ABSTRACT

CHENG, LEI. Soil Microbial Responses to Climate Change Factors: Impacts on Soil Carbon and Nitrogen Dynamics. (Under the direction of Shuijin Hu and H. David Shew.)

Predicting future climate is critically hampered by our limited knowledge of microbial responses to climate change factors. Climate change factors such as elevated atmospheric carbon dioxide (CO₂), ozone (O₃) and nitrogen (N) inputs can significantly alter plant growth and the availability of organic carbon (C) and N availability for soil microbes. However, the underlying mechanisms by which soil microbes respond to climate change-induced alterations in resource availability (for example C and N) remains poorly understood. This dissertation addresses two questions: 1) how do soil microbes respond to climate change factors in N-rich or N-aggrading systems such as agroecosystems? and 2) how do arbuscular mycorrhizal (AM) fungi mediate plant and ecosystem responses to climate change factors? Two hypotheses were proposed accordingly. The first hypothesis was that high N availability mediates microbial responses to CO₂-enhancement of C availability and exerts a major control over organic C turnover. A long-term field study conducted in no-till wheat-soybean system was used to test this hypothesis. Results obtained show that N availability critically influenced soil microbial responses to elevated CO₂ but not O₃. Elevated CO₂ significantly increased but O₃ reduced above-ground residue mass and residue N inputs. However, only elevated CO₂ significantly affected soil microbial parameters. While it only had marginal effects on microbial respiration in the first two years, elevated CO₂ significantly stimulated microbial biomass and decomposition in the third and fourth years when N availability increased, likely due to CO₂-stimulation of symbiotic N₂ fixation in soybean. These results suggest that high N availability in many agricultural soils may
accelerate organic C turnover and limit the potential of C sequestration in agroecosystems under future CO₂ scenarios. The second one proposed that the CO₂-enhancement of arbuscular mycorrhizal (AM) fungi facilitates organic C decomposition and subsequent plant N uptake, but elevated O₃ offsets the CO₂ effect. Three independent, but complementary experiments were designed to test this hypothesis. Results from both field and microcosm experiments demonstrate that the stimulation of AM fungi by elevated CO₂ as well as N additions significantly enhanced organic C decomposition. Results from stable isotope (δ¹⁵N) analyses also indicate elevated CO₂ increased mycorrhizally-mediated plant N acquisition from decomposing organic residues. However, the magnitude of mycorrhizally-mediated plant N uptake under elevated CO₂ varied significantly among different AM fungal species. These findings directly challenge the current view that the CO₂-stimulation of mycorrhizal fungi could increase soil C sequestration. These results also suggest that elevated CO₂ might alter the community composition of AM fungi and the feedbacks of plant and ecosystem responses.
Soil Microbial Responses to Climate Change Factors: Impacts on Soil Carbon and Nitrogen Dynamics

by
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DEDICATION

To my parents, my wife and my daughters!
Lei Cheng was born on August 6, 1978 and grew up in a China countryside (Hefei, Anhui Province), where he has developed an immense interest in the subject of soil and crops. In 1995, he chose the major of soil science in the Anhui Agricultural University as his undergraduate study. After graduation, he stayed at the Anhui Agricultural University as a full-time instructor and taught undergraduate students Pedology. In 2001, he started his graduate study to investigate the responses of belowground processes of agroecosystems to atmospheric CO₂ enrichment at the Institute of Soil Science, Chinese Academy of Sciences. In 2005, he joined the group of Dr. Shuijin Hu, a leading scientist in soil ecology and global change research, at the North Carolina State University to pursue his Ph.D. degree.

Lei Cheng has authored and co-authored more than 10 papers published in journals such as Ecology Letters and Environmental & Experimental Botany. During his Ph.D. studies, he had received a research fellowship from USDA-ARS Plant Science Research Unit, Raleigh, NC. He had also received several awards such as a student presentation award (in Agro-ecology section) from the Ecological Society of America.
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Chapter 1

Literature review
1.1 Introduction

Throughout their history, humans have interacted with and shaped Earth’s ecosystems for social and economic development. Over the past two hundred years, however, human alteration of the Earth’s atmosphere has accelerated more rapidly and extensively than at any other comparable periods of human history. Human activities (e.g. fossil fuel combustion and land use change) have significantly increased greenhouse gases such as carbon dioxide (CO₂), ozone (O₃) and nitrous oxide (N₂O) in the atmosphere at an unprecedented rates, potentially causing a significant change in climate (Solomon et al. 2007). Global atmospheric CO₂ concentration has been increased from approximately 270 µmol mol⁻¹ in 1750 to its current value of 380 µmol mol⁻¹ and is projected to reach as high as 700 µmol mol⁻¹ by the end of this century. Similar to atmospheric CO₂, the O₃ concentration in the troposphere has increased rapidly, from an estimated pre-industrial level of 38 nmol mol⁻¹ to the current level of 50 nmol mol⁻¹, a level that is already 25% higher than the threshold set for injury to sensitive plants (Fuhrer et al. 1997). Human activities have also increased reactive N (e.g. N₂O, NOₓ) in the atmosphere (Vitousek et al. 1997a; Galloway et al. 2004), resulting in a significant increase in the rate of atmospheric N deposition. Global atmospheric N deposition is expected to reach 195 Tg N yr⁻¹ in 2050, which is more than 6 times of the amount in 1860 (Galloway et al. 2004).

Elevated CO₂, O₃ and N inputs as well as other climate change factors (or global change factors) such as warming, biotic invasion and changes in precipitation may profoundly alter terrestrial ecosystem and constitute a potential threat to humanity (Wedin &
Tilman 1996; Vitousek et al. 1997; Körner 2000; Booker et al. 2009). Ecologists have long been interested in understanding how these global change factors affect the structure and functioning of terrestrial ecosystems and how ecosystems feed back to the ongoing climate change. So far, much attention has been directed towards the responses of aboveground processes to global change factors (Melillo et al. 1993; McLaughlin & Downing 1995; Curtis & Wang 1998; DeLucia et al. 1999). However, our ability to predict future climate change relies largely on our current incomplete understanding of belowground responses, especially soil microbes and microbially-mediated processes, to these global change factors (Hu et al. 1999; Bardgett et al. 2008; Heimann & Reichstein 2008).

This chapter (chapter I) provides an overview of current knowledge of global change effects on soil microbes with the emphasis on the impacts of elevated atmospheric CO₂ and O₃. Possible mechanisms that regulate soil microbial responses to several climate change factors (elevated CO₂ and O₃, climate warming, and N additions) are also discussed. Future research needs on plant-soil microbe interactions in a changing climate are also proposed.

1.2 Soil microbial responses to global change factors: current knowledge

1.2.1 Effects of elevated atmospheric CO₂

Both supply and demand for CO₂ regulate the rate of photosynthetic C assimilation of plants. With increasing atmospheric CO₂ concentrations, and therefore getting higher partial pressure of CO₂ within interspaces of plant leaves, photosynthesis increases steeply when Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase, the principal enzyme for C-
reduction) is present in saturating quantities (Farquhar & Sharkey 1982). Experimental evidence that elevated atmospheric CO$_2$ stimulates photosynthesis and plant growth is overwhelming (Drake et al. 1997; Curtis & Wang 1998; Kimball et al. 2002). Consequently, elevated CO$_2$ often increases C inputs belowground through enhancing rhizodeposition or litter inputs, increasing C availability to soil microbes (Rogers et al. 1994; Hungate et al. 1997b; Pendall et al. 2004). An increase in C availability induced by elevated CO$_2$ can significantly stimulate soil microbial growth and activities, as soil microbes are most commonly limited by C availability (Smith & Paul 1990; Zak et al. 2000b). Because soil bacteria and fungi differ in their C assimilation efficiency (Adu & Oades 1978; Griffiths et al. 1999), soil microbial community could shift to either fungi- or bacteria dominated in response to changes in the quantity and quality of substrates. Alterations in the microbial community structure and processes, in turn, can feed back to plants and atmospheric CO$_2$.

**Soil microbial biomass, activities and community structure**

*Microbial biomass.* Over the past two decades, a large number of studies have examined CO$_2$ effects on soil microbial biomass and activities, as summarized by several excellent reviews (Oneill 1994; Paterson et al. 1997; Sadowsky & Schortemeyer 1997; Hu et al. 1999; Zak et al. 2000b; de Graaff et al. 2006; Hu et al. 2006). Soil microbial biomass has been observed to increase at elevated CO$_2$ (Diaz et al. 1993; Zak et al. 1993; Rice et al. 1994; Hungate et al. 1997b; Dijkstra et al. 2005; Hu et al. 2005), but has also been detected to remain stable or decrease (Rice et al. 1994; Hungate et al. 1996; Jones et al. 1998; Hu et

al. 2001; Hu et al. 2005; Baronti et al. 2008). In a synthesis, Zak et al. (2000b) found that CO₂ enrichment caused an average increase by 19% in microbial biomass based on 45 observations. Among these observations, however, 62% of them showed a CO₂ stimulation effect, whereas 18% and 20% of them showed a negative and null response to elevated CO₂, respectively. Similar to the pattern summarized in Zak et al. (2000b), Hu et al. (2006) recently reviewed 135 reports published during the year of 2000 to 2006 and found that microbial biomass C and biomass N have been observed to increase under elevated CO₂ among 19 of 40 studies and 12 of 27 studies, respectively, but remained unchanged or even decreased in the remainder.

It is likely that some functional groups of soil microbes such as mycorrhizal fungi might be more responsive to elevated CO₂. Mycorrhizal fungi, especially arbuscular mycorrhizal (AM) fungi, have been traditionally considered as obligate biotrophs and obtain C solely from their host plants (Smith & Read 1997). Studies, either conducted in pot or field experiments over a wide range of biomes, have hitherto found that elevated CO₂ generally stimulates mycorrhizal fungal growth (Fitter et al. 2000; Treseder & Allen 2000; Treseder 2004; Alberton et al. 2005; Hu et al. 2006). For instance, Alberton et al. (2005) found that CO₂ enrichment caused an average increase by 21% in the abundance of AM fungi in a meta-analysis of 72 pot and field studies.

*Microbial activities.* Many studies have observed a CO₂ stimulation effect on soil microbial activities measured either in microbial respiration (Zak et al. 1993; Hu et al. 2001) or in enzymatic activities (Dhillion et al. 1996; Chung et al. 2006; Dorodnikov et al. 2009).
In a meta-analysis based on 30 studies, de Graaff et al. (2006) showed that microbial respiration increased, on average, by approximately 17% at elevated CO$_2$ in comparison to ambient CO$_2$. Microbial respiration offers a picture of the whole soil microbial community in response to elevated CO$_2$. Measurements on enzymatic activities can provide insights into how higher CO$_2$ affects specific functions of soil microbes (e.g. enzymes involved in C and nutrient transformations) (Dhillion et al. 1996; Chung et al. 2006). For example, the activity of 1,4-β-N-acetylglucosaminidase (chitin-degrading enzyme) at elevated CO$_2$ has been detected to increase significantly by 84% in comparison to ambient CO$_2$ (Chung et al. 2006), suggesting that CO$_2$ enrichment may significantly enhance the fungal biomass because chitin is the major component of fungal cell wall. Cellulose is a major compound in plant residues. The stimulation of activities of the cellulose-degrading enzymes has also been observed in some studies (Dhillion et al. 1996; Jones et al. 1998; Mayr et al. 1999; Chung et al. 2006; Dorodnikov et al. 2009), indicating that soil microbes at elevated CO$_2$ might facilitate the rate of cellulose degradation. Last, CO$_2$ enrichment can stimulate the activities of extracellular enzymes responsible for N and P metabolisms in soil (Dhillion et al. 1996; Moorhead & Linkins 1997; Ebersberger et al. 2003; Dorodnikov et al. 2009). Nevertheless, a null or negative CO$_2$ effect has also been detected either measured in microbial respiration (Hu et al. 2001; Billings & Ziegler 2005; West et al. 2006) or in extracellular enzyme activities (Larson et al. 2002; Kang et al. 2005; Austin et al. 2009).

The microbial community structure. Similar to microbial biomass and activities, soil microbial community structure under elevated CO$_2$ has also become a focus in the CO$_2$
research since 1990s. It has been proposed that increased C availability under elevated CO$_2$ would favor soil fungi to bacteria due to higher C use efficiency of fungi (Zak et al. 1996; Hu et al. 2001). To determine whether CO$_2$ enrichment could favor a fungi-dominant soil microbial community, I reviewed 22 studies (Table 1.1) that have directly evaluated changes in the whole microbial community at elevated CO$_2$ with a focus on fungi/bacteria ratios. These studies included a variety of woody, graminoid and herbaceous plant species, as well as grassland, scrub-oak and chaparral ecosystems. Experimental duration of these studies varied from a few weeks to 10 years. In the literature reviewed here, either modern molecular [e.g. DNA and PLFA (phospholipid fatty acid) analyses, see Table 1.1] or traditional techniques (plate counts) have been employed to assess shifts in the microbial community composition. Most of these studies have observed a significant increase in plant productivity (Hungate et al. 1997b; Jones et al. 1998; DeLucia et al. 1999; Carney et al. 2007). However, across the assembled data base, alterations in microbial community structure did not follow a consistent pattern with plant responses to elevated CO$_2$. The ratio of fungi to bacteria tended to increase at elevated CO$_2$ in some studies, but remained unchanged or even decreased in other reports (Table 1.1). These results are, in a large measure, independent of plant species, CO$_2$ concentrations, experimental duration, and experimental approaches. Among 17 observations using PLFA analyses, the most common tool used in these studies, only one reported a significant increase in the ratio of fungi to bacteria (Carney et al. 2007). In addition, CO$_2$ enrichment only caused an average increase by 8% with a coefficient of variation of 201% in fungi/bacteria ratios (Table 1.1). It is still unknown why the ratio of
fungi to bacteria is unresponsive to elevated CO$_2$. Many factors such as the quality and quantity of substrates (Griffiths et al. 1999) and soil temperature (Zhang et al. 2005) can be attributable to alterations in soil microbial community structure. A recent study has shown that the ratios of fungi to bacteria have a strong positive relationship with soil C:N ratios across a variety of biomes (Noah et al. 2009). However, we still lack sufficient evidence to link changes of soil C:N ratios to fungi:bacteria ratios under elevated CO$_2$, though soil C:N ratios have been found to be unresponsive to elevated CO$_2$ (Luo et al. 2006).

*Microbial feedbacks to atmospheric CO$_2$ concentrations*

The CO$_2$-induced changes in soil microbes can feed back to atmospheric CO$_2$, mainly through altering soil decomposition processes. The CO$_2$-stimulation of soil microbial activities observed in many studies (Zak et al. 2000b; de Graaff et al. 2006) suggests that soil microbes at higher CO$_2$ might respire more C back to the atmosphere, positively feeding back to the rising atmospheric CO$_2$. Elevated CO$_2$, on one hand, could stimulate the decomposition of plant-derived C such as root exudation and plant residues. For instance, it has been observed that soil microbes under elevated CO$_2$ decomposed more root-derived C in a annual grassland experiment in California (Hungate et al. 1997b), and in a mesocosm experiment using a range of young European tree species exposed to a gradient of elevated CO$_2$ treatments in England (Heath et al. 2005). On the other hand, the CO$_2$-stimulation of soil microbes could cause a priming effect on the decomposition of soil organic C (Cheng & Johnson 1998). In a scrub-oak ecosystem, microbial priming has been attributable to a 21% decline in total soil organic C contents at elevated CO$_2$ in comparison to ambient CO$_2$ in the
0-10 cm soil layer after six years of CO$_2$ fumigation (Carney et al. 2007). More importantly, CO$_2$-induced priming effects might also promote the decomposition of recalcitrant soil C pools (Langley et al. 2009), potentially altering long-term soil C stability (Fontaine et al. 2007).

By contrast, elevated CO$_2$ may reduce soil C losses through suppressing soil microbes, resulting in a negative feedback to the rate of increase in the atmospheric CO$_2$ concentration. In an annual California grassland after 5 years of CO$_2$ fumigation, Hu et al. (2001) found that elevated CO$_2$ significantly increased plant N uptake, reducing soil N availability to soil microbes at the late growing season. In addition, they also showed that elevated CO$_2$ did not alter below-ground respiration (the sum of root and heterotrophic respiration), but significantly reduced microbial respiration per unit of microbial biomass in the late growing season, suggesting that microbial decomposing processes were constrained due to lower N availability to soil microbes. It has also been proposed that elevated CO$_2$ could slow the rate of residue decomposition through reducing the litter quality (e.g. increasing C:N ratios of plant residues) to soil microbes (Strain & Bazzaz 1983). However, a meta-analysis of data based on decomposition of naturally senesced leaves in field experiments showed that N concentrations in leaf litter under elevated CO$_2$ reduced only by 7.1% (Norby et al. 2001), much less than the decline reported in green leaves (Couteaux et al. 1999). Accumulated evidence further indicated that these small changes in plant residue chemistry under elevated CO$_2$ did not alter residue decomposition significantly (Norby et al. 2001; Booker et al. 2005; Liu et al. 2009a).
Microbial feedbacks to plant growth

Soil microbes also critically modulate soil nutrient availability, particularly N, for plants. Changes in soil microbes can positively feed back to plant growth by increasing soil nutrient availability or facilitating plant nutrient uptake. Elevated CO$_2$ can stimulate heterotrophic respiration and subsequent nutrient release from organic matter. Increases in soil N mineralization have often been observed in CO$_2$ experiments (Zak et al. 1993; Hungate et al. 1997a). Moreover, elevated CO$_2$ can enhance plant nutrient uptake through stimulating the activities and functioning of symbiotic microbes. Experimental evidence showed that CO$_2$ enrichment can enhance plant N acquisition through stimulating mycorrhizal fungal growth (Treseder 2004; Alberton et al. 2005) and symbiotic N$_2$ fixation (Zanetti et al. 1996; Vogel et al. 1997; Hungate et al. 1999; Rogers et al. 2006; Tu et al. 2009).

Soil microbes under elevated CO$_2$ may also elicit a negative feedback to plant growth through decreasing soil nutrient availability. A few studies focusing on soil N cycling showed that elevated CO$_2$ can suppress either the rates of gross soil N mineralization (Berntson & Bazzaz 1997) or net soil N mineralization (Billings & Ziegler 2005). Additionally, it has been proposed that the CO$_2$-induced N losses through nitrification and denitrification would limit the response of plant growth to elevated CO$_2$ (Hu et al. 1999). A recent meta-analysis, however, showed that CO$_2$ enrichment caused a significant decline in NO$_3^-$ concentrations, nitrifying – and denitrifying- enzyme activity (Barnard et al. 2005), suggesting that nitrifiers and denitrifiers might have a lower competitive ability in acquiring
NO$_3^-$ and NH$_4^+$ than plants.

Soil microbial feedback to plant growth is largely time dependent. Over the short term, most studies indicate that soil microbes at elevated CO$_2$ might lead to positive feedback to plant growth through increasing N availability (Zak et al. 2000a; Barnard et al. 2005; de Graaff et al. 2006). Over the long term, however, a negative feedback may occur (Finzi et al. 2006; Reich et al. 2006) due to a progressively developed N limitation on plant responses to the rising CO$_2$. Initially proposed by Hu et al. (1999), then developed by Luo et al. (2004) and Hu et al. (2006), the progressive N limitation hypothesis predicts that the response of plant growth to elevated CO$_2$ will not sustain over the long term as soil N availability will be accumulated in plant biomass and soil organic matter. A key assumption underlying this hypothesis is that soil microbes at elevated CO$_2$ will release less N from litter or soil organic matter over time because the deficiency of N itself limits microbial growth and activities. However, only a few studies have so far observed a N limitation on plant responses to elevated CO$_2$ (Oren et al. 2001; Gill et al. 2002; Finzi et al. 2006; Reich et al. 2006). It remains unclear whether soil microbes such as mycorrhizal fungi can relieve the N limitation through enhancing plant N uptake via fungal hyphae.

1.2.2 Effects of elevated tropospheric O$_3$

Atmospheric O$_3$ can be both beneficial and harmful to organisms on Earth pending its location. In the stratosphere (upper atmosphere), the O$_3$ layer can reduce the penetration of ultraviolet light, protecting organisms on the Earth’s surface from ultraviolet radiation.
However, O\textsubscript{3} in the troposphere (ground-level) is considered an air pollutant and is toxic to many organisms, including for ozone-sensitive plants. Increasing O\textsubscript{3} concentrations in the troposphere have been mainly driven by the emission of NO\textsubscript{x} and CH\textsubscript{4}. As a powerful oxidant, O\textsubscript{3} can react with a diverse set of molecules within the cell wall and on the plasma membrane surface (Fiscus \textit{et al.} 2005; Booker \textit{et al.} 2009), resulting in a damage to plant leaves. Therefore, elevated tropospheric O\textsubscript{3} often suppresses photosynthesis and plant growth (Reich & Amundson 1985; Morgan \textit{et al.} 2003). It has been well documented that elevated O\textsubscript{3} can lead to a substantial decline in the aboveground biomass of forest and crop plants (Morgan \textit{et al.} 2003; Fiscus \textit{et al.} 2005; Booker \textit{et al.} 2009) and subsequent C allocation belowground (Andersen 2003; Grantz \textit{et al.} 2006). Decreased C availability belowground under elevated O\textsubscript{3} may also potentially affect soil microbes and associated processes.

\textit{Soil microbial biomass, activities and community structure}

\textit{Microbial biomass}. Compared to the CO\textsubscript{2} effects, the influences of elevated O\textsubscript{3} on soil microbial biomass, activities and community composition have received relatively less attention. Elevated O\textsubscript{3}, in contrast to elevated CO\textsubscript{2}, might be expected to suppress microbial growth and activities through reducing C supplies for microbes. Soil microbial biomass has been reported to decrease significantly under elevated O\textsubscript{3} in a wheat-soybean rotation experiment conducted in open-top chambers (Islam \textit{et al.} 2000), in a pot experiment using wheat plants (Chen \textit{et al.} 2009), in a California perennial bunchgrass (\textit{Elymus glaucus}) experiment using growth chambers (Yoshida \textit{et al.} 2001), and in the Rhinelander FACE
(free-air CO₂ and O₃ enrichment) forest experiment (Holmes et al. 2003). Moreover, experimental evidence showed an O₃-induced decline in mycorrhizal fungal biomass in several studies (Perezsoba et al. 1995; Yoshida et al. 2001; Kasurinen et al. 2005). However, contrasting results have also been found in other studies (Duckmanton & Widden 1994; Scagel & Andersen 1997; Yoshida et al. 2001; Larson et al. 2002; Kanerva et al. 2006). For instance, in a simulated meadow ecosystem continuously exposed to elevated O₃ for three years, Kanerva et al. (2006) showed that elevated O₃ did not change soil microbial biomass N in any of the experimental years. Soil fungal biomass has been found even to increase, rather than decrease, under higher O₃ concentrations in a pine tree experiment (Scagel & Andersen 1997) and in a bunchgrass experiment (Yoshida et al. 2001).

