AN ABSTRACT OF THE THESIS OF

Shannon M. Kimball for the degree of Master of Science in Botany presented on December 8, 1994. Title: The Effects of Ozone on the Growth, Rhizosphere Population Dynamics, and Mycorrhizal Colonization of Wheat.

Abstract approved:

Elaine R. Ingham

The influence of elevated ozone exposure on plant growth has been the subject of many studies. However, knowledge of ozone effects on the mycorrhizal colonization and rhizosphere population dynamics of exposed plants is limited.

The objectives of this study were to observe the effects of ozone on: 1) plant photosynthesis, and root and shoot growth, 2) mycorrhizal colonization of the experimental plant's root system, and 3) the population size and activity of bacteria and fungi, population size of protozoa and nematodes inhabiting the rhizosphere.

Spring wheat (*Triticum aestivum* L.) was exposed to ozone in controlled fumigation chambers within a greenhouse. An episodic ozone exposure profile was used, with ozone concentrations starting at a daily minimum of approximately 4 ng g^{-1} at 0500 hr, gradually increasing to an average maximum of approximately 120 ng g^{-1} by 1700 hr, then gradually decreasing to the original minimum concentration by 0500 hr the following day. Ozone concentration in control chambers was approximately 2 ng g^{-1}.

Two experiments were conducted. The plants in Experiment 1 were grown in a low nutrient, low organic matter soil. Ten plants were placed in each of four chambers: two chambers with additional ozone, and two control chambers. Five plants from each chamber were harvested 26 days after the initiation of exposure, and the remaining five plants in each chamber were harvested two weeks after the first harvest. The plants in

Experiment 2 were grown in soil high in organic matter and nutrients. Ten plants were placed in each of six chambers: two chambers with additional ozone, and two control chambers. All ten plants were harvested 35 days after the initiation of ozone exposure.

Results of Experiment 1 indicated that ozone did not influence plant photosynthesis, shoot, or root growth at either harvest date, when plants were grown in low nutrient, low organic matter soil . The activity and population size of bacteria and fungi, and the population size of cilliates and nematodes was not affected by ozone exposure at either harvest date. Compared to the controls, ozone caused an increase in numbers of flagellates and decrease in number of amoebae in the rhizosphere of exposed plants in the first harvest. A decrease in amoebae populations in the rhizosphere of ozone-exposed plants was also observed at the second harvest, but rhizosphere flagellate numbers were similar in control and ozone exposed plants. The standing crop of bacteria in the rhizosphere of plants exposed to ozone was reduced at the first harvest, but was similar to that of control plants at the second harvest. Below-ground respiration was higher in ozone-exposed plants, at the first harvest, but this difference was not observed at the second harvest. Vesicular-arbuscular mycorrhizal colonization was lower in the root system of ozone-exposed plants at both, the first and second harvest dates.

Results of Experiment 2 indicated that photosynthesis and the ratio of root:shoot dry tissue weight were lower in plants exposed to ozone, when plants were grown in high nutrient, high organic matter soil. Ozone caused an increase in dry weight of shoot tissue. Ozone did not affect the population size or activity of bacteria and fungi, or the population size of amoebae, cilliates or nematodes in the rhizosphere of exposed plants. However, a reduction in the number of flagellates in the rhizosphere of ozone-exposed plants was observed. The standing crop of rhizosphere bacterial biomass was not reduced by ozone exposure. Vesicular-arbuscular colonization was reduced in the root system of ozone-exposed plants.

The results show that ozone influences rhizosphere predator populations and mycorrhizal colonization, and the amount of nutrients and organic matter in the soil may affect the response of a plant and associated rhizosphere organisms to ozone. Changes in the population size of rhizosphere predators may significantly alter soil nutrient cycling, and reductions in rhizosphere predators may reduce nutrient availability for plants, because these organisms are responsible for a significant amount of nutrient mineralization. Reductions in mycorrhizal colonization may reduce the ability of mycorrhizal plants to obtain the nutrients and water they require for survival.

The long-term effects of ozone exposure on rhizosphere population dynamics must be further examined, to assess the influence of ozone on entire ecosystems that are at risk of being subjected to elevated levels of ambient ozone.

The Effects of Ozone on the Growth, Rhizosphere Population Dynamics, and Mycorrhizal Colonization of Wheat

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The Effects of Ozone on the Growth, Rhizosphere Population Dynamics, and Mycorrhizal Colonization of Wheat

1.0 Introduction

The concentration of ozone (O_3) in the troposphere of the earth has risen to potentially harmful levels in many areas due to accumulation of ozone precursor gasses, volatile organic compounds (VOC) and nitrogen oxides (NO_X) (U.S. Environmental Protection Agency, 1992, 1993; Aneja et al., 1993). Unlike stratospheric ozone concentrations, which are declining, concentrations of tropospheric ozone are increasing over many large regions of the United States. This increase has stimulated research concerning the effects of elevated ozone levels on a variety of responses, including plant health. Studies on plants indicate that ozone reduces plant productivity, alters carbon translocation to plant tissues, and may cause fatal injury at relatively high doses (Heagle et al., 1979; Heath, 1980; Heck et al., 1983; Reich and Amundson, 1985). Ozone may also affect the rhizosphere of an exposed plant, causing changes in the community structure of microorganisms that inhabit rhizosphere soil.

Bacteria, fungi, protozoa and nematodes form a complex foodweb in the soil around plant roots (Paul and Clark, 1990). Bacteria that feed on root exudate and sloughed material may be sensitive to alterations in the allocation of carbon to the root, as a result of ozone exposure. Changes in rhizosphere bacterial populations, particularly a decrease in number, may induce potentially harmful changes in the populations of other foodweb organisms. Since nutrient immobilization and mineralization are dependent on rhizosphere dynamics, these processes may be disrupted as an indirect result of elevated ambient ozone.

Mycorrhizal fungi form a mutualistic association with plant roots (Allen, 1991). Carbon from plant roots is provided to the mycorrhizal fungus, which the fungus uses for growth (Allen, 1991). In exchange for carbon, the fungus positively influences the host plant by increasing nutrient and water absorption, and disease resistance in the host plant

(Allen, 1991). Mycorrhizal fungi send fine, highly absorptive hyphae into the soil to absorb and transfer nutrients back to the host plant (Allen, 1991). A decrease in carbon allocation to root tissues, due to ozone exposure, may negatively impact the plant-fungus symbiosis. Consequently, lower levels of mycorrhizal colonization in roots of exposed plants may reduce the ability of an exposed plant to obtain nutrients, and reduce the plant's ability to compete with other plants, particularly non-mycorrhizae-requiring plants, for nutrients. For example, since most crop plants require mycorrhizae to obtain adequate nutrients for growth, the ability of crop plants to compete with weeds may be reduced without mycorrhizae (Weber et al., 1994).

The purpose of this investigation was to explore the below-ground changes that occurred in the rhizosphere of a plant exposed to an elevated level of ozone. The population size of active and total bacteria, active and total fungi, protozoa and nematodes were measured in the rhizosphere of both control (ambient ozone) plants and plants treated with elevated ozone. Colonization by mycorrhizal fungi was examined in the treated and control plants, to assess the effects of the treatment on this mutualistic symbiosis. Plant response to the ozone treatment was measured in terms of net photosynthesis, shoot and root biomass, and the ratio of root to shoot biomass. Spring wheat (*Triticum aestivum* L.) was used as the experimental plant because of its rapid growth, relative ozone sensitivity, and economic importance (Lehnherr et al., 1988; Farage et al., 1991).

The hypothesis that ozone exposure of wheat will decrease the population size of active bacteria, active fungi, protozoa and nematodes was tested. Mycorrhizal colonization in the roots of ozone-treated plants should also decrease. Net photosynthesis of plants exposed to ozone should decrease, along with root biomass, and the ratio of root to shoot biomass. Shoot biomass should be slightly lower in ozone exposed plants.

1.1 Tropospheric Ozone

Ozone is a highly reactive molecule, formed when localized emissions of nitric oxide and nitrogen dioxide (known together as NO_x), react with volatile organic compounds (VOCs) (Spicher et al., 1979). Ozone is formed excessively in and downwind of urban and industrialized areas. Motor vehicle exhaust, emissions from the use of solvents, and emissions from the chemical and petroleum industries are major sources of VOCs. NO_x is produced as a result of the combustion of fossil fuels. Motor vehicles and electricity generating stations are major sources of NO_x.

Surface ozone concentrations have been measured in various parts of the world since the late 1800s. Analysis of ozone levels measured 100 years ago in Paris indicated average hourly ozone concentrations of about 10 ng g^{-1}; currently, hourly ozone levels in the least polluted parts of Europe average between 20 and 45 ng g^{-1} (Volz and Kley, 1988; Janach, 1989). Ozone levels have been monitored in the United States in a variety of areas, ranging from fairly isolated locations to urban centers (Table 1). The National Ambient Air Quality Standards for Ozone, established by the Environmental Protection Agency in 1979, set the

Table 1. Highest second daily maximum eight-hour average ozone concentration in 1992 by metropolitan area (MA) (from U. S. Environmental Protection Agency, 1993).

MA	Ozone (ng/g)
Ann Arbor, Michigan	100
Indianapolis, Indiana	100
Los Angeles-Long Beach, California	178
Milwaukee, Wisconsin	118
Omaha, Nebraska	73
Philadelphia, Pennsylvania	123
Portland, Oregon	92
San Diego, California	128
Spokane, Washington	60

acceptable national ozone standard to "when the expected number of days per calendar year with maximum hourly average concentrations above 0.12ppm (120 ng g^{-1}) is equal to or less than one" (Federal Register, 1979). Hourly ozone concentrations typically fluctuate, reaching a maximum at mid-day. In Custer National Forest, Montana, and other areas characterized by low industrial activity, maximum hourly ozone concentrations were repeatedly measured at 70 ng g^{-1} over the period of one year, while the average hourly ozone concentration over the same period was 32 ng g^{-1} (Evans et. al., 1983). The maximum hourly ozone concentration per month, measured over a period of 2 years (1975-76), averaged 70 ng g^{-1} in White River, Utah (Singh et al., 1978). The average of all 1-h ozone values measured over this same time period was 37 ng g^{-1} (Singh, et al., 1978). Industrial activity is also low in the White River area. Maximum hourly ozone concentrations are notably higher in industrial areas. In and downwind of New York City, daily maximum ozone concentrations of 175-200 ng g^{-1} have been recorded (Spicher et. al., 1979). Due to the fact that ozone levels have been increasing over the last several decades these figures may be low estimates for current ozone concentrations. If high ozone concentrations have significant effects on biological systems, the formation of ozone in and around large urban areas, where ozone concentrations can reach
200-400 ng g^{-1}, represents the most critical aspect of the tropospheric ozone problem. Elevated ambient ozone concentrations in rural, agricultural areas may pose a threat to crop production, since ozone levels in these areas often exhibit higher sustained periods of elevated ozone concentrations than urban areas (Singh et al., 1978; Spicher et al., 1979).

1.2 Overview of Carbon Transport Through a C-3 Plant

The mechanism by which ozone induces damage to plant systems is complex (Saxe, 1991; Heath, 1980). Therefore, a review of the pattern of carbon transport through a plant under natural (no ozone) conditions is helpful in understanding the physiological alterations

that plants experience as a result of ozone exposure. The information presented in this section is more thoroughly described in general plant physiology texts, such as Salisbury and Ross (1992) or Tiaz and Zeiger (1991).

1.2.1 Carbon Fixation

Carbon fixation occurs when carbon dioxide enters the substomatal chamber, located in the mesophyll cell layer of a leaf. Carbon dioxide enters the chamber by moving through a stomatal opening in the leaf epidermis. The opening of stomata is regulated by osmotically controlled guard cells, which respond to physiological stimuli. Low levels of carbon dioxide within the substomatal chamber trigger a physiological change in the guard cells, causing them to swell and the stomata to open. Although diffusion of carbon dioxide into the leaf is beneficial, excessive water loss through open stomata (transpiration) may cause water stress. When leaves are subject to water stress there is a rise in abscisic acid in leaf tissues. This physiological change causes guard cells to shrink, closing stomata to prevent further water loss.

Carbon dioxide diffuses into leaf mesophyll cells and is incorporated into the three-carbon acid, 3-phospho-glyceric acid (3-PGA). The enzyme responsible for catalyzing this reaction, ribulose bisphosphate carboxylase (rubisco), adds carbon dioxide to the five-carbon substrate, ribulose-1,5-bisphosphate (RuBP) to form two 3-PGA. A molecule of 3-PGA may enter a series of reactions that form a cyclic pathway, collectively called the Calvin cycle, the photosynthetic carbon reduction cycle, or the C-3 photosynthetic pathway.

The Calvin cycle, which ultimately results in the regeneration of RuBP, involves three main phases: carboxylation, regeneration and reduction (Fig. 1). All three parts occur in the stroma of chloroplasts. Carboxylation, described above, consists of the addition of carbon dioxide and water to preexisting RuBP, to form two molecules of 3-

PGA, which is catalyzed by rubisco. Reduction involves the reduction of the carboxyl group in 3-PGA to an aldehyde group, forming 3-phosphoglyceraldehyde (3-PGaldehyde) and inorganic phosphate (P_i). RuBP may be regenerated from 3-PGaldehyde in the regeneration phase.

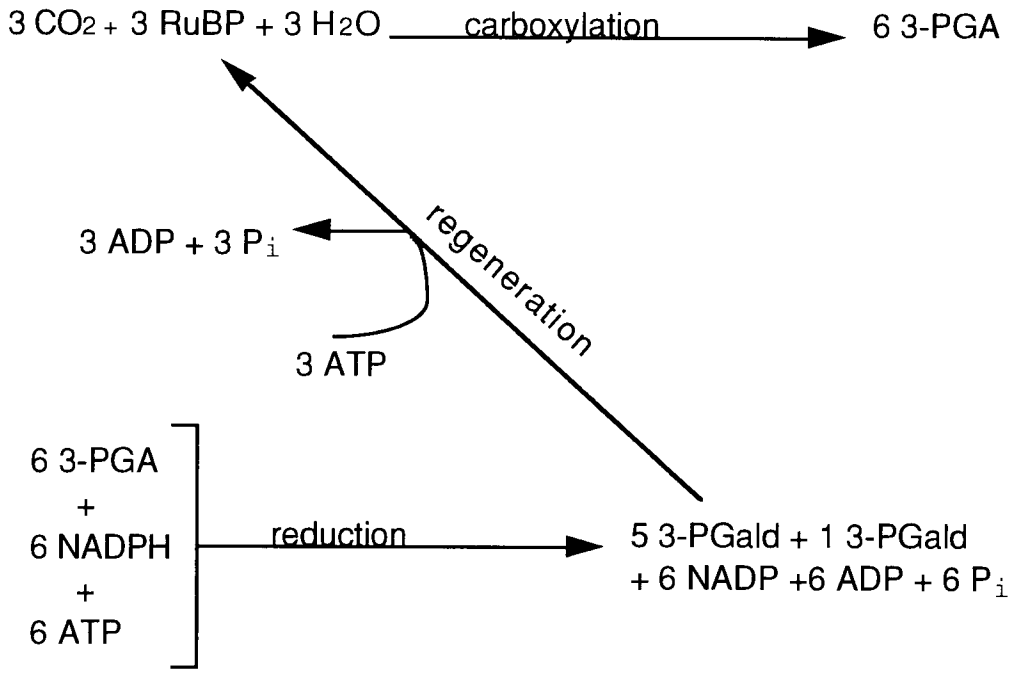


Fig. 1. General reactions of the Calvin cycle, emphasizing carboxylation, reduction, and regeneration (from Salisbury and Ross, 1992).

This phase is complex and involves the formation of several four-, five-, six- and seven-carbon phosphorylated sugar intermediates (Fig 2). Regenerated RuBP may also be used as a source of carbon for photorespiration, releasing carbon dioxide back into the atmosphere.

A carbon atom fixed and converted to 3-PGA, then reduced to 3-PGaldehyde, may take paths other than that of RuBP regeneration (Fig. 3). Some 3-PGaldehyde molecules may be used to form starch in chloroplasts. Other molecules of 3-PGaldehyde may be transported out of the chloroplasts, through an antiport carrier system, into the cytosol.

Molecules of 3-PGaldehyde may also be converted to dihydroxyacetone phosphate in the chloroplast. This compound may then be transported to the cytosol through the same antiport mechanism. Cytosolic 3-PGaldehyde and dihydroxyacetone phosphate are used to form mesophyll cell-wall polysaccharides or sucrose, some of which will be transported out of the mesophyll cell.

1.2.2 Carbohydrate Transport

Leaf mesophyll cells are located in close proximity to minor veins of the leaf phloem. Since sucrose need only pass through a maximum of two or three mesophyll cells before reaching a phloem transport cell (sieve element), export of sucrose to the phloem companion cells is highly efficient. Movement of sucrose within the mesophyll cell, through cytoplasmic streaming, accelerates sucrose transport from one cell to another. The transport between mesophyll cells is believed to be symplastic (through protoplasmic continuity between cells) or apoplastic (through cell wall connections outside of the protoplasm), depending on the plant species. Loading of sucrose into the phloem companion cells and sieve elements, referred to as "phloem loading", may occur through a variety of mechanisms, each resulting in phloem sap in which solutes are composed of ninety percent sucrose, as well as smaller amounts of amino acids, potassium, and trace amounts of several other sugars and elements (sodium, magnesium, calcium, iron, maganese, zinc, and copper).

Phloem loading is a selective process which actively transports sugars from mesophyll cells into companion cells, then into sieve elements through plasmodesmata via bulk flow (described below). Research has shown that the only sugars that accumulate in minor veins are those that are typically transported in the phloem, even when other sugars are added to leaf mesophyll cells, indicating selective transport is occurring (Salisbury and

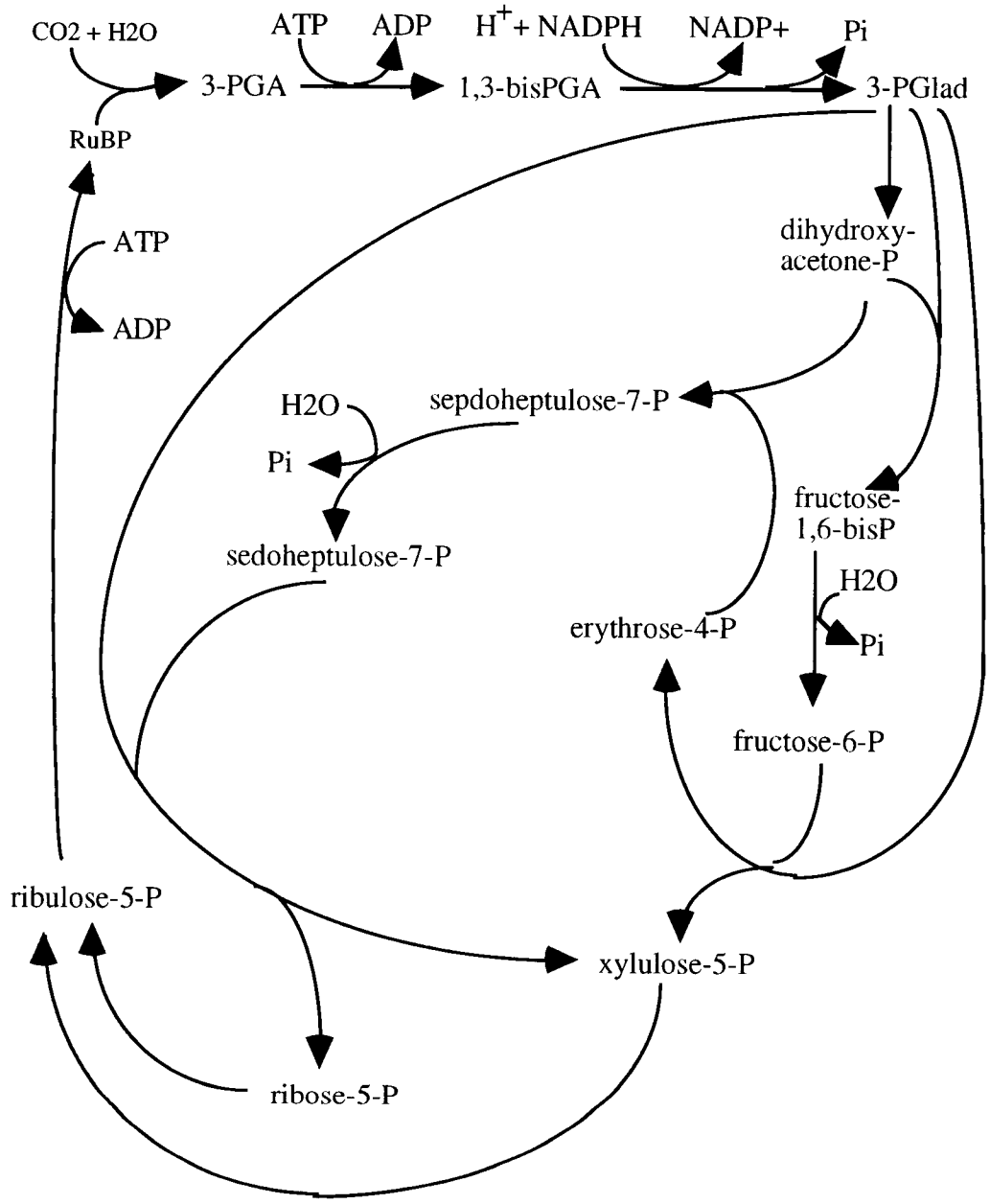


Fig. 2. Several 4-, 5-, 6-, and 7-carbon phosphorylated sugar intermediates are formed in the regeneration phase, which results in the regeneration of RuBP.

