Root biomass also followed a similar pattern of responses. Maximum root biomass was observed under N_2EC , and the minimum root biomass was found in N_0AC (Figure 4). At higher doses of nitrogen (N_2), concentrations of ten PLFAs and 6 PLFAs significantly increased in EC and AC, respectively. At the N_0 level, contents of the bacterial PLFA i16:0 increased under EC, but the contents of fungal PLFA 18:2 ω 6c and 18:1 ω 7c decreased. The biomarker for anaerobe 16:1 ω 9c DMA increased at EC with the N_0 level. PLFA contents of biomarkers for Gram-positive bacteria were higher than those of Gram-negative PLFA biomarkers at EC. The N_2 level indicated the presence of a biomarker for methanobacteria 16:1 ω 8c at EC; bacterial PLFA at 17:0 anteiso; and the Gram-negative bacterial PLFA biomarker 18:1 ω 5c at AC. However, these were not detectable under N_0 treatment. Anaerobes and fungal biomarkers were present, but their concentration dropped at EC compared to AC under N_2 .

3.3. Fungal/Bacterial Ratio Affected by CO_2 Enrichment and Nitrogen Application in *xTriticocereale* (Triticale)

The fungal-to-bacterial ratio showed opposite trends under EC at N_0 and N_2 levels. Fungal abundance decreased in N_2EC . The PLFA biomarkers specific to fungal community (18:1 ω 9c and 18:2 ω 6) showed a declining trend under EC; their abundance decreased under N_0EC treatment by ~14%, while at N_2EC , their abundance decreased further by ~38%. At N_0AC , the ratio was 0.16, while it increased to 0.19 at N_0EC ($p < 0.05$). On the other hand, for N_2 , the double dose of nitrogen application had no effect on this ratio (Table 3, Figure 5).



(a)



(b)

Figure 3. (a). Phospholipid fatty acid profile of soil samples in rhizosphere of *xTriticocereale* (Triticale) at ambient and elevated CO_2 and normal recommended nitrogen levels. (b). (Where, N_0 : normal recommended nitrogen; AC: ambient CO_2 ; EC: elevated CO_2 , N_2 : twice the normal recommended nitrogen amount).

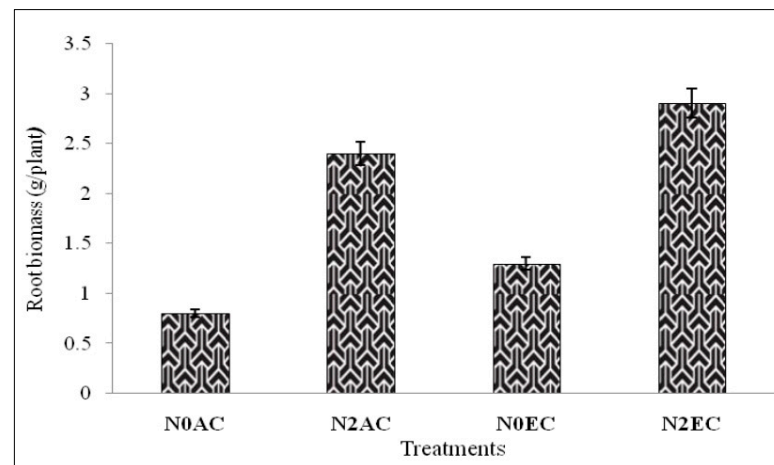


Figure 4. Root biomass of *xTriticocereale* (Triticale) influenced by ambient CO₂, elevated CO₂, and varying nitrogen levels. (Where, N₀: normal recommended nitrogen; N₂: twice the normal recommended nitrogen amount; AC: ambient CO₂; EC: elevated CO₂).

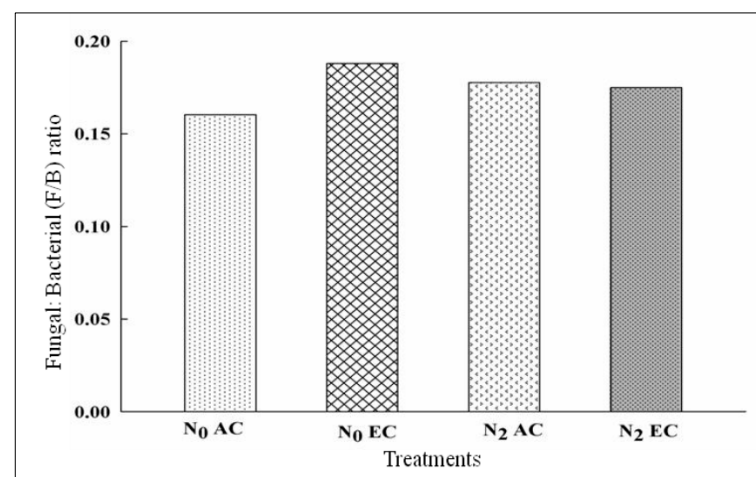


Figure 5. Fungal to bacterial ratio in rhizosphere of *xTriticocereale* (Triticale) at ambient CO₂, elevated CO₂, and varied nitrogen levels. (Where, N₀: normal recommended nitrogen; N₂: twice the normal recommended nitrogen amount; AC: ambient CO₂; EC: elevated CO₂).

