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The effects of stress on biomass, soluble sugar concentrations and VA mycorrhizal colonization in sugar maple seedlings (*Acer saccharum* Marsh.)

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Abstract

The effects of stress on biomass, soluble sugar concentrations and VA mycorrhizal colonization in sugar maple seedlings (*Acer saccharum* Marsh.)

Nadine Costanzo

Increasing ozone levels (0, 50, 100, 150, 200, 300 ppb) coupled with 80% shading and 50% defoliation were examined for two year-old sugar maple seedlings for one growing season (June - September). Biomass, soluble root carbohydrates and mycorrhizal colonization progressively decreased with increasing ozone stress. Shading had a negative effect on vesicles and coils at certain sampling periods. While defoliation negatively affected biomass and mycorrhizal colonization at the beginning of this study, towards the end, it increased biomass and mycorrhizal colonization and appeared to make those defoliated seedlings more tolerant to ozone. This experiment also showed how sensitive mycorrhizal fungi are to plant stress.

In a second experiment, low/high CO_2 (350/650 ppm) coupled with low/high O_3 (10/200 ppb) was examined for biomass, total soluble carbohydrates and mycorrhizal fungi of one month-old sugar maple seedlings for 61 days. By day 61, high CO_2 seedlings had the highest biomass, followed by control, high CO_2 - high O_3 and high O_3 though none of these results were significant. The same trends could be seen for the individual plant parts (second leaves, stems and roots) yet only high ozone had a significant effect on second leaves. For the carbohydrate analysis, high O_3 decreased soluble carbohydrate concentrations for the first leaves and stems though these results were not significant. No mycorrhizal colonization was found for any of the treatments. In both experiments, root

carbohydrates did not always give expected results pointing towards examining new factors that may affect mycorrhizal colonization within a host plant.

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Introduction

Throughout its existence, a plant may be subjected to many interacting stresses, both abiotic or biotic, natural, or human-induced, which affect its survival and reduce its growth below maximum attainable yields (Amthor and McCree, 1990; Kozlowski, 1991). A stress is defined as "any factor (in excess or limiting supply) that detrimentally affects any aspect of the plant's carbon balance" (Amthor and McCree, 1990). However, Anderson and Rygiewicz (1991) also see certain beneficial effects as possibly being "stressful" and have defined stress to include "any environmental factor with the capacity to elicit a chemical or physical change (metabolic adjustment), regardless of whether the change is beneficial or detrimental to the organism." Some stresses may affect the photosynthetic apparatus, which will therefore affect carbon fixation and/or translocation to the rest of the plant (Amthor and McCree, 1990). Usually, the effects of stress on plants are discovered after some visible symptom has become apparent (Vogt et al, 1993). As the stress becomes stronger or more chronic, the signs become more visible (Heath and Taylor, 1997). Such visible injuries may be the result of cellular death and show up as chlorosis or necrosis of the plant tissues (Heath and Taylor, 1997) and can eventually lead to such symptoms as leaf abscission or total crown dieback (Vogt et al, 1993). Moderate stresses may not be as visible, yet they may alter the biochemistry or physiology of the plants, leading to lowered rates of photosynthesis, transpiration or growth , or to the reallocation of carbon from the roots to the shoots (Heath and Taylor, 1997). The key to understanding and managing stress in plants is to detect and respond to early-warning signals before any aboveground effects become evident (Vogt *et al*, 1993). Because roots may either experience structural and functional changes not seen in the aboveground parts, or at a more rapid rate than the shoots, they make ideal indicators of stress. Three important early-warning indicators involving the belowground components are: changes

in plant biomass allocation, carbohydrate allocation to the roots and the relationship of a plant with its root symbionts. Some examples of photosynthetic stresses include: air pollutants such as ground-level ozone, elevated CO_2, shading, and defoliation.

Air Pollutants

Due to human activity, the world is changing at an alarming rate. As the human population continues to grow, gas emissions from agriculture, the burning of fossil fuels and industrial processes increases and exceeds the self-regulating capacity of the atmosphere (Manning and Tiedemann, 1995). As a result, it is important to study the possible effects of these gases on organisms including plants. Two of the most studied gases are ozone and carbon dioxide. Both gases result from the burning of fossil fuels.

Ozone

Ground-level ozone is a common air pollutant that forms over most of the industrialized world, such as Eastern Canada, the United States, Europe, and Japan, (Cooley and Manning, 1987). Naturally-occurring ozone levels are around 20 ppb, but they can go up to 50 ppb during daylight hours in the summertime (Mansfield and Pearson, 1993). Due to pollution, ozone peaks lasting several hours can reach 200 ppb in certain parts of the United States (Cooley and Manning, 1987). Ozone is considered to be a secondary pollutant because it is not produced directly as a result of human activities, but rather, it is the product of primary pollutants such as nitrous oxides and hydrocarbons that undergo reactions to form ozone when exposed to sunlight (Mansfield and Pearson, 1993). Since ground-level ozone is formed by photochemical means, it is found in higher concentrations during the summer months. These high ozone levels coincide with a plant's maximum growth period. During periods of maximum growth, exposure is

increased because a plant that is actively growing will keep its stomata open in order to photosynthesize (Reich and Amundson, 1985). Ozone is a very reactive pollutant with a very high oxidizing potential (Treshow and Anderson, 1989). As ozone enters the cell via the stomata it either reacts directly with the cell walls, or further degrades to form free radicals such as HO• or HO_2• which can increase the permeability (leakiness) of the cell membranes (Polle and Rennenberg, 1994). The stomatal guard cells may also become affected, decreasing stomatal conductance and thereby, decreasing photosynthesis (Jensen 1981; Chevone *et al*, 1990). Ozone can also affect the carbon balance in a plant by causing a certain amount of the fixed carbon to be invested in the formation of antioxidants such as glutathione (Smith *et al*, 1990: Schmeiden *et al*, 1993) or ascorbate (Polle and Rennenberg, 1993) to counteract the effects of ozone.

Exposure to moderate concentrations of ozone (less than 150 ppb) may inhibit root growth more than shoot growth (Letchworth and Blum, 1977; Tjoelker *et al*, 1993). Ozone also accelerates the senescence of older, more mature leaves, which are the carbon sources for the roots (Chevone *et al*, 1990). The result may be a decrease in the photosynthetic capacity in the older leaves relative to the younger leaves which might explain the decrease in carbon partitioning to the roots, (Cooley and Manning, 1987). At higher ozone concentrations, however, whole plant photosynthesis is inhibited and total biomass accumulation is substantially reduced (Letchworth and Blum, 1977).

Carbon Dioxide

The average concentration of CO_2 in the atmosphere has increased since the industrial revolution from 270 ppm to 355 ppm. Rogers *et al* (1994) predicted that levels will double to ~ 700 ppm by the year 2050. Whereas ozone is very detrimental to plant

growth, elevated CO_2 concentrations benefit plants. CO_2 is an essential component for plant growth since it is the carbon source for carbohydrate formation during photosynthesis. As photosynthesis is usually CO_2-limited, any increase in CO_2 available to a plant should improve its growth and development.

Elevated CO_2 allows for more efficient carbon fixation especially for C_3 plants such as most woody species, or agricultural crops such as soybean, wheat, rice and potato. Since C_3 plants undergo photorespiration as part of their photosynthetic process, they can lose between 20-50% of the carbon that is fixed (Rogers *et al,* 1994). The higher CO_2 actually stimulates photosynthesis because more CO_2 is available to compete against O_2 and catalyse with Rubisco so that more efficient carbon fixation results (Eamus and Jarvis, 1989; Rogers *et al,* 1994).