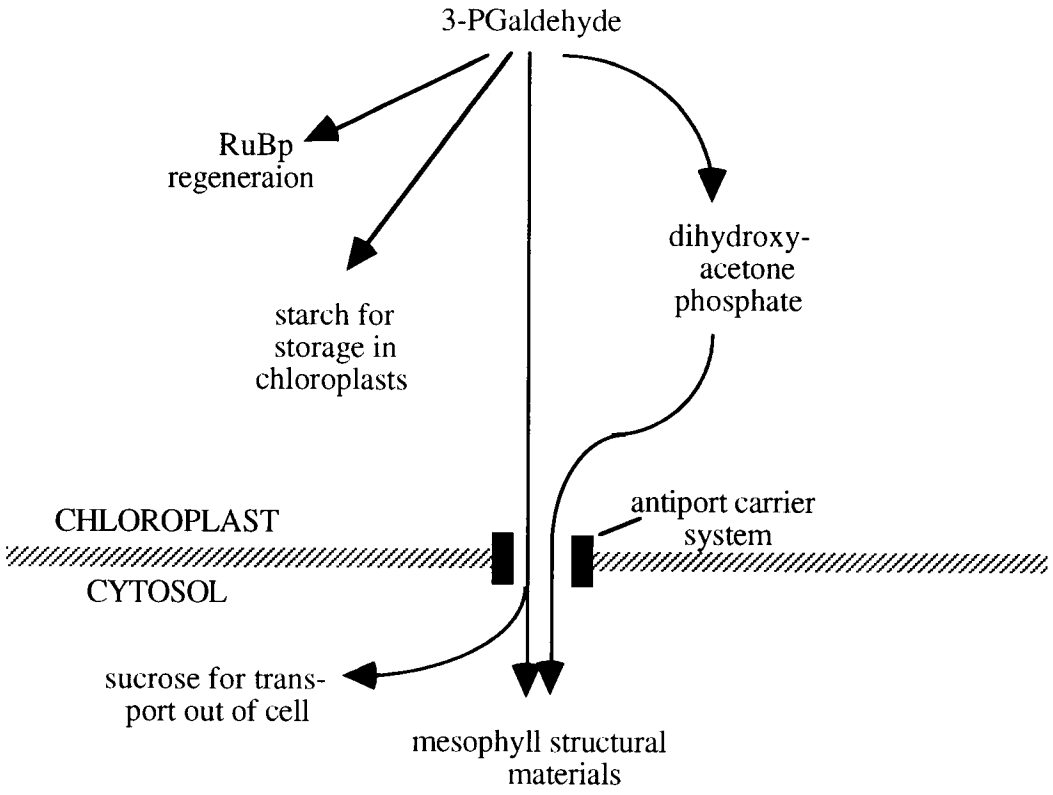


Fig. 3. Possible fates of 3-PGaldehyde, both within the chloroplast and in the cytosol.

Ross, 1992). Selective processes involving amino acid and mineral transport also take place. The transport of carbohydrates, amino acids and minerals is thought to be linked with a proton cotransport system, which would couple the diffusion of protons into a companion cell (after being actively pumped out of the cell) with transport of sucrose, amino acids or minerals.

Transport of sucrose from companion cells to sieve elements is thought to occur as a result of pressure-flow phenomena, described by E. Münch in Germany, in 1926 (from Münch in Salisbury and Ross, 1992). Münch's model describes a "source" and a "sink" osmometer that are connected with a tube. The source osmometer has a high solute concentration; the sink osmometer has a low solute concentration. If both osmometers are placed in a solution that is lower in solute concentration than that of the source osmometer,

water will move into the source osmometer, increasing the pressure within the source osmometer. This pressure is transferred through the connecting tube, to the sink osmometer, causing its pressure to build up. When the pressure inside the sink osmometer becomes more positive than that of the surrounding solution, water moves out into the surrounding solution, lowering pressure in the sink osmometer, and enabling more water to flow into the source osmometer. As a result of this flow, solutes and water are transported from source to sink much more rapidly than by simple diffusion of solutes.

Münch's system may be compared to a similar system within a plant, made up of solute rich phloem companion cells (source osmometer), phloem sieve elements (connecting tube), and various tissues that require sucrose for growth, maintenance or as storage (sink osmometer). Münch's theory explains the flow of solutes from companion cells to areas in the plant where carbon is used. This is a passive mechanism and is favored by most plant physiologists as a model for flow through phloem sieve tubes. Only metabolic energy necessary for the maintenance of sieve tube cells is required for bulk flow transport of solutes, such as sucrose, through the phloem.

The gradient of sucrose that drives the flow of sucrose through the phloem is potentially produced by sucrose metabolism in sink tissues. In storage tissues, sucrose may be converted to starch, and in growing tissue, sucrose is used as the energy source to drive growth. Both the conversion of sucrose to starch, and utilization of sucrose for respiration, enable sucrose transport to continue toward sink regions.

Phloem unloading of sucrose to sink tissues occurs via both the apoplast and symplast of sink cells. Growing and respiring sinks, such as meristems, roots, and young leaves, rapidly metabolize sucrose. Unloading in these tissues is primarily symplastic, through plasmodesmata. Sucrose is unloaded apoplastically into the cells of most storage organs, such as fruits (for example, apple and grape), roots (carrot) and stems (sugarcane). Symplastic unloading into storage organs has also been demonstrated in potato tubers

(Oparka, 1986). Developing embryos receive sucrose apoplastically, because there is no symplastic connection between the phloem of the mother plant and the embryo.

Carbon that is photosynthetically fixed by shoot cells, then converted to carbohydrates (primarily sucrose), may take various pathways through the plant body (Fig. 4). Photosynthate that is transferred from the leaf mesophyll may be translocated to above- or below-ground tissues, where these organic compounds are used to build cell material, or are respired to provide energy for metabolic cell functions. For example, water and nutrients are extracted from the soil by root epidermal cells, which require energy in the form of ATP to transfer these resources into root cells. The ATP generated for this transport process is obtained through respiration of sucrose. Water and nutrients are then transferred through cortical cells, into the xylem of the root. Xylem cells are the tubes through which water and nutrients are transported to plant shoot cells, where synthesis of organic compounds such as proteins, nucleic acids and porphyrin pigments (chlorophyll and cytochrome) may take place, in metabolically active cells. Plant roots are an important sink for translocated photosynthate (Fig. 4). Sucrose may be in demand as a respiratory substrate (see above), to build additional root tissue, to be transported to mycorrhizal fungi, or for storage in root cells, as starch.

Carbon allocation within a plant has been studied by Rygiewicz and Andersen (1994), by measuring the amount of ^{14}C translocated to the above-ground and below-ground portions of Ponderosa pine (*Pinus ponderosa* Laws.) seedlings, as well as the ^{14}C used in shoot and root respiration (Table 2). They report that the seedlings retained approximately 41.3% of the ^{14}C assimilated by the plant, while the remaining 58.6% was released through respiration. Approximately 24.7% of the retained ^{14}C was incorporated into shoot tissues, and approximately 16.7% was incorporated into below-ground tissues. Shoot and root respiration accounted for approximately 42.1 and 16.5% of the total respiration, respectively. The patterns of carbon allocation may vary with plant species and

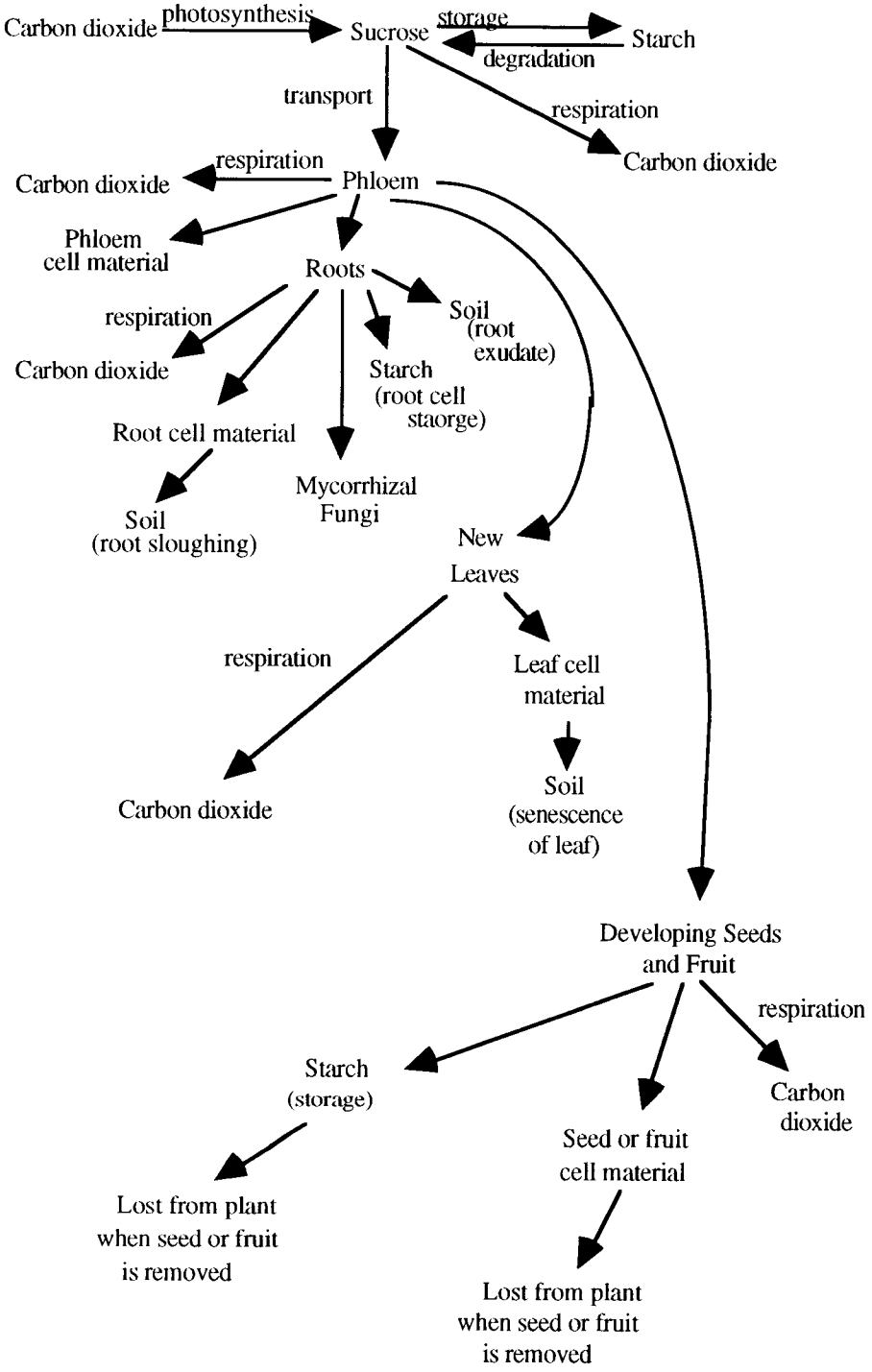


Fig. 4. General diagram of carbon flow in a plant. Carbon may be transported to and utilized or respired in various places throughout a plant.

stage of development. However, the study by Rygiewicz and Andersen (1994) provides a general quantitative model for the distribution of carbon within a plant.

Table 2. Percent labeled carbon retained and respired in above- and below-ground tissues (from Rygiewicz and Andersen, 1994).

Location of Carbon	% Retained	% Respired
Total Plant	41.3	58.6
Shoot	24.7	42.1
Root	16.7	16.5

1.2.3 Root Carbon Properties

Carbon transported to plant roots (Fig. 4) may be stored or used to form ATP, which energizes systems that regulate ion exchange in root cells (see above). Root carbohydrates may also be used to form carbon compounds for existing or new root cell structures. A significant amount of carbon is utilized through root respiration. Root respiration has been reported to release 49.4% of ^{14}C translocated to the roots of Ponderosa pine (*Pinus ponderosa* Laws.) over a 72 h period, while the remaining 50.6% was retained in coarse and fine roots (Rygiewicz and Andersen, 1994). Root respiration accounted for 60% of the ^{14}C translocated to faba bean (*Vicia faba*) roots (Paul and Kucey, 1981). The transport of carbohydrates to root cells is therefore essential for growth and metabolic activity of root cells, which obtain water and nutrients for use in growth and maintenance of cells throughout the plant.

Carbon may move into the soil through root exudation. Root exudation is a process by which organic substances move from the roots to the surrounding soil (Rovira, 1965). These substances may be secreted by root hairs, sloughed off of cell walls, released from cortical cell walls when attacked by microorganisms, solubilized from the mucigel sheath

produced by the root cap, or exuded as soluble material from intact living cells (Russell, 1977). The composition of root exudates includes carbohydrates, amino acids, organic acids, enzymes and many other compounds, such as auxins and choline (Rovira, 1965). Substances found in root exudates vary between species (Table 3).

Table 3. Primary components of root exudate from several plant species (from Rovira, 1965).

Reference	Species	Primary component	Secondary or trace component
Moody et al., 1987	Cowpea (*Vigna unguiculata*)	arabinose, galactose, glucose	fucose*, xylose, mannose, rhamnose, amino acids
	Wheat (*Triticum aestivum*)	arabinose, xylose, galactose, glucose	fucose*, mannose, rhamnose, amino acids
Bacic et al, 1986	Maize (*Zea may*)	galactose, fucose, arabinose, xylose, glucose	fucose*, mannose, amino acids
Chaboud and Rougier, 1984	Rice (*Oryza sativa)*	glucose, galactose, xylose, arabinose	fucose*, mannose, (amino acids not measured)

* Also called 6-deoxygalactose, fucose is a modified polysaccharide that gives recognition properties to the plasma membrane.

1.2.4 Carbon Allocation to Mycorrhizal Fungi

Mycorrhizal fungi form mutualistic symbioses with most land plants (Paul and Clark, 1990). Mycorrhizal fungi colonize plant roots and transport water and nutrients to the host plant by producing a network of fungal hyphae that extensively penetrates the soil (Allen, 1991). In exchange they obtain carbon from their host. Vesicular-arbuscular mycorrhizas (VAM) and ectomycorrhizas are two types of mycorrhiza that differ structurally from one another (Harley and Smith, 1983). VAM fungi penetrate the host root cells, forming vesicles and arbuscules within the cell (Allen, 1991). Ectomycorrhizal fungi form a sheath (mantle) over the root surface and penetrate between the root cortical cells, forming a

complex, branching network, called a Hartig net (Harley and Smith, 1983). Ectomycorrhizal fungi are capable of forming "net-shaped" mycelia by anastomosing with another compatible fungus (Allen, 1991). These mycelial networks are thought to be important to ecosystem dynamics through regulation of nutrient movement (Allen, 1991). VAM fungi have not been shown to form these mycelial networks (Harley and Smith, 1983).

By producing external hyphae that penetrate small soil pores, both VAM and ectomycorrhizae increase the volume of soil from which the host plant may obtain water and nutrients (Paul and Clark, 1990; Allen, 1991). This symbiosis is particularly important in soils where water and nutrients are limited. A mycorrhizal plant may successfully obtain enough water and nutrients to survive in conditions of limited resources, whereas a non-mycorrhizal plant may not, and subsequently become less competitive or senesce. Therefore, a disruption in the mutualistic association between a host plant and mycorrhizal fungus may alter the metabolic state of the plant, decreasing its ability to acquire resources that are essential for growth and survival.

Mycorrhizae have been shown to alter the natural patterns of carbon allocation in plants (Rygiewicz and Andersen, 1994). Rygiewicz and Andersen (1994) found that ^{14}C allocation to below-ground tissues was increased by 23% in mycorrhizal Ponderosa pine (*Pinus ponderosa* Laws.) seedlings, compared to non-mycorrhizal seedlings, and hyphal respiration accounted for 19.4% of the total respiration measured below ground. They suggest that fungal colonization has a significant effect on the amount of carbon that is translocated below-ground. Similar results were reported by Snellgrove et al. (1982) who noted that 7% more carbon was allocated to the roots of mycorrhizal *Allium porrum* L. than non-mycorrhizal plants. Paul and Clark (1990) report that colonization of three different host plant species (*Vicia faba, Glycine max,* and *Allium porram*) by the VAM fungus, *Glomus*, resulted in carbon allocation to the symbiont ranging from 10-14% of total fixed carbon, and increased host photosynthesis ranging from 8-21%. The distribution of

carbon in and flux of carbon through plant tissues and VA mycorrhizae have been measured in a soybean-*Glomus* association (Fig. 5) (Paul and Clark, 1990). The distribution of carbon to above- and below-ground plant tissues and fungal biomass (in percent of net uptake) and fluxes of ^{14}C (in mg ^{14}C day^{-1}) through these tissues are shown.

1.2.5 Carbon Translocation Between Plant Tissues

Carbohydrates may also be translocated between plant tissues via the xylem and phloem, although this type of transport is very limited. For example, during leaf senescence carbon products, in the form of amino acids, may be mobilized and transported in the phloem to growing tissues, such as developing seeds (Humphreys, 1988). Pate et al. (1977, 1979) has documented xylem transport of amides (asparagine and glutamine) to the developing fruit of white lupine (*Lupinus albus* L. cv. Ultra), suggesting transport of carbon from the phloem to the xylem before reaching the fruit tissue. Pate and coworkers (1979) also observed that phloem sap entering developing fruits was much more dilute in sucrose and richer in amino acids than the sap immediately exported from the leaves. This phenomena may occur due to phloem unloading of sucrose as the sap passes through the stem, coupled with the loading of amino acids into the phloem. These amino acids, coming from stored pools in the stems, must have originated from the xylem sap. Translocation of carbon products throughout the plant is an area of active research; elucidating the processes involved in carbon translocation will lead to a better understanding of whole-plant physiology.

1.3 Effects of Ozone on Plants

Ozone is an air pollutant that has been shown to significantly reduce plant growth and productivity (Heagle et al., 1979, 1983; Heck et al., 1983, Reich and Amundson, 1985).

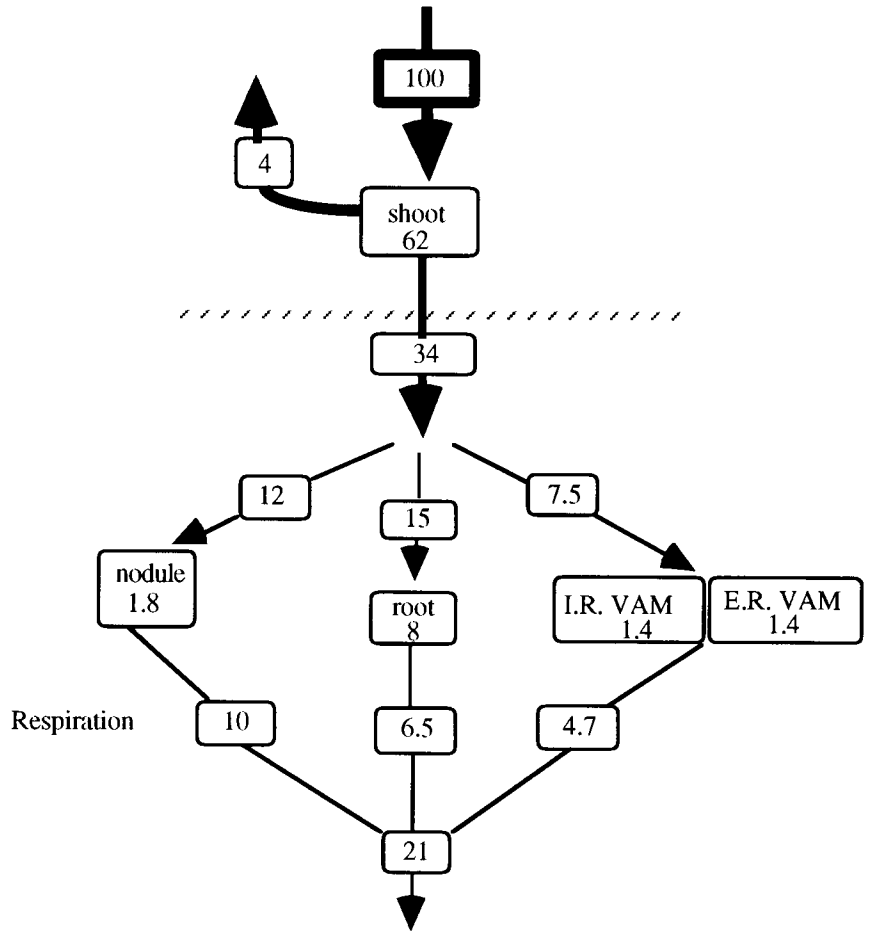


Fig. 5. Distribution of photosynthate in a 6 week old soybean-*Glomus* association (net uptake, 131 mg carbon-14/day). The sizes of the various components are shown within the boxes as percent of net uptake. The fluxes are shown on the arrows. ER VAM, Estimates of external root hyphae; IR VAM, intra root hyphae. (from Paul and Clark, 1990).

Ozone enters the open stomata of plant leaves where it comes into contact with the membrane of mesophyll cells. Ozone becomes adsorbed to the membrane surface, then is absorbed in the water phase of the cell wall were it reacts with water to form hydroxyl ion (OH^-), various free-radicals, including superoxide anion, and hydrogen peroxide (H_2O_2), which diffuse through the plasmalemma (Heath, 1980). Hydroxyl ion, hydrogen peroxide and superoxide anion disrupt normal cell function, due to their reactive nature (Alscher and Amthor, 1988; Heath, 1980; Chimiklis and Heath, 1975). Hydroxyl ion is by far the most

reactive of the three compounds, however the high potential of hydrogen peroxide and superoxide anion to react with each other, forming hydroxyl ion, makes them equally as harmful (Saran et al., 1988).

Hydroxyl ions oxidize membrane proteins and lipids, which results in an ionic imbalance of potassium ion (Heath, 1984). Potassium is required for activation of enzymes that are necessary for photosynthesis, respiration, and starch and protein synthesis. Maintaining particular levels of intracellular K^+ is also essential for the preservation of osmotic potential of cells and therefore cell turgor pressure. Heath and Castillo (1988) suggested that Ca^{+2} balance may also be affected by ozone and its products through altered membrane permeability. Calcium ion has long been recognized as important in intracellular function, regulating such processes as cytoplasmic streaming, cell elongation and division, and enzyme activation. Alteration of cytoplasmic levels of potassium and calcium ions by damage to the membranes that control their flux would obviously result in a disruption of many important cell functions. Activation of ion pumps to restore the levels of potassium and calcium ions in damaged cells may deplete ATP reserves, further damaging the affected cell (Heath, 1984).

Hydrogen peroxide disrupts normal cell function by deactivating enzymes that contain sulfhydrol groups (-SH) (Charles and Halliwell, 1980). Tanaka et al. (1982) demonstrated that exogenously supplied H_2O_2 inhibits light activated enzymes, causing a subsequent interruption in the Calvin cycle. The reaction of hydrogen peroxide with superoxide to form hydroxyl radical is perhaps the most detrimental role played by hydrogen peroxide in ozone injury, because of the extreme reactivity of the hydroxyl ion.