*Microbial activities.* Based on the predicted decline in C supplies, it is also expected that soil microbial respiration and enzymatic activities would decrease under elevated O₃. Experimental studies that have examined enzymatic activities, however, did not show clear pattern of responses of a diverse set of enzymes to elevated O₃. Chung et al. (2006) found that elevated O₃ significantly reduced the activity of cellulose-degrading enzymes (1,4-β-glucosidase) by 25% in comparison to ambient O₃ in a forest experiment in Rhinelander, Wisconsin, consistent with results from a previous work conducted at the same experiment site (Phillips et al. 2002). In a growth chamber experiment, Reddy et al. (1991) found that arylsulfatase, an enzyme that regulate the mineralization of soil organic S, decreased under extreme higher O₃ conditions (240 and 320 nmol mol⁻¹) in soils with a pH of 4.3. However, it has also been shown that elevated O₃ did not significantly affect the activities of many other
enzymes such as 1,4- β -xylosidase, phosphatase, 1,4-α-glucosidase, peroxidase, and phenol oxidase in these studies (Reddy et al. 1991; Larson et al. 2002; Chung et al. 2006). In addition, several studies have shown that soil microbial respiration remained largely unaffected by elevated O3 (Islam et al. 2000; Phillips et al. 2002; Chapman et al. 2005). Currently, it is still difficult to make generalizations on the basis of limited number of studies in the literature.

The microbial community structure. The influences of elevated O3 on soil microbial community composition have only recently received attention (Phillips et al. 2002; Kasurinen et al. 2005; Chung et al. 2006; Kanerva et al. 2008). The few studies available, however, indicate that results regarding the effect of elevated O3 on fungi/bacteria ratios were inconsistent. Using one O3-tolerant and one O3-sensitive genotype of Betula pendula Roth, Kasurinen et al. (2005) found that fungal/bacterial ratios did not change in any of these two clones due to the O3 effect. Recently, Kanerva et al. (2008) investigated the changes of the microbial community structure in a simulated meadow ecosystems exposed to elevated O3 for three years and found that the ratio of fungi to bacteria remained unchanged in the first year of the experiment, but decreased in the third year. Elevated O3 often increases the contents of recalcitrant compounds such as lignin in organic residues (Booker et al. 1996; Booker et al. 2005). Thus, it is likely that elevated O3 may alter the fungal community composition because soil saprotrophic fungi are major decomposers of those recalcitrant organic compounds. Chung et al. (2006) used a PCR-DGGE technique to identify the operational taxonomic units (OTU) of fungi in soil samples taken from the FACE forest
experimental site in Rhinelander, Wisconsin. They found that one unknown OTU, as a significant indicator of O₃ treatment, was present 44% in soil samples from elevated O₃, but it occurred only 6% of samples under ambient O₃. Nevertheless, it remains unknown whether and how alterations in fungal community composition under elevated O₃ impact soil organic C decomposition.

*Soil microbial feedback to plant growth*

Data demonstrating the effects of elevated O₃ on soil N dynamics and subsequent feedbacks to plant growth have also provided equivocal evidence. In the Rhinelander FACE forest experiment, elevated O₃ elicited a significant decline in gross soil N mineralization, but did not change the microbial N immobilization (Holmes *et al.* 2003, 2006), suggesting that soil N availability for plants reduced under higher O₃. The decline in soil N availability might explain the O₃-induced reduction in plant N uptake observed in a few studies (Haberer *et al.* 2007; Zak *et al.* 2007). However, a 3-year monitoring on soil N dynamics showed that O₃ had no impacts on soil N transformations in a meadow ecosystem (Kanerva *et al.* 2006). The unresponsiveness of the foliar N concentration or the total plant N uptake to elevated O₃ (Temple & Riechers 1995; Kytoviita *et al.* 2001; Tu *et al.* 2009) might also indicate a lack of O₃ effects on soil N mineralization.

*Soil microbial feedback to atmospheric CO₂ concentrations*

Although elevated O₃ often reduces the total C assimilation by plants, decreased C
availability (Andersen 2003; Grantz et al. 2006) and increased recalcitrant compounds in litter (Booker et al. 1996; Booker et al. 2005) under elevated O₃ would slow soil microbial decomposition processes, reducing C losses from soil. Few studies, however, have observed an O₃-induced suppressive effect on microbial decomposition processes. Instead, accumulated evidence has shown that both the rate of microbial respiration (Islam et al. 2000; Phillips et al. 2002; Chapman et al. 2005) and soil respiration (the sum of heterotrophic and root respiration measured in field) (Kasurinen et al. 2004; Pregitzer et al. 2006; Tingey et al. 2006) remained unaffected by elevated O₃, suggesting that microbial decomposition processes might be unresponsive to higher O₃ concentrations. Interestingly, reported studies have also indicated an O₃-enhancement of microbial decomposition under elevated CO₂ (Loya et al. 2003; Pregitzer et al. 2006), though this might be a temporary phenomenon (Alan et al. 2009). Evidently, mechanisms underlying the unresponsiveness of microbial decomposition to elevated O₃ are unknown, and more research is needed to examine whether the O₃ effects on soil microbial processes are confounded by other unseen factors.

1.3 Soil microbial responses to climate change factors: mechanisms

Soil microbes can be regulated by a diverse set of factors including soil temperature, moisture, oxygen, substrate quality and quantity, nutrient contents, soil animal predation, inhibitors, and other physical, chemical and biological conditions. The impacts of global change factors on soil microbes are mainly through their direct and/or indirect effects on the
factors listed above.

1.3.1 **Direct effects of global change factors on soil microbes**

*Climate warming*

The global surface temperature has been increased by approximately 0.6 °C during the past 100 years (Hansen *et al.* 2006) and this change would be much more rapid if we continue ‘business as usual ’ when it comes to greenhouse gas emissions (Cox *et al.* 2000). There is now ample evidence to show that climate warming can affect an array of ecosystems, from polar terrestrial to topical marine environments, and influence a broad range of organisms, from microorganisms to plants to animals (Walther *et al.* 2002; Root *et al.* 2003; Davidson & Janssens 2006). Generally, there are two thoughts on the effects of climate warming on soil microbes and associated processes. One thought maintains that current climate warming would stimulate soil microbial growth and activities, thus accelerating the rate of soil organic C decomposition. A positive relationship between temperature and soil microbial respiration has been well illustrated (Lloyd & Taylor 1994). Modeling and experimental evidence have documented a direct stimulation of higher temperature on soil microbial respiration (Jenkinson *et al.* 1991; Lloyd & Taylor 1994; Kirschbaum 1995; Melillo *et al.* 2002; Davidson & Janssens 2006; Hartley *et al.* 2008, 2009). The respiration rate of organisms in soil (mainly root and microbial respiration) would approximately double for every 10 °C increase in temperature. Another argument, however, states that soil microbes may acclimate or adapt to climate warming, therefore weakening the
positive effect of climate warming on soil respiration. Some experimental studies provided supporting evidence to show that soil microbial biomass and respiration did not respond to experimental warming (Jonasson et al. 1999; Giardina & Ryan 2000; Luo et al. 2001; Zhang et al. 2005; Heinemeyer et al. 2006; Bradford et al. 2008). However, the underlying mechanisms by which soil microbes acclimate to warming are not well understood. It is likely that temperature might be not the most limiting factor in those warming experiments that have observed a temperature insensitivity of microbial decomposition (Giardina & Ryan 2000; Luo et al. 2001; Bradford et al. 2008). In other words, the effects of warming on microbial decomposition might be obscured by other hidden factors such as the quality of substrate in an incubation study (Giardina & Ryan 2000), or plant growth in a field experiment (Luo et al. 2001; Zhang et al. 2005).

*N additions*

Soil microorganisms require N for their growth. Apart from C, H and O, N is the most abundant nutrient element existing in microorganisms (Duboc et al. 1995). Thus, changes in N availability can directly affect soil microbial growth and activities. Studies on N effects on soil microbes are plentiful, and most of them came from N addition experiments. However, because soil microbes are generally C-limited in many ecosystems (Waksman & Stevens 1929; Smith & Paul 1990; Vance & Chapin 2001), a lack of soil microbial responses to N additions is very likely to occur. Many studies have reported a null effect of N additions on soil microbial biomass and activities (Glendining et al. 1996;
Jonasson et al. 1996; Bardgett et al. 1999; Jonasson et al. 1999; Haase et al. 2008), and on microbial community structure (Zak et al. 2000a; Wiemken et al. 2001; Chung et al. 2007). Other studies even showed a negative effect of N additions on soil microbial biomass and activities (Smolander et al. 1994; Frey et al. 2004; Billings & Ziegler 2008), and microbial decomposition processes (Fog 1988; Knorr et al. 2005). However, N additions can have a stimulation effect on soil microbes under elevated CO$_2$ (Rice et al. 1994; Grayston et al. 1998; West et al. 2006), suggesting that soil microbes could be limited by N when C availability is high.

Elevated atmospheric CO$_2$ and O$_3$

Direct effects of elevated atmospheric CO$_2$ on soil microorganisms are negligible, because the CO$_2$ concentration in soil is at least one order of magnitude higher than the current or projected levels of atmospheric CO$_2$. Also, there is no available evidence to show that higher concentrations of tropospheric O$_3$ are able to directly affect soil microbial growth and activities, though the possibility is not excluded. Most likely, soil microbes are influenced indirectly through CO$_2$ or O$_3$-induced changes in belowground resources.

1.3.2 Indirect effects of global change factors on soil microbes

Global change factors can significantly affect soil microbes and their related processes through altering C availability (Zak et al. 1993; Zak et al. 2000b), N availability (Hu et al. 1999; Hu et al. 2001), soil moisture contents (Rice et al. 1994; Field et al. 1995;
Liu et al. 2009b), and grazing capability of soil animals (Jones et al. 1998; Lussenhop et al. 1998) singly or in combination.

**Carbon limitation on soil microbes**

It has long been thought that soil microbes are in general C-limited. Waksman & Stevens (1929) proposed that available C compounds rather than N and other nutrients are the limiting factor that controls soil microbes in peat soils. Since then, this idea that substrate availability limits soil microbial growth and activities has been restated and tested extensively (Stotzky & Norman 1961; Alexander 1977; Fog 1988; Smith & Paul 1990; Zak et al. 1993; Zak et al. 2000b; Hu et al. 2001; Vance & Chapin 2001; Allen & Schlesinger 2004). Consequently, increased C availability under elevated CO$_2$ and experimental warming would stimulate soil microbial growth and activities, whereas O$_3$-induced decline in C availability would suppress soil microbes. In the majority of CO$_2$ enrichment studies, elevated CO$_2$ enrichment consistently increased above- and belowground biomass in a wide range of biomes (Curtis & Wang 1998; Wand et al. 1999; Kimball et al. 2002). It has also been well documented that elevated CO$_2$ increased C inputs belowground through enhancing rhizodeposition or litter, increasing C availability to soil microbes (Rogers et al. 1994; Hungate et al. 1997b; Pendall et al. 2004). However, soil microbial responses to elevated CO$_2$ did not follow a similar pattern as the plant responses (Zak et al. 2000b; Hu et al. 2006). Variability in C availability is particularly insufficient to explain the negative responses of soil microbes to elevated CO$_2$ (Hungate et al. 1996; Hu et al. 2001; Billings & Ziegler 2005).
Similarly, the relationship between soil microbes and climate warming is not always positive (Giardina & Ryan 2000; Luo et al. 2001; Zhang et al. 2005). Therefore, there must be other factors involved in regulating soil microbial responses to global change factors.

**Nutrient limitation on soil microbes**

Nitrogen is generally the most abundant nutrient existing in soil microorganisms. Likely, soil microbial growth may also be constrained by N availability under the conditions with high C but low N. Even in relatively fertile soils, plant competitive N uptake can reduce soil N availability to a degree that limits soil microorganisms (Kaye & Hart 1997). Indeed, the assimilation of C and nutrients by soil microbes is an enzyme-catalysed process, which can be described by Michaelis-Menten kinetics (Michaelis & Menten 1913). Under a C limiting condition, increasing C availability would increase microbial assimilation of C substrate. If C supply is sufficient, soil microbes might switch to the N limiting conditions. Then soil microbes might become phosphorus (P)-limited when the enzymes that metabolize N also starts to saturate.

Experimental evidence, which clearly supports the proposed idea above, has shown that soil microbes responded strongly to C additions when N is abundant, but responded positively to N additions when C is sufficient (Vance & Chapin 2001). In other words, soil microbes could be N-limited when C availability is high. Clearly, this can occur in a higher CO₂ world, particularly in ecosystems where plant growth is often constrained by soil N availability. Increased C availability (Zak et al. 1993; Hungate et al. 1997b) and decreased
soil N availability (Hu et al. 2001; Finzi et al. 2007) under elevated CO$_2$ could gradually switch a C limiting condition to a N limiting condition for soil microbes. Several studies have documented a N limitation on soil microorganisms (Niklaus 1998; Hu et al. 2001; Barnard et al. 2006). It is note that soil microbes at elevated CO$_2$, in the short term, are usually limited by C availability, but are more likely constrained by N availability over the long term as N can be accumulated gradually in the standing biomass and recalcitrant N pools (Hu et al. 1999; Luo et al. 2004; Hu et al. 2006).

Possibly, other nutrients could also constrain soil microbial responses to elevated CO$_2$ if both C and N are not limited. Phosphorus is also a required nutrient for microbial growth. In most terrestrial ecosystems, however, soil microbes are usually not constrained by P (Stotzky & Norman 1961; Allen & Schlesinger 2004), though P could be a limiting factor for N transforming bacteria in coastal ecosystems (Sundareshwar et al. 2003). In a 3-year CO$_2$ experiment, Niklaus (1998) did not observe a P limitation on soil microbial biomass and activities in a calcareous grassland. In scrub-oak vegetation exposed to CO$_2$ for 7 years, Hungate et al. (2004) found that symbiotic N$_2$ fixation of a leguminous vine Galactia elliottii was initially stimulated, but suppressed later, by elevated CO$_2$. The decline in symbiotic N$_2$ fixation is likely owing to a Mo (Molybdenum) limitation on N$_2$ fixing bacteria associated with the leguminous plant (Hungate et al. 2004). Nutrient limitation of N$_2$ fixing bacteria can also be verified in experiments with non-N additions. A recent meta-analysis showed that elevated CO$_2$ significantly increased symbiotic N$_2$ fixation in studies (43 observations) of non-N nutrient additions, but had no impacts in 49 observations without any nutrient
additions (van Groenigen et al. 2006), suggesting that non-N mineral nutrients rather than C availability might limit the responses of N\textsubscript{2} fixing bacteria to elevated CO\textsubscript{2}.

**Soil water limitation on soil microbes**

Soil moisture contents may also mediate soil microbial responses to global change factors. Elevated atmospheric CO\textsubscript{2} often reduces stomatal conductance of leaves (Drake et al. 1997), increasing plant water use efficiency (Jackson et al. 1994; Field et al. 1995). Consequently, CO\textsubscript{2} enrichment may lessen the possible water limitation on soil microbes in arid and semi-arid systems (Rice et al. 1994). By contrast, climate warming might significantly affect soil microbes through decreasing soil water contents. In an experiment conducted in a tallgrass prairie ecosystem in the US Great Plains, an artificial warming of about 2 °C significantly increased plant biomass and C allocation belowground (Wan et al. 2005), but decreased soil moisture contents (Luo et al. 2001; Wan et al. 2002), suggesting that unresponsiveness of microbial biomass and activities to warming (Luo et al. 2001; Zhang et al. 2005) might be, at least in part, attributed to the limitation of soil moisture contents. In a recent study, Liu et al. (2009) showed that soil water content was a key factor regulating the responses of microbial respiration to experimental warming in a semiarid temperate steppe in northern China.

**Regulation by microbe-feeding soil fauna**

Soil microbial responses to changes in resource availability induced by global change
factors may be driven by top-down forces or grazing from soil animals (Wardle et al. 2004). Elevated CO$_2$ could increase the C transfer through the whole soil food web, resulting in a stimulation effect on soil protozoa, nematodes and collemmbola. The CO$_2$-enhancement of bacterial or fungal-feeding soil fauna, in turn, may suppress soil microbial populations (Klironomos et al. 1996; Jones et al. 1998; Lussenhop et al. 1998). Jones et al. (1998) showed that elevated CO$_2$ significantly increased C availability to soil microbes, but did not observe a CO$_2$-enhancement of soil microbial biomass. However, the total number of collemmbola, which are major consumers of soil fungi, at elevated CO$_2$ was significantly higher than that of ambient CO$_2$. In the rhizosphere, soil organisms are dominated by bacteria and bacteria-feeding protozoa and nematodes. Lussenhop et al. (1998) examined the effect of elevated CO$_2$ on soil biota in the rhizosphere and found that the CO$_2$-enhancement of soil protozoa accompanied the CO$_2$-induced decline in microbial biomass. These studies suggest that soil microbial responses to elevated CO$_2$ might be regulated by soil fauna predation.

1.4 Soil microbial responses to climate change factors: knowledge gaps

Despite decades of research on soil microbial responses to global change factors, uncertainties and challenges remain. One major theme of current global change research is to understand whether terrestrial ecosystems could act as a C sink or whether plant responses to elevated CO$_2$ could be sustained under future climate change scenarios. Though we have seen much progress in understanding the causes of changes in soil microbes under global change factors over the past decades, our knowledge of how soil microbes feed back to
global change factors remains poorly understood. Given that the key role soil microbes played in mediating soil organic C decomposition and nutrient availability for plants, this knowledge gap critically hampers our ability to predict microbial contributions to future atmospheric CO$_2$ concentrations and associated climate change.

Because plants in many natural or semi-natural ecosystems are in general N-limited (Vitousek & Howarth 1991), it has been proposed that soil N will be a major factor that limits long term ecosystem responses to elevated CO$_2$. Great attention, thus, has been directed towards N limitation of ecosystem responses to atmospheric CO$_2$ enrichment (Hu et al. 2001; Luo et al. 2004; de Graaff et al. 2006; Finzi et al. 2006; Reich et al. 2006). Likewise, most research on soil microbial responses to elevated CO$_2$ have also been conducted in N-limited ecosystems (Hu et al. 1999; Zak et al. 2000; Hu et al. 2006). In many managed or semi-managed systems, however, N availability is often high due to increasing N inputs from N fertilization and the incorporation of legume plants (Galloway et al. 2008; Vitousek et al. 2009). High N availability may sustain plant responses to rising CO$_2$ over long time frames and provides an opportunity for further C sequestration in soil (Kimball et al. 2002; Lal 2004; Reich et al. 2006). However, how soil microbes respond to elevated CO$_2$ in systems with increasing N availability, and what are their implications for long-term soil C dynamics in those systems remains unclear.

To date, most studies have emphasized on the influences of elevated CO$_2$ on changes in the whole soil microbial community. Less attention has focused on the responses of functional groups of soil microbes and their potential feedback to elevated atmospheric CO$_2$. 


AM fungi, ubiquitous in most soils, function on the basis of C fixed in their host plants and account for up to 20% of plant photosynthate (Jakobsen & Rosendahl 1990; Smith & Read 1997), thereby representing a critical link in terrestrial C cycling. It has been well documented that CO\textsubscript{2} enrichment can enhance mycorrhizal fungal growth over a wide range of biomes (Fitter \textit{et al.} 2000; Treseder & Allen 2000; Treseder 2004; Alberton \textit{et al.} 2005; Hu \textit{et al.} 2006), potentially increasing soil C sequestration through enhancing C flow via mycorrhizal symbioses (Sanders \textit{et al.} 1998; Rillig \textit{et al.} 1999b; Treseder & Allen 2000; Rillig \textit{et al.} 2001; Zhu & Miller 2003; Alberton \textit{et al.} 2005; Wilson \textit{et al.} 2009). Emerging evidence, however, has shown that AM fungi may also be involved, either directly or indirectly, in the decomposition of organic residues (Hodge \textit{et al.} 2001; Tu \textit{et al.} 2006). It is unknown whether and how CO\textsubscript{2}-enhancement of AM fungi impact organic C decomposition. Because AM fungi also have an important role in aiding plant N acquisition, whether and how AM fungi at elevated CO\textsubscript{2} affects plant N uptake from decomposing organic material remains to be investigated.

Aside from the abundant research on the effects of elevated CO\textsubscript{2} on soil microbes, the influences of other global change factors have increasingly received attention. Nevertheless, the majority of current work has centered on the effect of a single global change factor. Given the co-occurrence of elevated CO\textsubscript{2}, reactive atmospheric N deposition, and elevated tropospheric O\textsubscript{3}, relatively little research has examined the causes and consequences of the impacts of multiple global change factors on soil microbes.

In addition, current microbial ecology and global change research are facing many
challenges. First, most completed and current global change research, particularly CO₂ studies, have focused on grassland and forest ecosystems in temperature regions in developed countries. Little has been done in agro- and forest ecosystems in subtropical and tropical areas, where both plants and soil microbes are believed to be more responsive to elevated CO₂ (Melillo et al. 1993). Agroecosystems are particularly important because they not only provide the majority of global food needs, but have been suggested to have an enormous potential to sequester more C, particularly in no-till management systems (Paustian et al. 1997; West & Post 2002; Lal 2004). Experiments, designed to close the C budget in agroecosystems and to assess the changes in crop yields and food quality, are urgently needed. Second, the scale of the current research poses a big challenge to predicting future climate change. Studies that examined soil microbial responses to global change factors have been mainly carried out at small scales. For examples, most studies have been done in microcosms in laboratory greenhouses or in small area of the field within open top chambers. Experiments with larger scales conducted at community and ecosystem levels are desirable for future studies, but the experimental costs are much higher. To extrapolate experimental results into large scales and to scale up at regional and global levels, modeling techniques might be more practical in future research. Finally, our limited understanding of soil microbial responses to climate change factors may also be attributable to the lack of application of modern technology. Understanding the underlying mechanisms that regulate soil microbial responses often requires tools to track changes at a very fine scale. To uncover the mystery of biogeochemistry of functional soil microbial groups under global change
factors, therefore, it is essential to incorporate new technologies such as stable isotope probes and functional genomics in future studies.