Because elevated CO_2 allows for a more efficient photosynthesis, more carbohydrates can be produced by the plant. Usually, the excess carbohydrates are diverted to the roots which leads to larger root biomass, higher root to shoot ratios (O'Neill et al, 1987), and more efficient symbiotic relationships of the roots with organisms such as mycorrhizal fungi (Norby *et al,* 1986; Klironomos *et al,* 1996; Rillig *et al,* 1998). The increased growth efficiency allows plants to be better able to withstand environmental stresses (Norby *et al,* 1992). Also, the increase in carbohydrate production can lead to increased defense mechanisms within the plant since it has more energy to divert to secondary metabolite production (Rao *et al,* 1995).

Natural Stresses

In addition to human-induced stresses such as air pollutants, a plant must also endure natural stresses. Even if a plant were to grow in an environment free of anthropogenic stresses, it would still have to compete with other plants for water, nutrients and light. A plant may also have to withstand attacks from herbivores such as insects or mammals.

Defoliation

By chewing and sucking on living plant tissue, herbivores can damage plants by lowering productivity, and increasing mortality. As a result, overall plant fitness will be lowered. The physiological balance between the aboveground (energy producing) and the belowground (mineral acquiring) plant components will be upset (Dyer *et al*, 1991). As well, to maintain plant growth and survival, the plant tissue that has been removed or damaged will need to be repaired and replaced (Daft and El-Giahmi, 1978). As with many other photosynthetic stressors, such as ozone, this carbon will come at the expense of that which would normally be allocated to the roots. Ryle and Powell (1975), examined the effects of defoliation on photosynthate allocation to the roots. They found that upon removal of 50 % of the leaf biomass of unicum barley, aboveground biomass retained more photosynthate at the expense of the roots. Export of photosynthate to the meristematic tissues and young leaves nearly doubled while export to the roots was immediately reduced. As the new leaves developed and produced their own photosynthate, their demand for transported photosynthate decreased. Yet, even when the new leaves were fully functional, allocation to the roots was still severely reduced, (about 2-3 fold), and the roots never increased in biomass from their original mass at the start of this experiment. In another study by Lubbers and Lechowicz (1989), 50% removal of the

leaves from flowering *Trillium grandiflorum*, led to a decrease in biomass and carbon allocation to the belowground tissues (in this case, the rhizome) while the reproductive organs were left unaffected. It would therefore appear that the aboveground organs have priority over the belowground components when the plants become carbon stressed due to defoliation.

Shading

Plants require sunlight for photosynthesis. As more sunlight becomes available, a plant can produce more carbohydrates and biomass. When light is limited, growth may slow down or become inhibited. Plants requiring large amounts of light are said to be shade intolerant and may not survive low levels of sunlight. On the other hand, some plants, such as sugar maples, are shade tolerant and can persist in the forest understory for many years at very low levels light levels (Canham, 1988). During that time, they may experience growth suppression, having low photosynthetic rates (Walters *et al*, 1993). As a result, little carbohydrates and biomass is produced. The carbohydrate that is produced will most likely be allocated to the leaves at the expense of the roots in an effort to increase photosynthetic rates by increasing photosynthetic surfaces (Tjoelker *et al*, 1993).

Biomass/Carbon Allocation

Plant yield can be defined as the amount of dry matter produced (Cooley and Manning, 1987). Dry matter is composed mainly of carbon compounds (Cooley and Manning, 1987). Plant growth and yield are therefore the result of photosynthetically-fixed carbon and the allocation of this photosynthate (Cooley and Manning, 1987).

Plants undergoing increased stress levels are expected to produce less biomass than non-stressed plants (Cooley and Manning, 1987). This lowered production of biomass may be the result of carbon reserves being allocated to maintenance and repair as opposed to growth and reproduction (McLaughlin and McConathy, 1983; Gorrisen and Van Veen, 1988; Amthor and McCree, 1990; Geiger and Servaites, 1991). The photosynthetic stress reduces photosynthesis and the amount of carbohydrates that are produced (Cooley and Manning 1987). At low stress levels and for plants that have not yet set flowers or fruit, the plant may divert energy to leaves and stems at the expense of the roots (Jones and Mansfield, 1982; Okano *et al*, 1984; Amthor and McCree, 1990). In the case of carbohydrate partitioning, export of sugars from the source leaves decreases to the roots and lower stems, and increases to the younger leaves since the younger leaves have higher sink demands than roots (Cooley and Manning, 1987). This would appear to be an adaptive response to the reduction in photosynthate. This increase in photosynthate to the immature leaves may be because they will form the photosynthetic organs necessary for future production of new dry matter (through photosynthesis). As leaves expand, they go from being net importers to net exporters of photosynthate. During this transition, they both import and export photosynthate. When photosynthesis is inhibited, these leaves may become stronger sinks because their ability to consume photosynthate remains unchanged while their ability to produce photosynthate is lessened. At high stress levels, carbohydrate accumulation is greatly decreased. For perennial species, such as trees, it is the storage organs such as roots, which seem to suffer the most, (Cooley and Manning, 1987). This decrease in carbohydrates being allocated to the roots may have severe long-term effects on the plants, such as a decrease in the root respiration, in the colonization by beneficial root symbionts such as mycorrhizal fungi, (McCool and Menge, 1983; Ho and Trappe, 1984; Stroo *et al*, 1984) and in less efficient nutrient and

water-uptake by the plant which will, in turn, lead to an overall reduction in plant growth (Okano *et al,* 1984). A minimum amount of carbohydrates in the roots is needed for overwintering and subsequent regrowth. If the amount of carbohydrate is below the minimum, then the plant may not endure another season (Cooley and Manning 1987).

Effects of stress on VAM Anatomical Features

When the photosynthetic apparatus has been stressed, the flow of carbohydrates to the roots is reduced. This reduction in carbohydrate flow to the roots may have an effect on root symbionts such as the nodule-forming bacteria or mycorrhizal fungi (Vogt *et al,* 1993). This could lead to failure of the association, since reduced root exudates may not allow for the colonization, or subsequent development of the symbiont (Cooley and Manning, 1987).

Mycorrhiza is a mutualistic symbiosis that develops between fungi and plant roots. Plants benefit from mycorrhizal fungi in a number of ways. Mycorrhizae aid in the acquisition of nutrients, particularly phosphorus, but also nitrogen and trace minerals, such as copper and zinc (Smith and Read, 1997). The fungal hyphae, which are thinner than the roots and longer than the root hairs, grow out from the plant roots and into the soil away from the root zone (Smith and Read, 1997). These hyphae can therefore gather nutrients where the roots cannot reach. Similarly, the fungus helps prevent water stress, by obtaining water from outside the root zone (Smith and Read, 1997). Other important benefits from the fungal presence include the prevention of root disease (Klironomos and Kendrick, 1995) and improved survival of the plants in acid soils (Klironomos, 1995).

Mycorrhizal fungi benefit by getting their energy from the plant in the form of soluble sugars (Smith and Read, 1997). The vesicular-arbuscular mycorrhizal fungi cannot grow without the host, and must therefore be considered to be obligate symbionts (Smith and Read, 1997). The fungi cannot break down materials such as decomposing plant matter (dead wood and leaves) to obtain carbohydrates and must rely on their host to provide them (Smith and Read, 1997). The fungus can represent a considerable drain on the plant, using 4 - 20% of the plant's carbon resources (Smith and Read, 1997).

About 90% of all vascular plant species have vesicular - arbuscular mycorrhizae (VAM), a symbiosis that exists between a zygomycotic fungus, and the plant roots (Newsham et al, 1995). Examples of plants that contain VAM are agricultural plants, forest herbaceous species, and tropical trees. In temperate forests, trees that support VAM include maples, cherries, tulip-tree and ash. The name vesicular - arbuscular mycorrhizae derives from the main fungal structures (vesicles and arbuscules) that are found within the plant root (Smith and Read, 1997). The arbuscule is a finely branched intracellular structure that resembles a small bush (Smith and Read, 1997) and is thought to be the site of nutrient exchange from the fungus to the plant. It is therefore considered the most important structure of the whole VAM complex. In sugar maples, intracellular hyphal coils are the structure from which the arbuscules develop (Cooke *et al*, 1992). The function of these coils is not exactly known, however, it has been hypothesized that they work in a similar way to the arbuscule, that is, in nutrient exchange. Vesicles are swellings resembling a balloon that can be formed inter- or intra-cellularly. They appear to be organs for carbon storage, however, they may also serve as spores. Because they are carbon-rich, vesicles may behave as a carbon sink (Powell and Bagyaraj, 1986). VAM structures that are found outside of the plant roots include the extramatrical hyphae (used

to obtain nutrients and water), and spores (organs for carbon storage and reproduction - Powell and Bagyaraj, 1986).