Superoxide anion has a high potential for oxidation of thylakoid membrane components of the chloroplast (Alscher and Amthor, 1988). Superoxide can diffuse into the lumen side of the thylakoid membrane, where it may react with protons to form a more toxic molecular species: hydroperoxyl radical (HO_2) (Alscher and Amthor, 1988). Hydroperoxyl radical is highly reactive, potentially oxidizing thylakoid membranes or

enzyme components involved in Calvin cycle reactions. As a result, membrane disruption or enzyme denaturation may occur in cells with elevated levels of internal superoxide anion. Superoxide may react with hydrogen peroxide, as mentioned above, to form hydroxyl radical, causing further injury to the affected cell.

A single cell model system using the alga *Chlorella sorokiniana* was used to determine the time course of events that the cell experiences when exposed to ozone (Heath, 1980; Heath and Frederick, 1979; Chimiklis and Heath, 1975). The investigators found that under constant exposure, the algal cell sequentially experienced: (1) an increase in passive permeability to K^+ and simple carbon compounds, as well as membrane depolarization, (2) inhibition of active sugar transport, a further increase in passive permeability and a decline in the energy sources (ATP and sugar phosphates) within the cell, and finally (3) extreme alteration of membrane ultra-structure, and lipid oxidation (Heath and Castillo, 1988). It is apparent that ozone damage is detrimental to the structures necessary for photosynthesis.

Maintenance of cell homeostasis under oxidative conditions requires the reduction of oxidative compounds generated by ozone breakdown, as well as the generation of these reductive compounds (Alscher and Amthor, 1988). Such a mechanism enables a plant to tolerate low levels of pollutants such as ozone (Alscher and Amthor, 1988). Other tolerance mechanisms have been suggested by researchers (Queiroz, 1988, Alscher and Amthor, 1988; Tingey and Andersen, 1991). Queiroz (1988) suggested that ozone injury may induce the synthesis of metabolic enzymes damaged by free radical attack, allowing the plant to continue photosynthetic function. Alscher and Amthor (1988) also cite the ability of ozone exposed cells to resynthesize deactivated enzymes, as well as damaged membrane components (lipids and proteins), and subsequently restore the ionic balances across membranes following the completion of repair. Tingey and Andersen (1991) acknowledge that the available amount of any metabolic enzyme generally exceeds the demand for that enzyme under normal conditions. Therefore, it is possible that ozone

damage to enzymes within a mesophyll cell may be overcome through the function of reserve enzyme capacity (Tingey and Andersen, 1991).

The processes involved in cell repair and maintenance following ozone exposure significantly increase the demand for reductant (NADPH) and ATP in damaged cells (Alscher and Amthor, 1988). Pell and Brennan (1973) reported a significant increase in the content of ATP in bean (*Phaseolus vulgaris* L. var. Pinto) seedling leaves following ozone exposure, and an increase in respiration within 24 hrs of exposure. These results suggest that ozone stress may trigger respiration and ATP production for repair or tolerance processes. These mechanisms are energetically expensive and may damage plants by utilizing energy that, under natural conditions, would be allocated to other plant tissues, such as growing roots or shoots.

Because the highly reactive breakdown products of ozone may react with the chloroplast membrane, altering its permeability, the reactions of the Calvin cycle may be disrupted, slowing carbon fixation and carbohydrate synthesis (Heath, 1980). Therefore, elevated ozone may reduce plant growth by damaging enzymes and membrane structure within mesophyll cells necessary for photosynthesis. Research on a wide variety of plant species supports the suggestion that ozone exposure impairs plant photosynthesis (Miller et al., 1969; Barnes, 1972; Yang et al., 1983, Weber et al., 1993). Soybean (*Glycine max* cv. Hodgson) plants exposed to three different levels of ozone (50, 90, and 130 ng g^{-1}) for eight weeks showed reductions in whole plant photosynthesis ranging from 10-22% (Reich and Amundson, 1984). Reductions in net photosynthesis were observed in poplar (*Populus deltoides* x *trichocarpa*) after 20 days of exposure to 85 and 125 ng g^{-1} of ozone (Reich 1983). Exposure to ozone (70 and 100 ng g^{-1}) for nine days has been shown to reduce photosynthesis in wheat (*Triticum aestivum* var. Lemhi) (Lehnherr et al., 1988).

Plants have mechanisms which enable them to block or tolerate ozone penetration (Tingey and Andersen, 1991). Stomatal closing in response to elevated ozone completely or partially blocks ozone damage in a variety of species (Engle and Gabelman, 1966;

Thorne and Hanson, 1976). Exposure of oats (*Avena sativa* cv. Park) to ozone resulted in a reduction in the percentage of open stomata to 50% of that in control (no ozone) treatments (Hill and Littlefield, 1969). Stomatal closure was also recorded in cucumber (*Cucumis sativus* L., cv. Champion), and soybean [*Glycine max* (L.) Merr., cv. Harosoy 63] plants exposed to ozone for 6 hrs (Beckerson and Hofstra, 1979). Peas (*Pisum sativum* L. cv. Alsweet) exhibited stomatal closure following 2- and 8-hour exposures (Olszyk and Tibbitts, 1980).

The mechanism of stomatal response to gaseous air pollutants has been the subject of much discussion. Research on the effects of ozone on stomata indicates that ozone induces stomatal closure at concentrations above 150 ng g^{-1}(Thorne and Hanson, 1976; Olszyk and Tibbitts, 1980; Saxe, 1991). There are two theories which attempt to explain the stomatal mechanism: 1) the direct action of ozone on the stomatal complex may induce stomatal closure, and 2) the inhibition of photosynthesis and subsequent build-up of carbon dioxide within the mesophyll cell may trigger natural mechanisms of stomatal closure (Winner et al., 1988). Time course studies have been used to determine which mechanism is operating in plant cells under the influence of SO_2 (Winner et al., 1988; Alscher et al., 1987), but experiments of this type have not been undertaken for ozone. Results of SO_2-induced stomatal closure indicate that the inhibition of photosynthesis by the toxic breakdown products of SO_2 occurs first, and an increase in internal CO_2 brings about a subsequent stomatal response (Winner et al., 1988). Ozone may influence stomata in like manner since it has a similar effect on the photosynthetic system.

Early research on the effect of ozone on stomata indicated a direct effect of ozone on stomatal conductance at high concentrations (>250 ng g^{-1}) in various species (Hill and Littlefield, 1969; Bennett and Hill, 1973), resulting in lower leaf conductance. These studies concluded that ozone initially affected the stomates. However, recent research has indicated that internal CO_2 concentration does not decline with ozone-induced reductions in photosynthesis, as would be expected if stomates were directly affected by ozone (Weber et

al., 1993; Atkinson et al., 1988). Therefore, decreased stomatal conductance may be a result of ozone-induced disruptions in the photosynthetic apparatus (membranes and enzymes), which lead to an increase in internal CO_2 concentration, and subsequent stomatal closure. The environmental conditions in which an ozone-exposed plant is grown, such as temperature, water stress and atmospheric CO_2, may also affect the response of plant stomata to ozone (Weber et al., 1994).

Ozone injury may also be blocked through detoxification mechanisms are in place both before the pollutant enters and within the leaf mesophyll cell (Chameides, 1989; Lee et al, 1984). Elevated ascorbic acid levels in the extracellular water of plant leaves may play a role in decreasing a plant's sensitivity to ozone injury (Chameides, 1989). This hypothesis is supported by the work of Lee et al. (1984), who found higher levels of ascorbic acid in the leaves of ozone-resistant cultivars of soybean (*Glycine max* L. cv. Hood) than in ozone-susceptible cultivars (cv. Hark) after the plants were exposed to ozone. It has been suggested that ascorbic acid may protect mesophyll cells from ozone by scavenging toxic free radicals generated by ozone breakdown as it contacts water in the mesophyll cell membrane (Calabrese, 1980).

The scavenging of free radicals and hydrogen peroxide produced when ozone and water react is an important defense mechanism used by ozone affected cells (Alscher and Amthor, 1988; Lee and Bennett, 1982; Nakamo and Asada, 1981). Superoxide dismutase (SOD) has been implicated as an essential compound in the detoxification of superoxide anion (Lee and Bennett, 1982). Lee and Bennett (1982) found an increase in ozone tolerance in snap beans (*Phaseolus vulgaris* L.) correlated with an increase in young leaf levels of SOD. The reaction catalyzed by SOD results in the formation of hydrogen peroxide, which must also be deactivated to avoid cell injury (Fridovich, 1976). Catalase and peroxidase catalyze the divalent reduction of H_2O_2 to $2H_2O$ (Fridovich, 1976; Tolbert, 1981). Nakamo and Asada (1981) have supported the work by Calabrese (1980) by

showing that in spinach chloroplasts, hydrogen peroxide is scavenged by a peroxidase that uses ascorbate as the electron donor.

It is clear that in many plant species, ozone exposure results in a reduction of photosynthetic efficiency and, consequently, in the amount of available photosynthate (carbohydrates). The amount of photosynthate that is loaded into the phloem sieve elements and translocated to other plant tissues may subsequently be reduced. It is logical to suspect that sink tissues, such as areas of shoot and root growth, flowers, fruit and seeds may therefore be sensitive to alterations in carbon flow. The following section will explore recent research pertaining to the effects of ozone exposure on the allocation of photosynthate to sink tissues.

Carbohydrate compounds, such as sucrose, are required for the formation and growth of tissues throughout the plant. Ozone exposure reduces shoot growth by reducing the amount of assimilate available for growing leaves and stems (Heggestad et al., 1988; Heck et al., 1982; Laurence and Weinstein, 1981). Shoot biomass in *Populus tremuloides* Michx. exposed to ambient ozone was 8-24% less than in trees exposed to charcoal-filtered air (Wang et al., 1985). Hogsett et al. (1985a) observed a decrease in stem diameter, plant height and shoot dry weight in two varieties of *Pinus elliottii* Englem. (var. *elliottii* and *densa*) seedlings as a result of ozone exposure.

The development of seeds and fruit requires a substantial input of sucrose, glucose and fructose, which are used in starch and fat synthesis, and cell wall formation. A decrease in carbohydrates due to ozone exposure can be expected to reduce the level of these compounds in the seeds and fruit of exposed plants. Indeed, research on several species indicates that ozone reduces grain and seed yield (Heggestad et al., 1985; Kress and Miller, 1985) Kress and Miller (1985) found that grain sorghum (*Sorghum vulgare* Pers.) plants exposed to ozone displayed a significant reduction in total grain yield (kg ha^{-1}) and 100-seed weight. Seed yield in ozone treated soybean (*Glycine max* cvs. Williams and Corsoy) was linearly related to ozone dose, decreasing as ozone dose increased

(Heggestad et al., 1985). Following a short-term dose of $^{14}CO_2$, McLaughlin and McConathy (1983) observed an increase in foliar retention of photosynthate accompanied by decreased allocation to large pods of bush bean (*Phaseolus vulgaris* var. 290) as a result of ozone exposure. Allocation of photosynthate to small pods was increased in this study, however the researchers explain that these data can not be used as evidence for increased allocation to fruits because small pods represent a small fraction of the total sink for ^{14}C-assimilates.

Damage to plant tissues due to ozone exposure, such as ozone induced leaf senescence, necrosis (Wang et al., 1985; Heagle et al., 1983; Heck et al., 1983), or reductions in growth (Hogsett et al., 1985a; Wang et al., 1985) may reduce the amount of carbohydrate available to root tissues. Ozone reduces the flow of carbon to root tissues (Andersen et al, 1991; McLaughlin and McConathy, 1983; McCool and Menge, 1983). For example, the biomass of taproots, new root growth and starch concentration in coarse and fine roots was reduced the year following ozone exposure of ponderosa pine (*Pinus ponderosa* Dougl. ex. Laws.) seedlings, indicating a reduction in the amount of carbohydrate available for root growth (Andersen et al., 1991). McCool and Menge (1983) found that the relative amount of labeled ^{14}C fixed by tomato (*Lycopersicon esculentum* Mill. Heinz 1350') and moved to the roots was reduced in those plants exposed to ozone, indicating allocation to roots may be reduced as a result of ozone injury. Allocation of ^{14}C to root biomass was decreased in bush bean (*Phaseolus vulgaris* var. 290) as a result of ozone exposure, and the disruption in allocation to root tissues was observed to persist for at least 1 week after exposures ceased (McLaughlin and McConathy, 1983).

A reduction in the amount of carbon available to root cells may negatively influence root tissue by limiting the amount of photosynthate available for 1) production of ATP through respiration, 2) synthesis of compounds needed for cell maintenance and new root growth, and 3) storage in cell amyloplasts. A reduction in ATP synthesis through respiration may decrease the ability of root cells to regulate internal ion concentration and

nutrient uptake since ATP energizes the transport of compounds, such as potassium, nitrate and phosphate across root cell membranes (Salisbury and Ross, 1992). Carbon skeleton compounds that are used to synthesize cell material are essential for maintaining existing root cells, as well as new, developing root cells that will further penetrate and obtain resources from the soil. Decreased root storage reduces a plant's ability to rely on these carbon reserves in carbon-limited conditions. Ozone induced reductions in root carbon may therefore, negatively influence water and nutrient uptake by roots, decreasing the amount of these resources available for shoot growth and maintenance.

Research has shown that root colonization by VAM and ectomycorrhizal fungi is significantly reduced in ozone-exposed plants, relative to control, unexposed plants (McCool and Menge, 1983; Edwards and Kelly, 1992; McQuattie and Schier, 1992). Since mycorrhizal fungi rely on host-supplied root carbon for growth and maintenance, a decrease in this carbon supply, due to ozone exposure, may reduce the amount of mycorrhizal colonization in plant roots (McCool and Menge, 1983). McCool and Menge (1983) observed a reduction in the reducing sugars in root exudates concurrent with a decrease in mycorrhizal colonization of tomato (*Lycopersicon esculentum* Mill. 'Heinz 1350') exposed to ozone. A mycorrhizal plant that is exposed to ozone, and subsequently experiences a disruption in this symbiosis, may be subject to a reduction in nutrient and water uptake. Limited nutrient and water availability to shoot tissues limits the growth and metabolic function of shoot tissue cells, adding to the direct damage (reduced photosynthesis) produced by ozone exposure.

Understanding the influences of mycorrhizae and ozone alone on root characteristics aids in interpreting the effects of the two factors in combination (Fig. 6). A non-mycorrhizal plant that is not influenced by ozone is designated as a plant in a natural metabolic state. A mycorrhizal plant experiences an increase in carbon translocated below ground, an increase in soluble root carbohydrate concentration, a decrease in root exudation and a doubling of total below-ground respiration (Rygeiwicz and Andersen, 1994;

Thomson et al., 1986, Graham et al., 1981), compared to a non-mycorrhizal plant. A non-mycorrhizal plant that is exposed to ozone experiences an increase in foliar photosynthate retention, decrease in translocation of carbon to non-photosynthetic tissues, decrease in root starch concentration, decrease in root soluble sugar concentration, and decrease in non-structural carbohydrate content in roots (Andersen et al., 1991; Miller et al., 1989; Okano et al., 1984; McLaughlin and McConathy, 1983). A mycorrhizal plant that is subjected to ozone may experience primary ozone effects of photosynthetic impairment and decreased carbon allocation to roots, but may also be subject to a disruption in the mycorrhizal association, due to limited root carbon availability. A disruption in the mycorrhizal association my be detrimental to plants that rely on mycorrhizae to obtain limited resources, since a plant must overcome damage to photosynthetic apparatus as well as nutrient deficiency. A disruption in the mycorrhizal symbiosis may also increase root exudation (Graham et al., 1981), resulting in changes in rhizosphere organism populations that feed on this material. Rhizosphere responses to ozone will be discussed in the following section.

1.4 Response of Rhizosphere Organisms to Ozone Exposure

Processes of immobilization and mineralization of nutrients occur in rhizosphere soil surrounding plant roots. The metabolic processes of soil organisms, such as bacteria, fungi, protozoa and nematodes contribute to the cycling of nutrients, such as nitrogen and phosphorus, and carbon. The following section will explore the role of bacterial, fungal, protozoa and nematode populations in maintaining soil nutrient availability, as well as the potential impact of ozone exposure on these organisms. The information presented in sections 1.4.1-3 is more thoroughly described in general microbial ecology texts, such as Paul and Clark (1990) or Atlas and Bartha (1992).

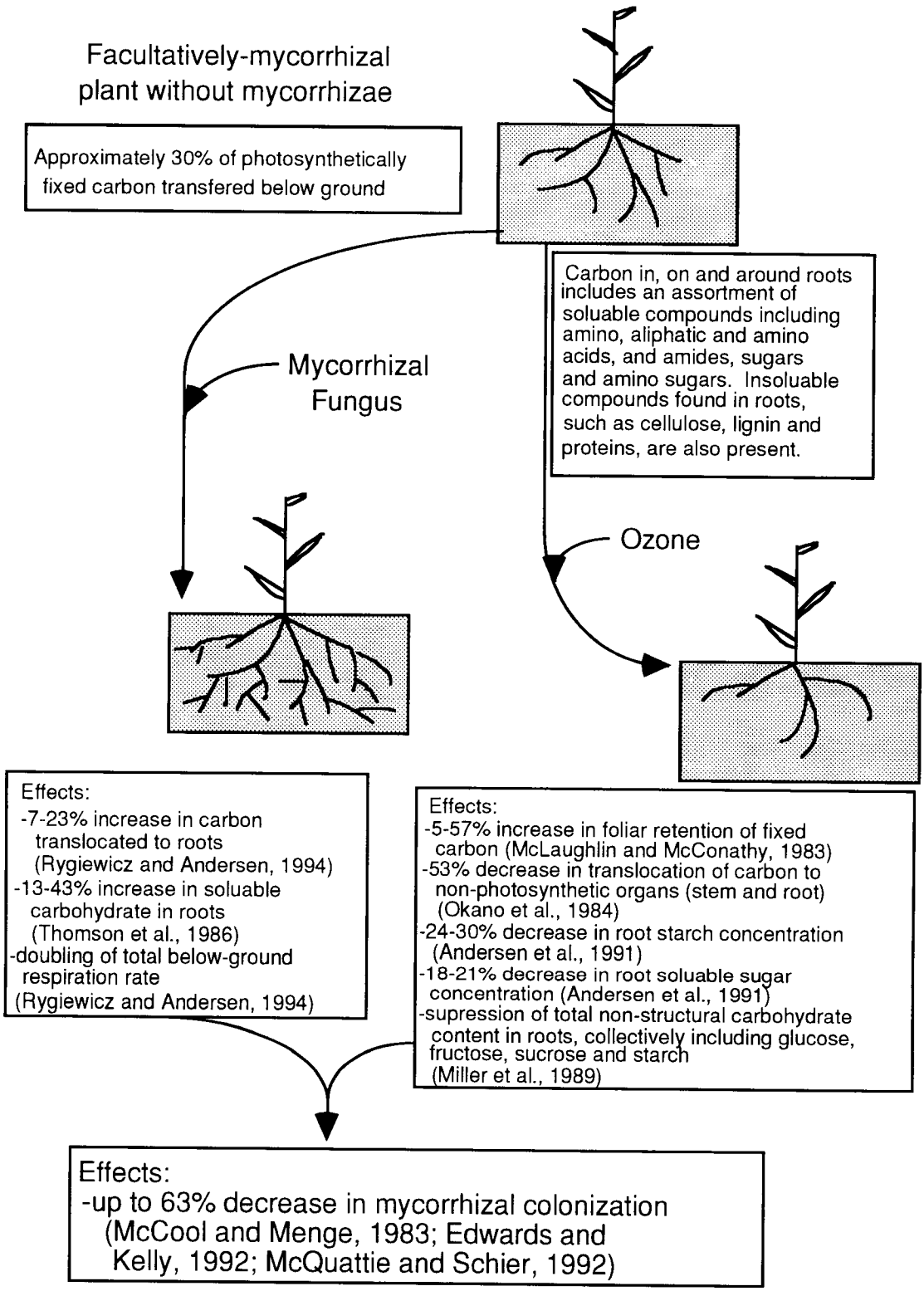


Fig. 6. Summary of the general effects of mycorrhizae and ozone, alone and in combination, on plant root characteristics.

1.4.1 Role of Soil Organisms in Nutrient Cycling

Nutrients from the soil may be absorbed by plant roots and incorporated into carbohydrate molecules to form plant components, such as cell wall material, enzymes or cellular organelles. When part of a plant dies and falls to the soil surface, when roots die and are sloughed, or when root exudates are released by the root system, the material is used by bacteria and fungi (primary consumers) in biosysnthesis, forming CO_2, microbial cells, and waste products. Bacteria and fungi produce enzymes, such as cellulases, amylases, and pectinases, that hydrolyze bonds and reduce plant compounds. A nutrient, such as nitrogen, is in organic form when it is linked to a carbohydrate molecule. When organic matter is used by a decomposing microorganism, nutrients may be 1) incorporated into the biomass of the decomposing organism, or 2) released into the soil nutrient pool in mineral form, or linked to more complex molecules, forming compounds such as amino acids and carbohydrates. "Mineralization" is the process of releasing inorganic nutrients into the soil as an organism breaks down or consumes organic material. Nutrients may be "immobilized" through the incorporation of nutrients into the metabolites or organic matter of a consuming organism, such as a decomposer. Immobilization of nutrients may also occur when a plant absorbs and incorporates a nutrient into plant material or metabolites.

Nutrients may be transferred through multiple trophic levels of the food-web that exists in soil (Ingham et al., 1986; Cole, et al., 1978; Coleman, et al., 1977). For example, soil protozoa and nematodes (secondary consumers) may feed on bacteria and fungi; protozoa and nematodes may then be ingested by soil arthropods (tertiary consumers). A portion of the nutrients in the ingested tissue are released into the soil nutrient pool at each trophic level transition. The cycle begins again when a soil organism, such as an arthropod, dies and is decomposed by bacteria and fungi, or when carbon dioxide is released through respiration. The immobilization of arthropod biomass by

bacteria and fungi completes the cycle of nutrients from primary consumer, to secondary consumer, to tertiary consumer, back to primary consumer.