1.5 References


Dorodnikov, M., Blagodatskaya, E., Blagodatsky, S., Marhan, S., Fangmeier, A. & Kuzyakov, Y. (2009). Stimulation of microbial extracellular enzyme activities by


### 1.6 Table

**Table 1.1**: Effects of elevated atmospheric CO$_2$ on the soil microbial community composition.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>CO$_2^a$</th>
<th>Year$^b$</th>
<th>Method$^c$</th>
<th>Fungi/bacteria ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grassland$^d$</td>
<td>700</td>
<td>4</td>
<td>DFS$^e$</td>
<td>N/A N/A Increase</td>
<td>(Hungate et al. 2000)</td>
</tr>
<tr>
<td>Grassland$^g$</td>
<td>700</td>
<td>6</td>
<td>DFS</td>
<td>N/A N/A Increase</td>
<td>(Rillig et al. 1999a)</td>
</tr>
<tr>
<td>Artemisia tridentata</td>
<td>700</td>
<td>0.2</td>
<td>DFS</td>
<td>N/A N/A Increase</td>
<td>(Kliromonos et al. 1996)</td>
</tr>
<tr>
<td>Model system$^f$</td>
<td>550</td>
<td>0.8</td>
<td>DNA</td>
<td>N/A N/A No change</td>
<td>(Jones et al. 1998)</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>720</td>
<td>0.1</td>
<td>DNA</td>
<td>N/A N/A No change</td>
<td>(Griffiths et al. 1998)</td>
</tr>
<tr>
<td>Triticum aestivum Liquidambar</td>
<td>720</td>
<td>0.3</td>
<td>DNA</td>
<td>N/A N/A No change</td>
<td>(Griffiths et al. 1998)</td>
</tr>
<tr>
<td>styraciflua</td>
<td>544</td>
<td>10</td>
<td>DNA</td>
<td>N/A N/A No change</td>
<td>(Austin et al. 2009)</td>
</tr>
<tr>
<td>Populus tremuloides</td>
<td>720</td>
<td>5.5</td>
<td>DNA</td>
<td>1.12 0.86 -23% (ns)$^i$</td>
<td>(Janus et al. 2005)</td>
</tr>
<tr>
<td>Populus tremuloides</td>
<td>720</td>
<td>5.5</td>
<td>DNA</td>
<td>0.66 0.96 44% (ns)$^j$</td>
<td>(Janus et al. 2005)</td>
</tr>
<tr>
<td>Arrhenatherum elatius</td>
<td>435</td>
<td>3</td>
<td>Ergosterol$^f$</td>
<td>N/A N/A Decrease</td>
<td>(Sonnemann &amp; Wolters 2005)</td>
</tr>
<tr>
<td>Dantonia richardsonii</td>
<td>712</td>
<td>4</td>
<td>Plate counts</td>
<td>N/A N/A Increase</td>
<td>(Grayston et al. 1998)</td>
</tr>
<tr>
<td>Adenostoma fasciculatum</td>
<td>550</td>
<td>8</td>
<td>Staining$^g$</td>
<td>0.28 0.41 45% (ns)</td>
<td>(Lipson et al. 2005)</td>
</tr>
<tr>
<td>Quercus alba</td>
<td>500</td>
<td>0.7</td>
<td>PLFA$^h$</td>
<td>0.18 0.22 22.2% (ns)</td>
<td>(Ringelberg et al. 1997)</td>
</tr>
<tr>
<td>Quercus alba</td>
<td>650</td>
<td>0.7</td>
<td>PLFA</td>
<td>0.18 0.14 -22.2% (ns)</td>
<td>(Ringelberg et al. 1997)</td>
</tr>
<tr>
<td>Grassland$^i$</td>
<td>610</td>
<td>1.5</td>
<td>PLFA</td>
<td>0.20 0.22 10.0% (ns)</td>
<td>(Insam et al. 1999)</td>
</tr>
<tr>
<td>Populus tremuloides</td>
<td>707</td>
<td>2</td>
<td>PLFA</td>
<td>0.19 0.21 11.6% (ns)</td>
<td>(Zak et al. 2000a)</td>
</tr>
<tr>
<td>Fagus silvatica /Picea abies Karst</td>
<td>566</td>
<td>4</td>
<td>PLFA</td>
<td>N/A N/A 0 (ns)</td>
<td>(Wiemken et al. 2001)</td>
</tr>
<tr>
<td>Grassland$^j$</td>
<td>600</td>
<td>3</td>
<td>PLFA</td>
<td>0.11 0.11 6.6% (ns)$^k$</td>
<td>(Ebersberger et al. 2004)</td>
</tr>
<tr>
<td>Grassland$^k$</td>
<td>600</td>
<td>3</td>
<td>PLFA</td>
<td>0.08 0.09 8.7% (ns)$^k$</td>
<td>(Ebersberger et al. 2004)</td>
</tr>
<tr>
<td>Betula pendula</td>
<td>729</td>
<td>3</td>
<td>PLFA</td>
<td>N/A N/A 16% (ns)$^l$</td>
<td>(Kasurinen et al. 2005)</td>
</tr>
<tr>
<td>Populus tremuloides</td>
<td>720</td>
<td>5.5</td>
<td>PLFA</td>
<td>0.10 0.13 28% (ns)$^l$</td>
<td>(Janus et al. 2005)</td>
</tr>
<tr>
<td>Populus tremuloides</td>
<td>720</td>
<td>5.5</td>
<td>PLFA</td>
<td>0.08 0.07 -10% (ns)$^l$</td>
<td>(Janus et al. 2005)</td>
</tr>
<tr>
<td>Scrub-oak vegetation</td>
<td>700</td>
<td>6</td>
<td>PLFA</td>
<td>0.11 0.17 48% *</td>
<td>(Carney et al. 2007)</td>
</tr>
<tr>
<td>Trifolium repens /Lolium perenne</td>
<td>600</td>
<td>9</td>
<td>PLFA</td>
<td>0.26 0.23 -10.4% (ns)</td>
<td>(Drissner et al. 2007)</td>
</tr>
<tr>
<td>Grassland$^m$</td>
<td>560</td>
<td>6</td>
<td>PLFA</td>
<td>0.14 0.15 13% (ns)$^m$</td>
<td>(Chung et al. 2007)</td>
</tr>
<tr>
<td>Grassland$^n$</td>
<td>450</td>
<td>3</td>
<td>PLFA</td>
<td>0.06 0.06 4.6% (ns)$^m$</td>
<td>(Kanerva et al. 2008)</td>
</tr>
<tr>
<td>Grassland$^o$</td>
<td>450</td>
<td>3</td>
<td>PLFA</td>
<td>0.07 0.07 2.2% (ns)$^m$</td>
<td>(Kanerva et al. 2008)</td>
</tr>
<tr>
<td>Grassland$^p$</td>
<td>600</td>
<td>6</td>
<td>PLFA</td>
<td>N/A N/A 8.0% (ns)</td>
<td>(Niklaus et al. 2003)</td>
</tr>
<tr>
<td>Populus grandidentata</td>
<td>692</td>
<td>0.4</td>
<td>PLFA</td>
<td>0.29 0.28 -3.8% (ns)</td>
<td>(Zak et al. 1996)</td>
</tr>
</tbody>
</table>
**Table 1.1 continued**

N/A: data not available, ns: non-significant, *: $P < 0.05$

| CO$_2$ exposure ($\mu$mol mol$^{-1}$). | Experimental duration (year). | The method used for analysis of microbial community structure. | Relative change (%), if data were not available to calculate the percentage change, we used ‘increase’, ‘decrease’ or ‘no change’ to describe the effects of elevated CO$_2$ on changes in the ratio of fungi/bacteria. | DFS: differential fluorescent staining technique. | Fungal biomass was calculated by determining the value of soil ergosterol; bacterial biomass was calculated using the total microbial biomass minus the fungal biomass. | Fungal biomass: grid-intersection method; bacteria biomass: bacteria stained with DAPI (4,6-diamidino-2-phenylindole) and then were counted by fluorescence microscopy. | PLFA: a molecular technique-phospholipid fatty acid analysis | 0-20 cm soil layer | 20-40 cm soil layer | Samples were taken in the Spring. | Samples were taken in the Summer. | Samples were taken in the 1$^{st}$ year of the experiment. | Samples were taken in the 3$^{rd}$ year of the experiment. | Data were pooled across two clones. |
Table 1.1 continued

\(^{p}\) Data were pooled across two N addition treatments and four plant species treatments.

\(^{q}\) Plant species including *Avena barbat*, *Bromus hordeaceus*, *Nassela pulchra*, *Lotus wrangelianus*, *Hemizonia congesta*.

\(^{r}\) Plant composition: *Cardamine hirsuta*, *Poa annua*, *Senecio vulgaris*, *Spergula avensis*.

\(^{s}\) Plant composition: *Elettaria cardamomum*, *Ficus benjamina*, *F. pumila*, *Heliconia humilis*, *Ctenanthe lubbersiana*, *Cecropia peltata*, *Epipremnum pinnatum*.

\(^{t}\) Plant species were dominated by *Bromus erectus* Huds.

\(^{u}\) Plant species included four C4 grasses (*Andropogon gerardii*, *Boutela gracilis*, *Schizachyrium scoparium*, and *Sorghastrum nutans*), four C3 grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata*, and *Poa pratensis*), four legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, and *Petalostemum villosum*), and four forbs (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa*, and *Solidago rigida*).

\(^{v}\) Plant species contained *Agrostis capillaris*, *Anthoxanthum odoratum*, *Fragaria vesca*, *Campanula rotundifolia*, *Ranunculus acris*, *Trifolium medium*, and *Vicia cracca*. 
Chapter 2

Nitrogen dependence of soil microbial responses to elevated CO$_2$ and O$_3$ in a wheat-soybean agroecosystem†

*Running title: Soil N, microbes and air CO$_2$ & O$_3$ enrichment*

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Keywords: Climate change, elevated CO₂, elevated O₃, microbial biomass C and N, microbial respiration, net N mineralization, no-till, soil C sequestration, soybean, wheat

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2.1 Abstract

Climate change factors such as elevated atmospheric CO$_2$ and O$_3$ can exert significant impacts on soil microbes and microbially-mediated ecosystem processes. However, the underlying mechanisms by which soil microbes respond to these environmental changes remain poorly understood. A prevailing hypothesis, which states that CO$_2$- or O$_3$-induced changes in C availability dominates microbial responses, successfully predicts outcomes in some cases, but fails in others. Using a long-term field study conducted in a no-till wheat-soybean system, we show that N availability critically influenced soil microbial responses to elevated CO$_2$ but not O$_3$. Elevated CO$_2$ significantly increased but O$_3$ reduced above-ground residue mass and residue N inputs. However, only elevated CO$_2$ significantly affected soil microbial parameters. While it only had marginal effects on microbial respiration in the first two years, elevated CO$_2$ significantly stimulated microbial biomass and decomposition in the third and fourth years when N availability increased, likely due to CO$_2$-stimulation of symbiotic N$_2$ fixation in soybean. Our results indicate that atmospheric CO$_2$ enrichment may create a positive feedback loop in which CO$_2$-enhancement of N availability stimulates microbial activities and CO$_2$ release from soil. These results also suggest that high N availability in many agricultural soils may accelerate organic C turnover and limit the potential of C sequestration in agroecosystems under future CO$_2$ scenarios.

Keywords

soil C sequestration, elevated CO$_2$, elevated O$_3$, global change, microbial biomass C and N, microbial respiration, net N mineralization, no-till, soybean, wheat
2.2 Introduction

Soil microbes critically affect plant and ecosystem responses to climate change by modulating organic carbon (C) decomposition and nutrient availability for plants. Experimental evidence accumulated over the last several decades has clearly shown that climate change factors such as CO\textsubscript{2} enrichment in the atmosphere can significantly alter plant growth (Ainsworth \textit{et al.}, 2002, Kimball \textit{et al.}, 2002) and the availability of organic C, nitrogen (N) and cation nutrients for microbes (Hungate \textit{et al.}, 1997, Hu \textit{et al.}, 2001, Cheng \textit{et al.}, 2010). Air pollutant O\textsubscript{3}, a greenhouse gas with demonstrated inhibitory effects on plant growth and resource allocation belowground, has been less well studied but is considered to have a potential influence on soil microbial processes (Andersen, 2003). Alterations in soil microbes can, in turn, profoundly influence soil C processes and the long-term potential of terrestrial ecosystems as a C sink in mitigating the rising CO\textsubscript{2} in the atmosphere. However, predicting exactly what these changes will be is essentially hampered by our limited understanding of the underlying mechanisms by which soil microbes respond to altered resource availability.

The current prevailing hypothesis, building on the dogma that soil microbes are generally C limited (Smith & Paul, 1990), predicts that elevated CO\textsubscript{2} stimulates soil microbial biomass and activities due to increased soil C availability (Zak \textit{et al.}, 1993, Hu \textit{et al.}, 1999, Zak \textit{et al.}, 2000), whereas higher O\textsubscript{3} suppresses them due to lower C allocation belowground (Islam \textit{et al.}, 2000, Phillips \textit{et al.}, 2002, Andersen, 2003). This broad hypothesis has been extensively tested over the past two decades for CO\textsubscript{2} but less so with O\textsubscript{3}
(Zak et al., 1993, Islam et al., 2000, Hu et al., 2001, Phillips et al., 2002, Langley et al., 2009, Evgenia et al., 2010). Though C availability to microbes has been commonly reported to increase under elevated CO₂ (Hungate et al., 1997, Pendall et al., 2004) and to decrease under elevated O₃ (Phillips et al., 2002, Andersen, 2003, Grantz et al., 2006), soil microbial responses to elevated CO₂ and O₃ have been inconsistent (Zak et al., 2000, Andersen, 2003, Hu et al., 2006). Hu et al. (2006) has recently reviewed 135 studies examining elevated CO₂ effects on a suite of soil microbial parameters such as microbial biomass and respiration. They found that microbial biomass C and microbial respiration have been observed to increase under elevated CO₂ in 19 of 40 studies and 20 of 38 studies, respectively, but remained unchanged or even decreased in the remainder. Despite considerable efforts in the last two decades, there is a lack of conceptual understanding of why and how these inconsistencies in CO₂ and O₃ effects on microbes occur.

Soil microorganisms may also be co-limited by soil N availability (Hu et al., 2001, Schimel & Weintraub, 2003). An alternative to the above hypothesis is that the relative availability of C vs. N primarily controls microbial responses to elevated CO₂ (Hu et al., 1999, 2001, Barnard et al., 2006). This hypothesis reasons that CO₂-enhancement of belowground C allocation and N accumulation in the standing plant biomass and recalcitrant organic matter would shift the relative availability of C and N for microbes in many natural and semi-natural ecosystems and prompts N limitation (Hu et al., 1999, Luo et al., 2004). Several CO₂ studies have documented N limitation on soil microorganisms (Hu et al., 2001, Barnard et al., 2006). In a California grassland exposed to CO₂ for 5 years, Hu et al. (2001)
observed that competitive plant N uptake significantly reduced soil N availability for microbes, limiting microbial decomposition over the short term. In many managed or semi-managed systems, however, human activities have increased N inputs remarkably through N fertilization and incorporation of legume plants (Galloway et al., 2008). Conceivably, increased N availability under elevated CO$_2$ would break the bottleneck of N limitation and stimulate microbial growth and decomposition. To our knowledge, however, no convincing experimental evidence has been reported because there is very limited information on long-term microbial responses to elevated CO$_2$ in N-rich, intensively managed ecosystems.

Many crop plants, particularly C$_3$ crops, are usually responsive to CO$_2$ and O$_3$ enrichments (Kimball et al., 2002, Fiscus et al., 2005, Cheng et al., 2010). For instance, it has been estimated that elevated CO$_2$ alone increased the shoot biomass of soybean and wheat by 48% and 16%, respectively (Ainsworth et al., 2002, Kimball et al., 2002), but elevated O$_3$ reduced them by 21% and 18% (Morgan et al., 2003, Feng et al., 2008). Also, elevated CO$_2$ has been often shown to ameliorate the O$_3$ effects on plants by reducing O$_3$ uptake and increasing C assimilation rates (Booker & Fiscus, 2005). However, whether the CO$_2$ and O$_3$-induced changes in plant biomass translate into alterations in soil C sequestration depends largely on the responses of soil microbial processes. Additionally, elevated CO$_2$ significantly increased symbiotic N$_2$ fixation in legumes such as soybean and peanut (Rogers et al., 2009, Tu et al., 2009), whereas elevated O$_3$ tended to reduce it (Tu et al., 2009). It has been suggested that high N availability in agro- and grassland ecosystems can sustain plant responses to the rising CO$_2$ over a long time frame and provide an
opportunity for soil C sequestration in soil in a higher CO\textsubscript{2} world (Kimball \textit{et al.}, 2002, Lal, 2004, Prior \textit{et al.}, 2005, Reich \textit{et al.}, 2006). Convincing evidence is still lacking, but soil microbial responses may be indicative for understating the long-term soil C dynamics in high N or N–aggrading ecosystems (Liu & Greaver, 2009, He \textit{et al.}, 2010).

Taking advantage of a long-term study examining climate change effects on soil C dynamics in a wheat-soybean agroecosystem, we continuously monitored a suite of soil microbial parameters for four years to ascertain the time course of microbial responses to elevated CO\textsubscript{2} and O\textsubscript{3}. Our objective was to determine whether and how changes in C and N availability induced by these climate change factors exert interactive controls over microbial biomass and activities. Specifically, we hypothesized that (1) the impact of elevated CO\textsubscript{2} and O\textsubscript{3} on soil C availability dominates microbial responses with elevated CO\textsubscript{2} increasing and O\textsubscript{3} decreasing microbial biomass and activities, (2) at the ecosystem level, elevated CO\textsubscript{2} enhances N availability for microbes while elevated O\textsubscript{3} offsets the CO\textsubscript{2} effect, and (3) elevated CO\textsubscript{2} and O\textsubscript{3}-induced effects on N availability critically affect microbial respiration and residue turnover.

\section*{2.3 Methods}

\textit{Site description}

We initiated a long-term field experiment in May 2005 to investigate the response of a wheat-soybean agroecosystem to elevated atmospheric CO\textsubscript{2} and O\textsubscript{3} using open-top field chambers (OTC). The experimental site is located at the Lake Wheeler Experimental Station,
5 km south of North Carolina State University, Raleigh, North Carolina, USA (35° 43’ N, 78° 40’ W; elevation 120 m). Annual mean temperature is 15.2 °C and annual mean precipitation is 1050 mm. The field had been left fallow for eight years prior to this study. Before CO₂ and O₃ treatments were initiated, the soil was repeatedly turned-over using a disc implement and rotovator. The soil is an Appling sandy loam (fine, kaolinitic, thermic Typic Kanhapludult), well drained with a pH of 5.5, and contained 9.0 g C and 0.86 g N kg⁻¹ soil when the experiment was initiated. Measurements on the initial soil microbial and chemical properties showed that the site was quite homogeneous (Table 2.1).

This experiment was a 2 × 2 factorial design with four treatments randomly assigned into four blocks. Four different trace-gas treatments were: (a) charcoal-filtered air and ambient CO₂ (CF); (b) charcoal-filtered air plus 1.4 times ambient O₃ and ambient CO₂ (O₃); (c) charcoal-filtered air plus 180 μl l⁻¹ CO₂ (CO₂); and (d) charcoal-filtered air plus 1.4 times ambient O₃ and 180 μl l⁻¹ CO₂ (CO₂ + O₃). The seasonal average daily concentrations of CO₂ and O₃ over the experimental duration are shown in Table 2.2. The purpose of filtration of ambient air with activated charcoal was to reduce the concentrations of ambient O₃ to levels considered nonphytotoxic to soybean and wheat plants. Ozone was deemed as a major air pollutant in this area, while other air pollutants such as NO₂ and SO₂ were below the phytotoxic levels at the experimental location (Booker et al. 2005).

Soybean [cv. CL54 RR (Year 1), Asgrow 5605 RR (Year 2 and 3) and SS RT5160N RR (Year 4)] was planted each spring followed by soft red winter wheat (Coker 9486) in the fall using no-till practices. Plants were exposed to reciprocal combinations of CO₂ and O₃.
within cylindrical OTCs (3.0 m diameter × 2.4 m tall) from emergence to physiological maturity. Carbon dioxide was released from a 14-ton liquid-receiving tank 24 h daily and monitored at canopy height using an infrared CO$_2$ analyzer (model 6252, Li-Cor Inc. Lincoln, NE, USA). Ozone was generated by electrostatic discharge in dry O$_2$ (model GTC-1A, Ozonia North America, Elmwood Park, NJ, USA) and dispensed 12 h daily (08:00-20:00 hours Eastern Standard Time) in proportion to concentrations of ambient O$_3$. The O$_3$ concentration in the chambers was monitored at canopy height with a UV photometric O$_3$ analyzer (model 49, Thermo Environmental Instruments Co., Franklin, MA, USA). During wheat growing seasons, each plot initially received 48 g NH$_4$NO$_3$ (equivalent to 24 kg N ha$^{-1}$) in November each year, followed by an additional input of 192 g NH$_4$NO$_3$ (equivalent to 96 kg N ha$^{-1}$) in March. Plots were treated with lime, K and P in November during the experiment according to soil test recommendations. During soybean growing seasons, plants were irrigated with drip lines to prevent visible signs of water stress, but no additional N fertilizers were applied. Upon senescence of the plants, all aboveground plant biomass in each chamber was harvested, soybean plants were divided into leaves, stems, husks and seeds, while wheat plants were separated as straw, chaff and seeds, then dried and quantified. Afterward, residues other than seeds were uniformly returned to their corresponding treatment plots and evenly distributed on the soil surface.

**Soil sampling**

The chamber plot was divided into two parts: the sampling area (an inner circular area
with a diameter of 2.4 m) and the border area (for purpose of reducing chamber effects, 0.3 m in width). To avoid taking soil samples from the same location, the sampling area was divided into 448 small subplots (10 × 10 cm). Soil sampling locations were determined using a random number generator and each subplot was sampled only once. In June and November of each year, corresponding to harvest time for each crop, we used a 5-cm diameter soil corer to take three soil cores to 20 cm depth in the center of three pre-determined subplots from each chamber. Additional three soil cores were taken from the border areas immediately to fill the holes in the sampling areas. Sample holes in border areas were refilled by soil cores taken just outside of each chamber. Soil cores were immediately separated into 0-5 cm, 5-10 cm and 10-20 cm depth fractions. Core sections were then pooled by the depth fraction into three soil samples per chamber. Soil samples were also collected at the mid-growing season to check whether microbial parameters were significantly different from those obtained at the end of the growing season. Soil samples (0-5 and 5-10 cm) were collected in each April of the first two years and each August of the last two years, corresponding to the maximal physiological activity of wheat and soybean plants. All samples were sealed in plastic bags, stored in a cooler and transported to laboratory.

Field-moist soils were sieved using a 4-mm screen and all visible residues and plant roots were carefully removed within 24 hours of the field sampling. A 10-g subsample was oven-dried at 105 °C for 48 h and weighed. All the soil and microbial data were calculated on the dry weight basis of soils.
Sample analyses

Residue N contents. Air-dried subsamples of plant residues (stems, leaves and husks of soybean; straw and chaff of wheat) were ground in a Tecator Cyclotec mill fitted with a 1-mm screen (FOSS, Eden Prairie, MN, USA). The C and N concentrations in various plant residue components were determined with a CHN elemental analyzer (Carla Erba and model 2400, Perkin Elmer Co., Norwalk, CT, USA). The amount of residue N input to soil was calculated by multiplying the dry mass with the N concentration of residue returned to each plot.

Soil microbial respiration. We determined soil heterotrophic respiration using an incubation-alkaline absorption method (Coleman et al. 1978). In brief, 20-g dry mass equivalent soil samples were adjusted to moisture levels of around 60% water holding capacity, placed in 1 L Mason jar, and then incubated at 25°C in the dark for 2 weeks. Respired CO$_2$ was trapped in 5.0 ml of 0.25 M NaOH contained in a beaker suspended in the jar. After the first week incubation, NaOH solutions were replaced with fresh solutions. The CO$_2$ captured in the NaOH solution was titrated with 0.125 M HCl to determine the amount of CO$_2$ evolved from the soil. Soil microbial respiration (SMR) rate was expressed as mg CO$_2$ kg$^{-1}$ soil d$^{-1}$ by averaging the data across two 1-wk incubations.

Soil microbial biomass C and N. Soil microbial biomass C (MBC) and biomass N (MBN) were determined using the fumigation-extraction method (Vance et al. 1987). Twenty g dry weight equivalent soil was fumigated with ethanol-free chloroform for 48 h, and then extracted with 50 ml of 0.5 M K$_2$SO$_4$ by shaking for 30 min. Another 20 g sample
of non-fumigated soil was also extracted with 50 ml of 0.5 M K$_2$SO$_4$. Soil extractable organic C in both fumigated and non-fumigated K$_2$SO$_4$ extracts was measured using a TOC analyzer (Shimadzu TOC-5050A, Shimadzu Co., Kyoto, Japan). Soluble N in the extracts of fumigated and non-fumigated soils was quantified on the Lachat flow injection analyzer after digestion with alkaline persulfate (Cabrera & Beare 1993). The differences in extractable organic C and inorganic N between fumigated and non-fumigated soils were assumed to be from lysed soil microbes. The released C and N were used to calculate MBC and MBN using a conversion factor of 0.45 ($k_{EC}$) and 0.45 ($k_{EN}$) (Jenkinson et al. 2004), respectively.