Most studies on the effects of stress in plants on mycorrhizae have used fungal colonization rates as indicators of stress (Vogt *et al*, 1993). The general conclusion is that as stress increases, mycorrhizal colonization decreases (Vogt *et al*, 1993). This is thought to reflect a reduction in the amount of sugar produced in the plant as a result of stress, reducing mycorrhizal fungal colonization (McCool and Menge, 1983; Ho and Trappe, 1984; Vogt *et al*, 1993).

Recently, researchers have begun to emphasize the importance of studying VAM structures in relation to plant health. In the past, spore counts and/or colonization rates were the only measurements used to assess the health of the VAM symbiosis. However, spore numbers can give false results because the spores may look alive, but, in fact, be dead. The ability to sporulate may not necessarily determine fitness and the spores may not even represent the real abundance of the fungi or their ecological contribution to the plants (Klironomos, 1995). Colonization rates can be the same or higher for a moderately stressed plant as compared to healthy plants, so when this method is used alone, it may be an unreliable indicator (Klironomos and Allen, 1995). More recently, scientists have been studying the ratios of vesicles to arbuscules as a means of determining the overall health of the symbiosis, since change in fungal structure may indicate a change in the function of the symbiont (Allen, 1991). It is possible that sometimes the "interests " of the plants may be in conflict with those of the fungus. It is sometimes the case that plants and mycorrhizal fungi negatively affect each other's growth and reproductive success (Bethlenfalvay *et al*, 1983).

Studies of sugar maples (Cooke *et al,* 1992; Cooke *et al,* 1993; Duckmanton and Widden, 1994; Klironomos 1995) have shown that, as stress increases, the abundance of vesicles and intracellular coils also increases while that of arbuscules decreases. Since stress can alter photosynthetic rates and the allocation of carbohydrates, this adjustment will have an effect on the mycorrhizal system (Duckmanton and Widden, 1994). In an unstressed plant, the higher ratio of arbuscules to vesicles and intracellular coils suggests that the fungus is actively transporting nutrients to the plant. When a plant undergoes moderate stress, it is assumed that a change in root carbohydrate allocation will be perceived by the VAM fungus. The fungus will therefore react by forming more vesicles and intracellular coils and fewer arbuscules. A change in these ratios shows that the fungus spends more energy on storage and long-term survival than on nutrient exchange, since the fungus is taking in carbohydrates, but is giving less in return. At this point, the fungus may further aid in the plant's decline (Anderson and Rygiewicz, 1995). A shift in the proportions of these VAM structures may therefore be an early-warning sign of the stress that is being experienced in the plant before external signs are visible. In the case of high stress levels, the overall VAM fungal colonization rates on the plant roots are expected to drop (Michelini *et al,* 1993), since there is not enough photosynthate available to support the symbiont.

Some recent vesicular-arbuscular mycorrhizal research focuses on studying the interactive outcome of different stressors on the VAM system. These stressors may have an antagonistic, or synergistic effect depending on whether their influence on the plant and its VAM association is reduced or amplified. Some research showing antagonistic effects of stressors is work done by Klironomos and Kendrick

(1995) where detritus and microarthropod grazing alone each had a negative effect on mycorrhizal health, (arbuscular numbers decreased). However, when the two "stressors " were both present, their negative effects were cancelled, and a positive effect on mycorrhizal health resulted (arbuscule numbers increased). An example of the synergistic effect of "pollutants" causing amplified stress on the mycorrhizal health, is a study done by Klironomos *et al*, (1996). Elevated levels of atmospheric carbon dioxide increased arbuscule numbers of the VAM fungus, allowing for more efficient nutrient uptake, compared to the control. However, when plants were grown with the increased nutrients as well as higher CO_2 levels, arbuscule numbers decreased. The environment is a dynamic system, with many factors affecting the organisms that live within it. Studying the interactions of different components within a system can therefore give a better picture of their true effects on a particular organism. Research is therefore needed which examines these interactions between environmental stresses on both plant physiology and the fungal response.

For our first experiment, we chose to examine the effects of increasingly high ozone levels on sugar maple seedlings, and the subsequent response of the mycorrhizal fungus to the stress. To our knowledge, this would be the first study that examined the effects of lethal concentrations (concentrations at which some seedlings would not survive by the end of the season) of ozone on the mycorrhizal system. We predicted that as ozone levels increased, plant biomass would decrease and less carbohydrates would be allocated to the roots. As a result, we predicted that the VAM fungus would react to this decrease in root carbohydrates by putting more energy into its own long-term survival. More vesicles and coils and less arbuscules would therefore follow. We predicted that as ozone concentrations increased, overall VAM fungal colonization rates would decrease.

Because a stress rarely occurs in isolation, we chose to add two other stresses (shading and defoliation) to the system. We predicted that the added stresses would further increase the sensitivity of the seedlings to the ozone thereby decreasing its lethal concentration. For those seedlings, we therefore predicted that VAM fungal colonization rates would decrease at even lower ozone concentrations than for seedlings that were exposed to the ozone alone.

For our second experiment, we chose to examine the effects of high ozone coupled with high CO_2 on the mycorrhizal system. Although there have been other studies that have examined the effects of the two stresses on the mycorrhizal system separately, (Carney *et al*, 1978; McCool and Menge, 1983; Ho and Trappe, 1984; Reich *et al*, 1986; Stroo et al, 1988; Duckmanton and Widden, 1994; Klironomos *et al*, 1996; Rillig *et al*, 1998) this would be the first study to examine the interacting effects of O_3 and CO_2 together, on mycorrhizal fungi. We predicted that the carbon dioxide would alleviate some of the effects of the ozone, making those seedlings more tolerant to the stress. For those seedlings, we predicted that their VAM fungi would have higher colonization rates with more arbuscules to vesicles and coils than for seedlings exposed to the high ozone alone.

Experiment 1. Effects of ozone, shading and defoliation on sugar maple seedling biomass, root soluble carbohydrates and VAM colonization

Methods and Materials

Two year-old nursery-grown (from the Berthierville Nursery) bare-root sugar maple (*Acer saccharum* Marsh.) seedlings were planted in eight inch pots containing soil obtained from the top 10 - 15 cm of a maple forest at the Centre de Recherches Acéricole de M.A.P.A.Q in Tingwick, Quebec. The seedlings were allowed to grow outdoors in their pots for one month, and were then randomly placed in open top ozone chambers in early June. Six chambers were used, one at each of the following ozone concentrations: 0, 50, 100, 150, 200 and 300 ppb. Shading was implemented by covering one half of each chamber with shading cloth that removed 80% of available light (1300 mol m^{-2} s^{-2} PPFD (unshaded), 290 mol m^{-2} s^{-2} PPFD (shaded)). One half of the seedlings were randomly chosen in each chamber to undergo the defoliation treatment. Defoliation was achieved by removing one-half of each leaf in mid-June , representing a 50% removal of leaf biomass. Defoliation was performed in mid-June because that is when sugar maples are most often attacked by herbivores such as the forest-tent caterpillar (Martineau, R. 1985, as quoted in Fortin, M. 1994). Trees were grown for 4 months and samples were taken at the end of June, July, August and September. At each sampling, we sampled at random, 6 seedlings from each treatment within each chamber for a total of 24 seedlings per chamber and 144 trees per sampling.