1.4.2 Nitrogen Cycling

Nitrogen is the fourth most common element in plant biomass. Inorganic nitrogen, in the form of nitrate (NO_3^-) and ammonium (NH_4^+), is absorbed by plant roots. Soil organisms that mineralize nitrogen are important in maintaining an adequate supply of NO_3^- and NH_4^+ to plants. The nitrogen cycle describes the pathway of nitrogen through processes of mineralization, immobilization, nitrification, and denitrification (Fig. 7). All three of these processes are driven by the metabolic activity of soil microorganisms. Organic nitrogen, in the form of proteins, amino sugars, and nucleic acids, is mineralized by microorganisms, to ammonia (NH_3) in a process called ammonification. Ammonia exists as ammonium ions (NH_4^+) in acidic to neutral aqueous environments. Ammonium in the soil may be immobilized through absorption by plant roots or assimilation by microorganisms.

Ammonium that is not incorporated into microbial biomass may have several fates (Table 4). One of these possible fates, nitrification, is a microbially mediated process that converts NH_4^+ to nitrite (NO_2^-) and nitrate (NO_3^{-2}). Plants readily assimilate NO_3^- and incorporate the nitrogen into amino acids, completing the cycle of nitrogen from organic to inorganic forms, and back to organic nitrogen. Nitrate may be subject to other pathways (Table 5). Of particular significance to the cycling of nitrogen between the soil and atmosphere is denitrification. Denitrification results in the production of N_2O and N_2 gas, which are subsequently released to the atmosphere. N_2 may be fixed from the atmosphere and returned to the soil or plant roots, by free-living or symbiotic bacteria, respectively. The incorporation of nitrogen into the biomass of soil microorganisms or plants completes the cycle of nitrogen, described here as starting with mineralization of detritus.

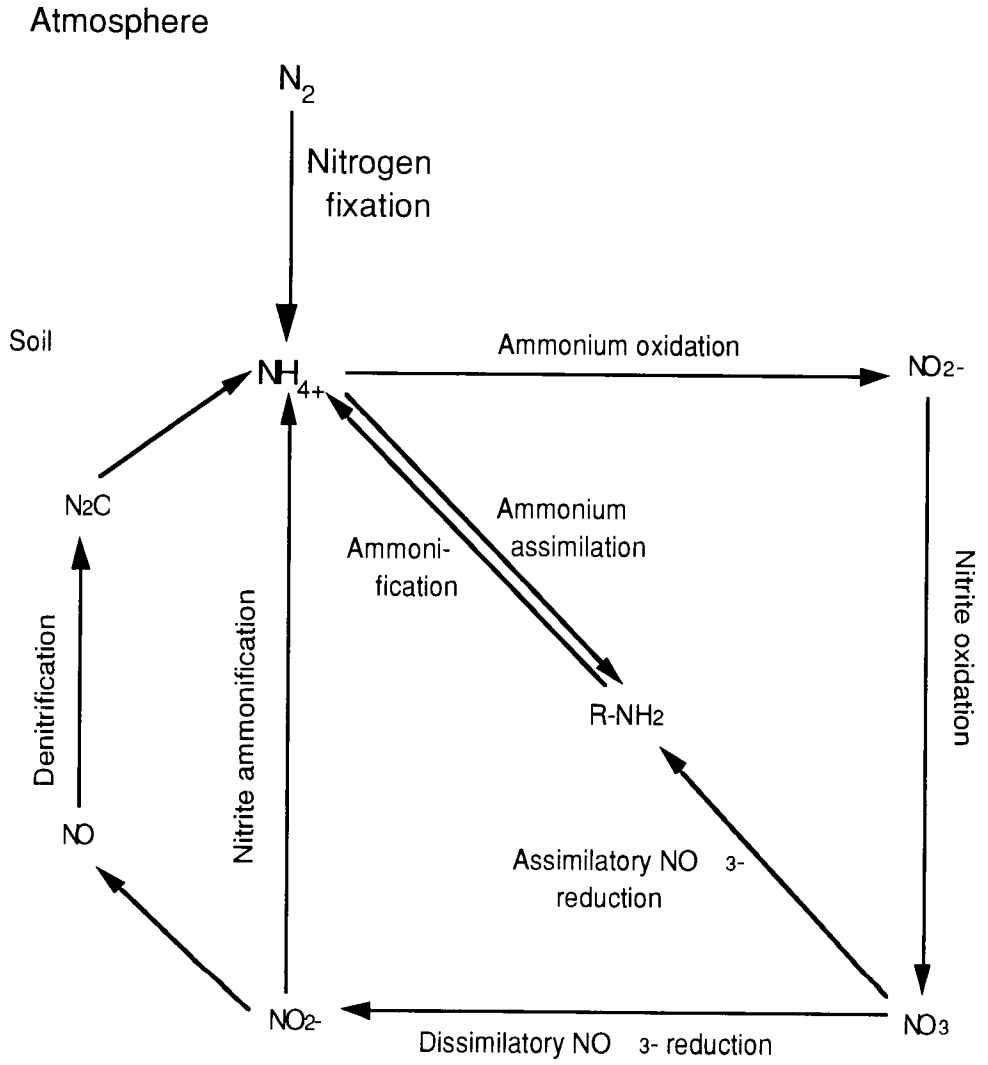


Fig. 7. The nitrogen cycle showing chemical forms and key processes in the cycling of nitrogen. Nitrogen fixation, nitrification, and denitrification are mediated by bacteria. $R\text{-}NH_2$ represents amino nitrogen in organic molecules (from Atlas and Bartha, 1992).

Table 4. Possible fates of NH_4^+ in the soil nutrient pool (from Paul and Clark, 1990).

1. Absorption by plant roots for amino acid formation
2. Adsorption to the carbohydrate exchange complex, where it may be exchanged for soil solution cations
3. Fixation in the interlayer portions of clay molecules, following collapse of the interlayer space due to drying
4. Formation of quinone-NH_2 complexes from reactions with soil organic matter
5. Volatilization into the atmosphere
6. Utilization as an energy source by autotrophic bacteria in nitrification

Table 5. Possible fates of NO_3^+ in the soil (from Paul and Clark, 1990).

1. Denitrification by microorganisms, followed by conversion to gaseous N_2O and N_2
2. Assimilation and synthesis of amino acids by microorganisms
3. Dissimilatory reduction by microorganisms, resulting in the formation of NH_4^+
4. Transport to deeper soil layers or ground water, through leaching
5. Transport off site, through water runoff
6. Accumulation in the soil, under fallow conditions

1.4.3 Carbon Cycling

The degradation of organic molecules by microorganisms contributes to the cycling of carbon (Fig. 8). The majority of carbon input to the soil comes from plant material, in the form of cellulose, hemicellulose, lignin, and protein. These compounds are metabolized by microorganisms, producing carbon dioxide, microbial biomass, by-products (such as simple hydrocarbons, vitamins, auxins, amino acids, gibberellins), and more-resistant soil humates. Carbon dioxide is released to the atmosphere and may continue to cycle, through photosynthetic carbon fixation by plants. Carbon in microbial biomass may be moved through additional trophic levels in organic form, or mineralized (i.e. respired when the organism dies) or is consumed by a microbial predator. By-products of microbial decomposition may be further degraded by other microbial populations, producing carbon dioxide. Soil humates mineralize very slowly, due to the

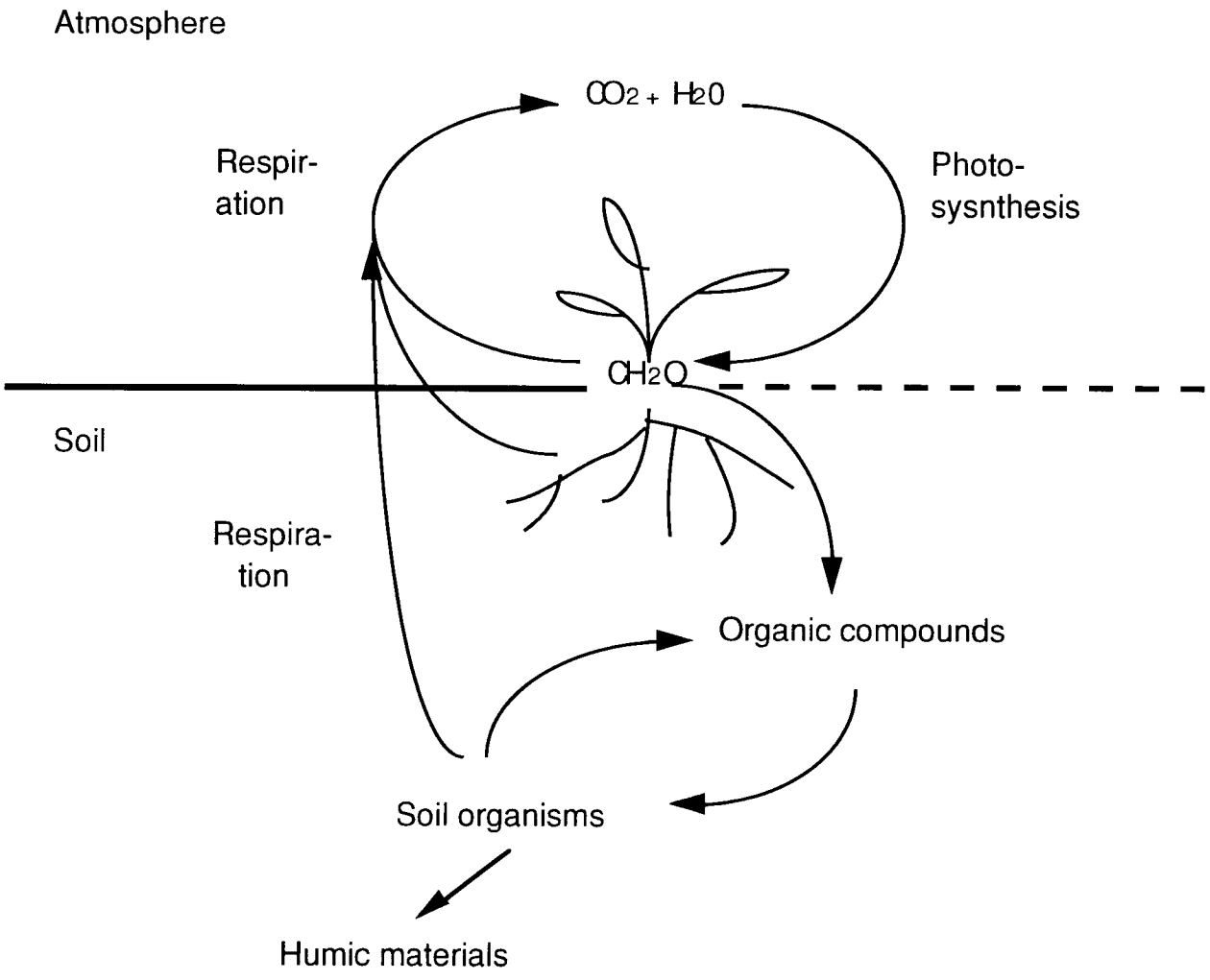


Fig. 8. The carbon cycle (from Atlas and Bartha, 1992).

complexity of their chemical structure, so are relatively persistent in soils, for example as humin.

1.4.4 Importance of Rhizosphere Population Structure

The importance of microorganisms to nutrient cycling processes cannot be overstated. Bacteria and fungi are primary consumers, responsible for converting plant material into biomass, CO_2, and a variety of by-products, including simpler organic

compounds and inorganic nutrients (Atlas and Bartha, 1992). Secondary consumers, including protozoa and nematode populations, feed on bacteria and fungi (Ingham et al., 1986; Cole, et al., 1978; Coleman, et al., 1977). A portion of bacterial and fungal biomass is immobilized by incorporation into predator biomass (Ingham et al., 1986). Ingested nutrients that are surplus to predator survival are excreted into the soil in mineral form, or linked to partially digested prey biomass as feces (Atlas and Bartha, 1992). Protozoa and nematodes are eaten by organisms in higher trophic levels, resulting in additional mineralization of nutrients (Ingham et al., 1986; Cole, et al., 1978; Coleman, et al., 1977). Nutrients are released at each trophic level transition, and the nutrients and carbon in the biomass of every soil organism is eventually mineralized through the trophic interactions that take place in below-ground communities (Atlas and Bartha, 1992).

Soil microorganism populations, such as bacteria and protozoa, form groups made up of similar individuals (Atlas and Bartha, 1992). Populations that with each other in a given location form a community (Atlas and Bartha, 1992). For example, populations of bacteria may interact, forming a bacterial community. Communities that interact, together with the abiotic environment, make up an ecosystem (Atlas and Bartha, 1992). Trophic interactions between communities of soil organisms, such as bacterial, fungal, protozoa and nematode communities, create complex belowground food webs (Ingham et al., 1986) (Fig. 9). Each population contributes to the maintenance of the community. The removal of a single population may affect the balance of the entire food web, resulting in an alteration of the community structure (Griffiths, 1994; Ingham et al., 1985). Communities that are altered by removal of a population may influence the structure of the ecosystem that they, along with other communities, comprise.

The primary role of bacteria and fungi in the soil food-web is to decompose organic compounds, resulting in the oxidation of a portion of the ingested organic carbon, and the immobilization of the remaining carbon and nutrients, such as nitrogen, sulfur, and phosphorus. Soil protozoa primarily ingest bacteria, using approximately 40% of

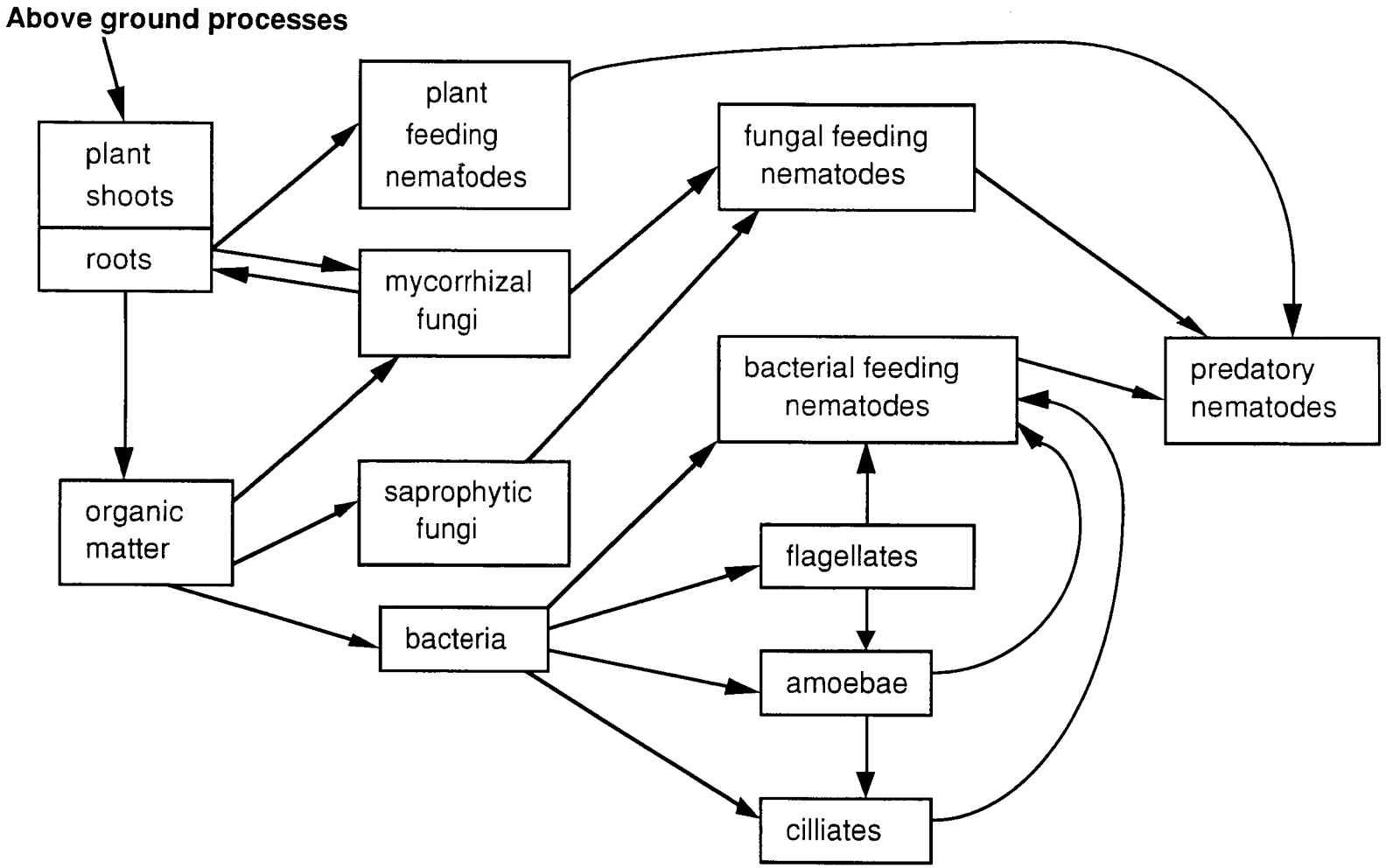


Fig. 9. Generalized soil foodweb diagram, showing the relationships between organism groups (from Ingham et al., 1986).

bacterial carbon and nitrogen for formation of biomass (Sleigh, 1989). Thirty percent of the remaining ingested bacterial carbon is respired, and 30% is excreted (Sleigh, 1989). Sixty percent of the remaining bacterial nutrients are excreted (Sleigh, 1989). Protozoa are clearly important members of the soil food web, responsible for the release of mineralized carbon, nutrients and simpler organic compounds into the soil.

Protozoa also affect soil nutrient flow indirectly, through non-trophic interaction with prey populations (Griffiths, 1994). The size and character of bacterial populations have been observed to be influenced by protozoa (Stout, 1980). The presence of protozoa can accelerate the turnover of microbial biomass, soil organic matter, and nutrients (Griffiths, 1994). Although the mechanisms behind the bacterial responses to protozoa have not yet been determined, the production of stimulatory compounds and grazing activity of protozoa have been suggested as one of the causes of changes in bacterial populations (Griffiths, 1994).

Nematodes, like protozoa, play an important role in soil carbon and nutrient flow, through trophic interactions within soil communities. Nematodes utilize bacteria, fungi, protozoa, plant root material, detritus, and/or other nematodes as a food source (Yeates, 1979). The grazing of microorganisms and plant roots by nematodes enhances mineralization of nutrients that are immobilized in their biomass (Yeates, 1979). Studies have shown that the presence of nematodes in soil increases mineral nitrogen concentrations in the soil, through grazing of the microflora and subsequent release of mineralized nutrients (Ingham et al., 1985; Opperman et al, 1993).

Soil organisms in higher trophic levels, such as soil arthropods, also contribute to nutrient and carbon cycling in soil ecosystems and are active members of the below-ground community. However, because the scope of this study is limited to the examination of bacterial, fungal, protozoa and nematode populations, a review of the importance of higher trophic level populations will not be included in this text.

1.4.5 Effects of Ozone on Rhizosphere Organisms

The direct effect of ozone on populations of rhizosphere organisms has been addressed to a limited extent. Research indicates that ozone penetration is probably restricted to the top 2 cm of the soil (Blum and Tingey, 1977). Molecules of ozone oxidize sites that are initially reduced in soil pores of the top soil layers (Turner et al., 1973). However, soil properties, such as water-filled pores and compaction, create resistance to ozone penetration to deeper soil layers (Turner et al., 1973). It is therefore probable that direct effects of ozone on rhizosphere organisms are negligible (Blum and Tingey, 1977).

Although the direct effects of ozone on rhizosphere organisms is unlikely, indirect ozone effects may pose a substantial threat to rhizosphere populations. Since bacterial and fungal populations utilize root exudate and sloughed material, an ozone-induced reduction in these compounds may be expected to produce a negative effect on the activity, population size, and diversity of these organisms. Recent research has suggested ozone induced changes in carbon allocation to plant roots as the cause for altered rhizosphere bacterial populations (Shafer, 1988). Ozone exposure has been observed to decrease the bacterial population size in the rhizosphere of a hybrid of sorghum (*Sorghum bicolor* (L.) Moench) and sudangrass (*Sorghum* x *drummondii* (Steudel) Millsp. and Chase), compared to control, unexposed plants (Shafer, 1988). More extensive research must be done to elucidate rhizosphere organism responses to ozone exposure.

The potential effects of ozone on soil organisms are clearly important to maintenance of both above-ground and below-ground function. Rhizosphere bacteria and fungi feed on carbon substrate input to the soil by plant roots, in the form of root exudates and sloughing (Atlas and Bartha, 1992). If carbon allocation to plant roots decreases root exudates and sloughing, the population size and diversity of bacteria and fungi may be reduced, due to a shortage of food. Protozoa feed primarily on bacteria, although fungi are a food source for a few species. Bacterial- and fungal-feeding nematodes feed on bacteria and fungi,

respectively. A decrease in the population size of bacteria and fungi may limit the food source for protozoa and nematodes, resulting in a reduction in the population size of these predators. Predatory organisms, such as protozoa and nematodes, have been shown to increase the amount of nitrogen available to plants, through mineralization of nitrogen in bacterial biomass (see section 1.5.4). A reduction in the numbers of protozoa and nematodes may decrease the amount of mineralization of nutrients, such as nitrogen (Ingham et al., 1985). A feedback to the plant may develop, due to inadequate soil nutrient supply, causing additional damage to a plant that has been exposed to ozone (Ingham et al., 1985).

1.5 Effects of Different Soil Types on the Response of Plants and Rhizosphere Organisms to Ozone Exposure

Plants need nutrients, such as ammonium, nitrate, phosphorous and sulfur for tissue growth and maintenance (Salisbury and Ross, 1992). If nutrients are abundant in the soil, a plant will be able to absorb nutrients through a relatively small root system. However, in a nutrient poor soil, a plant may have to develop a more extensive root system to penetrate the soil and extract nutrients. The development of an extensive root system requires a greater allocation of photosynthate to the roots than the development of a small root system, due to the production of more tissue. If the photosynthate supply to root tissues is limited, due to ozone exposure, the ability of a plant to synthesize root tissue may also be limited. If photosynthate is limited to a plant grown in nutrient poor soil, the plant may be unable to develop a root system that supplies the plant with adequate nutrients for survival.