**Net soil N mineralization.** Potential N mineralization was determined following a 4-wk incubation at 25 °C in the dark. Soil NH$_4^+$ and NO$_3^-$ in un-incubated and incubated subsamples (20-g each) were extracted with 50 ml of 0.5 M K$_2$SO$_4$ by shaking for 30 min. The concentrations of inorganic N were then measured on the Lachat flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA). Net mineralized N (NMN) was estimated by the difference in extractable total inorganic N (NH$_4^+$-N + NO$_3^-$-N) between incubated and un-incubated soil samples.

**Soil extractable C and N.** The concentration of organic C in non-fumigated soil extracts was used to represent soil extractable C. The extractable inorganic N referred to the sum of NH$_4^+$-N and NO$_3^-$-N in non-fumigated soil extracts.

**Statistical analysis**

A least significant difference (LSD) was applied to soil samples collected before gas
fumigation to examine the site variation at each soil layer (Table 2.1). We examined results for the entire experimental duration from 2005-2009, and used the linear mixed model (Littell et al. 2006) to test the main effects of CO₂, O₃ and the interaction of CO₂ and O₃, and whether these changed over time. We employed a set of covariance structures including compound symmetric model (CS), the first-order autoregressive model [AR (1)], and autoregressive with random effect to reduce autocorrelation. The p values for treatments and interaction terms were reported based on the covariance structure that minimized Akaike information criterion (AIC) and Bayesian information criterion (BIC) (Littell et al. 2006). Data for soil and microbial parameters from mid-seasons (Table 2.3) and residue N inputs were subjected to the analysis of variance using mixed models. To test for relationships between variables, we conducted a correlation analysis using all the data generated over the 4-year period. All statistical analyses were performed using the SAS 9.1 (SAS Institute, Inc., Cary, NC, USA). For all tests, \( P \leq 0.05 \) was considered to indicate a statistically significant difference.

2.4 Results

Residue N inputs

Over the 4-year period, elevated CO₂ increased the total amount of N in soybean plant residues by 23\% while O₃ decreased it by 13\% (Fig. 2.1). Neither treatment had a significant effect on wheat plant residue N (Fig. 2.1). The O₃ effect on residue N inputs primarily occurred in soybean with soybean residue N being reduced by 22\% in the fourth
year. No significant \( \text{CO}_2 \times \text{O}_3 \) interaction was observed on residue N inputs \((P > 0.1)\).

Soil microbial respiration

Over the 4-year period, atmospheric \( \text{CO}_2 \) enrichment increased soil microbial respiration (Fig. 2.2 and Table 2.4). Compared to ambient \( \text{CO}_2 \), microbial respiration under elevated \( \text{CO}_2 \) was 26%, 17% and 31% higher at 0-5, 5-10 and 10-20 cm soil layers, respectively. However, these increases were largely due to the \( \text{CO}_2 \) stimulation effects in the third and fourth years (Fig. 2.2). In the 0-5 cm soil layer, elevated \( \text{CO}_2 \) only increased microbial respiration by 9% in the first two years, but by 43% over the subsequent two years. Neither the \( \text{O}_3 \) effect nor the \( \text{CO}_2 \times \text{O}_3 \) interaction resulted in significant effects in any soil layers (Table 2.4).

Metabolic quotient of soil microbes (the respiration rate per unit of microbial biomass C) under elevated \( \text{CO}_2 \) increased by, on average, 16% (repeated measures mixed models; \( \text{CO}_2 \) effect: \( P = 0.003 \)), 9% \((P = 0.2)\), and 20% \((P = 0.02)\) at 0-5, 5-10, and 10-20 cm soil layers, respectively, over the 4-year period (Figure 2.3). The \( \text{CO}_2 \) effect on the metabolic quotient also changed considerably over time. In the 0-5 cm soil layer, \( \text{CO}_2 \) enrichment slightly increased the metabolic quotient by 7% in the first two years, but significantly increased it by 25% within the last two years.

Microbial biomass C and biomass N

Elevated \( \text{CO}_2 \) significantly enhanced both MBC and MBN in the 0-5 cm soil layer,
leading to an average increase of 8% (Fig. 2.4a) and 14% (Fig. 2.4d), respectively. Similar to SMR, the observed increase in MBC and MBN resulted primarily from CO₂-induced enhancement in the third and fourth years of the experiment (Fig. 2.4). Both MBC and MBN at elevated CO₂ remained unchanged in the top soil layer during the first two years of the experiment, but increased by 14% and 26%, respectively, within the last two years. The CO₂ effects were also significant for MBN in the 10-20 cm soil layer, but not significant for MBC (Table 2.4). However, neither O₃ nor the CO₂ × O₃ interaction had any significant impacts on MBC or MBN along the soil profile (Table 2.4).

**Net soil N mineralization**

Similar to the effects on SMR and MBN, CO₂ enrichment significantly stimulated the rate of net soil N mineralization at both 0-5 and 10-20 cm soil layers (Fig. 2.5a and 2.5c, and Table 2.4). On average, net mineralizable N (NMN) in elevated CO₂ plots was 13%, 5%, and 26% higher than those in ambient CO₂ plots, respectively, in the 0-5, 5-10, and 10-20 cm soil layers. Again, these effects were mainly due to the CO₂-induced increases within the last two years of the experiment (Fig. 2.5). Elevated CO₂ showed no impacts on net soil N mineralization in the first two years, but caused an average increase by 22%, 12% and 49%, respectively, in the 0-5, 5-10, and 10-20 cm soil layers. In contrast, neither the O₃ treatment effect nor the CO₂ × O₃ interaction were statistically significant (Table 2.4).
Neither CO\textsubscript{2} nor O\textsubscript{3} treatments had any significant impacts on soil extractable C in the whole soil profile, nor did the interactions between time and gas treatments over the 4-year period (Table 2.4 and Figure 2.6).

In general, elevated CO\textsubscript{2} tended to increase concentrations of total extractable inorganic N (Figure 2.7). Soil extractable N in elevated CO\textsubscript{2} plots increased by, on average, 17\%, 18\% and 8\%, respectively, in 0-5, 5-10, and 10-20 cm soil layers over the 4-year period. There were no significant effects of O\textsubscript{3} and the interaction of CO\textsubscript{2} and O\textsubscript{3} (Table 2.4).

Stratification of soil microbial parameters under elevated CO\textsubscript{2}

The time course of CO\textsubscript{2}-effects on various parameters along the soil profile was significantly different. In the top 5-cm deep soil sample, MBC fluctuated over the whole period (Fig. 2.4a), but SMR, MBN and NMN started to increase by the third year (Fig. 2.2a, 2.4d, and 2.5a). In the deep (5-10 and 10-20 cm) soil samples, MBC significantly decreased (Fig. 2.4b and 2.4c), MBN remained unchanged (Fig. 2.4e and 2.4f), but SMR and NMN increased (Fig. 2.2b, 2.2c, 2.5b and 2.5c) in the last two years of the study. Over the first two years of the experiment, all these parameters remained largely unaffected by CO\textsubscript{2} enrichment in the 5-10 and 10-20 cm soil layers (Fig. 2.2-2.5). By the third and fourth years, elevated CO\textsubscript{2} had no impacts on MBC in the deeper soil depths (Fig. 2.4b and 2.4c), but still significantly increased SMR and NMN (Fig. 2.4e, 2.4f, 2.5b and 2.5c).

The microbial parameters from soil samples collected at the mid-seasons were similar
with those at the harvest of the corresponding growing season and those results were shown in Table 2.3.

**Correlation analysis**

Correlation analysis, conducted among SMR, MBC, MBN, extractable C, extractable N, and NMN, showed that all six parameters were significantly correlated with each other, though the coefficients varied considerably (Table 2.5). Soil net N mineralization can best explain the variation of soil microbial respiration ($R^2 = 0.72$), microbial biomass C ($R^2 = 0.44$) and biomass N ($R^2 = 0.72$).

**2.5 Discussion**

Results obtained in our experiment demonstrate that the effect of elevated CO$_2$ on soil microbial biomass and microbial respiration significantly depends on both C and N availability (Fig. 2.2 and 2.3, Table 2.4). Microbial responses to elevated CO$_2$ have so far largely been considered in the context that soil microbes are C-limited (Smith & Paul, 1990, Zak et al., 2000) and plant growth is N-limited (Oren et al., 2001, Luo et al., 2004, Reich et al., 2006). Many experiments do show that increased C availability induced by elevated CO$_2$ stimulates soil microbial biomass and/or activities (Zak et al., 1993, Hungate et al., 1997, Hu et al., 2006, West et al., 2006). In the current study, elevated CO$_2$ significantly enhanced the productivity of both soybean and wheat in all four years, leading to an average increase of 21% in the dry weight of plant residues (Booker et al., unpublished data), comparable to our
previous results at this site (Booker et al., 2005). The resulting high residue inputs plus increases in root length (Booker et al., unpublished data) significantly increased C availability for microbes under elevated CO₂. However, microbial biomass and respiration did not increase significantly until the third year (Fig. 2.2a, 2.4a and 2.4d), indicating that C availability cannot solely explain the pattern of microbial responses to elevated CO₂.

Soil N availability may be another important factor that can significantly modulate soil microbial responses to elevated CO₂ (Hu et al., 2001, Schimel & Bennett, 2004). With the surface placement of residues in no-till systems, N existing in plant residues may gradually move into the soil profile, particularly the top soil layer, through leaching and decomposition processes. Higher N inputs through plant residues at elevated CO₂ (Fig. 2.1), which may stem from both CO₂-stimulation of N₂ fixation (Rogers et al., 2009, Tu et al., 2009) and plant N uptake (Hu et al., 2001, Finzi et al., 2002), can in turn increase soil N availability for microbes. It has been well documented that mineral N additions can stimulate decomposition, particularly for the non-lignin part of plant residues (Fog, 1988, Knorr et al., 2005). Non-lignin compounds (particularly cellulose) constitute the major part of crop residues such as wheat straw; thus increased soil N availability can significantly facilitate residue decomposition - particularly at the early stages of decomposition (Recous et al., 1995, Knorr et al., 2005). All exoenzymes responsible for disintegrating organic materials are N-rich proteins, and sufficient supplies of N for microbes may likely facilitate enzyme production (Fog, 1988, Schimel & Weintraub, 2003). In a nutrient-poor grassland, West et al. (2006) found that mineral N addition significantly stimulated microbial respiration at
elevated CO₂, similar to the CO₂-stimulation of microbial growth and activities with increasing N inputs observed in our study. At the opposite end of the spectrum, Hu et al. (2001) provided direct evidence of CO₂-induced N restriction on microbial decomposition in a N-limiting soil. Taken together, these results imply that the magnitude and direction of soil microbial responses to changes in C availability driven by elevated CO₂ might be in large part mediated by N availability.

The findings that increasing N availability stimulates microbial responses to elevated CO₂ may have significant implications for understanding residue turnover and soil C sequestration in agroecosystems under future climate change scenarios. In many natural and semi-natural ecosystems, the CO₂-induced stimulation of plant growth may not be sustained over time because of nutrient limitation (Luo et al., 2004, Reich et al., 2006). In agricultural ecosystems, however, N is typically not a limiting factor for plant growth due to the application of N fertilization and/or the incorporation of legume plants, and CO₂-stimulation of crop growth and biomass production is expected to be sustained (Ainsworth et al., 2002, Kimball et al., 2002, Peralta & Wander, 2008). Therefore, it has been suggested that elevated CO₂ can increase long-term C storage in agroecosystems, particularly in combination with no-tillage management (Paustian et al., 1997, West & Post, 2002, Lal, 2004). However, this assumption does not fully consider the C output from agroecosystems: unlike forest ecosystems where the standing biomass constitutes a major C pool, most agroecosystems have to accumulate C in soil for ecosystem C sequestration to occur. Consequently, the fate of returning residues will largely determine the potential of agroecosystem C sequestration.
Despite CO₂-enhancement of 21% in organic residue inputs at our site, no increase in soil C has been detected, even in the very top layer of soil that received all aboveground residues (Booker et al. unpublished). Similar results have been reported in the N-enriched SoyFACE soil in Illinois (Peralta & Wander, 2008). The close correlations between N availability and both microbial respiration and metabolic quotient under elevated CO₂ in our study (Fig. 2.2, 2.3 and Table 2.5) indicate that soil microbes became more active under higher CO₂ and the increasing N could further reinforce the impact of elevated CO₂ on soil microbes. Priming of microbial decomposition by enhanced N availability may, therefore, explain the unresponsiveness of soil C in both experiments.

Elevated O₃ often leads to a substantial decline in the aboveground biomass of O₃-sensitive plants (Morgan et al., 2003, Fiscus et al., 2005) and subsequent C allocation belowground (Andersen, 2003, Grantz et al., 2006). In the current study, the statistically significant 8% decline in plant residues primarily stemmed from O₃-reduction of soybean residues (by -13%) (P < 0.05) (Booker et al. unpublished data). The unresponsiveness of wheat to O₃ might be due to the relatively low O₃ concentrations during wheat growing seasons (Table 2.2) and comparatively low sensitivity of the cultivar to O₃ stress. The decrease in total residue N inputs under elevated O₃ (Fig. 2.1) likely resulted from O₃-induced reduction in residue biomass and symbiotic N₂ fixation in soybean plants (Tu et al., 2009). However, no significant O₃ effects were detected for any soil microbial parameters in this study (Table 2.4). These results suggest that N inputs through both fertilization and N₂ fixation in our system might overtake O₃-induced reduction of residue N as the major driver.
that affects soil microbes. Alternatively, these results might also suggest that the magnitude of reductions in both C and N likely dominates the subsequent effects on soil microbes. To our knowledge, there is only one published report with respect to the O₃ effect on soil microbes and decomposition in wheat-soybean agroecosystems. In an open top experiment under conventional tillage practice, Islam et al. (2000) found that elevated O₃ had no significant impacts on soil microbial respiration.

It is interesting to note that microbial parameters along the soil profile exhibited different patterns under elevated CO₂ (Fig. 2.2-2.5). No-till systems are characterized by vertical stratification of soil organic C and microbial biomass because of continuous residue surface placement (Beare et al., 1992, Prior et al., 2005). Rapid decreases in MBC and diminished CO₂ effects on MBC starting in the third year in deeper soil layers (Fig. 2.4b and 2.4c) seem to suggest that alteration in C availability caused by residue placement may dominate microbial responses. However, other parameters [SMR (Fig. 2.2c), MBN (Fig. 2.4f) and NMN (Fig. 2.5c)] did not decrease correspondingly with MBC and the CO₂ effects were significant (Table 2.4), suggesting that other factors may significantly exert controls. High correlations between MBN and SMR, and NMN in deeper soil depths (Table 2.5) suggest that N availability critically modulated microbial activities. In no-till systems, root-derived C is the primary source for deep soil C and CO₂-stimulation of both fine and deep roots has been proposed as a potential mechanism that facilitates C sequestration there (Prior et al., 2005). However, higher metabolic quotient (Figure 2.3), SMR (Fig. 2.2) and NMN (Fig. 2.5) under elevated CO₂ indicated that not only were microbes more active but also
organic C turnover was more rapid in the deeper soil layers. Consequently, high root production under elevated CO$_2$ might stimulate C losses from deep soil layers by priming decomposition of indigenous organic matter (Fontaine et al., 2007, Peralta & Wander, 2008, Langley et al., 2009). Long-term experiments are critically needed to examine whether the stimulation of SMR and NMN in our study is transient or will be sustained over time.

In summary, results obtained from this study showed that CO$_2$-induced alterations in soil C and N availability exerted interactive controls over soil microbes and microbially-mediated processes in the N-aggrading wheat-soybean system. While soil microbial biomass and activities were little affected by elevated CO$_2$ in the first two years, they significantly responded to CO$_2$ enrichment in the third and fourth years as more N became available. High N availability positively correlated with high microbial metabolism and organic C turnover. However, O$_3$ effects on soil C and N availability were likely insufficient in magnitude to produce detectable changes in the soil microbial parameters measured. These findings suggest that under future CO$_2$ scenarios, high N availability in many agricultural soils may accelerate organic C turnover, constraining the potential of C sequestration in agroecosystems.

2.6 REFERENCES


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### 2.7 Tables

**Table 2.1** Characteristics of soils at different experimental plots before gas fumigation.

<table>
<thead>
<tr>
<th>Soil depth (cm)</th>
<th>Experimental plot assignment</th>
<th>CF</th>
<th>O₃</th>
<th>CO₂</th>
<th>CO₂ + O₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil microbial respiration (mg CO₂ kg⁻¹ d⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>70.0 (5.0)</td>
<td>88.5 (13.4)</td>
<td>71.3 (7.2)</td>
<td>60.8 (7.9)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>40.6 (13.7)</td>
<td>43.2 (9.1)</td>
<td>36.3 (9.3)</td>
<td>43.1 (13.8)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>6.6 (1.9)</td>
<td>13.5 (2.8)</td>
<td>6.0 (1.9)</td>
<td>8.0 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Microbial biomass C (mg C kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>622 (63)</td>
<td>678 (49)</td>
<td>652 (38)</td>
<td>568 (43)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>262 (32)</td>
<td>326 (57)</td>
<td>355 (91)</td>
<td>363 (59)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>153 (22)</td>
<td>165 (17)</td>
<td>129 (7)</td>
<td>142 (10)</td>
<td></td>
</tr>
<tr>
<td>Microbial biomass N (mg N kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>70.1 (7.7)</td>
<td>73.2 (6.6)</td>
<td>73.2 (4.4)</td>
<td>64.2 (3.7)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>40.0 (13.7)</td>
<td>36.2 (8.3)</td>
<td>36.6 (8.5)</td>
<td>37.9 (8.6)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>13.2 (2.4)</td>
<td>15.9 (2.8)</td>
<td>12.7 (2.7)</td>
<td>13.7 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Extractable C (mg C kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>59.9 (3.9)</td>
<td>66.8 (2.7)</td>
<td>65.2 (4.0)</td>
<td>58.3 (5.1)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>55.4 (8.6)</td>
<td>66.3 (11.0)</td>
<td>59.8 (7.7)</td>
<td>58.9 (12.2)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>56.3 (4.3)</td>
<td>64.3 (3.9)</td>
<td>70.6 (8.6)</td>
<td>58.3 (9.0)</td>
<td></td>
</tr>
<tr>
<td>Extractable inorganic N (mg N kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>10.4 (1.5)</td>
<td>15.6 (4.4)</td>
<td>17.9 (7.7)</td>
<td>13.8 (3.2)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>8.6 (2.7)</td>
<td>17.1 (10.5)</td>
<td>17.1 (7.1)</td>
<td>15.8 (6.1)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>4.0 (1.4)</td>
<td>7.1 (3.8)</td>
<td>9.1 (3.8)</td>
<td>7.0 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Net N mineralization (mg N kg⁻¹ d⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>0.80 (0.05)</td>
<td>1.04 (0.16)</td>
<td>0.83 (0.11)</td>
<td>0.87 (0.20)</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>0.52 (0.24)</td>
<td>0.53 (0.14)</td>
<td>0.59 (0.19)</td>
<td>0.54 (0.17)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>0.06 (0.04)</td>
<td>0.12 (0.04)</td>
<td>0.09 (0.01)</td>
<td>0.09 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

Soil samples were taken on May 23, 2005. Values are means (n=4) with standard errors shown in parentheses. Except for soil microbial respiration at 10-20 cm soil layers, where microbial respiration under O₃ plots was higher than those under control (P = 0.031) and CO₂ + O₃ (P = 0.024) plots, all comparisons were not statistically significant (LSD, P > 0.05). CF, charcoal-filtered air and ambient CO₂; O₃, charcoal-filtered air plus 1.4 times ambient O₃ and ambient CO₂; CO₂, charcoal-filtered air plus 180 μl l⁻¹ CO₂; and CO₂ + O₃, charcoal-filtered air plus 1.4 times ambient O₃ and 180 μl l⁻¹ CO₂.
Table 2.2 The seasonal daily average (12 h) CO$_2$ and O$_3$ concentrations at the canopy height over the 4-year period.

<table>
<thead>
<tr>
<th>Crop</th>
<th>CO$_2$ (µl l$^{-1}$)</th>
<th>O$_3$ (nl l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient</td>
<td>Elevated</td>
</tr>
<tr>
<td>Soybean</td>
<td>376.0 ± 0.4</td>
<td>555.0 ± 0.7</td>
</tr>
<tr>
<td>Wheat</td>
<td>388.0 ± 0.4</td>
<td>547.0 ± 0.5</td>
</tr>
</tbody>
</table>

CF: charcoal filtered air. Values are mean ± S.E.
Table 2.3 Effects of elevated CO$_2$ on soil microbial respiration, microbial biomass C and N, extractable C and inorganic N, and net N mineralization during mid-growing seasons.