Biomass Allocation

Each seedling was divided into aboveground (leaves and stems) and belowground (root) parts, then small samples (~2 g of the fine roots) were removed from different sections of the root system, weighed and analyzed for sugar content and VAM

quantification. The plant parts were oven-dried for a minimum of 24h at 80°C and reweighed to obtain the dry weights and moisture content. In the case of the roots, the dry weight from the piece that was removed for the mycorrhizal and carbohydrate analysis was calculated from the known moisture content and was added to get the total root dry weight.

Soluble Sugar Analysis

Approximately 1g of fine root was removed, freeze-dried and ground to a fine powder. Ten mg of the sample was then weighed out and 0.5 ml of 70% methanol was added to it three times, giving a final volume of 1.5 ml methanol. The samples were then centrifuged for 10 minutes at 15 000g and the pellet was discarded. One hundred microliters of the supernatant were added to 400 µl of distilled water and 1.5 ml of anthrone (1.5g anthrone for every 100 ml of 99% sulfuric acid). The solution was vortexed and then incubated for 8 min at 100°C in a water bath. The solutions were cooled and the concentration of sugars was read colorimetrically with a spectrophotometer at 625 nm, using a standard curve of known glucose values.

VAM Morphology

The remaining portion of fine roots was washed and stored in F.A.A. (formalin acetic acid 50% alcohol (1:1:4.5) for a minimum of 24 h. The roots were autoclaved for 8 min in 10% KOH to remove the phenolics, rinsed with water, placed in 30% hydrogen peroxide for 1 h, rinsed in water, acidified in 1% HCl for 20 min and stained with 0.15% chlorazol black E for 20 min at 90° C.

The roots were mounted in glycerine jelly on slides and VAM structures (arbuscules, vesicles and coils) were quantified using the magnified intersect method (McGonigle *et al.*, 1990). One hundred intersects were observed for each plant, using a Nikon Optiphot microscope, equipped with differential interference contrast (DIC), to obtain percent colonization for each of the VAM fungal structures.

Statistics

Multiple regression analysis and descriptive statistics were performed using Systat version 5.03 (Wilkinson, 1990). The step-down backward elimination procedure was employed. Ozone concentration, shading, defoliation and the shading/defoliation interaction were the independent variables, while shoot biomass, root biomass, soluble carbohydrates and VAM fungal structures (arbuscules, coils and vesicles) were the dependent variables that were examined. Because the shading and defoliation treatments were either present or absent in each plant, they counted as dummy variables in this study.

Results

Seedling Appearance

After the first month, there was no visible difference among shaded/unshaded seedlings exposed to any of the ozone concentrations. By July, those seedlings growing at concentrations of 200 and 300 ppb O_3 were more yellow in appearance than those growing at the lower ozone concentrations. By the end of August, some seedlings exposed to the higher concentrations of ozone had stunted growth, mottled leaves, and senescent leaves that in some cases had already abscised. Seedlings that were growing at low ozone concentrations were healthy, having greener, larger leaves than their high ozone counterparts. The roots from the low ozone plants were very full and healthy in appearance, and had many fine roots. The roots from the high ozone plants were much smaller and had less fine roots than those of the other treatments. The seedlings from the intermediate ozone treatments were intermediate in health. These seedlings were somewhat smaller than the control plants, and somewhat less green. These differences became more pronounced at the end of the growing season. The defoliated treatment caused an extra shoot to develop that the intact seedlings did not produce.

Biomass

Ozone had a cumulative effect on biomass for both the roots and the shoots that became more pronounced as the growing season progressed. At the beginning of the experiment, the effects of ozone on biomass production were not apparent. Both control and shaded seedlings had similar growth patterns at all ozone levels (Figure 1A, 2A, Tables 1 and 2). At this stage however, defoliation (alone and in combination)

significantly reduced biomass for both the roots and the shoots (Figure 1A, 2A, Tables 1 and 2).

As the season progressed, the cumulative effects of ozone became more apparent on root and the shoot biomass for the month of July (Figure 1B, 2B, Tables 1 and 2). Although the R^2 value was very small for shoot biomass, the p-value for ozone effects were significant ($p< 0.05$) thus showing that ozone had a significant effect on seedling growth. The defoliation treatment also had a significant effect on root biomass. These results show that the defoliation treatment from the previous month still had a strong effect on root growth.

By the third month, (August), ozone was the only stress that had an effect on seedling growth for both the shoots and the roots (Figures,1C, 2C, Tables 1-2). At this stage, the seedlings that had undergone defoliation were no longer statistically different in size from the control or shade plants (Figure 1C, 2C, Tables 1-2).

By the fourth month (September), the slopes had become the steepest for both roots and shoots. The seedlings of the lower ozone treatments had practically doubled in size since the beginning of the experiment, whereas the seedlings from the higher ozone levels hardly grew at all (Figures 1D, 2D, Tables 1-2). The only other treatment to have a significant effect on seedling growth was defoliation which, increased shoot and root biomass compared with the control.

Soluble Sugar Analysis

For the August samples, ozone concentration was the only factor that had an effect on soluble sugars in the roots (Figure 3A, Table 3). For the month of September, ozone was the only treatment that had a significant effect on soluble carbohydrates allocated to the roots (Figure 3B, Table 3). Counter intuitively, for the 200 ppb treatment, many seedlings representing every treatment (except for the shading and defoliation combination treatment) had high carbohydrate concentrations (Figure 3B).