The effects of ozone on plants may be influenced by the type of soil in which the plant is grown. A plant that is exposed to ozone may experience a decrease in carbon allocation to roots (see section 1.2). If this plant is grown in nutrient rich soil the plant may be adequately supplied with resources it requires for tissue repair, growth and maintenance. If this plant is grown in nutrient-poor soil, the plant may not be able to obtain the resources

it requires for survival. An ozone-exposed plant grown in nutrient poor soil that relies on VAM fungi for nutrient uptake may be even more at risk for insufficient nutrient uptake, since the mycorrhizal association may be disrupted, due to ozone-induced lack of root carbon available for the fungal symbiont.

Alternatively, a mycorrhizal plant grown in nutrient poor soil and exposed to ozone may exhibit an increase in VAM colonization. In response to limited nutrient availability the plant may allocate more photosynthate below-ground to the fungal symbiont, to increase nutrient absorption. Consequently, photosynthate that would have been used for repair processes in shoot tissues may be reduced, resulting in a further reduction in photosynthesis.

The type of soil in which an ozone-exposed plant is grown may also affect populations of rhizosphere organisms. A soil that is low in carbon (organic matter) limits the food source for bacteria and fungi (Atlas and Bartha, 1992). A reduction in carbon supply to the rhizosphere, due to decreased root exudation and sloughing, may additionally reduce the food supply for the bacteria and fungi that inhabit the rhizosphere of a plant grown in carbon-poor soil. Reduced populations of bacteria and fungi may result in a decrease in the populations of protozoa and nematodes (see section 1.5.5). Reductions in microorganism populations may be larger in the rhizosphere of ozone-exposed plants grown in low organic matter soil than in high organic matter soil, due to lack of available carbon substrate for microorganisms.

The feedback which decreases plant nutrient availability, due to a reduction in mineralization by protozoa and nematodes (see section 1.5.5), may be increased if ozone-exposed plants are grown in a soil low in nutrients and organic matter for two reasons. First, the plant may be unable to obtain nutrients in a low nutrient soil, due to an ozone-induced reduction in root carbon allocation. If the plant is mycorrhizal, a disruption in the mycorrhizal symbiosis, due to limited carbon to the fungus, may further impair the plant's ability to obtain nutrients. Secondly, the rhizosphere organisms of the exposed plant will

experience a shortage of carbon substrate, decreasing the population size of bacteria and fungi. A subsequent reduction in the population size of protozoa and nematodes may result in decreased mineralization of nutrients, further decreasing the ability of the plant to obtain nutrients. Nutrients are needed to form molecules that will be used in the repair of photosynthetic tissue that has been damaged by ozone. A plant's inability to absorb nutrients reduces the formation of molecules to be used in repair, negatively affecting the plant's repair processes. The compounding of these negative effects may result in severe growth reduction or premature plant senescence.

1.6 Effects of Ozone on Spring Wheat

The sensitivity of spring wheat (*Triticum aestivum*) to moderate levels of ozone has been widely recognized (Adros et al., 1991; Farage et al. 1991; Lehnherr et al. 1988; Pleijel et al. 1991). Ozone decreases photosynthetic carbon fixation in leaves of spring wheat at ambient and elevated (15 ng g^{-1}, 30 ng g^{-1}, 70 ng g^{-1} and 100 ng g^{-1}) concentrations (Lehnherr et al. 1988). Reductions in shoot and root biomass have been reported for spring wheat exposed to ozone concentrations of 15 ng g^{-1} and 30 ng g-1 (Mortensen, 1990). Exposure to ozone has also been shown to significantly lower the grain yield in spring wheat (Pleijel et al. 1991; Fuhrer et al. 1989). Another study has indicated that both yield and growth of spring wheat decrease at elevated ozone concentrations (Adros et al., 1991).

Spring wheat is VA mycorrhizal, so the effects of ozone exposure on root VA mycorrhizal colonization may be of potential interest in exploring ozone effects on mycorrhizal symbioses. Available information shows that the amount of photosynthetically fixed carbon that is allocated by a host spring wheat plant to the fungal symbiont ranges from 4% to 17% (Paul et al., 1984). Therefore, a reduction in carbon allocation to root

tissues, due to exposure of spring wheat plants to ozone, may reduce the amount of VA mycorrhizal colonization in the root system of these plants.

Between 12 and 18% of the carbon photosynthetically fixed by spring wheat plants is translocated to the roots and released into the soil (Barber and Martin, 1976). A reduction in the amount of carbon allocated to the root system of spring wheat plants may significantly reduce the amount of carbon lost to the rhizosphere soil, reducing the amount of root exudate and sloughing material available for decomposition by microorganisms. Therefore, rhizosphere population dynamics may be potentially disrupted by exposing spring wheat plants to ozone. This disruption may reduce the mineralization of nutrients by predatory microorganisms, resulting in lower nutrient availability to the wheat plant. A subsequent decrease in growth due to the combined effects of direct ozone damage to photosynthetic tissues, and decreased nutrient acquisition may occur.

2.0 Objectives, Hypotheses and Proposed Mechanisms

The purpose of the current study is to determine the effects of elevated ozone on the growth, mycorrhizal colonization, and rhizosphere organism populations of ozone-exposed spring wheat (*Triticum aestivum* L.) plants. Specifically, the experiments were designed to study the effects of ozone on: 1) plant photosynthesis, and root and shoot growth, 2) mycorrhizal colonization of the experimental plant's root system, and 3) the population size and activity of bacteria and fungi, population size of protozoa, and population size of nematodes inhabiting rhizosphere soil.

Hypothesized effects of ozone on the above parameters are as follows. Ozone exposure of spring wheat plants is predicted to: 1) decrease plant photosynthesis, and root and shoot growth, 2) decrease mycorrhizal colonization in the root system, and 3) decrease the population size and activity of bacteria and fungi, population size of protozoa, and population size and alter community of nematodes inhabiting the rhizosphere. The mechanisms upon which these hypotheses are based will not be studied experimentally, but are proposed below.

Ozone exposure of spring wheat plants is predicted to decrease photosynthetic activity, root biomass, and VAM colonization, because photosynthetic activity is anticipated to be reduced in plants exposed to ozone, due to impairment of mesophyll cell function (see section 1.4). As a result of reduced photosynthetic activity, the exposed plants should experience a reduction in the production of carbohydrate compounds, and a subsequent reduction in carbon allocation to roots. A decrease in carbon available for symbiotic mycorrhizal fungi should result in lower levels of colonization in the roots of plants exposed to ozone, compared to control plants.

Changes in the rhizosphere due to ozone-induced changes in carbon allocation patterns are predicted to influence rhizosphere organism populations, because reductions in root carbon, due to ozone exposure, should decrease the amount of root exudate and

sloughing material available to rhizosphere bacteria and fungi. As a result, the populations of bacteria and fungi should be lower in the rhizosphere soil of plants that have been exposed to ozone, relative to control plants. Protozoa and nematodes, which feed on bacteria and fungi, should experience a reduction in population size, due to a reduction in the population size of their food source.

If reductions in the population size of protozoa and nematodes are observed, a negative feedback is expected to be observed on the growth of the exposed plant, because ozone-induced reductions in protozoa and nematodes should decrease the amount of nutrients mineralized from bacterial and fungal biomass, subsequently reducing the availability of nutrients in the soil. Nutrient absorption by the exposed plant should limit the plant's ability to repair the direct photosynthetic damage produced by ozone, further decreasing the plant growth.

The effects of ozone on plants and rhizosphere organisms described above are predicted to be amplified in low nutrient, low organic matter soil, relative to high nutrient, high organic matter soil, because a plant that is exposed to ozone and grown in soil low in nutrients and organic matter will: 1) be less likely to obtain the nutrients it requires for survival; 2) experience a greater reduction in carbohydrate synthesis; and 3) be more prone to an ozone-induced decrease in carbon allocation to roots than a plant grown in soil rich in nutrients and organic matter. Limited carbon allocation to roots should decrease the root biomass and VAM colonization of roots in plants exposed to ozone and grown in high nutrient, high organic matter soil more significantly than exposed plants grown in high nutrient, high organic matter soil.

Ozone-induced changes that occur in the rhizosphere of plants grown in low nutrient, low organic matter soil, are predicted to be more pronounced, relative to the changes occurring in nutrient rich soil, because the carbon supply to bacteria and fungi is further limited. As a result, the populations of rhizosphere bacteria and fungi in ozone exposed plants grown in low nutrient, low organic matter soil should experience a greater decrease

in response to ozone exposure than the populations of bacteria and fungi in the rhizosphere of ozone-exposed plants grown in high nutrient, high organic matter soil. Protozoa and nematode populations should decrease in response to lower numbers of bacteria and fungi, resulting in decreased mineralization. A reduction in the mineralization of nutrients should further decrease the ability of the ozone-exposed plant to obtain nutrients, resulting in a greater reduction in plant growth when grown in low nutrient, low organic matter soil.

3.0 Materials and Methods

3.1 Plant Culture

Wheat (*Triticum aestivum* var. 'Classic') plants were used in this study. Sterile seedlings were obtained by the following method. Wheat seeds were aeseptically germinated by placing approximately 30 seeds on damp, sterile filter paper, inside a sterile petri plate. Approximately 15 petri plates were used to germinate 450 seeds, in each experiment. Significantly more seeds were germinated than needed, to account for the possibility of non-viable seeds or contamination.

Approximately 3 days after the initiation of germination the seedlings were planted in pots made of polyvinyl chloride (PVC) pipe. The pots were 14.7 cm high and 10.9 cm in diameter. The pots were filled with soil to 1.4 cm from the rim. Three seeds were planted in each pot in experiment 1, and five seeds were planted in each pot, in Experiment 2 (Fig. 10). All seeds were sowed approximately 1.4 cm deep into the soil.

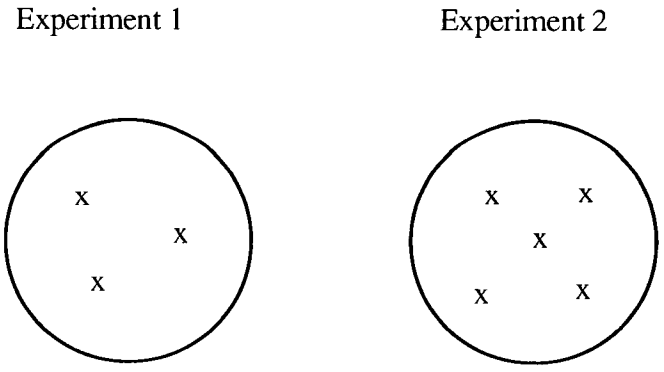


Fig. 10. Planting pattern of seedlings within each pot, where seedling position is represented by "x" and large circles represent the perimeter of the pot.

The pots were saturated with Hoaglund's nutrient solution (see Appendix) immediately after planting, then watered with tap water throughout the remainder of the study approximately every 48 hr (hot days often required more frequent wetting). Hoaglund's nutrient solution was substituted every third watering. Mesh screen in the bottom of each pot allowed liquid to drain while retaining soil.

3.2 Soil

Two different soil types were tested in this study. The first experiment utilized a low organic matter, low clay soil, Millican soil. This soil has been extensively characterized by both the Biotechnology and Ecological Site Assessment Programs within ERL-C (Table 6). The second experiment utilized Millican soil enhanced with organic material. Peat was mixed with Millican soil, in a cement mixer (1 part peat: 9 parts Millican soil). The soil was stored in 32 gallon plastic refuse containers, lined with large trash bags.

Table 6. Chemical and physical analysis of low organic matter Millican soil and Millican soil amended with organic matter (Biotechnology and Ecological Site Assessment Programs, ERL, Corvallis).

Parameter	Millican soil	
	low organic matter	high organic matter
pH	7.1	5.0
Phosphorus (ppm)	14.9	22.1
Potassium (ppm x 10)	4.2	4.8
Calcium (m_{eq}/ 100 g)	4.9	8.9
Magnesium (m_{eq}/ 100 g)	2.2	3.1
Organic matter (%)	1.2	5.1
Cation exchange capacity (m_{eq}/ 100 g)	8.7	15.2
Ammonium (ppm)	3.0	14.4
Nitrate (ppm)	4.5	6.1
Sand (%)	82.2	77.6
Silt (%)	14.1	12.1
Clay (%)	2.5	5.2

3.3 Growth Conditions

The plants were placed in chambers located inside a single, unshaded greenhouse. The chambers were 90 cm high x 90 cm deep x 135 cm wide, and rested on benches approximately 90 cm above the greenhouse floor. Light in the greenhouse was supplemented from 0500 h to 2100 h by 450 W high intensity mercury discharge lamps. Photoperiod in the green house and chambers was 12 hr days / 12 hr nights and PAR was approximately 330 $\mu mol\ m^{-2}\ s^{-1}$. The average temperature in each chamber was 23°C, and the average daily minimum and maximum temperatures were approximately 17 and 29°C, respectively. The average relative humidity in the chambers ranged from 30% to 50% relative humidity.

3.4 Ozone Treatments

Exposures to air with additional ozone took place within the chambers. The pattern of ozone administered mimicked the ozone concentration pattern often seen in and downwind of urban areas. Ozone was administered in an episodic pattern by a programmable exposure control system which uses a HP 41CV hand-held computer (Hewlett-Packard, Corvallis, Oregon). Ozone concentration within the treatment chambers started at a daily minimum of approximately 4 $ng\ g^{-1}$ at approximately 0500 hrs, gradually increased to an average maximum of approximately 120 $ng\ g^{-1}$ by 1700 hrs, then gradually decreased to the original minimum concentration by 0500 hrs the following day (Fig. 11) (Hogsett et al., 1985b).

3.5 Experimental Design

Two separate experiments were carried out, utilizing different soil types and experimental designs.

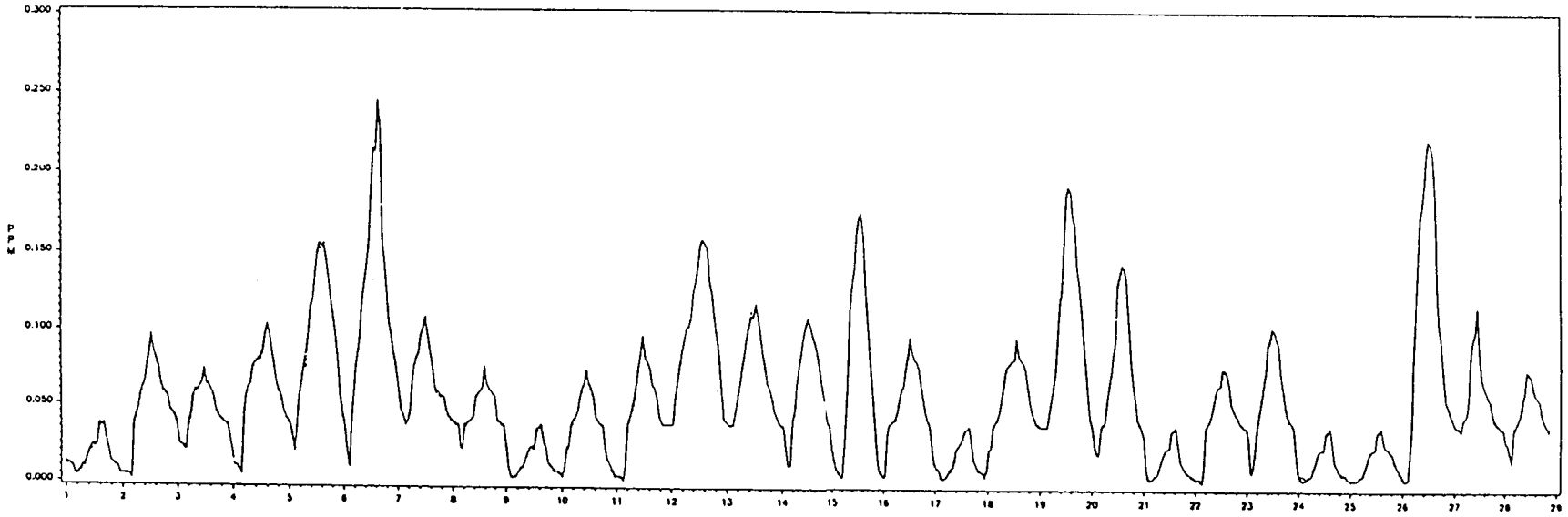


Fig. 11. Twenty-eight day episodic ozone profile.

3.5.1 Experiment 1

The first experiment utilized two exposure chambers for each treatment: ozone and no ozone. Ten replicate pots were placed in each chamber. Extra pots were planted and placed in each chamber to allow for seedling mortality. After 26 days of exposure, five pots were removed from each chamber for analysis. Two weeks later the remaining five pots in each chamber were removed for analysis. The two harvest dates represented a harvest date before and after seed set in the wheat plants.

3.5.2 Experiment 2

The second experiment utilized three exposure chambers for each treatment: ozone and no ozone. Ten replicate pots were placed in each chamber. Extra pots were prepared and placed in each chamber to allow for seedling mortality. After 35 days of exposure, all ten pots in each chamber were removed for analysis.

3.6 Photosynthesis and Soil Respiration Measurements

Photosynthetic rate ($\mu mol\ m^{-2}s^{-1}$) was recorded using a portable LI-COR photosynthesis system, model 6200, with 0.25 l cuvette (Li-Cor Inc., Lincoln, Nebraska). The LI-COR 6200 also recorded the temperature, humidity, and rates of CO_2 depletion or accumulation inside the cuvette at the time each measurement was taken. Photosynthesis measurements were taken for a period of 60 s, using the youngest fully expanded leaf on the tallest plant in each pot. Ambient CO_2 concentrations were less than 380 $\mu l\ l^{-1}$ (characteristic of natural CO_2 levels) before measurements were taken. The temperature and humidity were constant throughout the measurement period. Photosynthetic rates were computed and stored using the LI-COR system datalogger and Quatro Pro software. Photosynthesis measurements were taken approximately 2-8 hrs before plant harvest.

Soil respiration rate for each replicate in Experiment 1 was recorded using the LI-COR 6200 system (Li-Cor Inc., Lincoln, Nebraska). Soil respiration was not recorded in Experiment 2. CO_2 evolution through the mesh screen covering the bottom of each pot was measured over a 60 s period, using a specially designed adapter that fit firmly over the bottom of the pot base. This measurement accounted for approximately half of the total below-ground respiration, since evolution of CO_2 from the top of the pot was not measured. The LI-COR cuvette was removed and tubes that allow air from the analyzer into the specially designed adapter, and from the cuvette to the analyzer were securely connected, retaining the integrity of the closed system. Adjustments were made to correct for system volume. Soil respiration rates were computed and stored using the LI-COR system datalogger and Quatro Pro software and are expressed as nmol $pot^{-1}s^{-1}$. Soil respiration measurements were taken immediately before plant harvest.

3.7 Plant Harvest

Plants and soil were removed from each replicate pot by tipping the pot upside-down and shaking the pot until the soil was loosened from the sides. The soil and wheat plants gently fell from the pot onto a large piece of plastic that was spread over the top of a work-table. The bulk soil, which fell easily from the root system when the plant was lifted, was pushed to the side of the work-space. Rhizosphere soil was defined as soil adhering to roots (Atlas and Bartha, 1992), and was obtained by gently shaking the remaining soil from the root system, onto the workspace. A sample of rhizosphere soil (approximately 100 g) was placed in a labeled polyethylene bag for analysis of bacterial, fungal, protozoa and nematode population estimates. Labels consisted of the chamber number, and pot number within each chamber.

In the laboratory, a 5 g sample of each rhizosphere soil sample was weighed, oven dried (48 h at 85°C), then reweighed, to determine the dry weight of each soil sample. Ten

(Experiment 1) or 50 (Experiment 2) grams of rhizosphere soil was weighed out onto a milk filter for nematode extraction (Anderson and Coleman, 1977). The milk filters were folded and taped with an adhesive label, to retain soil and designate the soil sample number.

The shoot material from each replicate pot was severed just above the adventitious roots. The shoot material was stored in a paper bag, labeled with the appropriate chamber and pot number designation. The shoot tissue was oven dried (48 h at 85°C), then weighed to 0.01 g, to determine the dry weight of shoot tissue for each replicate.

The root system from each pot (including root fragments that were broken from the main root mass during separation) was placed in a small, labeled polyethylene bag. A root subsample was obtained from each replicate for VA mycorrhizal analysis. The root subsample was weighed, then placed between damp paper towels, and stored in a labeled polyethylene bag. The remaining root system from each replicate pot was weighed to 0.01 g, oven dried (48 h at 85°C), then reweighed to obtain the dry weight of root tissue for each replicate pot.

Root and shoot tissue measurements were normalized so comparisons could be made between Experiment 1 and 2, by multiplying the shoot weight per pot in the second experiment by a factor of (3/5).

3.8 Rhizosphere Assays

Approximately 24 hr after plant harvest, a 1:10 dilution of soil from each soil sample was prepared by placing one gram of soil in a dilution tube containing 9 ml of sterile phosphate buffer (Herzberg et al., 1978). Phosphate buffer was prepared by adding 28 ml of 0.2 M monobasic phosphate buffer (2.7 g monobasic phosphate in 100 ml distilled H_2O) to 72 ml of 0.2 M dibasic phosphate buffer (3.5 g anhydrous dibasic phosphate in 100 ml distilled H_2O). The 1:10 dilution was shaken for 5 minutes, and a 10^{-2} dilution

prepared by adding 1 ml of the 1:10 dilution to another dilution tube containing 9 ml of sterile phosphate buffer. One ml of the 10^{-2} dilution was transferred to a small, labeled test tube for total bacteria estimates. For total and active fungal estimates and active bacteria estimates, 0.5 ml of the 1:10 dilution was transferred to another small, labeled test tube. Sample dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared for analysis of protozoan population size.

To estimate the biomass of active bacteria (Lodge and Ingham, 1991), total fungi, and active fungi (Ingham and Klein, 1984) per gram dry weight of soil, fluorescein diacetate (FDA) working solution was prepared by adding 1 ml of FDA stock solution (2 mg of fluorescein in 10 ml of acetone, dissolved for 30 minutes) to 99 ml of phosphate buffer. A 0.5 ml aliquot of the 1:10 dilution (see above) was stained with 1 ml of FDA working solution, for approximately 3 minutes. One ml of alkaline phosphate buffer agar (1.5 g agar in 100 ml dibasic phosphate buffer) was added to the FDA-soil suspension, the aliquot mixed, and a few drops placed on a slide with known well depth. A coverslip was placed over the well, forming an agar film. The length of active fungal hyphae were measured using epifluorescent microscopy. Three transects through the agar film on the slide were made, using 250 X magnification, and the length of the FDA-stained hyphae recorded for each transect. Average diameter of the observed hyphae was recorded for each sample. Total fungal hyphae were then estimated, using phase-contrast microscopy. Three transects were made through the slide using 250 X magnification. The total length of all hyphae in each transect, and approximate average diameter, was observed and recorded. Active bacterial estimates were made using epifluorescent microscopy. Five fields were examined at 450 X magnification, and the number of apple-green rod-shaped or cocci bacteria were observed and recorded for each field.