<table>
<thead>
<tr>
<th>Month</th>
<th>Year</th>
<th>0-5 cm Ambient CO$_2$ (mg CO$_2$ kg$^{-1}$ d$^{-1}$)</th>
<th>0-5 cm Elevated CO$_2$ (mg CO$_2$ kg$^{-1}$ d$^{-1}$)</th>
<th>5-10 cm Ambient CO$_2$ (mg CO$_2$ kg$^{-1}$ d$^{-1}$)</th>
<th>5-10 cm Elevated CO$_2$ (mg CO$_2$ kg$^{-1}$ d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>1</td>
<td>28.9 (2.8)</td>
<td>37.7 (4.1)</td>
<td>19.0 (2.4)</td>
<td>20.3 (1.7)</td>
</tr>
<tr>
<td>April</td>
<td>2</td>
<td>51.6 (4.4)</td>
<td>59.1 (4.4)</td>
<td>28.4 (1.3)</td>
<td>30.0 (2.1)</td>
</tr>
<tr>
<td>August</td>
<td>3</td>
<td>51.5 (1.7)</td>
<td>52.5 (2.9)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>August</td>
<td>4</td>
<td>51.5 (4.8)</td>
<td>91.1 (9.5)**</td>
<td>24.7 (1.6)</td>
<td>37.7 (3.0)*</td>
</tr>
<tr>
<td>Microbial biomass C (mg C kg$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April</td>
<td>1</td>
<td>394 (25)</td>
<td>457 (26)</td>
<td>264 (24)</td>
<td>270 (17)</td>
</tr>
<tr>
<td>April</td>
<td>2</td>
<td>488 (54)</td>
<td>521 (56)</td>
<td>162 (9)</td>
<td>161 (8)</td>
</tr>
<tr>
<td>August</td>
<td>3</td>
<td>430 (36)</td>
<td>484 (24)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>August</td>
<td>4</td>
<td>239 (13)</td>
<td>321 (18)**</td>
<td>139 (10)</td>
<td>142 (9)</td>
</tr>
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<td>Microbial biomass N (mg N kg$^{-1}$)</td>
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<tr>
<td>April</td>
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<td>43.3 (2.5)</td>
<td>52.7 (3.7)</td>
<td>24.8 (2.7)</td>
<td>25.8 (2.8)</td>
</tr>
<tr>
<td>April</td>
<td>2</td>
<td>53.1 (3.6)</td>
<td>57.8 (3.5)</td>
<td>28.4 (2.3)</td>
<td>28.8 (1.4)</td>
</tr>
<tr>
<td>August</td>
<td>3</td>
<td>52.7 (3.4)</td>
<td>54.3 (3.1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>August</td>
<td>4</td>
<td>51.6 (2.3)</td>
<td>69.5 (4.6)**</td>
<td>28.4 (1.6)</td>
<td>28.4 (1.7)</td>
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<tr>
<td>Extractable organic C (mg C kg$^{-1}$)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>79.6 (5.7)</td>
<td>70.8 (4.0)</td>
<td>69.0 (5.4)</td>
</tr>
<tr>
<td>April</td>
<td>2</td>
<td>67.4 (3.5)</td>
<td>73.5 (3.8)</td>
<td>58.2 (4.6)</td>
<td>55.1 (4.3)</td>
</tr>
<tr>
<td>August</td>
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<td>84.5 (5.2)</td>
<td>84.3 (5.1)</td>
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<tr>
<td>August</td>
<td>4</td>
<td>76.0 (4.3)</td>
<td>78.2 (2.6)</td>
<td>50.2 (2.8)</td>
<td>51.4 (2.9)</td>
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<tr>
<td>Extractable inorganic N (mg N kg$^{-1}$)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>13.4 (1.8)</td>
<td>12.7 (1.8)</td>
<td>11.9 (2.0)</td>
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<tr>
<td>April</td>
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<td>7.3 (0.5)</td>
<td>8.0 (0.5)</td>
<td>4.2 (0.3)</td>
<td>4.0 (0.3)</td>
</tr>
<tr>
<td>August</td>
<td>3</td>
<td>6.0 (0.3)</td>
<td>7.2 (0.3)*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>August</td>
<td>4</td>
<td>7.7 (0.2)</td>
<td>9.5 (0.4)**</td>
<td>3.9 (0.2)</td>
<td>4.6 (0.3)</td>
</tr>
<tr>
<td>Net N mineralization (mg N kg$^{-1}$ d$^{-1}$)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April</td>
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<td>0.52 (0.04)</td>
<td>0.63 (0.04)</td>
<td>0.18 (0.02)</td>
<td>0.22 (0.02)</td>
</tr>
<tr>
<td>April</td>
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<td>0.55 (0.04)</td>
<td>0.55 (0.03)</td>
<td>0.28 (0.02)</td>
<td>0.28 (0.02)</td>
</tr>
<tr>
<td>August</td>
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<td>0.41 (0.02)</td>
<td>0.43 (0.02)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>August</td>
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<td>0.67 (0.05)</td>
<td>0.87 (0.03)*</td>
<td>0.33 (0.02)</td>
<td>0.36 (0.03)</td>
</tr>
</tbody>
</table>

ND: not determined. April/Year 1: wheat season, April/Year 2: wheat season, August/Year 3: soybean season, August/Year 4: soybean season. *(P < 0.05) or **(P < 0.01) denote statistically significant effects of CO$_2$ treatment at specific sampling date, ANOVA.
Table 2.4 *P* values of analyses of repeated measures linear mixed models of CO₂, O₃ and time effects, and all interactions over 4 years.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Depth (cm)</th>
<th>CO₂</th>
<th>O₃</th>
<th>Time</th>
<th>CO₂ × O₃</th>
<th>Time × CO₂</th>
<th>Time × O₃</th>
<th>Time × CO₂ × O₃</th>
</tr>
</thead>
<tbody>
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<td>SMR</td>
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<td><strong>0.012</strong></td>
<td>0.160</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.469</td>
<td><strong>0.003</strong></td>
<td><strong>0.046</strong></td>
<td>0.179</td>
</tr>
<tr>
<td></td>
<td>5-10</td>
<td>0.149</td>
<td>0.615</td>
<td><strong>0.017</strong></td>
<td>0.159</td>
<td>0.412</td>
<td>0.922</td>
<td>0.449</td>
</tr>
<tr>
<td></td>
<td>10-20</td>
<td><strong>0.044</strong></td>
<td>0.576</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.817</td>
<td>0.121</td>
<td>0.853</td>
<td>0.965</td>
</tr>
<tr>
<td>MBC</td>
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<td><strong>0.026</strong></td>
<td>0.140</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.558</td>
<td>0.319</td>
<td>0.838</td>
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</tr>
<tr>
<td></td>
<td>5-10</td>
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<td>&lt; <strong>0.001</strong></td>
<td>0.883</td>
<td>0.961</td>
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<td>0.975</td>
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<tr>
<td></td>
<td>10-20</td>
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<td>&lt; <strong>0.001</strong></td>
<td>0.397</td>
<td>0.803</td>
<td>0.958</td>
<td>0.740</td>
</tr>
<tr>
<td>MBN</td>
<td>0-5</td>
<td><strong>0.025</strong></td>
<td>0.630</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.135</td>
<td><strong>0.018</strong></td>
<td>0.174</td>
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</tr>
<tr>
<td></td>
<td>5-10</td>
<td>0.820</td>
<td>0.080</td>
<td><strong>0.002</strong></td>
<td>0.445</td>
<td>0.940</td>
<td>0.349</td>
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<tr>
<td></td>
<td>10-20</td>
<td><strong>0.040</strong></td>
<td>0.526</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.578</td>
<td>0.810</td>
<td>0.998</td>
<td>0.995</td>
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<tr>
<td>Extr-C</td>
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<td>0.457</td>
<td>&lt; <strong>0.001</strong></td>
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<td>0.781</td>
<td>0.759</td>
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<tr>
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<td>&lt; <strong>0.001</strong></td>
<td>0.106</td>
<td>0.988</td>
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<td>0.968</td>
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<tr>
<td></td>
<td>10-20</td>
<td>0.528</td>
<td>0.266</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.054</td>
<td>0.985</td>
<td>0.971</td>
<td>0.998</td>
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<tr>
<td>Extr-N</td>
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<td>&lt; <strong>0.001</strong></td>
<td>0.328</td>
<td>0.749</td>
<td>0.933</td>
<td>0.791</td>
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<tr>
<td></td>
<td>5-10</td>
<td><strong>0.004</strong></td>
<td>0.386</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.248</td>
<td>0.176</td>
<td>1.000</td>
<td>0.955</td>
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<tr>
<td></td>
<td>10-20</td>
<td>0.430</td>
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<td>&lt; <strong>0.001</strong></td>
<td>0.871</td>
<td>0.088</td>
<td>0.908</td>
<td>0.891</td>
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<tr>
<td>NMN</td>
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<td><strong>0.002</strong></td>
<td>0.940</td>
<td>&lt; <strong>0.001</strong></td>
<td>0.796</td>
<td><strong>0.011</strong></td>
<td>0.949</td>
<td>0.518</td>
</tr>
<tr>
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<td>0.422</td>
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<td>0.350</td>
<td>0.453</td>
<td>0.926</td>
<td>0.974</td>
</tr>
</tbody>
</table>

Significant effects (*P* ≤ 0.05) are shown in bold text. SMR, soil microbial respiration; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Extr-C, soil extractable organic carbon; Extr-N, total soil extractable inorganic nitrogen; NMN, net mineralizable nitrogen.
**Table 2.5** Linear correlations ($R$) among microbial respiration, microbial biomass C and N, extractable C and N, net mineralizable N of soils over the 4-year period.

<table>
<thead>
<tr>
<th></th>
<th>SMR</th>
<th>MBC</th>
<th>MBN</th>
<th>Extr-C</th>
<th>Extr-N</th>
<th>NMN</th>
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<td>0.826***</td>
<td>0.419***</td>
<td>0.605***</td>
<td>0.847***</td>
</tr>
<tr>
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<td>0.511***</td>
<td>0.581***</td>
<td>0.664***</td>
<td></td>
</tr>
<tr>
<td>MBN</td>
<td>1</td>
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<td>0.687***</td>
<td>0.846***</td>
<td></td>
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<tr>
<td>Extr-C</td>
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<td>0.565***</td>
<td>0.449***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extr-N</td>
<td></td>
<td>1</td>
<td>0.581***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

*** denotes significance at $P < 0.0001$ (n = 384). SMR, soil microbial respiration; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; Extr-C, soil extractable organic carbon; Extr-N, soil extractable total inorganic nitrogen; NMN, net mineralizable nitrogen.
2.8 Figures

Figure 2.1 Total residue N inputs under elevated CO₂ and O₃. The amount of residue N inputs was calculated by multiplying residue N concentrations with the dry weight of plant residues. Soybean residue N inputs were the sum of residue N contents from four soybean growing seasons, whereas wheat residue N inputs were the sum of the first three wheat growing seasons because the placement of wheat residues from the last growing season lagged behind our last soil sampling date. Values are given as mean ± s.e.m. (n = 4). Different letters denote statistically significant treatment effects, ANOVA.

Figure 2.2 Effects of CO₂ enrichment on soil microbial respiration. (a) 0-5 cm soil layer. (b) 5-10 cm soil layer. (c) 10-20 cm soil layer. Data represent means (n = 8) ± s.e.m.

Figure 2.3 Effects of CO₂ enrichment on metabolic quotient of soil microbes at the 0-5 (a), 5-10 (b) and 10-20 (c) cm soil layers. Data represent means (n = 8) ± s.e.m. The metabolic quotient of soil microbes (the respiration rate per unit of microbial biomass C) was calculated by using the soil microbial respiration data divided by soil microbial biomass C.

Figure 2.4 Effects of CO₂ enrichment on soil microbial biomass C and N. Microbial biomass C: (a) 0-5 cm soil layer, (b) 5-10 cm soil layer and (c) 10-20 cm soil layer. Microbial biomass N: (d) 0-5 cm soil layer, (e) 5-10 cm soil layer and (f) 10-20 cm soil layer. Data represent
Figure 2.5 Effects of CO$_2$ enrichment on net soil N mineralization. (a) 0-5 cm soil layer, (b) 5-10 cm soil and (c) 10-20 cm soil layer. Data represent means (n = 8) ± s.e.m.

Figure 2.6 Effects of CO$_2$ enrichment on soil extractable organic C at the 0-5 (a), 5-10 (b) and 10-20 (c) cm soil layers. Data represent means (n = 8) ± s.e.m.

Figure 2.7 Effects of CO$_2$ enrichment on total soil extractable inorganic N at the 0-5 (a), 5-10 (b) and 10-20 (c) cm soil layers. Data represent means (n = 8) ± s.e.m.
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Figure 2.5 Effects of CO₂ enrichment on net soil N mineralization. (a) 0-5 cm soil layer, (b) 5-10 cm soil layer and (c) 10-20 cm soil layer. Data represent means (n = 8) ± s.e.m.
Figure 2.6 Effects of CO₂ enrichment on soil extractable organic C at the 0-5 (a), 5-10 (b) and 10-20 (c) cm soil layers. Data represent means ($n = 8$) ± s.e.m.
Figure 2.7 Effects of CO$_2$ enrichment on soil total extractable inorganic N at the 0-5 (a), 5-10 (b) and 10-20 (c) cm soil layers. Data represent means (n = 8) ± s.e.m.
Chapter 3

Mycorrhizal mediation of organic carbon decomposition under elevated CO$_2$, O$_3$ and N inputs
3.1 Abstract

Over the past three decades, the rising atmospheric CO$_2$ has prompted an increasing number of studies to assess whether terrestrial ecosystems could act as a carbon (C) sink under current and future CO$_2$ scenarios. Considerable attention has recently focused on the impact of elevated CO$_2$ on mycorrhizae, particularly arbuscular mycorrhizae (AM), assuming that the stimulation of mycorrhizae by elevated CO$_2$ would enhance soil C sequestration through facilitating soil aggregation and reducing C losses. However, the impact of climate change-induced alteration in AM fungi on organic C decomposition has not been carefully assessed. Here we investigated the mycorrhizal mediation of soil organic C decomposition under multiple climate change factors by combining dual $^{13}$C/$^{15}$N labeling of organic residues with hyphae in-growth bag techniques. Results obtained show that the stimulation of AM fungi by elevated CO$_2$ as well as N inputs significantly increased organic C decomposition. Results from our stable isotope ($\delta^{15}$N) analyses also indicate that elevated CO$_2$ increased mycorrhizally-mediated plant N acquisition from decomposing residues. These findings directly challenge the current view that the CO$_2$-stimulation of mycorrhizal fungi could increase soil C sequestration. They also suggest that AM fungi might shortcut the soil N cycle by increasing plant N acquisition from decomposing residues under elevated CO$_2$, especially in N-poor soils, thereby alleviating N limitation on ecosystem responses to elevated CO$_2$. 
Keywords: arbuscular mycorrhizal fungi, *Avena fatua*, climate change, elevated CO\textsubscript{2}, elevated O\textsubscript{3}, \textsuperscript{15}N, N additions, N limitation, N uptake, organic C decomposition, *Triticum aestivum*
3.2 Introduction

Arbuscular mycorrhizal (AM) fungi, which form intimate associations with more than 80% of land plants, can supply their host plants with nutrients in return for carbohydrates—the product of photosynthesis (Smith & Read 1997). It is estimated that a plant can allocate up to 20% of its photosynthate to associated AM fungus (Jakobsen & Rosendahl 1990). Thus, changes in plant photosynthesis and growth induced by climate change factors can critically affect the growth and functioning of AM fungi. Over the past three decades, increasing atmospheric CO$_2$ has prompted a large number of studies examining the influences of elevated atmospheric CO$_2$ on mycorrhizal fungi, assuming that CO$_2$-stimulation of photosynthesis and plant growth would enhance mycorrhizal growth. Experimental results have hitherto found that elevated CO$_2$ in general stimulated mycorrhizal fungal growth over a wide range of biomes (Fitter et al. 2000; Treseder & Allen 2000; Treseder 2004; Alberton et al. 2005; Hu et al. 2006). In a meta-analysis, Treseder (2004) showed that elevated CO$_2$, on average, caused an 87% increase in the percentage of root colonization by AM fungi across 6 field studies. Based on the synthesis of 72 pot and field studies, Alberton et al. (2005) found that CO$_2$ enrichment caused an average increase by 21% in the abundance of AM fungi. However, the functioning of AM fungi and their feedbacks to ecosystem processes under elevated CO$_2$ and other climate change factors have received very limited attention.

It is widely suggested that the CO$_2$-enhancement of AM fungi may increase soil C sequestration through enhancing C flux from plant to soil via mycorrhizal symbioses (Sanders et al. 1998; Rillig et al. 1999; Treseder & Allen 2000; Rillig et al. 2001; Zhu &
Miller 2003; Alberton et al. 2005; Wilson et al. 2009). There are two major proposed mechanisms that could account for the contribution of AM fungi to the C accumulation in soil C. First, AM fungi might expand soil C pools through depositing slow cycling compounds such as chitin, the primary component of fungal cell walls (Gleixner et al. 2002; Zhu & Miller 2003), and glomalin, a glycoprotein produced by AM hyphae (Wright & Upadhyaya 1998; Rillig et al. 1999; Rillig et al. 2001). Second, AM fungi might increase soil C storage and stability through the interactions of their filamentous external hyphae with soil mineral particles and organic materials (Miller & Jastrow 1990; Tisdall et al. 1997). Results from many experimental studies have shown that elevated CO$_2$ significantly increased the length of external hyphae (Sanders et al. 1998; Treseder 2004; Alberton et al. 2005), the production of glomalin (Rillig et al. 1999; Rillig et al. 2001), and soil aggregation (Rillig et al. 1999; Six et al. 2001), thus potentially increasing soil C sequestration. Though AM fungi under elevated CO$_2$ can lead to a larger amount of C flow to belowground, whether AM fungi can contribute to soil C sequestration depends on the effect of AM fungi on the net C fluxes between soil and atmosphere. On one hand, a large fraction of C allocated to the external hyphae can recycle quickly back to the atmosphere (Friese & Allen 1991; Johnson et al. 2002; Staddon et al. 2003; Heinemeyer et al. 2006). On the other hand, increased easily degradable C compounds through AM fungi may stimulate the activities of soil decomposers, potentially promoting the rate of soil organic C decomposition (Hodge et al. 2001; Tu et al. 2006; Talbot et al. 2008). So far, however, few studies have quantitatively assessed the role of mycorrhizal fungi, especially AM fungi, in soil organic C decomposition.
Hyphal transfer of N (mainly NH$_4^+$ and NO$_3^-$) from soil to plant has been well documented (Ames et al. 1983; Hawkins et al. 2000; Hodge et al. 2001; Govindarajulu et al. 2005; Tanaka & Yano 2005; Tu et al. 2006; Leigh et al. 2009). It has long been suggested that mycorrhizal fungi at elevated CO$_2$ can assist plant N uptake (Constable et al. 2001; Gamper et al. 2005; Chen et al. 2007), especially under N limiting conditions. However, the majority of soil N is in complex organic forms which are not available either by most plants (Schimel & Bennett 2004) or AM fungi (Read & Perez-Moreno 2003). Emerging evidence has showed that AM fungi might have the ability to directly capture N from decomposing organic material (Hodge et al. 2001; Leigh et al. 2009), potentially alleviating N limitation of plant response to elevated CO$_2$ through enhancing plant N uptake from organic pools via fungal hyphae.

Other global change factors such as atmospheric N deposition and elevated tropospheric O$_3$ can also alter the growth and functioning of mycorrhizal fungi (Andersen 2003; Treseder 2004). Mineral N additions, on one hand, may increase AM fungal growth when soil N is a limiting factor for plant growth (Treseder & Allen 2002; Tu et al. 2006). On the other hand, extra N inputs may either directly suppress AM fungal growth or indirectly reduce plant C allocation to AM fungi when initial soil N availability is sufficient (van Diepen et al. 2007). In contrast to the CO$_2$, elevated O$_3$ might suppress AM fungal growth through reducing C allocation belowground (Andersen & Rygiewicz 1995). Again, scant data is available on how changes in AM fungi induced by elevated O$_3$ and N inputs affect soil organic C decomposition and subsequent hyphal N transfer. Given that the co-occurrence of
elevated CO₂, reactive atmospheric N deposition, and elevated tropospheric O₃ concentration, we still lack the evidence of whether and how these global change factors, single and in combination, influence the mycorrhizally-mediated organic material decomposition and the subsequent plant N acquisition.

The aim of this study was to investigate the mycorrhizal mediation of soil organic C decomposition under elevated CO₂, O₃ and N inputs in two separate, but complementary experiments. A technique based on dual ¹³C/¹⁵N labeled Panicum virgatum (a perennial C₄ grass) shoot materials was used to quantify decomposition of soil- and residue-derived organic C and to trace the hyphal transfer of ¹⁵N from decomposing residues to plants. In a microcosm experiment, we assessed the effect of AM fungi on soil organic C decomposition under elevated CO₂ and N additions. We hypothesized that both elevated CO₂ and N additions would stimulate AM fungal growth in a N-poor soil, promoting the rate of organic C decomposition and plant N uptake from decomposing organic material via fungal hyphae. In the field study, we examined the influence of hyphae ingrowth on soil organic C decomposition under elevated CO₂ and O₃. Our hypothesis was that the CO₂-enhancement of AM fungi would facilitate organic C decomposition, while elevated O₃ would offset the CO₂ effect.

3.3 Methods

Experiment 1: the microcosm experiment

This experiment was conducted in the USDA Air-Quality CO₂ facility at North
Carolina State University. The facility consists of 20 continuously stirred tank reactor (CSTR) chambers for exposure of plants to CO₂ (Booker et al. 2000; Hu et al. 2005). Each CSTR (1.2 m diameter × 1.4 m tall) is covered with transparent Teflon film material. A blower system was used to supply a constant flow of charcoal filtered air through each CSTR during the experiment. The air in each CSTR was continuously moved out to reduce the heating effects of chambers. For CSTRs assigned to an elevated CO₂ treatment, compressed CO₂ was mixed with air and dispensed to chambers using a rotameter to maintain CO₂ concentrations at a target level. To monitor CO₂ concentrations, a small quantity of gas was extracted from each CSTR and directed through Teflon lines into an adjacent laboratory using computer-activated solenoid valves, and then measured automatically using infrared analyzers (model 6252, LiCor Inc., Lincoln, NE, USA) every two minutes. A computer collected and averaged CO₂ data for analysis.

We established a split-split plot experiment with whole-plots in a randomized complete block design. The whole-plot treatments were two atmospheric CO₂ levels (ambient and elevated CO₂) with four replicates. The split-plot factors were two N supply levels (with and without N addition) which were further split into mycorrhizae and non-mycorrhizae control. Eight CSTR chambers were blocked into four blocks. Either an ambient CO₂ (380 μmol mol⁻¹) or elevated CO₂ (ambient control + 200 μmol mol⁻¹) was randomly assigned to each CSTR chamber within each block. A microcosm unit was placed in each chamber and used to manipulate N and mycorrhizal treatments.

The microcosm unit was composed of four equal-size compartments with each
compartment measuring $13 \times 14 \times 15$ cm (width \times depth \times height). Each compartment was filled with a 3.2 kg soil mixture of steamed sandy loam soil and steamed quartz sand (1:1 w/w). Two compartments of one row were received additional N during growing period (N addition), the other two was treated as the no-N addition treatment. Within each N level, one compartment was inoculated with 100.0 g soil inoculum; the other was treated as non-mycorrhizal control, but also received 100.0 g autoclaved soil inoculum. To correct possible difference in microbial communities (Koide & Li 1989), one hundred ml filtered washing of soil inoculum (without mycorrhizal propagules) was added to non-mycorrhizal control. The AM fungal species were trap-cultured from a North Carolina Agricultural Research Station at Clayton, NC, USA, and were then pot-cultured in the greenhouse (Sylvia 1994). The seven species identified from this mixture were *Acaulospora scrobiculata*, *Acaulospora koskei*, *Gigaspora margarita*, *Glomus etunicatum*, *Glomus intraradices*, *Paraglomus occultum* and *Scutellospora pellucida*. The total C and N contents in the growth medium were 1.4 g C and 0.1 g N per kg of soil, respectively.

We employed a new technique based on dual $^{15}$N/$^{13}$C labeled shoot tissue of *Panicum virgatum* (switchgrass, a perennial C4 grass) to quantify the rate of organic C decomposition and the hyphal N transfer from decomposing residues to plants. Switchgrass plants were grown in containers filled with steamed quartz sand, fertilized with $^{15}$N-enriched Hoagland nutrient solutions. We then harvested the shoots of switchgrass, dried and chopped into small segments (ca. 1 cm length). A hyphae in-growth bag filled with a mixture of 1.20 g of plant residues and 120.0 g of steamed soil (the same soil used for plant growth), was sandwiched
in each compartment of the microcosm unit. The hyphae ingrowth bag (9 × 14 cm) was made of a nylon mesh with a pore size of 20μm (Tetko/Sefar MESH, Sefar America, NY, USA), which only allowed AM fungal hyphae rather than plant roots to penetrate through. The initial values of δ¹³C and δ¹⁵N of the residues were −12.8‰ and 6,982‰, respectively.

Thirty seeds of *Avena fatua* L. (wild oats, an annual C₃ grass; S&S Seeds, Inc., Carpinteria, CA, USA) were sown into each compartment and then thinned after emergence so that each compartment had 22 healthy plants. The CO₂ fumigation started immediately following seed germination. Before seedling emergence, each compartment received 50 ml Hoagland nutrient solution containing 5 mg N kg⁻¹ soil of NH₄NO₃, but without phosphorus. During the growing season, mineral N of 12.5 mg N kg⁻¹ soil (NH₄NO₃, equivalent to 2.5 g N m⁻²) was added to the N treatment at the 4th and 7th week after seedling emergence. Microcosms were watered with deionized water daily. Plants were allowed to grow for 10 weeks, and then above-ground plant parts were cut to the soil surface at final harvest. Roots were carefully separated from the growth medium and thoroughly washed with tap waters.