Figure 1. Shoot Biomass for June through September

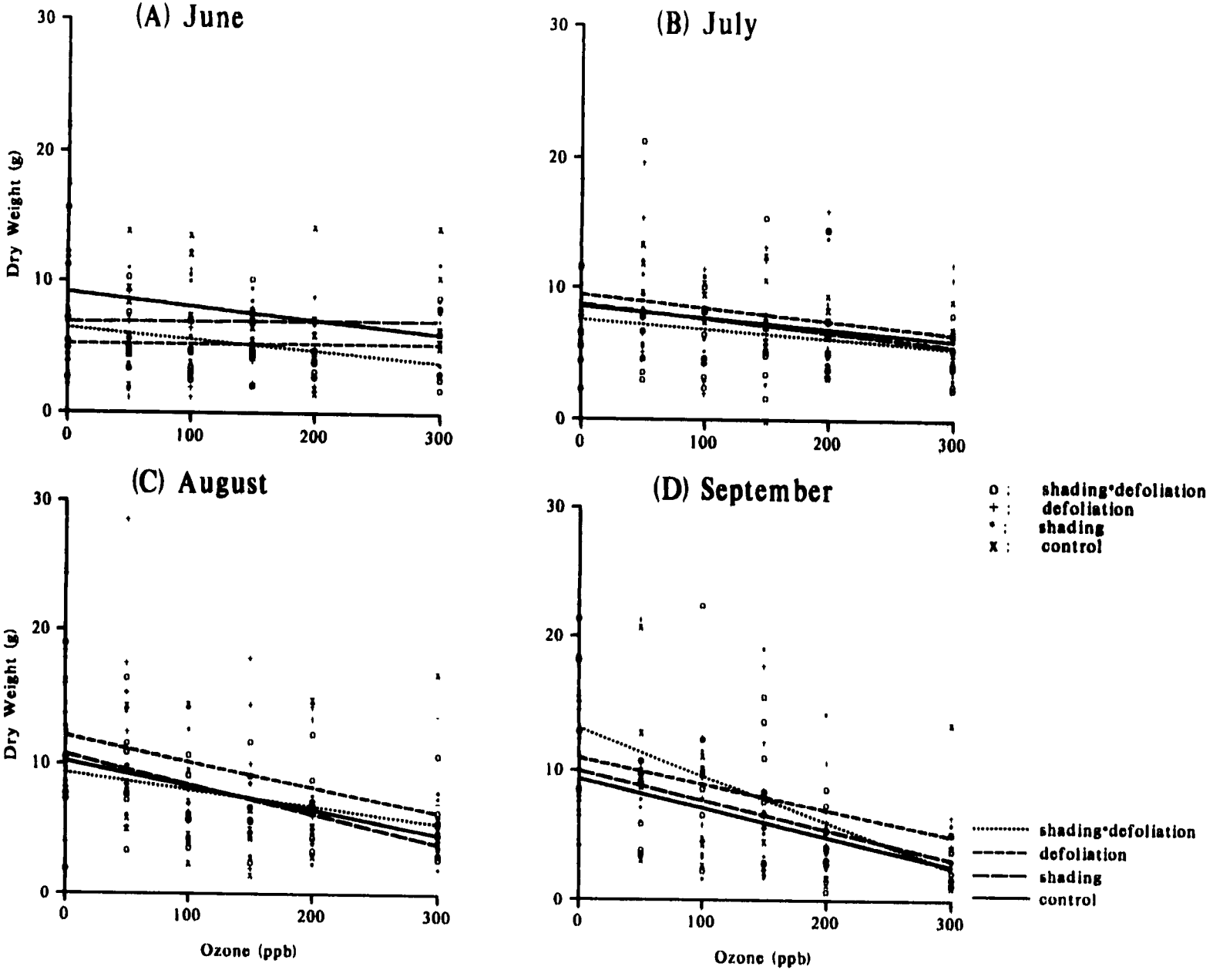

(A) June
(B) July
(C) August
(D) September
Dry Weight (g)
Ozone (ppb)
0
10
20
30
100
200
300
o : shading*defoliation
+ : defoliation
* : shading
x : control
shading*defoliation
defoliation
shading
control

Figure 2. Root biomass for June through September.

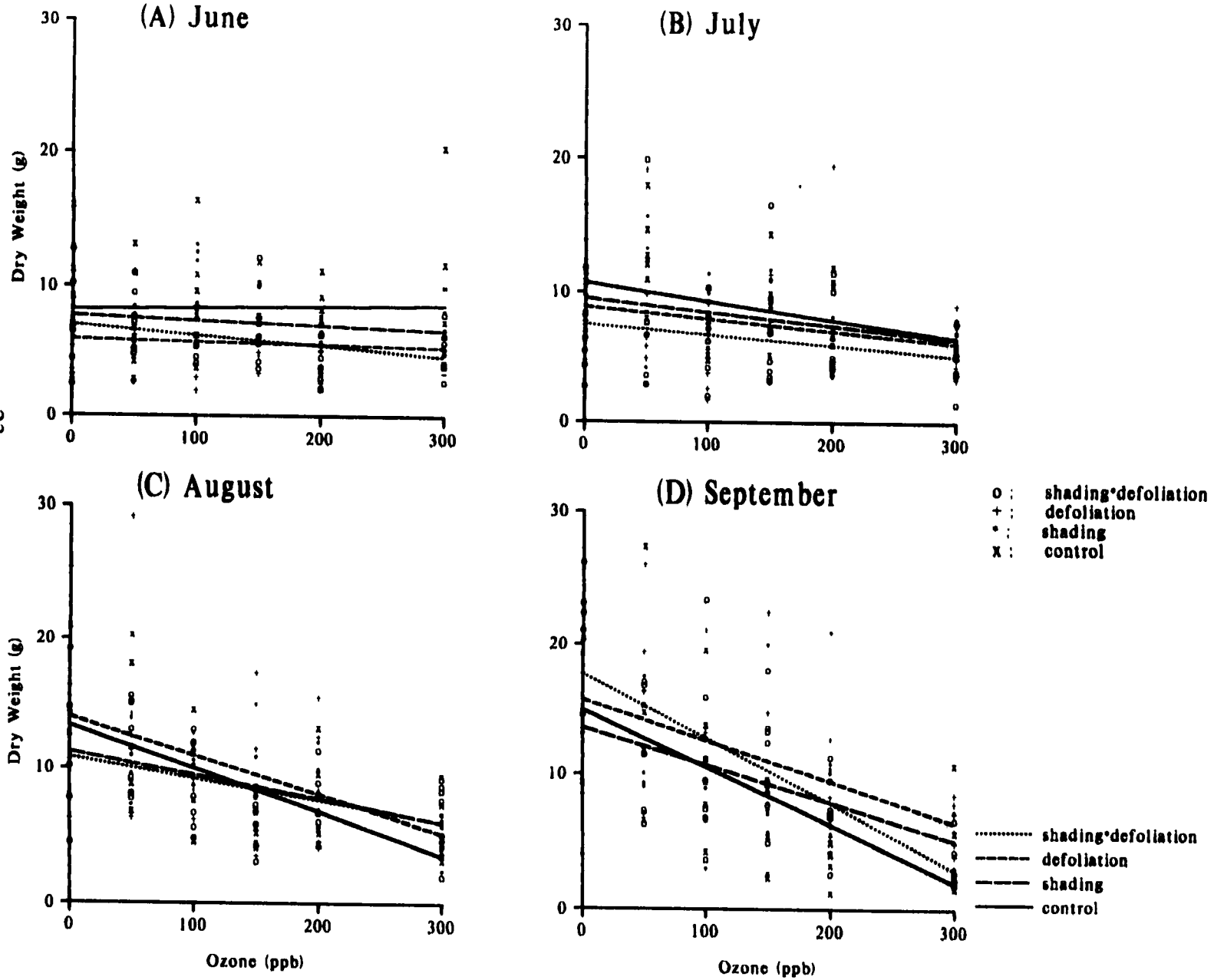

(A) June
(B) July
(C) August
(D) September
Dry Weight (g)
Ozone (ppb)
0
10
20
30
100
200
300
o : shading*defoliation
+ : defoliation
* : shading
x : control
shading*defoliation
defoliation
shading
control

Table 1. Regression equations at the start and end of stepwise backward elimination for shoots

O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
June	y = -0.005 O - 0.791 Sh - 2.529 Df + 0.863 Sh*Df (.106) (.322) (.002) (.443)	**0.114**	y = -0.005 O - 2.103 Df + 7.967 (.104) (.000)	**0.107**
July	y = -0.009 O - 0.128 Sh + 0.716 Df -1.398 Sh*Df (.005) (.887) (.418) (.263)	**0.078**	y = -0.009 O + 8.565 (.005)	**0.056**
August	y = -0.021 O - 0.037 Sh + 1.786 Df - 0.999 Sh* Df (.000) (.975) (.130) (.548)	**0.170**	y = - 0.021 O + 1.286 Df + 10.507 (.000) (.122)	**0.165**
September	y = -0.025 O - 0.556 Sh + 1.908 Df - 0.435 Sh* Df (.000) (.975) (.130) (.548)	**0.252**	y = - 0.025 O + 1.674 Df + 9.98 (.000) (.029)	**0.250**

Table 2. Regression equations at the start and end of stepwise backward elimination for roots