To estimate the total number of bacteria in each soil sample, one ml of fluorescein isothiocyanate (FITC) solution was added to a small test tube containing 1 ml of the 10^{-2} soil suspension (prepared above), and stained for at least 3 minutes (Babiuk and Paul,

1970). Fluorescein isothiocyanate solution was prepared by adding 2 mg of fluorescein isothiocyanate to 10 ml of FITC mix. Fluorescein isothiocyanate mix was prepared by combining 1.02 ml of Na_2CO_3 (0.053 g Na_2CO_3 in 1 ml of distilled H_2O), 4.49 ml of PO_4 buffer (0.2 M, pH 7.2; see recipe above), and 4.49 ml of physiological saline (85% solution). Using a sterile 1 ml syringe, the entire sample was filtered through a sterile, non-fluorescent (iraglan-black stained), 25 mm diameter, 0.2 μm pore size Nuclepore polycarbonate filter on a Sweenex filter holder. Using a sterile 1 ml syringe, 1 ml of sodium carbonate solution (5.3 g Na_2CO_3 in 100 ml distilled water) was filtered through the sample on the filter holder, to rinse excess stain from the sample. Using another sterile 1 ml syringe, 1 ml of 5% pyrophosphate solution (5 g pyrophosphate in 100 ml distilled water) was filtered through the sample on the filter holder, to quench extraneous fluorescence. Using the same syringe, 1 to 2 volumes of air were pushed through the filter to make certain all liquid had passed through. The Sweenex filter was then opened and the Nuclepore filter removed with forceps, and placed on a labeled slide. These filters were stored in a slide box.

Numbers of total bacteria were determined for each filter using epifluorescent microscopy. Brightly fluorescent, apple-green bacteria were counted at 1000 X magnification. Care was taken to ensure only morphologically distinct (cocci or rod-like) bacterium were counted. The number of bacteria in 10 fields was recorded, along with a record of the dilution used, area counted, and magnification used.

Protozoan population structure was determined for each soil sample using soil dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} (Darbyshire et al., 1974). A 24-well tissue culture plate was labeled and prepared for each sample by pipetting 0.5 ml of molten soil extract agar in each well. Soil extract agar was prepared by mixing a ratio of 1 part soil: 9 parts distilled water, letting the soil settle from the mixture, and using the supernatant to prepare 2% agar. A 0.5 ml aliquot of the 10^{-1} dilution was placed in each of the top four wells of the tissue culture plate, and the row labeled as the 10^{-1} dilution designation. This

procedure was repeated for the next five dilutions, using the next five rows in the tissue culture plate. The tissue culture plate, containing four 0.5 ml replicate samples per dilution, was incubated at room temperature (22°C) for 4-7 days. Following incubation, each well was mixed, a drop placed on a microscope slide, and covered with a glass coverslip. One transect of the coverslip was examined, using phase-contrast microscopy at 250 X magnification, and the presence or absence of flagellates, amoebae, and ciliates noted. Using a most probable number table, numbers of each group in the sample were determined.

Nematode populations for each soil sample were estimated through the following procedure (Anderson and Coleman, 1977). Soil was weighed onto milk filters (see experimental design), placed on a Baerman extractor funnel, the funnel tube clamped, and the extractor filled with water and covered with a plastic disc to prevent evaporation. The nematodes were extracted for four days. Care was taken to maintain the water level in the funnel above the soil. After four days, the funnel clamp was released and 40 - 50 ml of the water drained into a vial. The vials were stored at 4°C in a refrigerator. The nematodes were counted by carefully pouring the top two-thirds of the vial into one Rodac plate (counting dish). The bottom one-third of the vial was gently mixed, then poured into a second Rodac plate. Using a dissecting scope, the total number of nematodes in each sample were counted and recorded. The first 30 nematodes in each sample were picked from the counting dish, using a fine dental pick, and placed in a drop of distilled water on a slide, covered with a glass coverslip, and heat fixed by passing a flame under the slide for approximately 1 second. Each nematode was then identified to genus, using phase contrast microscopy, and the genus recorded with the sample number.

The standing crop of bacterial biomass was calculated for both control and ozone treatments in Experiment 1 and 2, to account for the bacteria preyed upon by rhizosphere protozoa and nematodes in the 24 hour period before each harvest. The estimated number of bacteria consumed by predatory protozoa and nematodes per day was added to the total

bacterial biomass, the sum of which was the total standing crop of bacteria for the 24 hr period prior to harvest.

Vesicular-arbuscular fungal mycorrhizal colonization in the root system of each root subsample was measured (Phillips and Hayman, 1970). Each root subsample was placed in a small, labeled vial. Ten percent KOH was added to clear the roots of dark colored compounds within the cytoplasm for 15 hrs. Trypan blue was added to stain fungal hyphae for approximately 24 hrs. Roots were rinsed with distilled water and stored, until examination, in labeled vials filled with distilled water. Twenty-five 1 cm segments were removed from each subsample and aligned parallel in a single row on a microscope slide. A transect was made across the slide, perpendicular to the root segments, and VA mycorrhizal colonization noted at the intersection of each root segment with the transect. Percent colonization for each root sample was then calculated as the number of colonized segments per 25 segments.

3.9 Statistical Analysis

An analysis of variance was be used to determine significant differences between control and ozone-exposed treatments. A p-value of greater than 0.05 was chosen to indicate that there was no treatment effect. Therefore, statistical significance for a treatment effect was indicated by $p \leq 0.05$. Treatment effects that were less apparent might not have been detected using such a strict level of significance, yet these responses were biologically interesting and warranted further exploration. Therefore, a trend in differences between treatments was be noted, and indicated by $0.05 \leq p \leq 0.10$. Statistical analyses were performed using Statgraphics statistical software (Manguistics, Inc).

4.0 Results

All comparisons were made between ozone-exposed plants and control, unexposed plants. Experiment 1 sample dates were 26 and 40 days after the initiation of ozone exposure, while in Experiment 2, samples were taken only once, 35 days after ozone exposure was initiated.

4.1 Plant Responses

Plant photosynthesis was not significantly reduced by ozone exposure in the first experiment on either the first or second harvest dates (Table 7; Fig. 12). Plant photosynthesis was significantly ($p \leq 0.05$) reduced due to ozone exposure in the second experiment (Table 7; Fig. 13). Ozone caused a mean reduction of 3.34 $\mu mol\ m^{-2}s^{-1}$ in plant photosynthesis (Table 7).

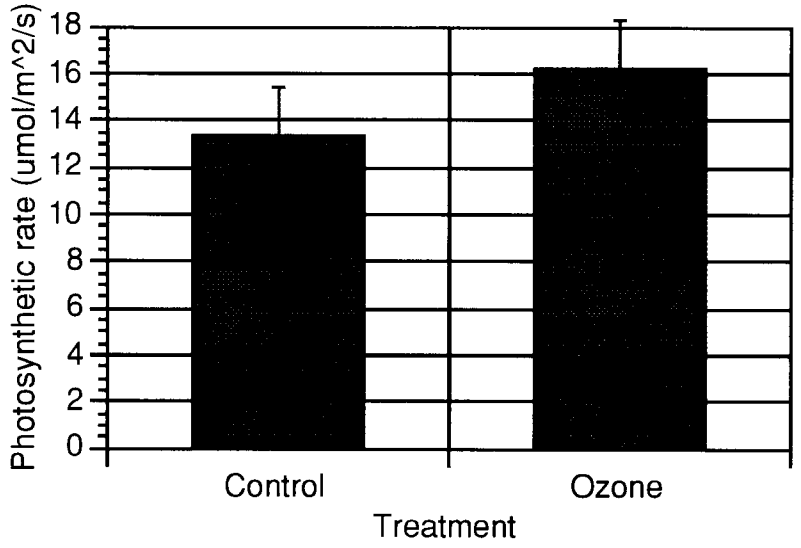
Table 7. Effects of ozone on photosynthesis of wheat plants.

Experiment	No. days of exposure	Mean photosynthetic rate ($\mu mol\ m^{-2}s^{-1}$) Ozone	Control	Ozone effect on photosynthetic rate (+ / - $\mu mol\ m^{-2}s^{-1}$)	Sign. level
1	26	16.19	13.31	+2.79	0.44
1	40	15.94	19.06	-3.12	0.38
2	35	16.00	19.34	-3.34	0.05[a]

[a] denotes a statistically significant difference at 95% confidence level (LSD F-test)

Ozone did not significantly affect the dry weight of above-ground plant tissue (shoot dry weight) in the first experiment, at either harvest date (Table 8; Fig. 14). However, shoot weight was significantly ($p \leq 0.05$) increased by ozone exposure in the second experiment (Table 8; Fig. 15). Ozone caused a mean increase of 0.59 g in shoot dry

a.



b.

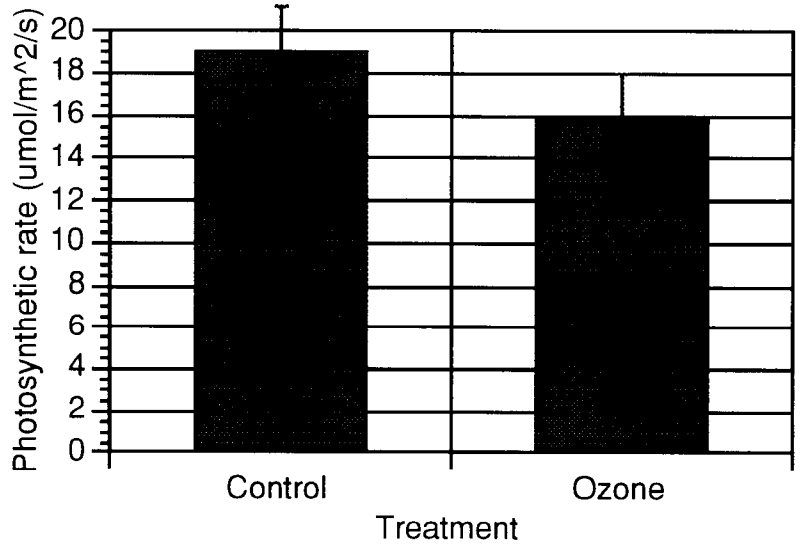


Fig. 12. Effects of ozone on photosynthetic rate (μmol m^{-2} s^{-1}) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

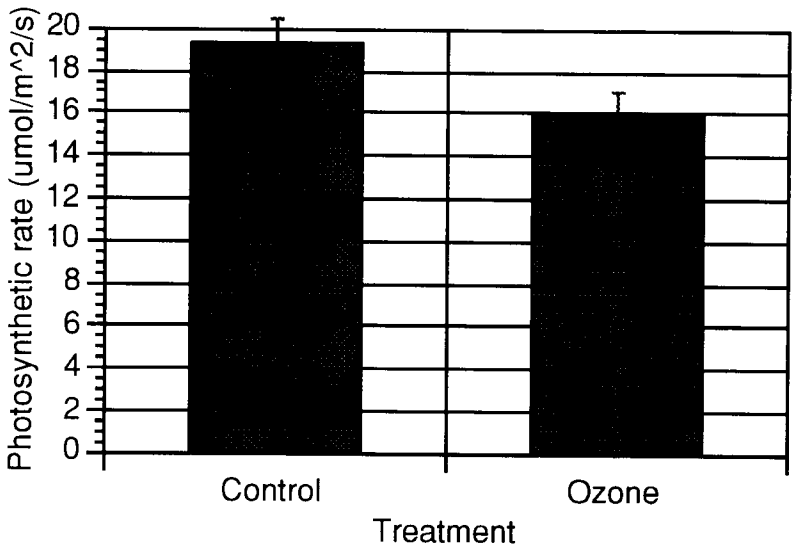
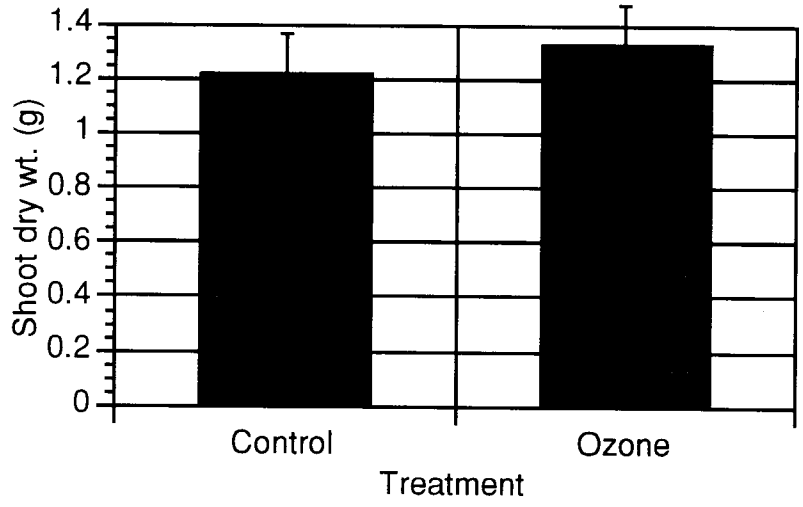


Fig. 13. Effects of ozone on photosynthetic rate (μmol $m^{-2}s^{-1}$) in Experiment 2 (bars represent one SE).

weight, in the second experiment. Root dry weight was not significantly influenced by ozone exposure in Experiment 1 or 2 (Fig. 16, 17). The ratio of root tissue to shoot tissue (root:shoot) was not significantly different between treatments in Experiment 1, on either the first or second harvest date (Table 9; Fig. 18). In the second experiment ozone produced a highly significant ($p=0.007$) reduction in root:shoot (Table 9; Fig. 19). The mean root:shoot in ozone exposed pots was 30% less than the mean root:shoot in control pots, in Experiment 2.

Below-ground respiration measurements estimated approximately half of the total below-ground respiration, including both root and microbial respiration. Below-ground respiration measurements in the first harvest of Experiment 1 were higher in the ozone treated plants ($p\leq 0.10$), however the difference was not statistically significant ($p\leq 0.05$) (Fig. 20a). Ozone did not significantly affect below-ground respiration in the second harvest of Experiment 1 (Fig. 20b). Below-ground respiration was not measured in the second experiment.

a.



b.

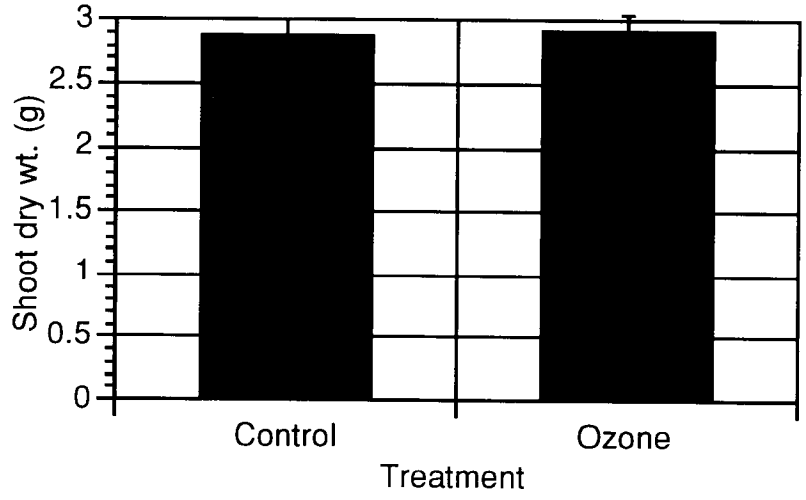


Fig. 14. Effects of ozone on shoot dry weight (g) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

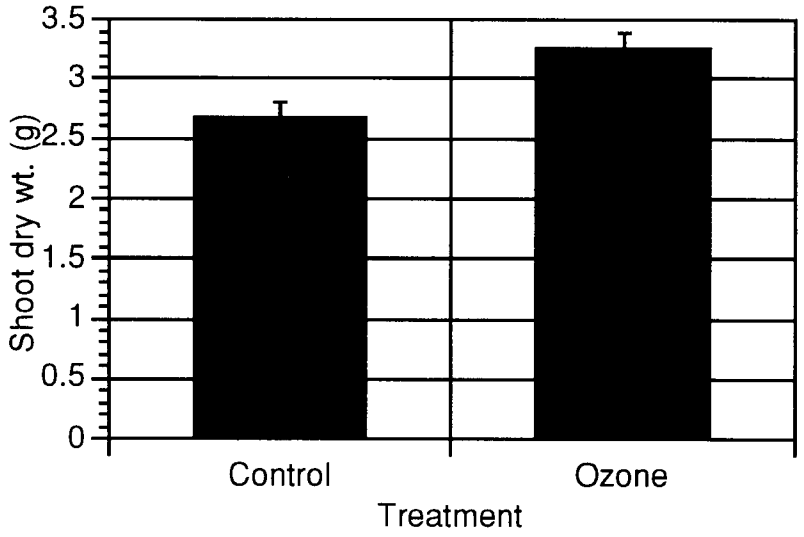


Fig. 15. Effects of ozone on shoot dry weight (g) in Experiment 2 (bars represent one SE).

4.2 VAM Responses

Ozone reduced the percent of vesicular-arbuscular mycorrhizal colonization of plant roots in both the first and second experiments (Table 10; Fig. 21, 22). In the first experiment VAM colonization was reduced by 14.0% in the first harvest, and 20.8% in the second harvest ($p \leq 0.10$ and $p \leq 0.05$, respectively), as a result of ozone exposure. Ozone caused a significant ($p \leq 0.05$) decrease in VAM colonization in the second experiment, reducing root colonization by 26.1% (Table 10).

4.3 Rhizosphere Responses

Results from rhizosphere assays in the first harvest of Experiment 1 indicate that ozone-exposure resulted in an increase in flagellate populations and a decrease in amoebae populations at the first harvest ($p \leq 0.10$, Fig. 23a, 24a). Rhizosphere soil in ozone-

exposed plants had a mean of 23,988 flagellates gdw^{-1} (gram dry weight of soil), while a mean of 6457 flagellates gdw^{-1} was observed in the rhizosphere of control, unexposed plants. Conversely, a mean of only 5 amoebae gdw^{-1} were found in the rhizosphere of ozone-exposed plants, while 123 amoebae gdw^{-1} were observed in the rhizosphere of control plants. At the second harvest, flagellate populations in the ozone-exposed treatment were not significantly different than in the control treatment ($p>0.10$, Fig. 23b). The trend of decreased amoebae populations in response to ozone was again observed at the second harvest ($p\leq0.10$, Fig. 24b). Rhizosphere soil of control plants contained a mean of 813 amoebae gdw^{-1}, while only 324 amoebae gdw^{-1} were observed in the rhizosphere of ozone-exposed plants. Populations of total and active bacteria, total and active fungi, cilliates, and nematodes were not reduced as a result of ozone exposure in either the first or second harvest of Experiment 1 (Table 11).

Table 8. Effects of ozone on shoot dry weight of wheat plants.

Experiment	No. days of exposure	Mean shoot dry weight (g)		Treatment effect on shoot dry weight (+/- g)	Sign. level
		Ozone	Control		
1	26	1.33	1.22	+0.11	0.67
1	40	2.95	2.87	+0.08	0.72
2	35	3.26	2.67	+0.59	0.03[a]

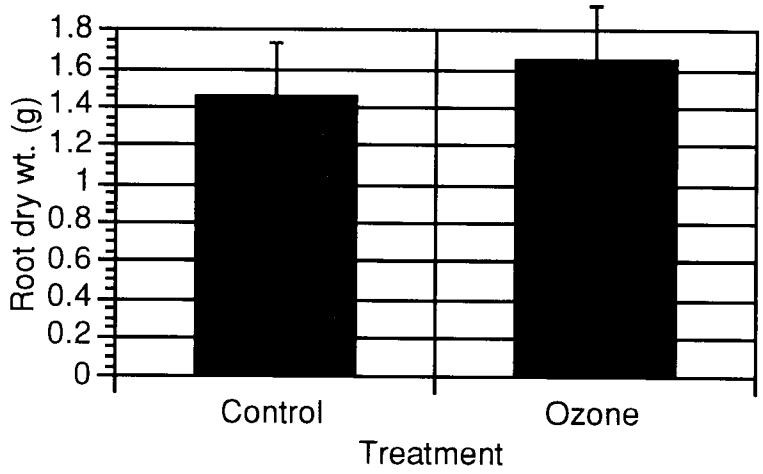
[a] denotes a statistically significant difference at 95% confidence level (LSD F-test)

Table 9. Effects of ozone on root/shoot ratio of wheat plants.

Experiment	No. days of exposure	Mean root/shoot ratio		Treatment effect on root/shoot ratio (+/-)	Sign. level
		Ozone	Control		
1	26	1.23	1.21	+0.02	0.92
1	40	0.68	0.68	0.00	0.94
2	35	1.06	1.53	-0.47	0.007[a]

[a] denotes a statistically significant difference at 99% confidence level (LSD F-test)

a.



b.