A subsample of approximately 0.5 g of fresh root segments was taken randomly and cleared in 5% (w/v) KOH, acidified in 1% (v/v) HCl, and then stained with acidic glycerol-trypan blue solution. The stained roots were used to examine mycorrhizal colonization using the gridline-intersect method (Giovannetti & Mosse 1980). Briefly, stained root segments were evenly dispersed on a Petri dish with gridlines and the total number of infected and non-infected root segments was counted at 40 × using a dissecting microscope.

Total root and shoot biomass of each compartment were determined after being dried.
at 70 °C for 48 hours. Shoots were then cut into pieces and ground into fine powder using an 8000-D Mixer Mill (SPEX CertiPrep Inc. Metuchen, NJ, USA). Shoot N concentrations and N isotope ratios (\(^{14}\)N and \(^{15}\)N) were determined using a Thermo Finnigan Delta Plus continuous flow isotope ratio mass spectrometer (CF-IRMS, Bremen, Germany). Isotope ratios (\(\delta\)) were expressed by

\[
\delta^{15}\text{N (‰)} = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1,000
\]  

(1)

where \(R_{\text{sample}}\) is the molar isotope ratio of \(^{15}\)N/\(^{14}\)N in samples and \(R_{\text{standard}}\) is the ratio \(^{15}\)N/\(^{14}\)N in atmospheric N\(_2\), which is 0.0036765. Sample \(^{15}\)N concentrations were calculated from isotope ratios and total N content (Hu et al. 2001). The amount of plant \(^{15}\)N acquisition mediated by mycorrhizae (mg \(^{15}\)N kg\(^{-1}\)) was calculated by subtracting \(^{15}\)N in non-mycorrhizal samples from \(^{15}\)N in mycorrhizal samples.

Hyphae ingrowth bags were collected immediately following the harvest. Twenty (20.0) grams of incubated soil mixture samples were then taken from each bag, oven-dried at 65 °C and ground into fine powder before total C and \(^{13}\)C analyses on the mass spectrometer. The net C losses (mg C kg\(^{-1}\)) resulting from mineralization during 10-week growing period were calculated by subtracting the total C content in the incubated samples from the initial value (5.34 ± 0.016 g C kg\(^{-1}\)). The mycorrhizally-enhanced total C losses (MCL, mg C kg\(^{-1}\) d\(^{-1}\)) were calculated using the following equation:

\[
\text{MCL (mg C kg}^{-1} \text{ d}^{-1}) = (\text{net C losses in mycorrhizal samples} - \text{net C losses in non-mycorrhizal samples})/ \text{incubation days (2)}
\]

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To estimate the net losses of residue-derived C by the mycorrhizal effect, we first computed the mean proportion \( f_R \) of the residue C in the incubated samples and the standard error (SE) of the \( f_R \) using a single isotope, two-source mixing model (Phillips & Gregg 2001). The linear mixing model can be described by the following mass balance equations:

\[
\bar{\delta}_M = f_R \bar{\delta}_R + f_S \bar{\delta}_S \tag{3}
\]

\[
f_R + f_S = 100\% \tag{4}
\]

where \( f_R \) and \( f_S \) represent the mean proportion of residue C and soil C, respectively, and \( \bar{\delta}_M \), \( \bar{\delta}_R \) and \( \bar{\delta}_S \) denote the mean isotopic signatures (\( \delta^{13} \)C, which is the \( ^{13} \)C content of a sample relative to the reference standard Vienna PeeDee belemnite) for the mixture M (incubated samples of soil/residue mixture), source R (residue, which is \(-12.8 \pm 0.05\%\)) and source S (soil, which is \(-21.2 \pm 0.26\%\)). To calculate the mean proportion of source residue in the mixture, equation (3) was rearranged and inserted into equation (2) that was further rearranged to solve for \( f_R \): the mean proportion of source residue in the mixture can be calculated as

\[
f_R = \frac{\bar{\delta}_M - \bar{\delta}_S}{\bar{\delta}_R - \bar{\delta}_S} \tag{5}
\]

The variance for \( f_R \) can be estimated by a first-order Taylor series approximation using partial derivatives as:

\[
\sigma_{f_s}^2 = \frac{1}{(\bar{\delta}_R - \bar{\delta}_S)^2} \left[ \sigma_{\bar{\delta}_M}^2 + f_R^2 \sigma_{\bar{\delta}_R}^2 + (1 - f_R)^2 \sigma_{\bar{\delta}_S}^2 \right] \tag{6}
\]

where \( \sigma_{\bar{\delta}_M}^2 \), \( \sigma_{\bar{\delta}_R}^2 \) and \( \sigma_{\bar{\delta}_S}^2 \) represent variances of the mean isotopic signatures for the mixture M.
and source R and S, respectively. Using the equation (5), we considered the variability of isotopic signatures of both sources and mixture in estimating the variance of $f_R$. The amount of residue-derived C remaining in incubated samples (soil/residue mixture) was calculated by using $f_R$ multiplied the total C contents. The mycorrhizally enhanced losses of residue-derived C (mg C kg$^{-1}$ d$^{-1}$) were calculated using the equation (2).

We employed linear mixed-effects models (LME) to analyze the data using the software SAS 9.1 (proc MIXED, SAS Institute Inc., Cary, USA) (Littell et al. 2006), as our experiment contained both fixed and random effects and the LME allowed us to handle fixed and random effects easily. For effects of elevated CO$_2$, N inputs and mycorrhizal fungi on plant biomass, the model used in the LME analysis is

$$y_{ijkl} = \mu + b_i + c_j + n_k + (c \times n)_{jk} + m_l + (c \times m)_{jl} + (n \times m)_{kl} + (c \times n \times m)_{jkl} + b_{ij} + b_{ijk} + \varepsilon_{ijkl}$$

(7)

where $y_{ijkl}$ is the root or shoot biomass; $\mu$ the intercept, fixed effects; $b_i$ the block, fixed effects; $c_j$ the CO$_2$ treatment (whole plot), fixed effects; $n_k$ the N treatment (split-plot), fixed effects; $(c \times n)_{jk}$ the CO$_2$ and N interaction, fixed effects; $m_l$ the AM fungi treatment, fixed effects; $(c \times m)_{jl}$ the CO$_2$ and AM fungi interaction, fixed effects; $(n \times m)_{kl}$ the N and AM fungi interaction, fixed effects; $(c \times n \times m)_{jkl}$ the interaction of CO$_2$, N and AM fungi, fixed effects; $b_{ij}$ the CO$_2$ within block, random effects; $b_{ijk}$ the interaction of CO$_2$ and N within block, random effects; and $\varepsilon_{ijkl}$ the random experimental error. For the data of mycorrhizal infection rate, mycorrhizally-enhanced plant $^{15}$N uptake, and mycorrhizally-enhanced losses of total C contents, we first performed the procedure of UNIVARIATE of SAS 9.1 to check
the normality. If the data violated the normality assumption, log-transformed data were used to improve homogeneity of variance. Similarly, the LME model is as follows:

\[ y_{ijk} = \mu + b_i + c_j + n_k + (c \times n)_{jk} + b_{ij} + \varepsilon_{ijk} \]  

(8)

The significance of the net losses of residue-derived C by AMF was determined using t-test.

For all tests, differences between treatments were considered significant when \( P \leq 0.05 \).

**Experiment 2: the field experiment**

The experimental site is located at the Lake Wheeler farm of North Carolina State University, Raleigh, NC, USA (35° 43' N, 78° 40' W; elevation 120 m) with an annual mean temperature of 15.2 °C and an annual mean precipitation of 1050 mm. The field had been left fallow for eight years prior to this study. Before gas fumigation, the soil was repeatedly turned-over using a disc implement and rotovator. The soil is an Appling sandy loam (fine, kaolinitic, thermic Typic Kanhapludult), well drained with a pH of 5.5, and contained 9.0 g C and 0.86 g N kg\(^{-1}\) soil when the experiment was initiated. Measurements on the initial soil microbial and chemical properties showed that the field site before the experiment was quite homogeneous.

This experiment was a 2 × 2 factorial design with four treatments randomly assigned into each of four blocks. Four different trace-gas treatments were: (a) charcoal-filtered air (control), (b) charcoal-filtered air plus 1.4 times ambient O\(_3\) (elevated O\(_3\)), (c) charcoal-filtered air plus 180 \(\mu\)l l\(^{-1}\) CO\(_2\) (elevated CO\(_2\)), and (d) charcoal-filtered air plus 1.5 times ambient O\(_3\) and 180 \(\mu\)l l\(^{-1}\) CO\(_2\) (elevated CO\(_2\) and O\(_3\)). The 12-h daily average CO\(_2\)
concentrations were 382 and 552 μl l⁻¹ CO₂ for low (a and b) and high (c and d) CO₂ treatments, respectively. The daily average O₃ concentrations for low (a and c) and high (b and d) O₃ treatments were 21 and 59 nl l⁻¹ O₃, separately. The purpose of filtration of ambient air with activated charcoal was to reduce the concentrations of ambient O₃ to levels considered nonphytotoxic to soybean and wheat plants. Ozone was deemed as a major air pollutant in this area, while other air pollutants such as NO₂ and SO₂ were below the phytotoxic levels at the experimental location (Booker et al. 2005).

Soybean [Glycine max (L.) Merr.] was planted each spring followed by winter wheat (Triticum aestivum L.) in the fall using no-till practices. Plants were exposed to reciprocal combinations of CO₂ and O₃ within cylindrical open-top chambers (OTC, 3.0 m diameter × 2.4 m tall) from emergence to physiological maturity. Carbon dioxide was released from a 14-ton liquid tank 24 h daily and monitored at canopy height using an infrared CO₂ analyzer (model 6252, Li-Cor Inc. Lincoln, NE, USA). Ozone was generated by electrostatic discharge in dry O₂ (model GTC-1A, Ozonia North America, Elmwood Park, NJ, USA) and dispensed 12 h daily (08:00-20:00 hours EST) in proportion to concentrations of ambient O₃. The O₃ concentration in the chambers was monitored at canopy height with a UV photometric O₃ analyzer (model 49, Thermo Environmental Instruments Co., Franklin, MA, USA). During wheat growing seasons, each plot initially received 48 g NH₄NO₃-N (equivalent to 22 kg N ha⁻¹) in November each year, followed by an additional input of 192 g NH₄NO₃-N (equivalent to 90 kg N ha⁻¹) in March. During soybean growing seasons, plants were irrigated with drip lines to prevent visible signs of water stress, but no additional N
fertilizers were applied. Upon senescence of the plants, all aboveground plant biomass in each chamber was harvested and divided into leaves, stems and reproductive organs, then dried and weighted. Afterward, residues other than seeds were uniformly returned to their corresponding treatment plots and evenly distributed on the soil surface.

A hyphae ingrowth bag (pore size: 20 µm) was used to assess the influence of fungal hyphae on organic C decomposition. At the same time, a root ingrowth bag (pore size: 1.6 mm) was employed to collect fine wheat roots for determination of mycorrhizal colonization of roots. Both ingrowth bags were 12 cm long and 8 cm wide and filled with a mixture of sandy-loam soil and dual $^{15}$N/$^{13}$C labeled shoot tissue of *Panicum virgatum* (100g soil: 1.20g residues). The initial mixture of soil and residue had 6.38 g kg$^{-1}$ C, 0.23 g kg$^{-1}$ N, -15.38‰ of δ$^{13}$C, and 7174‰ of δ$^{15}$N. On January 8, 2008, we placed a total of 96 ingrowth bags (48 each for hyphae or roots) in the ground of 16 experimental plots, and harvested them at February 12, March 18, and April 24 (every five weeks), respectively. Within each plot, the 6 in-growth bags were randomly buried at 0-5 cm soil layer within two rows of wheat plants.

On each sampling date, we collected one root ingrowth bag and one hyphae ingrowth bag from each plot. For root ingrowth bags, we carefully picked up the live root segments from soil for determining the mycorrhizal fungal colonization. For hyphae ingrowth bags, a 20.0g subsamples were taken, dried at 65 °C and ground in to fine powder using an 8000-D Mixer Mill (SPEX CertiPrep Inc. Metuchen, NJ, USA). Total C and N, $^{13}$C and $^{15}$N were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa
20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at University of California, Davis.

The percentage of the total C and N remaining in the soil mixture was calculated as the total amounts of C or N in samples after the field incubation divided by the initial values before the field incubation. A constant overall fractional C loss rate (k) can be estimated from: \( X = X_0 e^{-kt} \), where t is the duration of the incubation experiment, \( X \) and \( X_0 \) are the remaining of organic C content at time t and time 0, respectively. We also used the instantaneous fractional loss rate \( k' = 1 - e^{-kt} \) to compare the decomposition rates at different sampling dates.

The field data with time series measurements were analyzed using repeated measures mixed model (Littell et al. 2006). The mixed model for repeated measures is

\[
y_{ijklm} = \mu + t_j + c_k + o_l + (co)_{kl} + (ct)_{kl} + (ot)_{kl} + (cot)_{kl} + b_i + e_{ijklm} \tag{9}
\]

where \( \mu \) is the intercept, fixed effects; \( t_j \) the fixed effect for time (month); \( c_k \) the fixed effect for CO\(_2\) treatment; \( o_l \) the fixed effect for O\(_3\) treatment; \( (co)_{kl} \) the CO\(_2\) and O\(_3\) interaction, fixed effects; \( (ct)_{kl} \) the fixed effect for the interaction of time and CO\(_2\) treatment; \( (ot)_{kl} \) the fixed effect for the interaction of time and O\(_3\) treatment; \( (cot)_{kl} \) the CO\(_2\), O\(_3\) and time interaction, fixed effects; \( b_i \) the block random effects; and \( e_{ijklm} \) the random experimental error. We used a set of covariance structures including compound symmetric model (CS), the first-order autoregressive model [AR (1)], and autoregressive with random effect to reduce autocorrelation. The \( p \) values for treatments and interaction terms were reported based on the covariance structure that minimized Akaike information criterion (AIC) and Bayesian
information criterion (BIC) (Littell et al. 2006). In the field experiment, no significant O₃, O₃ × CO₂ effects and their interactions with time were detected ($P > 0.05$). Therefore, the data was reported as the mean of eight ambient CO₂ and eight elevated CO₂ plots, respectively. All data were also subjected to ANOVA using GLM at each sampling date. All statistical analyses were performed using the SAS 9.1 software (SAS Institute Inc. Cary, NC). The significant differences were determined at the 95% probability level.

3.4 Results

Experiment 1

Elevated CO₂ and N additions significantly stimulated the growth of A. fatua, increasing the total biomass by 32% (CO₂ effects: $P < 0.001$) and 54% (N effects: $P < 0.001$), respectively (Fig. 3.1). Mycorhizal fungi also had a significant impact on the shoot biomass ($P < 0.001$; Fig. 3.1a), but not on the root biomass ($P = 0.382$; Fig. 3.1b).

On average, the percentage of root length colonized by mycorrhizal fungi increased by 48% ($P = 0.017$; Fig. 3.2) under elevated CO₂ in comparison to the ambient CO₂. N inputs also significantly enhanced mycorrhizal root colonization ($P < 0.001$; Fig. 3.2), leading to an average increase by 80%.

Mycorrhizal fungi significantly enhanced shoot $^{15}$N of A. fatua ($P < 0.001$: 9.8 and 11.0 mg $^{15}$N m⁻² for non-mycorrhizal and mycorrhizal treatments, respectively). This mycorrhizal effect was also significantly stimulated by elevated CO₂ ($P = 0.050$; Fig. 3.3) and N additions ($P = 0.047$; Fig. 3.3). The mycorrhizally-enhanced losses of the total C
(calculated using the total C losses from mycorrhizal samples minus those from non-mycorrhizal samples) were significantly higher under elevated CO₂ \( (P = 0.029; \text{Fig. 3.4}) \) and N additions \( (P = 0.035; \text{Fig. 3.4}) \). A stable isotope mixing model was used to estimate the proportion of residue-derived C in the loss of total soil C from hyphae ingrowth bags. Modeling results illustrated that elevated CO₂ caused a marked increase in the loss of residue-derived C due to the mycorrhizal effect both under no-N and N additions (Fig. 3.5).

*Experiment 2*

Elevated CO₂ significantly stimulated mycorrhizal fungal growth, leading to an average increase by 110% in mycorrhizal colonization of wheat roots across the last two sampling dates (10 and 15-wk field incubation) (Repeated measures mixed model: CO₂ effects, \( P < 0.008; \) Figure 3.6). Also, the CO₂-stimulation effect on mycorrhizae varied considerably over time (Repeated measures mixed model: the CO₂ × time interaction, \( P = 0.069; \) Fig. 3.7), with the relative CO₂ effect increasing with time.

The total C remaining in hyphae ingrowth bags was 13% lower under elevated CO₂ than under ambient CO₂ across three samples dates (Repeated measures mixed model: CO₂ effect, \( P < 0.001; \) the CO₂ × time interaction, \( P = 0.087; \) Fig. 3.7). The instantaneous fractional C loss rate \( (k') \) of samples incubated for 5, 10 and 15 weeks (0.25, 0.29 and 0.39, respectively) under elevated CO₂ was 29%, 41%, and 80% higher than those of ambient CO₂. Elevated CO₂ also significantly reduced \( \delta^{13} \text{C} \) of incubated samples (Repeated measures mixed model: CO₂ effect, \( P = 0.004; \) Fig. 3.8). By the end of 15 weeks, hyphal ingrowth
caused a 1.3‰ depletion of δ¹³C in incubated samples under elevated CO₂, while only a 0.8‰ depletion under ambient CO₂ compared to the initial value of δ¹³C. Modeling results obtained from a single isotope, two sources model, also revealed that the loss of residue-derived C were consistently higher under elevated than ambient CO₂. By the end of the 15-wk field incubation period, elevated CO₂ led to a loss of residue-derived C by 53% relative to the original residue C mass, while ambient CO₂ only caused a loss of 34% in residue-derived C (Fig. 3.9).

CO₂ enrichment also significantly reduced total N remaining in incubated samples (Repeated measures mixed model: CO₂ effect, \( P = 0.006; \) Fig. 3.10); and this CO₂ effect varied enormously among three sampling dates (Repeated measures mixed model: CO₂ × time, \( P = 0.066; \) Fig. 3.10). The total N contents decreased steeply under both ambient and elevated CO₂ in comparison with the initial value by the end of 5 weeks; but there was no significant difference between two CO₂ treatments (\( P > 0.05 \)). Under ambient CO₂, total N in hyhal ingrowth bags incubated for 10 and 15 weeks increased by 33% and 20%, respectively, compared to samples incubated for 5 weeks (Fig. 3.10). Under elevated CO₂, the percentage of increases in total N was only 18% and 13% for samples incubated for 10 and 15 weeks, respectively (Fig. 3.10). Elevated CO₂ also tended to reduce the δ¹⁵N of incubated samples over the sampling period (Repeated measures mixed model: CO₂ effect, \( P = 0.170; \) CO₂ × time, \( P = 0.268; \) Fig. 3.11).
3.5 Discussion

Results obtained in the microcosm experiment clearly show that the stimulation of AM fungi by elevated CO$_2$ as well as N additions significantly enhanced shoot $^{15}$N contents from decomposing organic material in a N-poor soil (Fig. 3.3). Lower total and residue-derived N in hyphae ingrowth bags under elevated CO$_2$ observed in the field (Fig. 3.10 and Fig. 3.11) also suggest that AM fungi under CO$_2$ enrichment may increase N transfer from both inorganic and organic pools to their host plants.

It has been well documented that AM fungi can acquire large amounts of inorganic and simple organic N from soil and transport them to their host plants (Ames et al., 1983, Govindarajulu et al., 2005, Hawkins et al., 2000, Hodge et al., 2001, Leigh et al., 2009, Tanaka et al., 2005, Tu et al., 2006). But most soil N (> 99%) exists in organic form, and AM fungi have long been considered not being able to use organic N directly (Smith and Read 1997). Using $^{15}$N labeling and root exclusion techniques, however, Hodge et al. (2001) demonstrated that an AM fungus, *Glomus hoi*, can directly acquire N from decomposing grass leaves in a microcosm experiment. A recent study also shows that N transferred from organic material by *G. hoi* and *G. intraradices* could account for approximately 20% of total plant N uptake (Leigh et al. 2009). These findings are consistent with results presented in the current study using a putative AM community consisting of multiple AM fungal species (Fig. 3.3) or in field (Fig. 3.11). It remains unclear how climate change factors impact mycorrhizal mediation of plant N uptake from complex organic materials. Climate change factors such as CO$_2$ enrichment and atmospheric reactive N deposition have been also shown to considerably
affect AM fungal growth (Alberton et al., 2005, Fitter et al., 2000, Hu et al., 2006, Treseder, 2004), potentially altering mycorrhizal mediation of plant N acquisition. For instance, a significant increase in mycorrhizally mediated plant N uptake has been observed in *Trifolium repens* in a CO2-enrichment experiment (Gamper et al., 2005) and in *Avena fatua* in a microcosm study with mineral N inputs (Tu et al., 2006). Higher mycorrhizally-enhanced shoot 15N contents (Fig. 3.3) and lower δ15N values within hyphae ingrowth bags (Fig. 3.11) under elevated CO2 observed in this work suggest the CO2-led increase in mycorrhizally mediated plant N uptake might originate, at least in part, from decomposing organic material.

The findings that elevated CO2 promotes mycorrhizal contribution to plant N uptake from organic N pools may have an important ramification that transcends current global change studies. In many natural and semi-natural ecosystems, plant growth is often limited by soil N availability (Vitousek & Howarth 1991), though the total amount of organic N in soil is at least two orders of magnitude higher than that of the annual requirement of plants. This has led to the hypothesis that N limitation on plants growing under elevated CO2 might be exacerbated over time, as soil available N under CO2 enrichment can be progressively transferred to living plant tissues and soil organic matter (Hu et al., 1999, Luo et al., 2004). A significant N limitation on plant responses to elevated CO2 has already been detected in a loblolly pine forest in the 4th years of Duke Forest CO2 experiment (Finzi & Schlesinger 2003) and a perennial grassland after 4 to 6 years of CO2 fumigation (Reich et al., 2006). Although the CO2-led fine root proliferation could temporarily and partially lessen the N limitation on plant growth by reaching wider and deeper soil inorganic N pools (McKinley et
only an enhancement of N uptake from the vast soil organic N pool can sustain plants under the rising CO$_2$ in N limiting conditions over the long term. Our finding that the CO$_2$-stimulation of AM fungi could facilitate plant N acquisition from decomposing organic residues indicates that mycorrhizal fungi may potentially alleviate N limitation on plant responses to elevated CO$_2$ through mining N directly from soil organic N pools. The results also suggest that elevated CO$_2$ may speed up the internal N cycling by short-cutting the conventional N cycle via mycorrhizal symbioses in the plant-soil system.