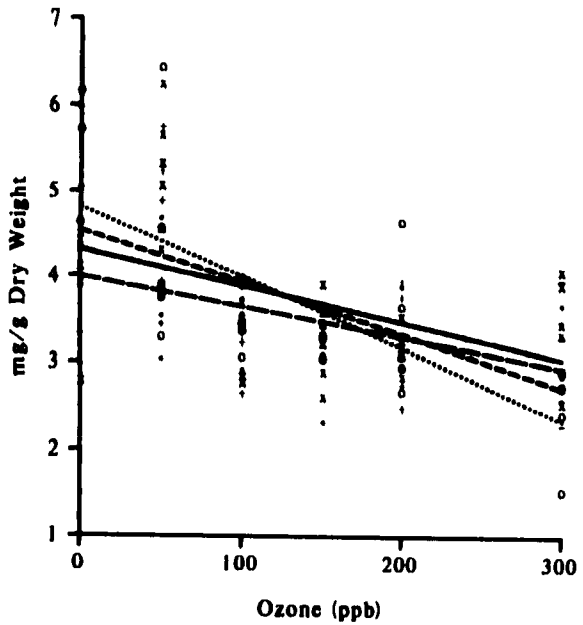
O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
June	y = -0.003 O - 1.154 Sh - 2.784 Df + 1.482 Sh*Df (.194) (.116) (.000) (.156)	**0.131**	y = - 2.048 Df + 7.823 (.000)	**0.102**
July	y = -0.011 O - 0.702 Sh + 1.200 Df -0.486 Sh*Df (.000) (.396) (.148) (.677)	**0.137**	y = -0.011 O - 0.945 Sh - 1.433 Df + 10.364 (.000) (.106) (.014)	**0.136**
August	y = -0.028 O - 0.465 Sh + 0.589 Df - 0.011 Sh* Df (.000) (.680) (.604) (.995)	**0.263**	y = - 0.028 O + 13.227 (.000)	**0.259**
September	y = -0.037 O - 0.584 Sh + 2.413 Df - 0.978 Sh* Df (.000) (.643) (.054) (.575)	**0.362**	y = - 0.037 O + 1.913 Df + 14.402 (.000) (.028)	**0.250**

Figure 3. Soluble sugar concentrations in the roots for the months of August and September.

(A) August



(B) September

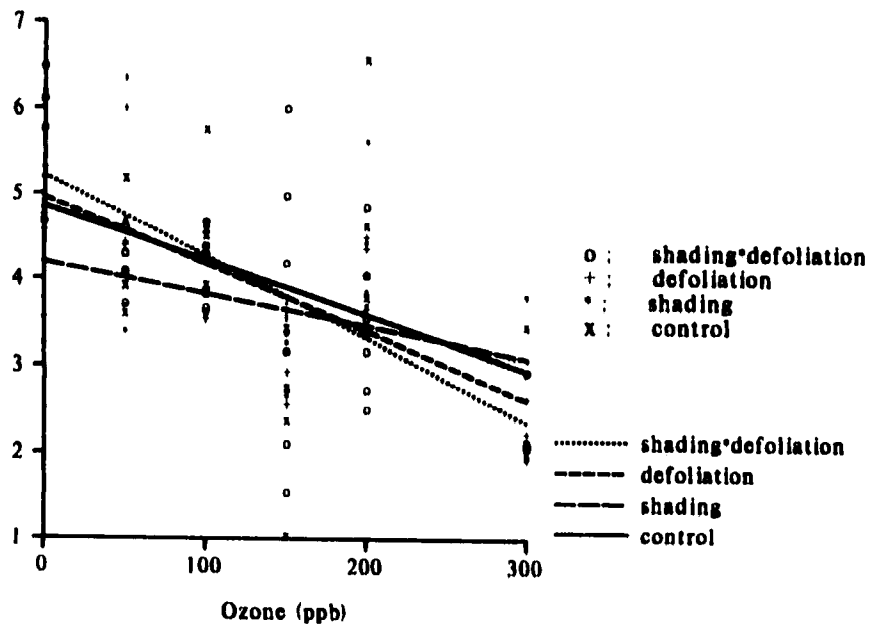


Table 3. Regression equations at the start and end of stepwise backward elimination for Total Root Soluble Sugars

O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
August	y = -0.006 O - 0.231 Sh - 0.025 Df + 0.348 Sh*Df (.000) (.305) (.911) (.277)	**0.300**	y = - 0.006 O + 4.468 (.000)	**0.287**
September	y = -0.008 O - 0.363 Sh - 0.112 Df +0.526 Sh*Df (.000) (.209) (.691) (.175)	**0.398**	y = -0.008 O + 4.980 (.000)	**0.382**

Mycorrhizal Colonization

Colonization rates for the beginning of the season were very low, and were therefore omitted from the results section. For the month of July, ozone, shading and defoliation had an effect on arbuscule production (Figure 4A, Table 4). For coil production, ozone was the only treatment that had a significant effect (at $p < 0.05$). There was no significant difference for the 3 other treatments, however, trends seem to show that defoliation might have had an effect as well (Figure 5A, Table 5). Although vesicle levels were very low, ozone, shading and defoliation all had significant negative effects on lowering the production of vesicles (at $p< 0.05$; Figure 6A, Table 6).

For the month of August, ozone continued to have a significant effect on production of the mycorrhizal structures that were studied (Figure 4-6B, Tables 4-6). However, R^2 levels for both coils and vesicles were very low.

For the month of September, colonization levels were at their highest for the lower ozone treatments. The slopes for the abundance of arbuscules were steepest in September for all the different treatments (Figure 6C, Table 6). Ozone was the only treatment to have a significant effect on arbuscule production. The other treatments (shading, defoliation, and shading/defoliation) had no significant effect (at $p < 0.05$; Figure 4, Table 4). (However, the trends in the data seem to imply that defoliation slightly increased arbuscule production, and shading slightly decreased arbuscule production compared to the control. The combination treatment (shading and defoliation) seemed to give results similar to the control (Figure 4, Table 4).

For coils, ozone was once again, the only treatment to have a significant effect on their development (at $p < 0.05$), but the slopes were not as steep, nor as obvious as they were for the arbuscules (Figure 5, Table 5) Vesicle levels were once again, very low for the month of September but ozone and shading had a negative effect on vesicle production (Figure 6, Table 6).

Figure 4. Percent occurrence of arbuscules for the months of July through September