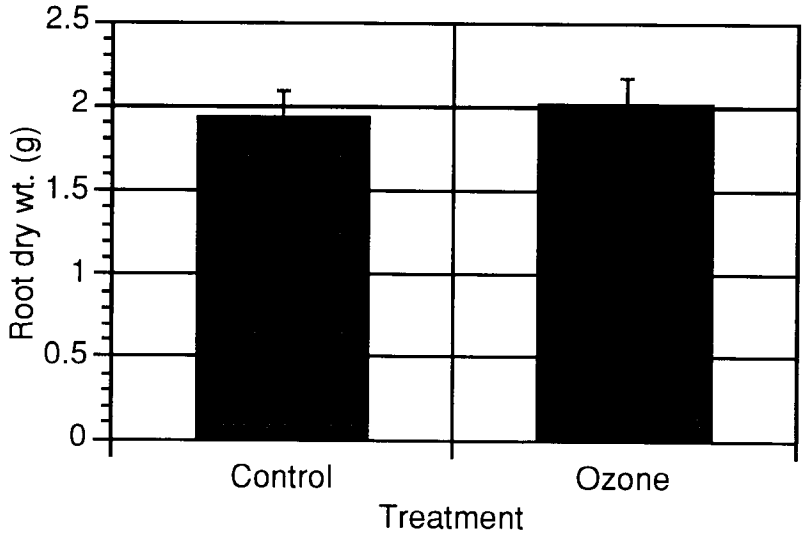


Fig. 16. Effects of ozone on root dry weight (g) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

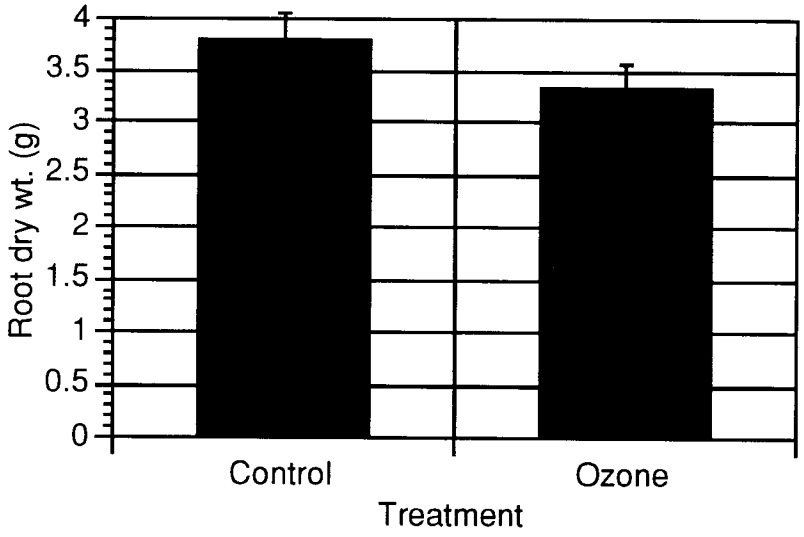


Fig. 17. Effects of ozone on root dry weight (g) in Experiment 2 (bars represent one SE).

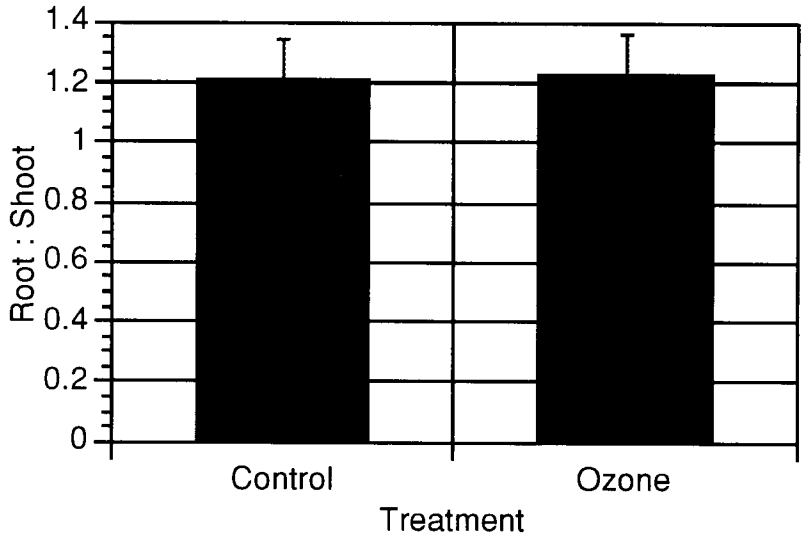
Significant differences or trends in the populations of rhizosphere flagellates in ozone vs. control pots were detected in the second experiment (Table 11). A trend of reduced flagellate populations in response to ozone was observed in the rhizosphere soil ($p \leq 0.10$) of the experimental wheat plants (Fig. 25). Ozone was not shown to affect rhizosphere populations of fungi, bacteria, amoebae, cilliates or nematodes (Table 11).

Ozone increased the standing crop of bacterial biomass in the rhizosphere at the first harvest of Experiment 1 ($p \leq 0.10$), although this increase was not significant. The standing crop of bacterial biomass in the rhizosphere of ozone-exposed plants was not affected by ozone in the second harvest of Experiment 1. Ozone also did not influence the standing crop of bacterial biomass in the second experiment.

4.4 Influences of Soil Type on Ozone Responses

To assess the influence of added soil organic matter on the responses of wheat plants and rhizosphere organisms to ozone, the results obtained from day 40 of the first

a.



b.

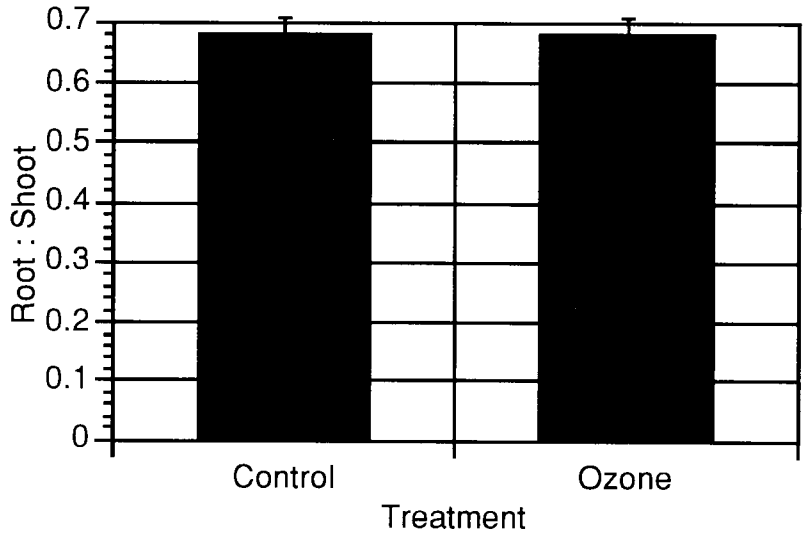


Fig. 18. Effects of ozone on root:shoot at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

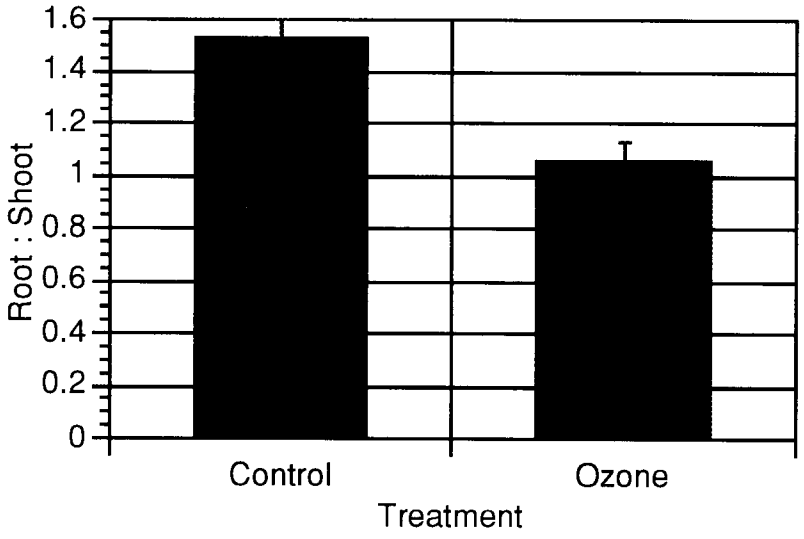


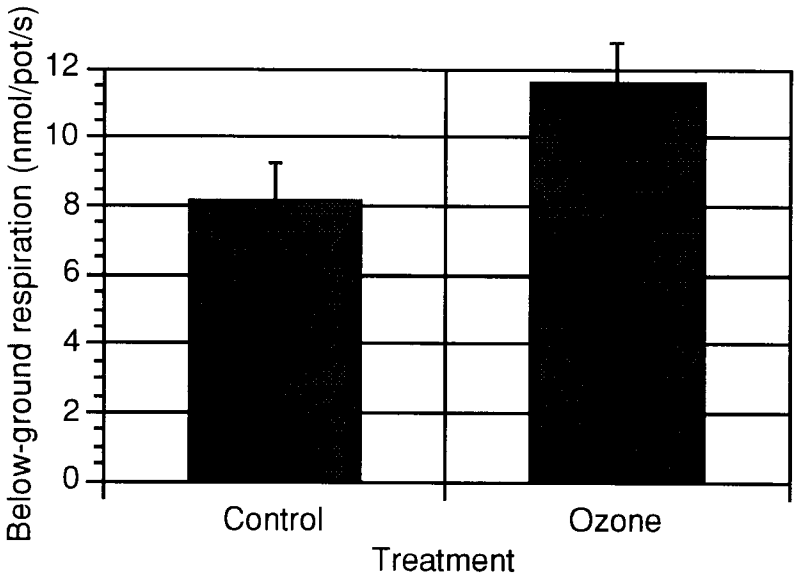
Fig. 19. Effects of ozone on root:shoot in Experiment 2 (bars represent one SE).

experiment were compared with the results obtained in the second experiment (Table 12). Although the experiments took place at different seasons, conditions within the climatic controlled chambers in Experiment 2 were very similar to those in Experiment 1. The humidity, photoperiod and PAR (approximately 330 μmol m^{-2} s^{-1}) were similar in both experiments. Warm, sunny climatic conditions outside the greenhouse occurred during the first experiment. As a result, temperatures within the chambers in the first experiment were slightly higher (3-4°C) than those in the second experiment.

Three seedlings were planted in each pot in the first experiment, whereas five seedlings were planted in each pot in the second experiment. Therefore, more root material per pot occurred in Experiment 2 as compared to Experiment 1. To compare of results in the two experiments, the root and shoot dry weight was normalized per plant.

The root dry weight per plant was not affected by ozone exposure when the plants were grown in either low nutrient, low organic matter soil, or high nutrient, high organic matter soil. Shoot dry weight was increased in the plants that were exposed to ozone and

a.



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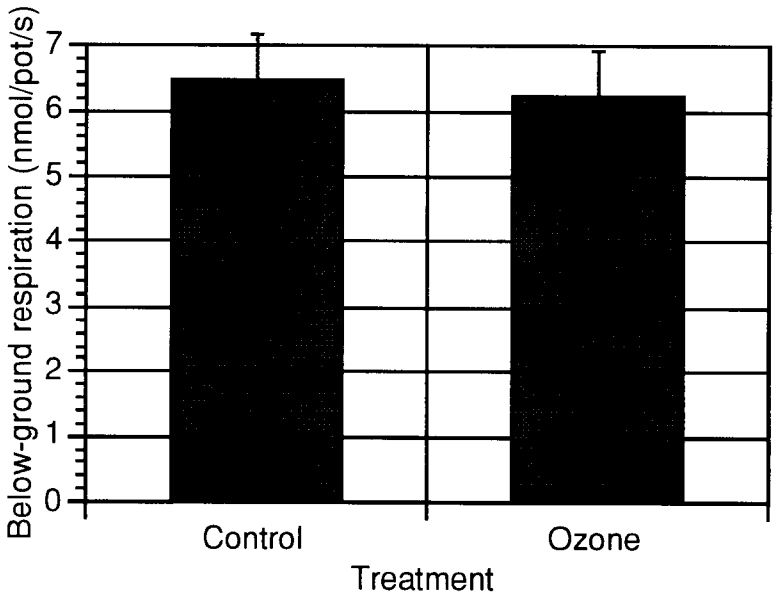


Fig. 20. Effects of ozone on approximately half of the below-ground respiration (nmol $pot^{-1}s^{-1}$) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

grown in high nutrient, high organic matter soil. However, an increase in shoot dry weight due to ozone exposure was not observed in the plants grown in low nutrient, low organic matter soil. The ratio of root:shoot was reduced by ozone exposure when plants were grown in high nutrient, high organic matter soil, but this reduction was not observed when plants were grown in low nutrient, low organic matter soil.

Table 10. Effects of ozone on VAM colonization of wheat plants.

Experiment	No. days of exposure	Mean % VAM colonization		Treatment effect on VAM colonization (+/- %)	Sign. level
		Ozone	Control		
1	26	50.8	64.8	-14.0	0.07[a]
1	40	55.2	76.0	-20.8	0.01[b]
2	35	39.5	65.6	-26.1	0.003[c]

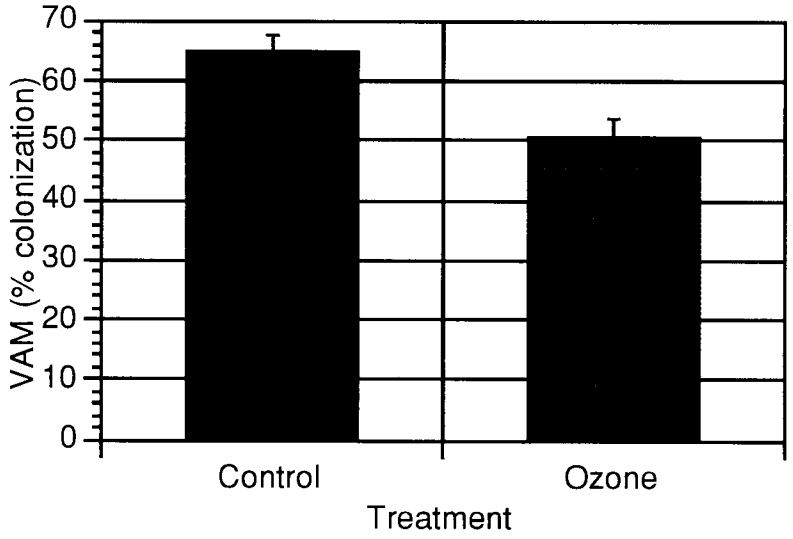
[a] denotes a statistically significant effect at 90% confidence level (LSD F-test)
[b]denotes a statistically significant effect at 95% confidence level (LSD F-test)
[c]denotes a statistically significant effect at 99% confidence level (LSD F-test)

Table 11. Mean effects of ozone (+ or -) and p-values (LSD F-test) for the significance of ozone effects on microorganisms in the rhizosphere of wheat plants.

Parameter	Significance level					
	Exp. 1 (1st harvest)		Exp. 1 (2nd harvest)		Exp. 2	
	Mean	p-value	Mean	p-value	Mean	p-value
Active fungi	+9.73	0.46	+4.52	0.70	-5.87	0.78
Total fungi	-1.48	0.98	-2.87	0.97	-114.49	0.33
Active Bacteria	+0.30	0.39	+0.01	0.99	-0.56	0.18
Total Bacteria	-0.09	0.99	-1.12	0.13	+0.35	0.54
log Flagellates	+0.57	0.06	-0.08	0.78	-0.55	0.10
log Amoebae	-1.40	0.09	-0.40	0.08	+0.12	0.79
log Cilliates	+0.63	0.34	-0.11	0.83	+0.25	0.22
Nematodes	+1.41	0.32	+0.28	0.91	-0.30	0.34

A significant ($p \leq 0.05$) reduction in photosynthesis occurred following ozone exposure, when plants were grown in high nutrient, high organic matter soil. A reduction

a.



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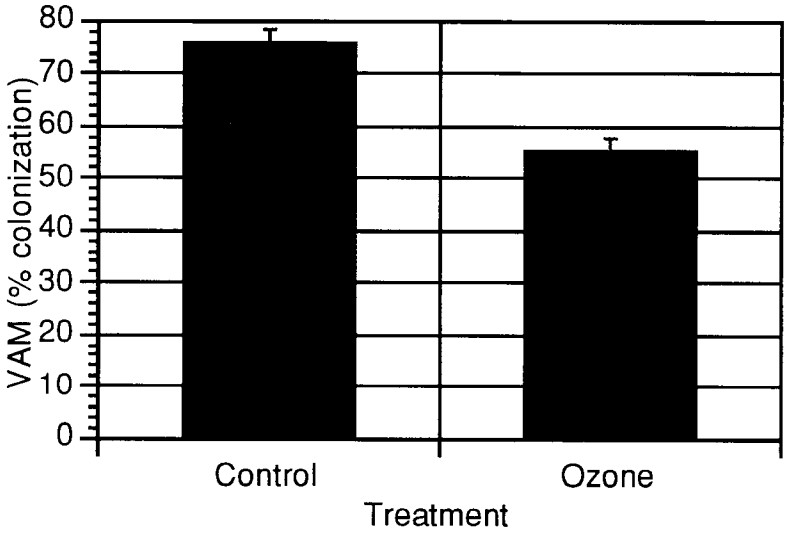


Fig. 21. Effects of ozone on vesicular-arbuscular mycorrhizal colonization (% colonization) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

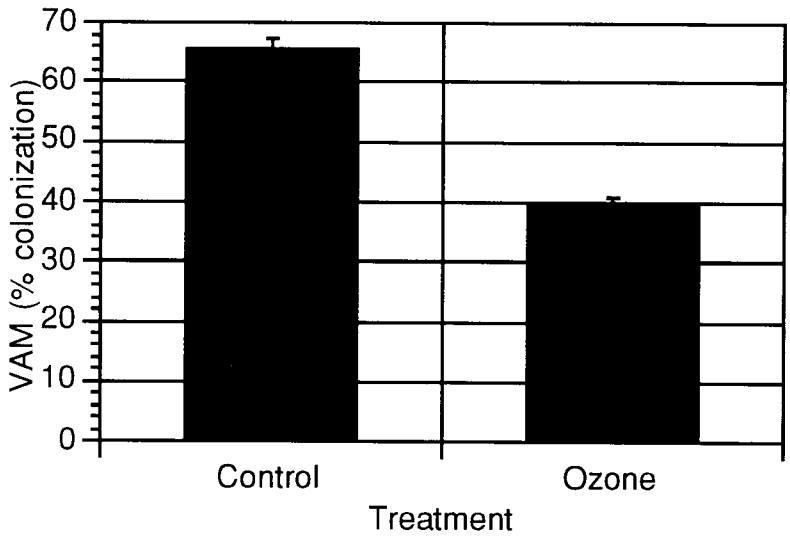


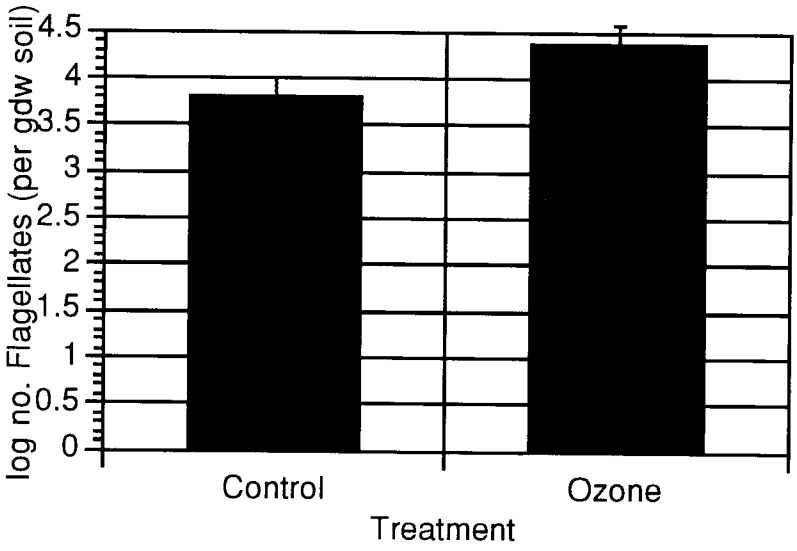
Fig. 22. Effects of ozone on vesicular-arbuscular mycorrhizal colonization (% colonization) in Experiment 2 (bars represent one SE).

in photosynthesis also occurred when the plants were grown in low nutrient, low organic matter soil, but this ozone-induced reduction was not significant ($p \geq 0.10$).

Ozone-induced changes in rhizosphere populations of exposed plants grown in low nutrient, low organic matter soil were different than those of plants grown in high nutrient, high organic matter soil. A reduction in amoebae in the rhizosphere of ozone-exposed plants grown in low nutrient, low organic matter soil was not observed when the plants were grown in high nutrient, high organic matter soil. Populations of total and active bacteria, total and active fungi, cilliates and nematodes did not show significant responses to ozone exposure in either low nutrient, low organic matter, or high nutrient, high organic matter soil

The only trend that was apparent in both experiments was a reduction in VAM colonization of roots as a result of ozone exposure. VAM colonization was reduced by 20.8% in the roots of plants exposed to ozone, and grown in low nutrient, low organic matter soil. A 26.1% reduction in VAM colonization was observed in the roots of ozone-

a.



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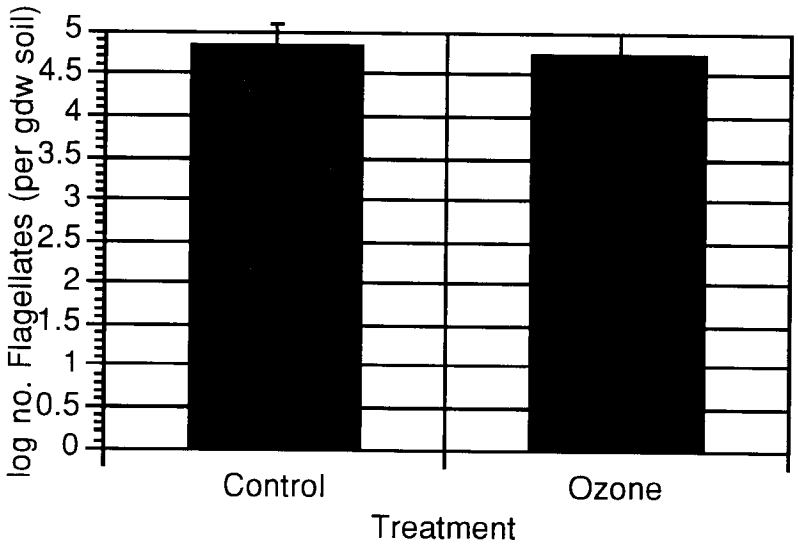
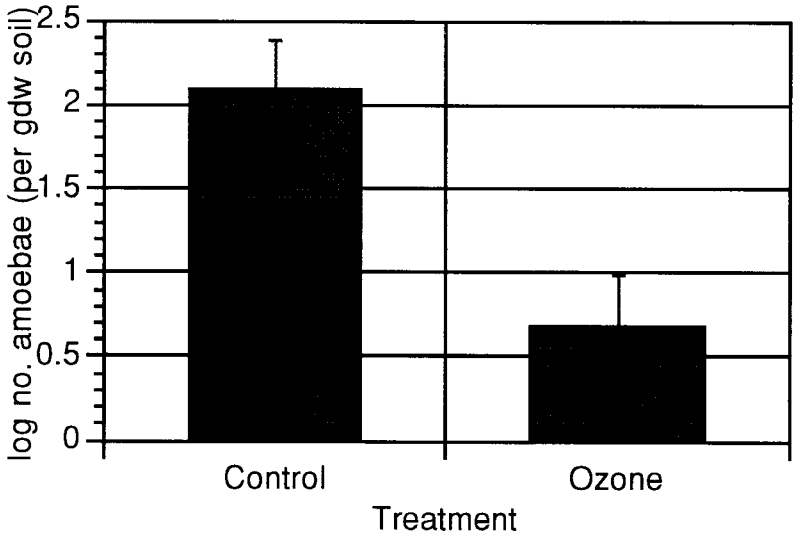


Fig. 23. Effects of ozone on the log number of flagellates (per gdw of soil) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

a.



b.