3.4. Responses in Gram Positive and Gram Negative Bacterial Distribution in CO₂ Enrichment and Differential Doses of Nitrogen Application in *xTriticocereale* (Triticale)

Bacterial distributions in terms of Gram (–ve) and Gram (+ve) bacteria were studied using PLFA biomarkers (Figure 6). Gram negative biomarkers (16:1ω7c and 18:1ω7c) and Gram-positive biomarkers (15:0 anteiso; 16:0 and 17:0 anteiso) varied in the peak area. The peak area of Gram negative bacteria decreased with the addition of N, but that of Gram positive bacteria increased with N application. In rhizospheric soil samples of N₀, no significant change in the population of Gram (+ve) bacteria could be observed under both AC and EC. However, the population of Gram (–ve) bacteria significantly ($p < 0.001$) decreased at N₀EC compared to N₀AC. At the N₂ level, similar patterns of response were observed in terms of suppression of Gram-negative bacteria under EC conditions compared to AC. Overall, in both N levels (N₀ and N₂), elevated CO₂ was found to have negative impacts on the Gram-negative bacterial population.

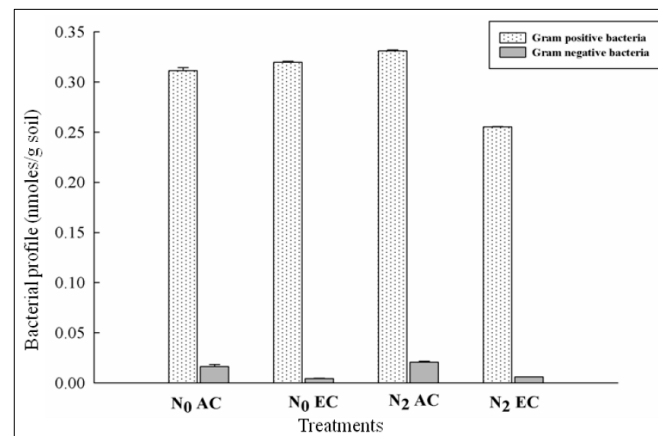


Figure 6. Bacterial profile in rhizosphere of *xTriticocereale* (Triticale) at ambient CO₂; elevated CO₂ and varying nitrogen levels. (Where, N₀: normal recommended nitrogen; N₂: twice the normal recommended nitrogen amount; AC: ambient CO₂; EC: elevated CO₂).

3.5. Shannon–Weiner Diversity Index (H') as Influenced by CO₂ Enrichment and Differential Doses of Nitrogen in *xTriticocereale* (Triticale)

The recommended nitrogen (N₀) dose under AC was recorded with minimum diversity, while the diversity was higher in rhizospheric samples under EC from both N doses. The soil of AC with double N doses showed moderate diversity (Figure 7). For the H' diversity index, which is derived from all PLFA biomarkers responses, maximum diversity was noticed under N₂EC, while the minimum diversity was recorded under N₀AC (Figure 7).

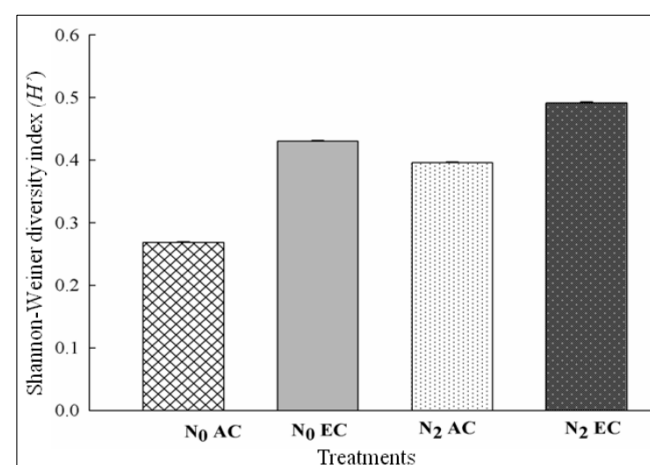


Figure 7. Shannon–Weiner diversity index (H') of rhizospheric samples of *xTriticocereale* (Triticale) at ambient and elevated levels of CO₂ and varying nitrogen levels. (Where, N₀: normal recommended nitrogen; N₂: twice the normal recommended nitrogen amount; AC: ambient CO₂; EC: elevated CO₂).