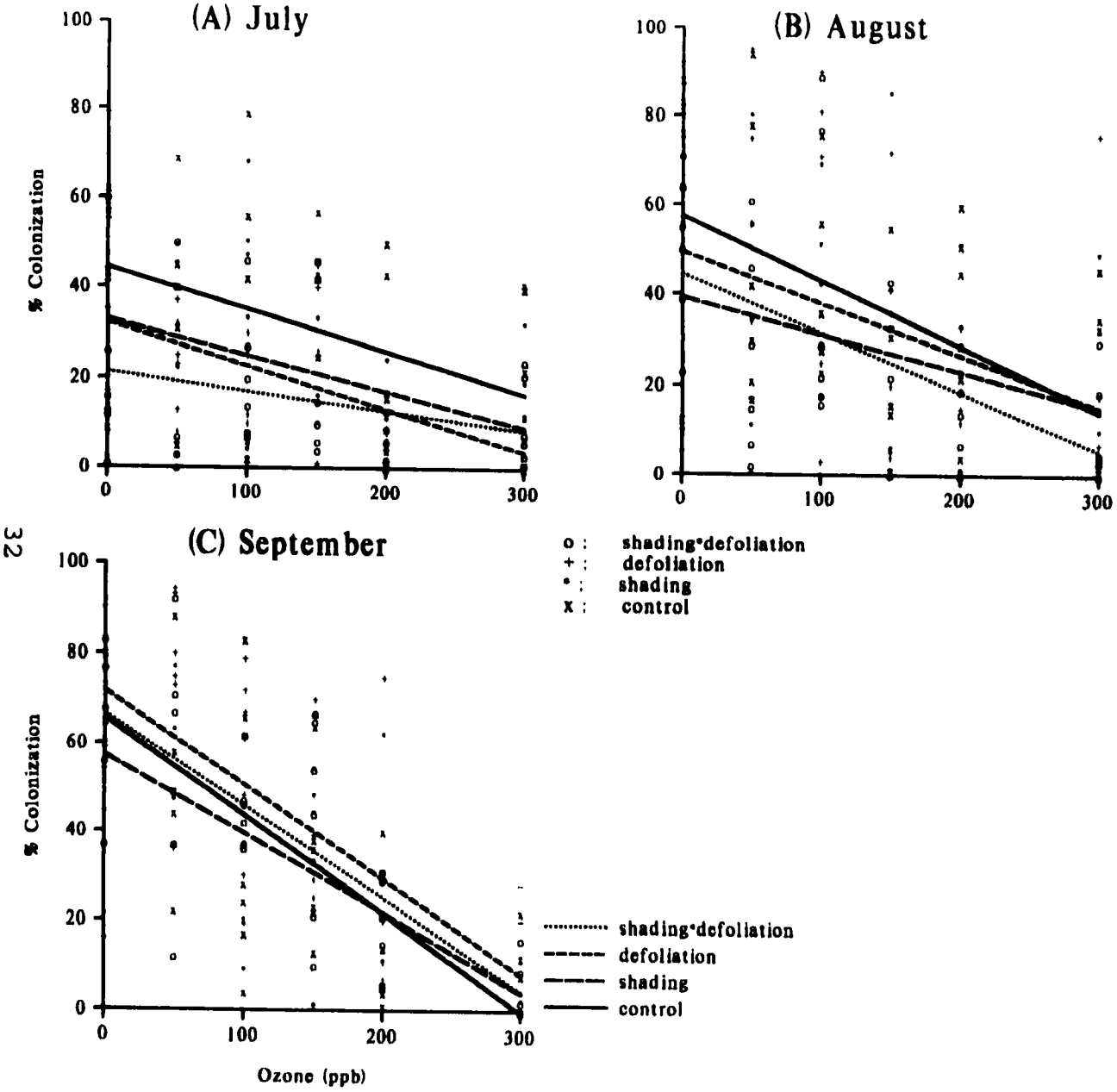

(A) July
(B) August
(C) September
% Colonization
Ozone (ppb)
0
20
40
60
80
100
0
100
200
300
o : shading*defoliation
+ : defoliation
• : shading
x : control
shading*defoliation
defoliation
shading
control

Table 4. Regression equations at the start and end of stepwise backward elimination for Arbuscule Colonization

O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
July	y = -0.077 O - 9.716 Sh - 12.556 Df - 5.939 Sh*Df (.000) (.022) (.003) (.318)	**0.238**	y = -0.077 O - 6.726 Sh - 9.607 Df + 40.836 (.000) (.025) (.001)	**0.233**
August	y = -0.116 O - 9.591Sh - 4.003 Df + 2.629 Sh*Df (.000) (.106) (.504) (.757)	**0.201**	y = - 0.116 O - 8.267 Sh + 51.725 (.000) (0.052)	**0.198**
September	y = -0.205 O - 2.878 Sh + 7.171 Df - 1.583 Sh* Df (.000) (.360) (.045) (.821)	**0.496**	y = - 0.204 O + 6.47 Df + 61.982 (.000) (.066)	**0.492**

Figure 5. Percent occurrence of coils for the months of July through September

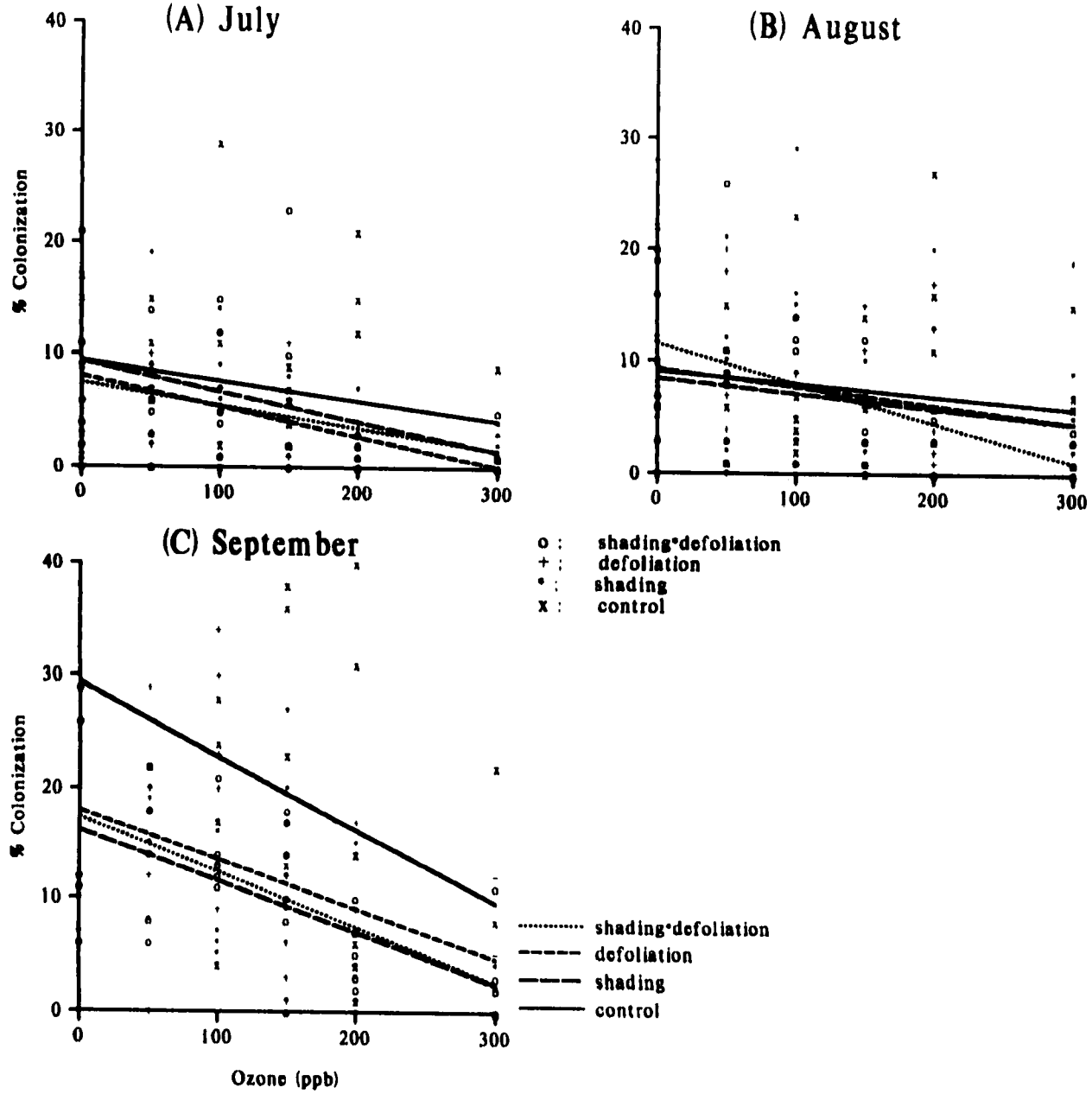

(A) July
(B) August
(C) September
% Colonization
Ozone (ppb)
0
10
20
30
40
100
200
300
o : shading*defoliation
+ : defoliation
* : shading
x : control
shading*defoliation
defoliation
shading
control

Table 5 Regression equations at the start and end of stepwise backward elimination for Coil Colonization

O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
July	y = -0.022 O - 1.240 Sh - 2.556 Df + 1.574 Sh*Df (.000) (.022) (.003) (.318)	**0.151**	y = -0.022 O - 1.777 Df + 9.463 (.000) (.071)	**0.146**
August	y = -0.019 O - 0.903 Sh + 0.584 Df - 0.408 Sh*Df (.002) (.595) (.735) (.868)	**0.075**	y = - 0.019 O + 9.966 (.000)	**0.068**
September	y = -0.044 O + 1.697 Sh + 3.691 Df - 2.971 Sh* Df (.000) (.360) (.045) (.247)	**0.267**	y = - 0.044 O + 2.175 Df + 15.100 (.000) (.090)	**0.259**

Figure 6. Percent occurrence of vesicles for the months of July through September