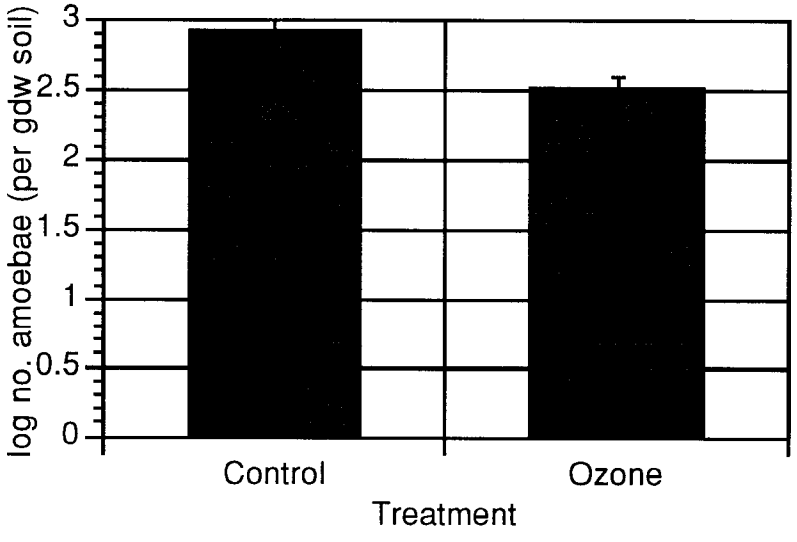


Fig. 24. Effects of ozone on the log number of amoebae (per gdw of soil) at the first (a) and second (b) harvest dates of Experiment 1 (bars represent one SE).

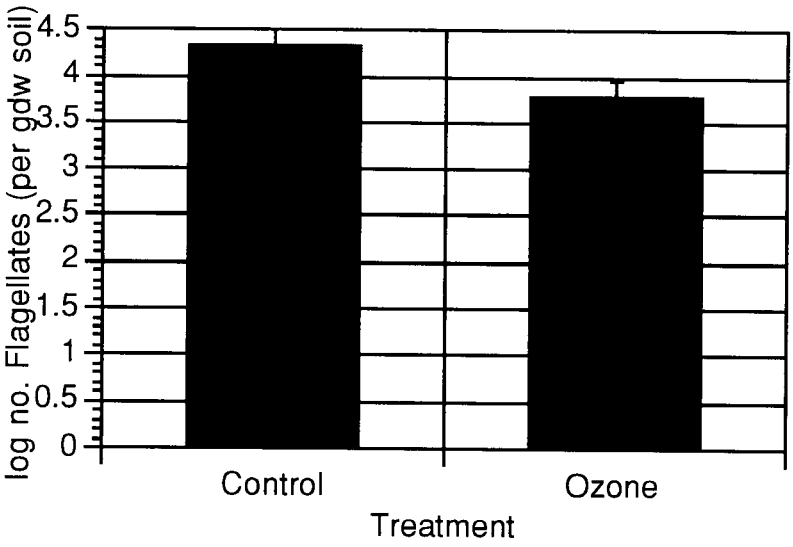


Fig. 25. Effects of ozone on the log number of flagellates (per gdw of soil) in Experiment 2 (bars represent one SE).

exposed plants grown in high nutrient, high organic matter soil. The ozone-induced reduction in root colonization was higher in plants grown in high nutrient, high organic matter soil than in plants grown in low nutrient, low organic matter soil.

The overall effects of varying soil nutrients and organic matter on wheat plants and rhizosphere microorganisms was summarized (Table 13). Shoot tissue dry weight was similar in both experiments, but root tissue dry weight was higher overall in the second experiment. Therefore, the ratio of root to shoot tissue was higher in experiment two. Active and total fungal biomass were higher in the second experiment, but active bacterial biomass was similar in both experiments. Total bacterial biomass was lower in the second experiment than in the first experiment. Levels of flagellates, amoebae and cilliates were similar in Experiment 1 and 2. The numbers of nematodes per gram dry weight of soil was higher in the first experiment than in the second experiment. A decrease in percent colonization of roots by VAM fungi occurred in both experiments (see above), and a greater decrease in colonization occurred in Experiment 2 than in Experiment 1.

Table 12. Influence of ozone on parameters in Experiment 1 and 2.

Parameter	Mean ozone induced increase (+) or decrease (-) Exp. 1	p-value	Exp. 2	p-value
Photosynthesis ($\mu mol/m^2s^1$)	-3.12	0.38	-3.34	0.05
Shoot wt. (g)	+0.08	0.72	+0.59	0.03
Root wt. (g)	+0.12	0.77	-0.48	0.25
Root:Shoot	0.00	0.94	-0.47	0.01
Active fungi (µg/gdw)	+4.52	0.70	-5.87	0.78
Total fungi (µg/gdw)	-2.91	0.97	-114.49	0.33
Active bacteria (µg/gdw)	+0.01	0.99	-0.58	0.18
Total bacteria (µg/gdw)	-1.12	0.13	+0.35	0.54
log Flagellates	0.08	0.78	-0.55	0.10
log Amoebae	-0.40	0.08	+0.12	0.79
log Cilliates	-0.11	0.83	+0.25	0.22
Nematodes (#/gdw)	+0.28	0.91	-0.30	0.34
VAM (% colonization)	-20.80	0.01	-26.13	0.003

Table 13. Mean values for parameters in Experiment 1 and 2.

Parameter	Mean Experiment 1	Mean Experiment 2
Photosynthesis ($\mu mol/m^2$ s)	17.50	17.67
Shoot wt. (g)	2.89	2.97
Root wt. (g)	1.97	3.57
Root:Shoot	0.68	1.30
Active fungi (µg/gdw)	15.42	38.51
Total fungi (µg/gdw)	222.30	303.50
Active bacteria (µg/gdw)	1.69	1.57
Total bacteria (µg/gdw)	20.61	7.36
log Flagellates	4.78	4.05
log Amoebae	2.71	2.47
log Cilliates	2.47	2.01
Nematodes (#/gdw)	0.99	0.28
VAM	65.60	52.54

5.0 Discussion

Ozone exposure influenced the growth, photosynthetic rate, mycorrhizal colonization, and rhizosphere organism populations of wheat plants. However, some predictions did not occur, which leads to questions about how well the mechanisms of ozone effects are understood.

5.1 Photosynthesis

Previous studies have documented photosynthetic impairment by ozone exposure to occur in many plant species, including wheat (Adros et al., 1991; Farage et al. 1991; Reich and Amundson, 1985; Heath, 1980). Ozone is believed to enter open plant stomata, adsorb to mesophyll cell membranes, and react with water to form hydroxyl ion, superoxide ion and hydrogen peroxide. These products damage membranes and enzymes that are essential for the function of photosynthetic cells, reducing the efficiency of these cells (see section 1.4). It was intriguing that the wheat plants in Experiment 1 showed no photosynthetic response to ozone. Results of the effect of ozone exposure on photosynthesis in the second experiment indicated that ozone significantly ($p \leq 0.05$) reduced photosynthesis in exposed wheat plants. Although ozone exposure induced a decrease in photosynthetic rate of similar magnitude in the second harvest of Experiment 1 and in Experiment 2, the treatment effect observed in the first experiment is not significant (Table 7). This discrepancy can be explained by the degrees of freedom in each experiment. The degrees of freedom were lower in the first experiment (1 and 2) than in the second experiment (1 and 4), resulting in less statistical power in the first experiment.

Different levels of organic matter used in the two experiments may explain the different photosynthetic responses to ozone that occurred. The soil used in the first experiment was a sandy soil, low in organic matter (Millican soil). This soil may have

dried quickly after watering, because the soil pore size in sandy soil is relatively large, and water molecules are unable to form bonds with relatively low charged, sand particles (Jury et al, 1991). Consequently, the wheat plant roots in this Experiment 1 may have been water-limited, especially during the warmest hours of the day, causing the plants to close stomata to prevent water loss. The peak hours of ozone concentration, in both experiments, occurred between 1200 and 1900 hr. These hours correspond to the warmest hours of the day, so stomatal closure may have taken place during 1200 and 1900 hr. Because ozone enters a plant through stomata, stomatal closure may have enabled the plant to avoid ozone damage to mesophyll cells and a subsequent decrease in photosynthesis.

In the second experiment the Millican soil was amended with organic matter by adding commercial peat in a ratio of 1 part peat to 9 parts Millican soil. The added organic matter may have increased the soil water holding capacity (Jury et al., 1991), increased water availability to the wheat plant roots, and prevented mid-day stomatal closure.

These results suggest that the level of soil organic matter in which ozone-exposed plants are grown may be important in predicting the plant's response to ozone. Plants grown in low organic matter soil may be able to avoid ozone damage, particularly when high ozone concentrations occur concurrently with high temperatures. Drought stress may cause a plant to avoid ozone penetration, and subsequently evade ozone-induced cell damage. This suggestion is supported by the work of Pell et al. (1990), who demonstrated that ozone injury was reduced in radish plants grown in soil with reduced moisture content.

Alternatively, if ozone was able to enter the stomata of exposed plants and damage photosynthetic apparatus, in Experiment 1, the damage may have been repaired efficiently enough to overcome any loss in photosynthetic rate. The ozone-exposed plants may have allocated photosynthate to shoot tissue to repair damage produced by ozone. Ozone-induced changes in the roots or rhizosphere may suggest that carbon allocation to shoot tissues for repair processes took place. Changes in mycorrhizal colonization and

rhizosphere organism populations occurred in the first experiment, possibly suggesting a shift in carbon allocation due to ozone exposure.

5.2 Shoot and Root Growth

Ozone-induced reductions in partitioning of tissue dry weight to roots have been previously observed (Andersen et al., 1991; McLaughlin and McConathy, 1983). Allocation of carbon to the roots of an ozone exposed plant may be reduced due to increased carbon demand in the shoot for photosynthetic tissue repair. The results obtained in Experiment 2 support this theory, as the ratio of dry root material to dry shoot material was reduced by ozone exposure. However, the root:shoot ratio in ozone-exposed plants was lower than that of control plants because of an ozone-induced increase in shoot dry weight, not a reduction in root dry weight. This result is puzzling because previous studies have documented ozone-induced reductions in root dry weight (Andersen et al., 1991, McLaughlin and McConathy, 1983). Previous research has also shown that shoot tissue dry weight is reduced as a result of ozone exposure (Heggestad et al, 1988; Hogsett et al., 1985a). The wheat plants in Experiment 2 may have shown an increase in shoot tissue due to ozone exposure due to allocation of photosynthate to shoot tissues. By allocating carbon to build more photosynthetic tissue (leaf tissue) the plant may have been able to compensate for the ozone-induced photosynthetic cell damage.

Ozone exposure did not affect the dry weight of shoot or root tissue, or the ratio of root to shoot dry tissue weight in either harvest of the first experiment. As discussed in the previous section, the plants in Experiment 1 may have been drought stressed, causing stomatal closure. Therefore, the plants may have avoided ozone cell damage, and subsequent changes in carbon allocation. If drought-stress did not cause stomatal closure, and ozone was able to penetrate the stomata of exposed plants in Experiment 1, the lack of

an effect of ozone on the dry weight of shoot and root tissue indicated that ozone did not influence these parameters under conditions of low organic matter.

5.3 Below-ground Respiration

Previous studies have shown that ozone decreases below-ground respiration (Edwards, 1991). This decrease has been attributed to an ozone-induced reduction in photosynthate allocation to plant roots (Gorissen et al. 1991). Reduced root respiration, as a result of ozone exposure, has been documented (Edwards, 1991; Hofstra et al., 1981). Edwards (1991) suggested that ozone also decreased microbial respiration, due to an ozone-induced reduction in root exudation, decreasing organic material for microbial consumption.

Below-ground respiration measurements in the first harvest of the Experiment 1 indicated that ozone increased the below-ground respiration of exposed plants. Below-ground respiration was not affected in the second sample date of Experiment 1. The results of both experiments contradict the results of previous studies, which show a decrease in below-ground respiration due to ozone exposure (Edwards, 1991; Gorissen et. al, 1991). Two hypotheses that suggest a mechanism for reduced carbon allocation to root tissues may explain this observation. First, since photosynthetic measurements are taken at one particular point in time, it is possible that photosynthesis may have been negatively affected at times other than when measurements were taken, subsequently reducing carbon fixation, reducing allocation of carbon to root tissues. Secondly, it is possible that the ozone-exposed plants repaired ozone damage to shoot material, keeping the photosynthetic rate equal to control plants, while reducing the amount of root carbon allocated below-ground. This reduction in root carbon may have resulted in senescence of some of the root material, increasing organic material available for microbial metabolism, and subsequently increasing the below-ground respiration in ozone-exposed plants. However, the root weight in ozone

exposed plants was not reduced by ozone, as would be expected if root senescence had occurred. An ozone-induced effect on below-ground respiration was not observed in the second harvest of Experiment 1. It is possible that the organic matter which increased microbial respiration in the first harvest was rapidly consumed, so was not present at the time of the second harvest.

5.4 VAM Colonization

Ozone-induced reductions in carbon compounds available to the root system of exposed plants may have reduced the ability of the fungus to form a mycorrhizal symbiosis, in Experiments 1 and 2. The colonization of wheat plant roots by VA mycorrhizal fungi was reduced by ozone exposure in both harvest dates of the first experiment, and in the second experiment. These results support previous studies that have shown ozone exposure to reduce mycorrhizal colonization in a variety of plant species (McQuattie and Schier, 1992; Edwards and Kelly, 1992; McCool and Menge, 1983). The decrease in VAM colonization due to ozone was greater in the second harvest date (20.8%) than in the first harvest date (14.0%), suggesting that the negative impact of ozone on mycorrhizal colonization increased with time.

The implications of reduced VAM colonization in mycorrhizal plants due to ozone exposure are important to long-term plant health. When water and nutrients are in limited supply and cannot be absorbed by plant roots, a facultatively mycorrhizal plant relies on its mycorrhizal partner to obtain these resources. In conditions of limited water and nutrients, a reduction in fungal colonization may severely reduce the plant's ability to obtain sufficient resources for survival. Damage to the photosynthetic system of a plant, produced by ozone, combined with a reduced ability to obtain water and nutrients when these resources are in short supply, may lead to severe tissue destruction and senescence.

Colonization by mycorrhizal fungi alters root cell membrane permiability, resulting in reduced root exudation (Graham et al., 1981). Reduced VAM colonization in plants exposed to ozone may therefore increase the amount of exudate material released into the rhizosphere. Since bacteria and fungi ingest this root exudate material, their populations may have avoided the decrease in their populations, that was hypothesized in these experiments, by feeding on root exudate material released by the mycorrhizal wheat plants that had experienced an ozone-induced disruption in the mycorrhizal symbiosis.

5.5 Soil Microorganisms

Previous research on the effects of ozone exposure on rhizosphere microorganism populations is limited. Ozone has been shown to reduce bacterial populations in the rhizosphere of exposed plants (Shafer, 1988). However, ozone effects on populations of saprophytic fungi, protozoa, and nematodes have not been examined.

Ozone exposure changed the populations of flagellates and amoebae in the rhizosphere of wheat plants, in Experiment 1 (Fig. 23,24,25). Although statistically significant ($p \leq 0.05$) responses to ozone were not observed in the rhizosphere organism populations, several interesting trends were evident. In the first harvest date of the first experiment a greater number of flagellates per gram dry weight of soil ($p \leq 0.10$) (Fig. 23a), and a lesser number of amoebae per gram dry weight of soil ($p \leq 0.10$) (Fig. 24a) were observed in the rhizosphere of ozone-exposed plants, than in control plants. Amoebae and flagellates both rely on bacteria as a primary food source, so the opposing responses of these two organism groups suggests that the competitive ability of amoebae may have been decreased by ozone exposure, while the competitive ability of flagellates may have been enhanced. Another possible explanation for the increase in flagellate and decrease in amoebae populations in the rhizosphere of ozone-exposed plants is that the amoebae populations may have been negatively affected by ozone, enabling the flagellate population

to grow and fill the trophic niche vacated by members of the amoebae population. The standing crop of bacterial biomass in the rhizosphere of ozone-exposed plants was higher than that of control plants, and the below-ground respiration was higher in plants exposed to ozone, although neither of these differences were significant ($p \geq 0.10$). The flagellate population may have increased in the rhizosphere of ozone-exposed plants as a result of increased food availability.

The amoebal population in the second harvest of Experiment 1 was also reduced by ozone exposure ($p \leq 0.10$) (Fig. 24b), but the flagellate population was not affected (Fig. 23b). It is possible that the food source for flagellates became limited, perhaps due to ozone exposure, decreasing the ability for flagellates to fill the trophic niche left open by the reduced amoebae population. This suggestion is supported by measurements of the standing crop of rhizosphere bacterial biomass. The standing crop of bacterial biomass was not higher in the rhizosphere of ozone-exposed plants, as was observed in the first harvest.

Amoebal populations were not influenced by ozone in the second experiment, so results from the this experiment suggest different mechanisms occur in high organic matter soil. In contrast to the results of Experiment 1, the number of flagellates per gram dry weight of soil decreased in the rhizosphere of plants exposed to ozone ($p \leq 0.10$) in Experiment 2. Since the soil used in Experiment 2 was higher in organic matter than the soil used in the Experiment 1, it is possible that the soil composition influenced the effect of ozone on rhizosphere flagellates, by affecting the flagellate's food source. Elevated organic matter may have maintained the rhizosphere bacterial population at a level high enough to keep the amoebal population from experiencing a food shortage, as may have been the case in Experiment 1. The amoebae may have then out-competed the flagellates for food in Experiment 2, causing the flagellate populations to decrease.

5.6 Implications of Rhizosphere Alterations

The results of this study indicate that ozone exposure may have severe effects on the long-term health of a plant's associated rhizosphere microorganisms, and these effects may be altered by the soil type in which a plant is grown. Two primary ozone-induced changes observed in this study could influence plant growth: reduced rhizosphere amoebae populations and reduced colonization by vesicular-arbuscular mycorrhizae.

By reducing the number of rhizosphere amoebae in ozone-exposed plants, as was observed in the first experiment, ozone exposure could have long-term effects on mineralization in soils. Amoebae prey primarily on bacteria, mineralizing nutrients that are locked in bacterial biomass (Paul and Clark, 1990). Reduced mineralization of nutrients, such as nitrogen, sulfur, and phosphorus, may limit the availability of plants to obtain enough nutrients for survival, causing reduced growth or premature senescence in plants exposed to ozone for an extended period of time.

The influence of ozone on the mycorrhizal colonization of plant roots is important. Conditions of limited water and nutrients occur in almost every ecosystem at some time. The reduced ability of mycorrhizal plants to obtain these resources, due to ozone exposure, suggests that the health of mycorrhizal plants may be increasingly threatened when conditions of low nutrient and water availability, and high ambient ozone are combined. If these conditions persist for several years, the productivity of an entire ecosystem, such as a grassland or an agricultural field, may be reduced.

The implications of reduced carbon fixation over large areas has been given much attention over the past decade, as the issue of global warming and its link to elevated atmospheric carbon dioxide levels has become a major concern. Since ozone levels are rising in many rural areas, and a relationship has been suggested between elevated ambient ozone and decreased carbon fixation by plants, decreasing ambient ozone levels may be important in regulating tropospheric carbon dioxide.

5.7 Research Needs

The results of this study indicate that ozone influences rhizosphere dynamics when exposure periods are as short as 26 to 40 days. Studies that monitor rhizosphere responses to ozone exposure over longer time periods are needed to assess long-term ozone effects. Future studies that focus on the effects of ozone on the growth, photosynthesis, mycorrhizal colonization and rhizosphere organism populations of a perennial plant species, over a period of several years, may be of interest, to investigate the cumulative effects of elevated ozone concentrations on these variables.

The effects of ozone on plants grown in different soil types and at different soil moisture contents should also be explored. The results of this study suggest that ozone affects plants and rhizosphere organisms differently under different levels of soil nutrients and organic matter. The moisture level in these soils may have affected the plant's response to ozone. Future research should examine ozone effects under a wide range of soil types, including soils high in clay, sand, or organic matter. Monitoring the moisture content in these soils may help explain any soil-related differences in ozone effects.

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APPENDIX

APPENDIX

Table 14. Hoaglund's nutrient solution is made by preparing a 50-fold water dilution of Hoaglund's stock solution. Hoaglund's stock solution is prepared by combining Stock solutions A and B.

Stock Solution	Ingredient	Amount (per/l)
A	Magnesium Nitrate $(Mg(NO_3)_2) \cdot 6H_2O$ FW=256.41	6.5 g
	Calcium Nitrate $(Ca(NO_3)_2) \cdot 4H_2O$ FW=236.15	16.0 g
	Sequestrene 330 FE	2.5 g
B	Ammonium Nitrate $(NH_4\ NO_3)$ FW=136.09	8.0 g
	Potassium Phosphate (Monobasic) (K_2HPO_4) FW=136.09	1.2 g
	Potassium Phosphate (Dibasic) $(K_2HPO_4) \cdot 3H_2O$ FW=174.18	1.4 g
	Potassium Sulfate (K_2SO_4) FW=174.27	1.5 g
	Sodium Sulfate (Na_2SO_4) FW=142.04	1.7 g
	Minor Element Stock Solution (recipe below)	50 ml
	Methyl Blue	
Minor Element	Boric Acid (H_3BO_3) FW=61.83	14.0 g
	Molybdic Acid $(MoO_3 \cdot 2H_2O)$ FW=179.97	0.1 g
	Hampene Mn (12%)	9.5 g