3.6. Principal Component Analysis (PCA) of PLFA Responses Using *xTriticocereale* (Triticale) Soil Microbial Community as Influenced by Ambient and Elevated Levels of CO₂ and Varying Nitrogen Levels

All the responses of PLFA were further analyzed through Principal component analysis. Principal component analysis (PCA) expressed substantial and significant compositional variations in soil microbial communities among all four treatments. The first principal component (PC1) explained about 91.66% of the total variation (Figure 8). Also, all four treatments were segregated into two different quadrants, wherein the carbon dioxide effects were more visible than the different doses of nitrogen applications. The treatments N₀AC and N₂AC appear in one quadrant and the other two treatments of elevated CO₂ appear in

a separate quadrant. Although the effects of CO₂ exposure may be indirect, significant and differentiable variations were explained in terms of rhizospheric microbial compositions.

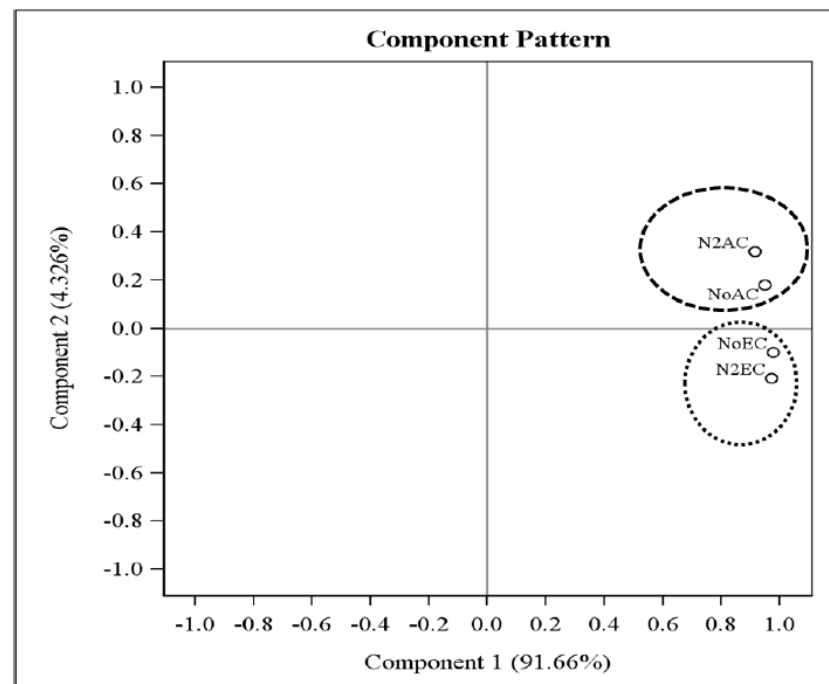


Figure 8. Principal component analysis (PCA) of PLFA responses using *xTriticocereale* (Triticale) soil microbial community influenced by ambient CO₂, elevated CO₂ and varying nitrogen levels. (Where, N₀: normal recommended nitrogen; N₂: twice the normal recommended nitrogen amount; AC: ambient CO₂; EC: elevated CO₂).

4. Discussion

Using *xTriticocereale* (Triticale) as the test crop in the present experiment, we investigated the dynamics of phospholipid fatty acids in rhizospheric soil by examining the soil microbial structure's composition and content during a 50% anthesis period. Living cells comprise fatty acids, which have hydrophobic tails at one end; another OH-group is linked with the hydrophilic head of a phosphate group. Therefore, these lipids become asymmetric in nature and possess both hydrophobic and hydrophilic regions. Different types of microbes contain different types of fatty acids in their membrane and, thus, their abundance can be studied through PLFA biomarkers (specific to different microorganisms), which allows information to be gained about the abundances of different groups of microbes in soil. Our finding that there is a higher microbial content under EC and a non-significant effect exerted by N is in agreement with studies performed by Hungate et al. (1996) [23]. Also, Zak et al. (1993) [24] reported significantly higher microbial biomass, plant C assimilation, fine root turn over and higher rates of net N mineralization under elevated atmospheric CO₂. Another report by Van Ginkel et al. (2000) [25] depicted a 42% increase in the microbial biomass under EC in *Lolium perenne*. An increase in microbial biomass responses may be attributed to the resultant priming effect by EC. Thus, an altered microbial community may alter these functions as well. As microbes are the crucial players in bio-geochemical cycling, EC can change soil functions and, thus, in future climate change conditions, soil may become a carbon-emitting source either in the form of CO₂ or CH₄ and act as a form of carbon sinks through sequestration [26].