Results obtained in both microcosm and field experiments also show that the CO$_2$-enhancement of AM fungi significantly facilitated the rate of organic C decomposition (Fig. 3.4 and Fig. 3.7). It has long been suggested that the CO$_2$-led increases in AM fungal growth could increase soil C sequestration through enhancing C flux from plant to soil and improving soil aggregation (Sanders et al. 1998; Rillig et al. 1999; Treseder & Allen 2000; Rillig et al. 2001; Zhu & Miller 2003; Alberton et al. 2005; Wilson et al. 2009). However, this potential depends largely on the effect of AM fungi on the balance of soil C inputs and outputs. Though AM fungi have been traditionally considered obligate biotrophs and having no saprotrophic capabilities (Smith & Read 1997; Read & Perez-Moreno 2003), emerging evidence suggested that AM fungi might play an important role in degrading plant residues (Hodge et al., 2001, Leigh et al., 2009, Tu et al., 2006). Hodge et al. (2001), using a compartment to separate fungal hyphae from plant roots, showed that the hyphae of *Glomus hoi* can grow extensively towards an organic patch labeled with $^{13}$C and $^{15}$N, leading to a remarkable decreases in both $^{13}$C and $^{15}$N contents in the organic patch. Tu et al. (2006)
recently reported that the stimulation of AM fungal growth by N inputs significantly facilitated the degradation of particular organic C derived from newly added plant residues in a N-poor soil. Results obtained in the current study (Fig. 3.4 and Fig. 3.7) are consistent with these two studies, indicating that AM fungi might be either directly or indirectly involved in decomposition of organic material. The current study advances our understanding of the effects of AM fungi on organic C decomposition and shows that these effects can be significantly altered by climate change factors such as elevated CO₂ and N deposition.

What are possible reasons explaining the mycorrhizal enhancement of C losses under CO₂ enrichment and N additions observed in this study? One hypothesis, initially proposed by Hodge et al. (2001), is that AM fungi may have the saprotrophic capability to directly degrade complex organic material. Thus, increased organic C decomposition in the current study might be a direct consequence of CO₂-led increases in degradation of complex organic molecules by AM fungi. It has been well documented that both ericoid (ERM)- and ecto (ECM)- mycorrhizal fungi are able to directly attack nutrient-containing complex organic polymers (e.g. a review by Read et al. 2003). Analyses of ¹⁵N from both the present study (Fig. 3.3 and Fig. 3.11) and previous work (Hodge et al., 2001, Leigh et al., 2009) suggest that AM fungi might be also directly involved in capturing nutrients from organic materials. Decomposers such as bacteria and saprotrophic fungi can produce a suite of exoenzymes that are needed for the complete degradation of complex organic polymers (Swift et al. 1979). Experimental evidence showed that AM fungi are able to produce cellulase during the early stage of penetration of host plants (Garcia-Garrido et al. 1992). However, it is not known
whether AM fungi can behave similarly as soil decomposers.

Another possible mechanism is that enhanced C losses under elevated CO₂ may result from increased microbial activities in the mycosphere (the area influenced directly by fungal hyphae), a phenomenon known as the priming effect (Kuzyakov et al. 2000). The CO₂-stimulation of soil respiration has been frequently reported (Bernhardt et al., 2006, Carney et al., 2007, Hungate et al., 1997, Janssens et al., 1998, Langley et al., 2009, Luo et al., 1996, Trueman et al., 2005, Van Kessel et al., 2000), which has been often attributed to the rhizospheric priming effect driven by root exudates (Cheng, 1999, Langley et al., 2009, Zak et al., 2000b). Like the exudation of organic compounds by roots, mycorrhizal hyphae can also secret a large amount of easily degradable substrates available for soil decomposers within mycosphere. Enhanced mycorrhizal growth, as evidenced by CO₂-led increases in internal (Fig. 3.2 and Fig. 3.6) and external hyphae (Sanders et al. 1998), thus may fuel the activities of soil decomposers, leading to a priming effect on decomposition of plant residues and/or soil organic matter (Fontaine et al., 2007, Kuzyakov et al., 2000, Langley et al., 2009, Talbot et al., 2008). Because the average length density of AM fungal hyphae is often 100 times higher than that of typical roots (Jakobsen et al. 1994) and the diameter of fungal hyphae (< 5 μm) is far smaller than that of fine roots (ca. 0.2-2 mm), AM fungal hyphae can proliferate in much larger soil spaces than plant roots. Results presented in the current study thus suggest that the mycospheric priming effect might account for a larger proportion of CO₂-induced soil respiration than the rhizospheric priming effect.

The mycorrhizally-enhanced organic C decomposition at elevated CO₂ can
theoretically occur in many terrestrial ecosystems, particularly the vast agro- and grassland ecosystems where arbuscular mycorrhizas predominate (Read & Perez-Moreno 2003). Soils in agro- and grassland ecosystems store nearly the same amount of C as is currently present in the atmosphere (Jobbagy & Jackson 2000). Under future climate change scenarios, even a small change in C stocks of agricultural and grassland soils driven by AM fungi might significantly alter the atmospheric CO$_2$ concentrations. It is uncertain whether the observed phenomenon in the current study can be applied to the ERM-dominated healthland and ECM-dominated boreal and temperate forest ecosystems. But, given that the well demonstrated saprotrophic capabilities of both ERM and ECM (Read & Perez-Moreno 2003) and the CO$_2$-stimulatin effect on both fungi (Alberton et al., 2005, Treseder, 2004), our results may also be generalized to soils of many healthland and forest ecosystems. This must be done with caution while extending observed C losses driven by the CO$_2$-enhancement of mycorrhizal fungi into future climate change scenarios at the global scale. However, results from the present study do provide insights into possible C losses due to the CO$_2$-stimulation of mycorrhizal fungi and the feedback to climate change.

3.6 References


3.7 Figures

**Figure 3.1** Effects of elevated CO$_2$, N addition and arbuscular mycorrhizal fungi on plant biomass (Experiment 1). a, Shoot biomass of *Avena fatua*. b, Root biomass of *Avena fatua*. In both panels, each bar represents mean dry weight ± s.e.m. (n=4). –AMF-N, neither arbuscular mycorrhizal (AM) inoculum nor mineral nitrogen was added; +AMF-N, only AM inoculum was added; -AMF+N, only mineral N was added; AMF+N, both AM inoculums and mineral N were added.

**Figure 3.2** Mycorrhizal colonization on roots of *Avena fatua* under elevated CO$_2$ and N additions (Experiment 1). For non-mycorrhizal treatments, the percentage of mycorrhizal root colonization was almost zero and excluded in this figure. Values are means ± s.e.m. (n=4).

**Figure 3.3** The net mycorrhizal effect on plant $^{15}$N uptake under elevated CO$_2$ and N additions (Experiment 1). Mycorrhizally-enhanced shoot $^{15}$N uptake by *Avena fatua* was calculated as shoot biomass $^{15}$N in mycorrhizal samples minus that in non-mycorrhizal samples. Data represent means ± s.e.m. (n=4).

**Figure 3.4** Mycorrhizally-enhanced total C losses from hyphae ingrowth bags under elevated CO$_2$ and N additions (Experiment 1). Data represent mean ± s.e.m. (n=4). We first calculated
the net C losses (mg C kg\(^{-1}\)) from mineralization during 10-week growing period by subtracting the measured total C content after incubation from the total C content before incubation in hyphae ingrowth bags. We then estimated the net mycorrhizal effect on C losses (mg C kg\(^{-1}\) d\(^{-1}\)) from hyphae ingrowth bags by: (the net C losses in mycorrhizal samples - the net C losses in non-mycorrhizal samples) / 70.

**Figure 3.5** Mycorrhizally-enhanced losses of residue-derived C under elevated CO\(_2\) and N additions (Experiment 1). The proportion of residue derived C was estimated using a single isotope, two-source mixing model. Mathematical equations employed in calculation were presented in the text.

**Figure 3.6** Mycorrhizal colonization on roots of *Triticum aestivum* (Experiment 2). Values represent mean ± s.e.m., n=8.

**Figure 3.7** The percentage of C remaining in hyphae ingrowth bags under elevated CO\(_2\) (Experiment 2). Values are mean ± s.e.m., n=8.

**Figure 3.8** Changes in soil δ\(^{13}\)C in hyphae ingrowth bags under elevated CO\(_2\) (Experiment 2). Values are mean ± s.e.m., n=8.

**Figure 3.9** The proportion of residue-derived C relative to the total C under elevated CO\(_2\)
(Experiment 2). Values are mean ± s.e.m., n=8.

**Figure 3.10** The percentage of N remaining in hyphae ingrowth bags under elevated CO₂ (Experiment 2). Values are mean ± s.e.m., n=8.

**Figure 3.11** Changes in soil $\delta^{15}$N in hyphae ingrowth bags under elevated CO₂ (Experiment 2). Values are mean ± s.e.m., n=8.
Figure 3.1 Effects of elevated CO$_2$, N addition and arbuscular mycorrhizal fungi on plant biomass (Experiment 1). a, Shoot biomass of *Avena fatua* L. b, Root biomass of *Avena fatua* L. In both panels, each bar (n = 4) represents mean dry weight ± s.e.m. –AMF-N, soil without arbuscular mycorrhizal (AM) inoculum and mineral nitrogen inputs; +AMF-N, soil inoculated with AM fungi but without nitrogen inputs; -AMF+N, soil without AM inoculum but with N inputs; AMF+N, soil added with both AM inoculums and mineral nitrogen.
Figure 3.2 Mycorrhizal colonization on roots of *Avena fatua* under CO$_2$ and N treatments (Experiment 1). For non-mycorrhizal treatments, the percentage of mycorrhizal root colonization was almost zero and excluded in this figure. Values are means ± s.e.m.; n=4.
Figure 3.3 The net mycorrhizal fungal effect on plant $^{15}$N uptake under elevated CO$_2$ and N additions (Experiment 1). Mycorrhizally-enhanced shoot $^{15}$N uptake by *Avena fatua* was calculated as shoot biomass $^{15}$N in mycorrhizal samples minus shoot biomass $^{15}$N in non-mycorrhizal samples. Data represent means ± s.e.m.; n=4.
Figure 3.4 Mycorrhizally-enhanced total C losses from hyphae ingrowth bags under elevated CO₂ and N additions (Experiment 1). Data represent mean ± s.e.m; n=4. We first calculated the net C losses (mg C kg⁻¹) from mineralization during 10-week growing period by subtracting the measured total C content after incubation from the total C content before incubation in hyphae ingrowth bags. We then estimated the net mycorrhizal effect on C losses (mg C kg⁻¹ d⁻¹) from hyphae ingrowth bags by: (the net C losses in mycorrhizal samples - the net C losses in non-mycorrhizal samples) / 70.
Figure 3.5 Mycorrhizally-enhanced losses of residue-derived C under elevated CO$_2$ and N additions (Experiment 1). The proportion of residue derived C was estimated using a single isotope, two-source mixing model. Mathematical equations employed in calculation were presented in the text.
Figure 3.6 Mycorrhizal colonization on roots of *Triticum aestivum* (Experiment 2). Values represent mean ± s.e.m., n=8.
Figure 3.7 The percentage of C remaining in soil due to hyphal ingrowth (Experiment 2). Values are mean ± s.e.m., n=8.
Figure 3.8 Changes in soil $\delta^{13}$C in hyphae ingrowth bags (Experiment 2). Values are mean ± s.e.m., n=8.
Figure 3.9 The proportion of residue-derived C relative to the total C after field incubations (Experiment 2). Values are mean ± s.e.m., n=8.
Figure 3.10 The percentage of N remaining in soil due to hyphal ingrowth (Experiment 2). Values are mean ± s.e.m., n=8.
Figure 3.11 Changes in soil $\delta^{15}$N in hyphae ingrowth bags (Experiment 2). Values are mean ± s.e.m., n=8.
Chapter 4

Species of arbuscular mycorrhizal fungi differentially influence host nitrogen acquisition under elevated CO$_2$ and O$_3$
4.1 Abstract

Plants are generally limited by N availability in many natural and semi-natural ecosystems and their responses to atmospheric CO₂ enrichment can be constrained by N deficiency over the long term. It has been suggested that arbuscular mycorrhizal fungi (AMF), which form mutualistic symbioses with more than 80% of land plants, may potentially alleviate this N limitation. Experimental evidence from our previous study showed that a manipulated AMF community can significantly increase plant N uptake from decomposing plant residues under elevated CO₂ and N additions in a N-poor soil. However, it remains unknown whether different AMF species differ in their ability for N acquisition. In this study, we examined how AMF species from three taxonomic families mediate N acquisition of their host plants (*Avena fatua*) under elevated CO₂ and O₃. We show that both elevated CO₂ and AMF significantly increased plant N uptake and reduced soil extractable inorganic N. Elevated CO₂ and AMF also had a significant interactive impact on shoot biomass N of *A. fatua* and N acquisition from decomposing residues. Under elevated CO₂, AMF species exhibit different capacities for N acquisition from decomposing residues with *Gi. margarita* being significantly higher than *A. morrorriae* and *G. clarum*. Neither the O₃ effect nor the O₃ × AMF interactions were significant on plant growth or N uptake. These results emphasize the need to consider the influences of changed AMF communities on plant and ecosystem responses to elevated CO₂ in future global change research.

**Key words:** arbuscular mycorrhizal fungi, *avena fatua*, elevated CO₂, elevated O₃, ^{15}N, N
limitation, N uptake, organic C decomposition
4.2 Introduction

A major uncertainty in predicting future terrestrial ecosystem C balance is whether the CO\textsubscript{2}-enhancement of plant growth will be sustained over time. It has been suggested that limited soil N availability could constrain the magnitude of CO\textsubscript{2}-induced biomass accumulation in terrestrial ecosystems (Diaz \textit{et al.} 1993; Hu \textit{et al.} 1999; Hu \textit{et al.} 2001; Oren \textit{et al.} 2001; Luo \textit{et al.} 2004; Reich \textit{et al.} 2006), because plants in many natural and semi-natural ecosystems are generally N-limited (Vitousek & Howarth 1991). Soil N availability has been observed to decrease under elevated CO\textsubscript{2} in a number of previous studies (Hungate \textit{et al.} 1999; Hu \textit{et al.} 2001; Williams \textit{et al.} 2001; Finzi & Schlesinger 2003; Schneider \textit{et al.} 2004; Billings & Ziegler 2005; Reich \textit{et al.} 2006). However, plants under elevated CO\textsubscript{2} may also evolve a suite of strategies to offset the influences of N limitation on plant growth such as increasing plant N use efficiency (Drake \textit{et al.} 1997), producing a larger fine-root system to capture N from wider or deeper soil volumes (Zak \textit{et al.} 2000; McKinley \textit{et al.} 2009), and enhancing N release from organic pools via stimulation of microbial activities (Zak \textit{et al.} 1993; Hungate \textit{et al.} 1999; Dijkstra \textit{et al.} 2008). Arbuscular mycorrhizal fungi (AMF), which form mutualistic associations with more than 80\% of land plants, could also be important, as AMF play a vital role in assisting plant N acquisition (Ames \textit{et al.} 1983; Johansen \textit{et al.} 1992; Hawkins \textit{et al.} 2000; Hodge \textit{et al.} 2001; Govindarajulu \textit{et al.} 2005; Tanaka & Yano 2005; Tu \textit{et al.} 2006; Leigh \textit{et al.} 2009).

It has been well documented that plants grown under CO\textsubscript{2} enrichment often allocate to their roots and associated mycorrhizal fungi (Hungate \textit{et al.} 1997; Fitter \textit{et al.} 2000),
which stimulates fungal growth (Treseder 2004; Alberton et al. 2005). In a meta-analysis based on 72 field and microcosm experiments, Alberton et al. (2005) showed that elevated CO₂ caused an average increase by 21% in AM fungal abundance. The CO₂-enhancement of AM fungi has led to the proposition that mycorrhizal fungi may facilitate plant N uptake, partially offsetting N limitation on plant growth responses to elevated CO₂ (Luo et al. 2004). But experimental evidence explicitly examining mycorrhizal mediation of plant N uptake under elevated CO₂ is still lacking. Several studies have investigated the role of AMF in plant N acquisition indirectly by comparing N contents in mycorrhizal and non-mycorrhizal plants or only compared N uptake by mycorrhizal plants with different CO₂ treatments (Syvertsen & Graham 1999; Gavito et al. 2000; Gamper et al. 2005; Chen et al. 2007). Yet the results are inconclusive and the underlying mechanisms remain unclear.

Most N in soils exists in the complex organic molecules, which cannot be utilized directly by plants roots. It has been shown that AMF could have the ability to directly mine N from decomposing organic material (Hodge et al. 2001; Leigh et al. 2009). Likely, AMF under elevated CO₂ may assist plant N acquisition from the vast organic N pools. So far, there is little evidence to support this hypothesis most likely because common techniques utilized in most CO₂ research can not differentiate and quantify the mycorrhizal contribution of plant N uptake from organic pools. In the previous chapter (Chapter III), by combining dual $^{13}$C/$^{15}$N labeling and hyphae ingrowth techniques, we demonstrated that plants treated with an AM fungal community can acquire more N from decomposing plant residues under elevated CO₂. However, AMF communities can also be changed under elevated CO₂.
(Klironomos et al. 1998; Wolf et al. 2003), which may further induce subsequent changes in mycorrhizal functioning (Gamper et al. 2005; Johnson et al. 2005). For instance, Klironomos et al. (1998) reported that elevated CO$_2$ increased the percentage of arbuscular and hyphal colonization on roots of *Artemisia tridentate* plants inoculated with either *Glomus intraradices* or *G. etunicatum*, but not those inoculated by *Acaulospora enticulata* or *Scutellospora calospora*. If different AMF species differed in their ability to acquire N from organic N pools, mycorrhizal contribution of plant N acquisition would be changed under a shifted AMF community structure in future climate change scenarios. To date, very few studies have examined the interspecific variation in mycorrhizal mediation of plant N uptake under elevated CO$_2$.

In contrast, elevated O$_3$ often reduces C allocation belowground in O$_3$-sensitive plants (Andersen 2003), potentially suppressing mycorrhizal fungal growth (Perezsoba et al. 1995; Yoshida et al. 2001; Andersen 2003) and altering the fungal community composition (Andersen & Rygiewicz 1995; Cairney & Meharg 1999; Andersen 2003). For example, AM fungal colonization on roots of *Elymus glaucus* decreased approximately 30% under elevated O$_3$ in a perennial grassland (Yoshida et al. 2001). Mycorrhizal fungi has also been found to reduce plant N uptake under higher O$_3$ concentrations (Haberer et al. 2007). Again, few studies have carefully assessed differences in AM fungal species’ contribution to plant N acquisition under elevated O$_3$.

We initiated a microcosm experiment examining the influences of elevated CO$_2$ and O$_3$ on mycorrhizal mediation of plant N acquisition using different AMF species treatments.
and *Avena fatua* L. (wild oats) as the model plant. *A. fatua*, native to Eurasia, was introduced into California 200 years ago (Talbot *et al.* 1939) and now is considered one of the worst annual weeds in agricultural fields and an invasive species in many temperate grasslands (Thurston & Phillipson 1976; Cousens *et al.* 1991; Malmstrom *et al.* 2005). In general, *A. fatua* is readily colonized by AM fungi and responsive to climate change factors such as atmospheric CO$_2$ enrichment (Hu *et al.* 2005; Menge & Field 2007). *A. fatua* plants typically have high tolerance to environmental stresses (Cousens *et al.* 1991; Malmstrom *et al.* 2005), but it is not known whether they are tolerant to elevated O$_3$ concentrations. We hypothesized that 1) AMF species differ in their ability to acquire N from soil and decomposing organic residues, 2) elevated CO$_2$ enhances, but elevated O$_3$ reduces the mycorrhizal contribution to plant N acquisition with the magnitude of gas effects varying among distinct AMF species.

4.3 Methods

This experiment was conducted in the USDA Air-Quality CO$_2$ facility at North Carolina State University, Raleigh, NC, USA. The facility includes a $9 \times 12$ m bay holding 20 continuously stirred tank reactors (CSTR) for exposure of plants to CO$_2$ and O$_3$. The CSTR (1.2 m diameter $\times$ 1.4 m tall), designed for rapid gas mixing and full light penetration, is a cylindrical chamber enclosed with transparent materials. To lower ambient O$_3$ concentrations, a blower system was used to supply a constant flow of charcoal filtered air through each CSTR. The air in each CSTR was continuously replaced to reduce the heating effects of chambers. For CSTRs assigned to an elevated CO$_2$ treatment, compressed CO$_2$ was
mixed with air and dispensed to chambers 24 hours daily using rotometers to maintain CO₂ concentrations at a target level. Ozone was generated by electrostatic discharge in dry O₂ (model GTC-1A, Ozonia North America, Elmwood Park, NJ, USA) and dispensed to chambers 12 hours daily (08:00-20:00 EST). To monitor CO₂ and O₃ concentrations, a small quantity of gas was extracted from each CSTR and directed through Teflon lines into an adjacent laboratory using computer-activated solenoid valves, and then CO₂ and O₃ were measured automatically using either an infrared analyzer (model 6252, LiCor Inc., Lincoln, NE, USA) and a UV photometric O₃ analyzer (model 49, Thermo Environmental Instruments Co., Franklin, MA, USA) for every two minutes, respectively.

**Experimental design and treatments**

This study was set up using a split-plot design with whole-plots in a RCBD (randomized complete block design). The whole-plots were three gas treatments (ambient control, elevated O₃ and elevated CO₂) with four replicates; the split-plot factors were six different AM fungal species treatments (Table 4.1). Plants were exposed to charcoal-filtered air (CF, 20 nmol O₃ mol⁻¹ + 380 µmol CO₂ mol⁻¹), CF air plus 60 nmol O₃ mol⁻¹ and CF air plus 200 µmol CO₂ mol⁻¹, which correspond to ambient control, elevated O₃ and elevated CO₂ treatments, respectively. Twelve CSTR chambers were blocked into four blocks with three gas treatments randomly assigning to each CSTR chamber within each block.
Hyphae ingrowth cores

A hyphae ingrowth core was used to determine the effect of AM fungi on plant acquisition of $^{15}\text{N}$ from decomposing plant residues. Hyphae ingrowth cores were constructed by cutting PVC pipe (4 cm inner diameter) into cylinders 10 cm tall. The bottom of the cylinder was sealed with a plastic cover. Two windows totaling 50% of the surface area of the cylinder were opened in two sides and then were covered with a nylon mesh (Tetko/Sefar MESH, Sefar America, NY, USA). The nylon mesh had a pore size of 20 μm, allowing fungal hyphae rather than plant roots to penetrate. Ingrowth cores were placed in the center of containers (15 cm in diameter, 13 cm in depth), which were filled with 2.0 kg soil mixture of steamed sandy loam soil and steamed quartz sand (1:1 w/w) as a plant growth medium. Two hundred grams (200.0g) of the same soil mixture were thoroughly mixed with 2.00 g of $^{15}\text{N}$ enriched shoot tissues of Panicum virgatum (ca. 1 cm length), and added to the ingrowth cores. The initial N content and $^{15}\text{N}$ enrichment (i.e. the value of $\delta^{15}\text{N}$) of plant residues were 0.7% and 13240‰, respectively. The concentrations of total N and extractable inorganic N of the soil mixture were 230 and 6.4 mg per kg soil, respectively.

AM fungal species

To determine the interspecific differences in mycorrhizal mediation of plant N acquisition under elevated CO$_2$ and O$_3$, three AMF species (A. morroriae, Gi. margarita, and G. clarum) were chosen as single species treatments. Two putative AMF communities with different species composition (see Table 4.1) were also included. Each container
associated with the corresponding fungal species treatment was inoculated with 80.0 g of soil inoculums containing the corresponding fungal species. The non-mycorrhizal control also received 80.0 g of autoclaved soil inoculums to correct potential differences in soil nutrient level and 50 ml of filtered washing of soil inoculum (without mycorrhizal propagules) to adjust possible differences in microbial communities (Koide & Li 1989). The AM fungal species were trap-cultured from a field at Goldsboro, NC, USA, and were then pot-cultured in the greenhouse (Sylvia 1994).