Many studies have reported mixed responses, with few expressing significant changes and some non-significant changes in PLFA contents under elevated CO₂ and higher doses of nitrogen. A report by Drisner et al. (2007) [27] expressed that the total PLFA content

increased by ~25% in the EC compared to the content (~57.38 nmol/g soil) at AC. Our findings are also in accordance with this report. The priming effect exerted by the plants under EC may be the reason behind the boost in microbial growth under EC compared to AC. However, contrary to these reports, no significant alteration in the total PLFA contents was reported by Feng et al. (2010) [28] under high N and EC conditions in Duke Forest sites. Analysis of fungal-to-bacterial ratios in our study revealed that the bacterial community dominated in the microbial group compared to the fungal community, and fungal abundance decreased with additional nitrogen fertilization. This might have occurred because the higher N dose potentially mitigated the investment made by plants in finer roots and associated with mycorrhizal fungi, because, at higher doses of N, the uptake of nutrients becomes less critical [29]. Feng et al. (2010), in the Duke Forest FACE experiment, reported decreased F/B ratios in soil [28]. This was in accordance with our findings, which confirm that fungi are negatively affected by N fertilization in comparison to bacteria. Another experiment by Billings and Ziegler (2008) at the Duke FACE site reported reduced fungal PLFA abundance [30]. They observed that fungal PLFA was 2.8 times more abundant in non-fertilized soil than in fertilized soil. These findings can be explained by reduced C-allocation to finer roots for fungal colonization.

Recently, fresh plant materials have been preferred over Gram negative bacteria as the food source, while old soil organic matter (SOM) has been preferred over Gram positive bacteria. Therefore, we expected more Gram negative populations under EC owing to them having more exudates from the roots under this condition; however, the result was the opposite. Instead, the population of Gram negative bacteria was seen to decline with a concurrent increase in Gram-positive bacterial abundance under EC, irrespective of N addition. The soil's physico-chemical parameters might have ruled out this, rather than the types of C sources used. The present study revealed no effect on the actinomycetes population. Ringelberg et al. (1997) also reported similar findings, identifying only a subtle and non-significant alteration in Gram negative bacteria and actinomycetes under EC [31]. This could be ascribed to their slower growth in comparison to bacteria and fungi. Our findings are in accordance with Drigo et al. (2010) [32]. Fatty acid biomarkers, which are specific to anaerobes and Gram-negative bacteria, decreased under EC along with N application. Conversely, eukaryotes increased under the same conditions. The results from the PLFA performed by Montealegre et al. (2002) indicated that the abundance of eukaryotes in the rhizosphere increased under EC, whereas the abundance of prokaryotes was not affected [33]. They all reported that slow growers are the least affected under EC. In the present study, microbial diversity was mainly affected by CO₂ levels, but N addition also played a role in altering this diversity. Although the effects of CO₂ exposure may be indirect, significant and differentiable variations in terms of rhizospheric microbial compositions were explained. However, long-term studies are needed to identify clear findings regarding the interaction between these two factors and their impact on the diversity in rhizospheric samples and clarify whether these effects/alterations are transient or continuous.

5. Conclusions

The results from this study depict how changing CO₂ levels in combination with high levels of nitrogen affect the soil microbial community. Microbial traits, such as biomass content and diversity index, were more significantly influenced by elevated CO₂. Rhizospheric microbial community analysis using a fungal/bacterial ratio (F/B) showed that the bacterial community dominated in soil under EC and higher N applications. Elevated CO₂ and higher N levels may have minimal effects on the population dynamics of actinomycetes owing to their slow growth compared to bacteria and fungi. The present

investigation found a non-significant impact on rhizospheric soil microbial diversity in response to short-term N addition in a tropical agro-ecosystem. However, elevated CO₂ levels even during short-term exposure significantly affected the soil rhizospheric microbial community structure in terms of both diversity and abundance. The interaction of these drivers can lead to positive feedback in soil with increased biomass and soil respiration and decreased fungal population. As alterations in microbial community composition occur at a very slow rate or on the micro-scale, long-term studies are required to determine whether the observed changes will continue over time and become more pronounced or not. In the future, long-term studies are required to uncover clear findings regarding the interaction of these two factors on the microbial community structure in rhizospheric samples. Long-term interaction studies could clarify whether these effects/alterations are transient or continuous. Finally, it can be inferred that elevated CO₂ has a pronounced effect on soil microbial communities, apart from the population of actinomycetes.

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