*Plant material and harvest*

Twenty-four seeds of *Avena fatua* L. (S&S Seeds, Inc., Carpinteria, CA) were sown into each container and then thinned after emergence so that each pot had 18 healthy plants. Gas fumigation started immediately following seed germination. Before seedling emergence, each compartment received 50 ml low-phosphorus Hoagland nutrient solution containing 5 mg N kg⁻¹ soil. During the growing season, mineral N of 12.5 and 5 mg N kg⁻¹ soil (NH₄NO₃) was then added at the 3th and 6th week, respectively. Both containers and ingrowth cores were watered with distilled water daily. The plants were allowed to grow for 10 weeks, and then above-ground plant parts were cut to the soil surface at the final harvest. Roots were carefully separated from the growth medium and washed several times using tap waters, and a subsample of fresh roots was sampled for examination of mycorrhizal colonization on plant roots.
Measurements and analyses

Gas exchange measurement. Net photosynthesis was determined in situ using a portable photosynthesis system (Model 6400, Li-Cor Inc. Lincoln, NE, USA). Measurements were made on the second fully expanded leaf down from the top of the plant canopy on three plants per container per chamber between 10:30 and 13:30 h (EST) when ambient photosynthetic photon flux density was larger than 1000 µmol m\(^{-2}\) s\(^{-1}\) at the seventh week. Due to the time limitation for making the measurements, net photosynthesis was only measured in three mycorrhizal fungal treatments (non mycorrhizal control, one single species treatment A. morrorrae, and a multiple species treatment A. morrorrae + Gi. margarita + G. clarum) for each chamber. Net photosynthetic rate of leaves was measured at 580 µmol CO\(_2\) mol\(^{-1}\) in elevated CO\(_2\) chambers, and at 380 µmol CO\(_2\) mol\(^{-1}\) for both elevated O\(_3\) and ambient control chambers. The CO\(_2\) concentration was controlled by the Li-Cor CO\(_2\) injection system, and leaf temperature was maintained at around 28 °C.

Mycorrhizal colonization. A subsample of approximately 0.5 g of fresh root segments was taken randomly and cleared in 5% (w/v) KOH, acidified in 1% (v/v) HCl, and then stained with the acidic glycerol-trypa blue solution. The stained roots were used to examine the percentage of fungal colonization using the gridline-intersect method (Giovannetti & Mosse 1980). In brief, stained root segments were evenly dispersed on a Petri dish with gridlines and the total number of infected and non-infected root segments was counted at 40 × using a dissecting microscope.

Soil extractable inorganic N. Soil extractable total inorganic N was estimated using
the method described previously (Hart et al. 1994). Twenty grams (20.0) of dry weight equivalent soil subsamples were weighed and extracted using 1 M KCl solution. The concentrations of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) in the extracts were determined on a Lachat flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA). The total amount of inorganic N was reported by combining \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) concentrations.

**Plant biomass, shoot N and \( ^{15} \text{N} \).** Root and shoot biomass were determined after drying at 70 °C for 48 hours. Shoots were then cut into pieces and ground into fine powder using an 8000-D Mixer Mill (SPEx CertiPrep Inc. Metuchen, NJ, USA). Shoot N concentrations and N isotope ratios (\( ^{14} \text{N} \) and \( ^{15} \text{N} \)) were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at University of California, Davis. Isotope ratios (\( \delta \)) were expressed by

\[
\delta^{15} \text{N} (\%o) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1,000 \tag{1}
\]

where \( R_{\text{sample}} \) is the molar isotope ratio of \( ^{15} \text{N}/^{14} \text{N} \) in samples and \( R_{\text{standard}} \) is the ratio \( ^{15} \text{N}/^{14} \text{N} \) in atmospheric N\(_2\), which is 0.0036765. Sample \( ^{15} \text{N} \) contents were calculated from isotope ratios and total N content (Hu et al. 2001). The mycorrhizally-enhanced shoot \( ^{15} \text{N} \) acquisition was calculated using total \( ^{15} \text{N} \) contents in mycorrhizal samples minus those in non-mycorrhizal samples.
Statistical analyses

Data were subjected to analysis of variance using the Mixed Model procedure of SAS (Littell et al. 2006) with block × gas as a random part, block, gas, AMF, and gas × AMF as fixed effects. A least significant difference (LSD) test was also run on all data. The significant differences were determined at the 95% probability level. Statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC, USA).

4.4 Results

Leaf photosynthesis and plant biomass

Elevated CO\textsubscript{2} significantly stimulated net photosynthetic rate of leaves of *Avena fatua*, leading to an average increase by 66% (Fig. 4.1 and Table 4.2). Correspondingly, plant production was significantly higher under elevated CO\textsubscript{2} than ambient control, with an average increase by 46% and 41%, respectively, in shoot and root biomass (Fig. 4.2 and Table 4.2). In addition, the interaction between CO\textsubscript{2} and AMF was significant for net photosynthetic rate of plant leaves, but not for plant biomass (Table 4.2). However, both leaf photosynthesis and plant biomass remained unaffected by elevated O\textsubscript{3} (Fig. 4.1, Fig. 2.2 and Table 4.2).

Plants inoculated with either a single AMF species (*A. morrorriae*; 31%; \(P = 0.001\)) or multiple AMF species (*A. morrorriae* + *Gi. margarita* + *G. clarum*; 46%; \(P < 0.0001\)) had significantly higher rates of net photosynthesis than those in non-mycorrhizal control (Fig. 4.1). Both aboveground and belowground biomass also varied significantly among AMF
species treatments (Fig. 4.2, Table 4.2).

_Mycorrhizal colonization_

Compared to the ambient control, elevated CO$_2$ increased mycorrhizal colonization of roots, on average, by 22% (Fig. 4.3, $P = 0.039$). In particular, elevated CO$_2$ caused an increase of 32% in root colonization by the fungus _Gi. margarita_; this relative CO$_2$ effect was larger than those of every other four AMF species treatments (Fig. 4.3). However, neither the O$_3$ effect nor the O$_3$ × AMF interaction was significant (Fig. 4.3, Table 4.2).

The extent of root colonization also differed significantly among AMF species treatments (Fig. 4.3, Table 4.3). Fungus _G. clarum_ had the largest mean percentage of root colonization (52%) and _Gi. margarita_ had the lowest (5%). Root colonization with multiple AMF species treatments was lower than species _G. clarum_, but was significantly larger than those with _A. morrorriae_ and _Gi. margarita_ (Fig. 4.3).

_Total soil extractable inorganic N_

Total soil extractable inorganic N at elevated CO$_2$ decreased significantly by nearly 32% across all AMF species treatments, but remained unchanged at elevated O$_3$ in comparison with the ambient control (Fig. 4.4 and Table 4.2). Neither the CO$_2$ × AMF nor the O$_3$ × AMF interaction was significant for soil extractable N (Table 4.2). Fungal species significantly affected soil extractable N (Fig. 4.4 and Table 4.2). Soil extractable N under species _Gi. margarita_ remained unchanged, but was reduced significantly under the other...
four AMF species treatments in comparison with non-mycorrhizal control (Fig. 4.4 and Table 4.3).

Plant N and N uptake

Compared to the ambient control, elevated CO\(_2\) reduced the concentrations of shoot N by 21% across all AMF species treatments (Fig. 4.5a and Table 4.2), but enhanced total shoot biomass N by 16% (Fig. 4.5b and Table 4.2). The CO\(_2\) × AMF interaction was significant for the shoot biomass N, but not for the shoot N concentrations (Table 4.2). Elevated O\(_3\) had no impact on either shoot N concentrations or shoot biomass N (Fig. 4.5 and Table 4.2).

Both shoot N concentrations and shoot biomass N varied significantly among AMF species treatments (Fig. 4.5 and Table 4.2). However, the pattern of shoot N concentrations under AMF species treatments differed from that of shoot biomass N (Fig. 4.5). Compared to the non-mycorrhizal control, shoot N concentrations remained unchanged with *Gi. margarita*, but decreased significantly in all other four AMF species treatments (Table 4.3). Except for the single species treatment *A. morrorriae*, however, shoot biomass N increased significantly in all AMF species treatments compared to the non-mycorrhizal treatment (Table 4.3).

Mycorrhizally-enhanced plant \(^{15}\)N uptake

On average, elevated CO\(_2\) significantly increased mycorrhizally-enhanced shoot \(^{15}\)N uptake by 27% in comparison to the ambient control (Fig. 4.6 and Table 4.2). Furthermore,
the CO₂ × AMF interaction was significant for mycorrhizally-enhanced shoot ¹⁵N uptake (Table 4.2). It is surprising to note that mycorrhizally-enhanced shoot ¹⁵N uptake under the single species treatment Gi. margarita was 114% higher under elevated CO₂ than the ambient control (Fig. 4.6, P = 0.008). Similar to other parameters, elevated O₃ had no significant impact on mycorrhizally-enhanced shoot ¹⁵N uptake (Fig. 4.6 and Table 4.2).

The mycorrhizally-enhanced shoot ¹⁵N uptake also differed significantly among five AMF species treatments (Fig. 4.6 and Table 4.2) with the fungus Gi. margarita contributing the largest amount of ¹⁵N to plants and A. morrorriae the least.

4.5 Discussion

It has long been recognized that AMF can transfer substantial amounts of inorganic and simple organic N (e.g. amino acids) from soil to their host plants (Ames et al. 1983; Johansen et al. 1992; Hawkins et al. 2000; Govindarajulu et al. 2005; Tanaka & Yano 2005; Tu et al. 2006). This phenomenon was also observed in the current study, as most AMF species treatments significantly reduced soil extractable inorganic N (Fig. 4.4 and Table 4.3). The relatively high soil extractable inorganic N under Gi. margarita (Fig. 4.4) seems to suggest that Gi. margarita has a low capability to obtain N in the inorganic N pool. However, both the concentration and the total N contents of shoots under Gi. margarita were higher than under other AMF species treatments (Fig. 4.5), suggesting that Gi. margarita may employ different strategies other than simple N uptake from the soluble N pool. Traditionally, AMF have been considered obligate biotrophs and are unable to utilize N
directly from complex organic molecules (Smith & Read 1997; Read & Perez-Moreno 2003). Results from an experiment by Hodge et al. (2001), however, indicated that AM fungus, *G. hoi* might have saprotrophic capability and could directly acquire N from decomposing grass leaves. A recent study showed that N uptake from a plant residue patch by two *Glomus* species *G. hoi* and *G. intraradices* can account for nearly 20% of total plant N (Leigh et al. 2009). In most natural ecosystems, however, AMF communities consist of multiple AMF species which often belong to different AMF families (Johnson et al. 1992; Rosendahl 2008). Results presented in this study showed that plants inoculated with either single AMF species or multiple species treatments can also acquire N directly from degrading plant residues (Fig. 4.6), suggesting that this function might be common among all AMF.

It is interesting to note that the mycorrhizal contribution of plant N uptake evidently was not related to mycorrhizal colonization in this study (Fig. 4.3 and Fig. 4.5b). Much evidence has demonstrated that the mycorrhizal contribution of plant phosphorus (P) acquisition and plant growth is not correlated with the extent of AM colonization on plant roots (McGonigle & Fitter 1988; Fitter et al. 2000; Smith et al. 2004). Using compartmented pots and $^{33}$P, Smith et al. (2004) showed that mycorrhizal P uptake might be related to the development and effectiveness of the external hyphae in acquiring P and their effect on P transporter expression, but not to mycorrhizal colonization. It has been reported that there is a strong positive relationship between hyphal length density and mycorrhizal N uptake from decomposing organic residues (Hodge et al. 2001). We speculate that differences in hyphal length density and the efficiency of external hyphae in absorbing N might be possible reasons

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explaining the disparities in N uptake among different AMF treatments in the current study. Experimental evidence has clearly established that external hyphae account for the majority of fungal biomass of *Gigaspora* spp., while most fungal biomass in *Glomus* spp. exist in internal hyphae (hyphae growing inside the root) (Hart & Reader 2002; Maherali & Klironomos 2007). Results that *G. clarum* had high and *Gi. margarita* low root colonization (Fig. 4.3) agree very well with the two previous observations, thus suggesting that *G. clarum* might produce low, while *Gi. margarita* yield high external fungal biomass.

Probably, observed variation in AMF species’ contribution to plant N uptake at elevated CO$_2$ (Fig. 4.5b) might also be a result of CO$_2$-led changes in growth and activities of fungal external hyphae. Compared to other AMF species treatments, the soluble inorganic N pool might contribute less to the CO$_2$-led increase in plant N uptake under *Gi. margarita*, as the concentrations of extractable inorganic N in both ambient and elevated CO$_2$ under *Gi. margarita* were higher than under other AMF species (Fig. 4.4). Higher mycorrhizal-enhanced shoot $^{15}$N uptake under *Gi. margarita* than under other AMF species (Fig. 4.6) suggests that *Gi. margarita* might contribute more to the CO$_2$-led increases in plant N acquisition from decomposing organic materials. We conceive that two major mechanisms might account for the CO$_2$-led variation in mycorrhizal contribution to plant N acquisition observed in the current study. First, some AMF might have saprotrophic capability (Hodge et al. 2001) but not all species have equal capabilities under elevated CO$_2$. The second approach may be due to the mycospheric priming effect that the CO$_2$-stimulation of fungal external hyphae (Sanders et al. 1998) can export a large number of easily degradable
compounds, thereby fueling the activities of soil decomposers and in turn increasing mineralization of organic N. Thus, either the CO$_2$-induced changes in the quantity of mineralized N or the subsequent hyphal N transfer could result in variation in plant N acquisition. Clearly, both hypotheses remain to be investigated further.

The findings that different AMF species under elevated CO$_2$ differed in their ability to utilize organic N pools may have important implications in the global N cycling under future CO$_2$ scenarios. A central theme in the global change ecology is whether plant growth will be limited by soil nutrients under future CO$_2$ scenarios. Experimental evidence has shown that forest and grassland ecosystems could be constrained by limited soil N availability under CO$_2$ enrichment conditions (Oren et al. 2001; Finzi & Schlesinger 2003; Reich et al. 2006). What remains unknown is whether AMF under elevated CO$_2$ could assist plant N uptake, particularly from organic N pools, partially alleviating the N limitation on plant responses to elevated CO$_2$ (Luo et al. 2004; Hu et al. 2006; Tu et al. 2006). Results obtained in this study (Fig. 4.6) indicate that some AMF species such as *Gi margarita* may increase plant N acquisition from organic N pools, but others such as *A. morrorriae* and *G. clarum* may not. Under future CO$_2$ scenarios, AMF communities could be altered (Klironomos et al. 1998; Wolf et al. 2003; Gamper et al. 2005; Johnson et al. 2005). Thus, whether AMF could alleviate the N limitation on plant responses to elevated CO$_2$ in a large part rests on the changes in the AMF community structure. Results from two multiple species treatments (Fig. 4.6) suggest that if AMF communities were dominated with species with low efficiency in acquiring N from organic pools like *A. morrorriae* and *G. clarum*, they would
be less effective in offsetting the N limitation on plant responses to elevated CO$_2$ in many natural and semi-natural ecosystems. Only where species with high capabilities in acquiring N from organic pools such as *Gi margarita* dominated in natural AMF communities of many ecosystems, plants under future CO$_2$ scenarios might be less constrained by limited soil N availability.

In contrast to our expectations, however, neither plant growth nor nutrient uptake was responsive to elevated O$_3$. The lack of plant growth response to elevated O$_3$ was likely a consequence of the unresponsiveness of plant photosynthesis to higher O$_3$ concentrations. Elevated tropospheric O$_3$ often induces a series of oxidative reactions in leaf apoplastic and symplastic spaces, leading to a repression of photosynthesis or even cell death (Pell *et al.* 1997; Rao & Davis 2001; Fiscus *et al.* 2005; Booker *et al.* 2009). The lack of O$_3$ effects on *A. fatua* suggests that plants must have the ability to limit the ozone-induced damage to their leaves. In other words, *A. fatua* plants used for this study might be O$_3$-tolerant under the concentrations of 80 ppb O$_3$ in the current study. The ozone tolerance of *A. fatua* plants might be related to the source of seeds. The seeds used in the present study were collected from a wild population in California, USA, where the historically high ambient O$_3$ may select O$_3$-insensitive genotypes. Those ozone-tolerant plants may produce large amounts of antioxidants such as ascorbic acid and glutathione to remove O$_3$ or other oxidative molecules (Burkey 1999; Burkey & Eason 2002; Fiscus *et al.* 2005; Booker *et al.* 2009). For example, Burkey *et al.* (2002) found that enriched ascorbic acid in the leaf apoplast was a major mechanism responsible for the O$_3$ tolerance in a genotype of *Phaseolus vulgaris*. 
Mycorrhizal fungi may increase the performance of plants under environmental stresses by stimulating plant nutrient uptake (Smith & Read 1997; Bai et al. 2008). However, both plant N (Fig. 4.5b) and P (data not shown) uptake also remained unchanged under elevated O₃ in different AMF species treatments, suggesting that the observed O₃ tolerance of A. fatua in this study might be the cause rather than consequence of the lack of mycorrhizal responses to elevated O₃.

In summary, results obtained in this study show that elevated CO₂ and AMF had an interactive impact on total shoot biomass N and shoot ^1⁵N acquisition from decomposing organic residues, but not on soil extractable total inorganic N. Different AMF species showed distinct capabilities in acquiring N with Gi. margarita having higher efficiency in taking up N from decomposing organic materials, but lower efficiency in absorbing N from the soluble inorganic N pool than A. morrorriae and G. clarum. However, both plant growth and N uptake under AMF species treatments remained unchanged under elevated O₃, suggesting that A. fatua plants may be tolerant to higher O₃ concentrations. Our findings suggest that future research should not overlook any changes in the AMF community composition by climate change and their potential feedback to plant growth.

4.6 References


4.7 Tables

Table 4.1 AM fungal species treatments.

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<th>Species</th>
<th>AMF treatments</th>
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<tr>
<td>G. rosea</td>
<td></td>
</tr>
<tr>
<td>Scutellospora pellucida</td>
<td></td>
</tr>
<tr>
<td>S. heterogama</td>
<td></td>
</tr>
<tr>
<td><strong>Family Glomeraceae</strong></td>
<td></td>
</tr>
<tr>
<td>Glomus clarum</td>
<td></td>
</tr>
<tr>
<td>G. mosseae</td>
<td></td>
</tr>
</tbody>
</table>

The non-mycorrhizal control (N-AMF) received 80.0 g of autoclaved soil inoculum consisting of eight fungal species, and 50 ml filtered washing of soil inoculum without mycorrhizal propagules. Each single species treatment received 80.0 g soil inoculum. The three species treatments (A + Gi + G) were inoculated with 26.7 g of A. morroriae, Gi. margarita, and G. clarum soil inocula, respectively. The eight species treatment included 10.0 g soil inoculum of each species. *E denotes five additional fungal species except for A. morroriae, Gi. margarita, and G. clarum.
Table 4.2 P-values of analysis of variance of CO$_2$, O$_3$, AMF and their interactive effects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CO$_2$</th>
<th>O$_3$</th>
<th>AMF</th>
<th>CO$_2$ × AMF</th>
<th>O$_3$ × AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net photosynthesis</td>
<td><strong>0.0002</strong></td>
<td>0.8870</td>
<td>&lt; <strong>0.0001</strong></td>
<td><strong>0.0323</strong></td>
<td>0.0624</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.9866</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.1420</td>
<td>0.6181</td>
</tr>
<tr>
<td>Root biomass</td>
<td><strong>0.0002</strong></td>
<td>0.9102</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.0743</td>
<td>0.1894</td>
</tr>
<tr>
<td>AM colonization</td>
<td><strong>0.0394</strong></td>
<td>0.1499</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.3200</td>
<td>0.0534</td>
</tr>
<tr>
<td>Soil extractable N</td>
<td><strong>0.0091</strong></td>
<td>0.6165</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.7650</td>
<td>0.9805</td>
</tr>
<tr>
<td>Shoot [N]</td>
<td><strong>0.0004</strong></td>
<td>0.6105</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.5860</td>
<td>0.3997</td>
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<tr>
<td>Shoot biomass N</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.7383</td>
<td>&lt; <strong>0.0001</strong></td>
<td><strong>0.0346</strong></td>
<td>0.6769</td>
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<tr>
<td>Net $^{15}$N uptake</td>
<td>0.3673</td>
<td>0.6858</td>
<td>&lt; <strong>0.0001</strong></td>
<td><strong>0.0426</strong></td>
<td>0.5623</td>
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</tbody>
</table>

Significant effects ($P \leq 0.05$) are shown in bold text.
**Table 4.3** Comparisons among AMF species treatments.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Net photosynthesis</td>
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<td>b</td>
<td></td>
<td>b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoot biomass</td>
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<td>c</td>
<td>b</td>
<td>c</td>
<td>cd</td>
<td>d</td>
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<tr>
<td>Root biomass</td>
<td>a</td>
<td>c</td>
<td>b</td>
<td>d</td>
<td>cd</td>
<td>b</td>
</tr>
<tr>
<td>AM colonization</td>
<td></td>
<td>b</td>
<td>a</td>
<td>d</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>Soil extractable N</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Plant [N]</td>
<td>c</td>
<td>a</td>
<td>c</td>
<td>b</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Shoot biomass N</td>
<td>a</td>
<td>a</td>
<td>c</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Net $^{15}$N uptake</td>
<td>a</td>
<td>c</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

Different letters for each variable represent statistically significant differences ($P \leq 0.05$) after an analysis of variance. Abbreviations of AMF species treatments are shown in Table 1.
4.8 Figures

**Figure 4.1** Effects of AMF species, elevated CO$_2$ and O$_3$ on net photosynthesis of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species. Other species treatments were not measured due to the time limitation.

**Figure 4.2** Effects of AMF species, elevated CO$_2$ and O$_3$ on plant shoot (a) and root (b) biomass of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.

**Figure 4.3** Effects of AMF species, elevated CO$_2$ and O$_3$ on AM colonization on roots of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.

**Figure 4.4** Effects of AMF species, elevated CO$_2$ and O$_3$ on total soil extractable inorganic N. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.

**Figure 4.5** Effects of AMF species, elevated CO$_2$ and O$_3$ on shoot N concentrations (a) and total shoot N contents (b). Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
**Figure 4.6** Effects of AMF species, elevated CO$_2$ and O$_3$ on mycorrhizally-mediated $^{15}$N acquisition of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
Figure 4.1 Effects of AMF species, elevated CO\(_2\) and O\(_3\) on net photosynthesis of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species. Other species treatments were not measured due to the time limitation.
Figure 4.2 Effects of AMF species, elevated CO$_2$ and O$_3$ on plant shoot (a) and root (b) biomass of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
Figure 4.3 Effects of AMF species, elevated CO$_2$ and O$_3$ on AM colonization on roots of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
Figure 4.4 Effects of AMF species, elevated CO$_2$ and O$_3$ on total soil extractable inorganic N. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
Figure 4.5 Effects of AMF species, elevated CO$_2$ and O$_3$ on shoot N concentrations (a) and total shoot N contents (b). Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.
Figure 4.6 Effects of AMF species, elevated CO$_2$ and O$_3$ on mycorrhizally-mediated $^{15}$N acquisition of *Avena fatua*. Data represent means (n=4) ± s.e.m. See Table 1 for abbreviations of AMF species.