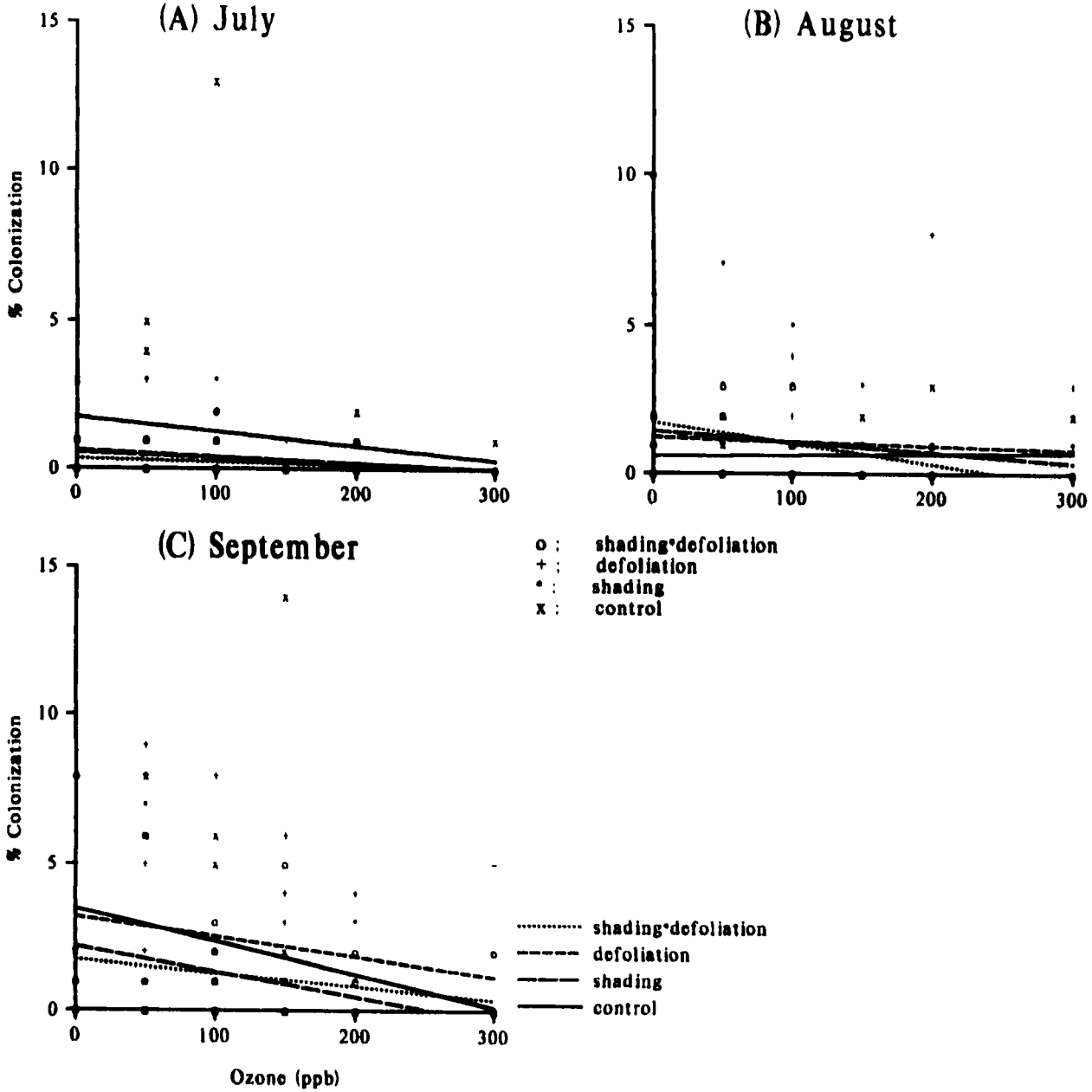

(A) July
(B) August
(C) September
% Colonization
Ozone (ppb)
0
5
10
15
100
200
300
o : shading*defoliation
+ : defoliation
• : shading
x : control
shading*defoliation
defoliation
shading
control

Table 6 Regression equations at the start and end of stepwise backward elimination for Vesicle Colonization

O = Ozone, Sh = Shading, Df = Defoliation, Sh*Df = Shading*Defoliation

(X) = p-Values

Month	Regression Equation Start	R^2	Regression Equation End	R^2
July	y = -0.003O - 0.773 Sh - 0.806 Df + 0.662 Sh*Df (.024) (.015) (.011) (.137)	**0.104**	y = -0.003 O - 0.773 Sh - 0.806 Df + 0.662 Sh*Df + 1.451 (.024) (.015) (.011) (.137)	**0.104**
August	y = -0.003 O - 0.321 Sh + 0.404 Df - 0.554 Sh*Df (.002) (.595) (.735) (.868)	**0.044**	y = - 0.003 O + 1.263 (.000)	**0.028**
Sept.	y = -0.010 O - 0.949 Sh + 1.213 Df - 1.123 Sh* Df (.001) (.218) (.111) (291)	**0.149**	y = - 0.010 O - 1.556 Sh + 3.895 (.001) (.004)	**0.132**

Discussion

Shoot and Root Biomass

After the first month, ozone had no apparent effect on biomass. This may be because sugar maples are very tolerant to ozone and can endure relatively high concentrations (Reich *et al,* 1986, Laurence *et al,* 1996). A short-term study of one month's duration therefore would give insufficient time to show any effects. However, the sugar maple seedlings were probably undergoing biochemical and physiological changes due to the ozone stress that were not monitored (Chevone *et al,* 1990).

In this study, shading did not have any apparent negative effect on shoot or root biomass. Sugar maples are very shade-tolerant (Tjoelker *et al,* 1993) normally germinating and growing under a closed canopy of ~ 2% photosynthetically active radiation (PAR) (Canham, 1988). It was found in a study by Ellsworth and Reich (1992) that as light availability is increased from 1 - 15%, photosynthetic capacity is increased by 75-80%. The 20% natural light available in this study may have been more than sufficient for normal growth of the seedlings resulting in no significant difference in growth compared to the control.

These results contrast with those of Tjoelker *et al,* (1993) who found that shading (7% irradiance) significantly decreased biomass (especially root biomass) of sugar maple seedlings exposed to ~ 115 ppb ozone levels for 37 days. On the contrary, seedlings exposed to 45% irradiance with the same level of ozone were not affected by ozone. The difference between their study and ours, was that their light conditions were somewhat higher than our shade conditions. As a result, they used much lower light levels than we

did for their shade conditions which most probably prevented the seedlings from producing enough carbohydrates to combat the effects of ozone.

Defoliation had a very significant effect on plant biomass. For the aboveground biomass this was expected, since the defoliation was administered only two weeks prior to the first sampling. As a result, the seedlings did not have sufficient time to recover lost photosynthetic biomass. It is interesting to note however, that the root biomass was higher for the intact plants (control and shading). The two weeks following defoliation would have been a period of active root growth. Because the defoliated plants were recovering from the damage, they may not have had the resources to allocate to the roots. The combined effect of the reduced photosynthetic surface and the need to replace lost tissues would result in the roots from the defoliated seedlings having lower biomass than the ones from the intact plants.

By July, plants exposed to ozone began to show significant signs of decline. Those seedlings growing at concentrations of 200 and 300 ppb O_3 were more yellow in appearance than those growing at lower concentrations and their root and shoot biomass was significantly lower. These results suggest that the seedlings grown at higher ozone concentrations had reduced photosynthetic abilities compared to the seedlings exposed to the lower ozone concentrations. The seedlings were probably also allocating more energy to maintenance and repair from the ozone damage, than to actual growth and biomass production. Shading once again, did not have any significant effects on growth. Defoliated plants continued to have lower biomass, though not as much so as the previous month. This demonstrates that the defoliated seedlings were beginning to recover and replace the biomass that was removed at the beginning of the experiment. However, the

roots of the seedlings that underwent defoliation still seemed to be affected by the treatment. It is probable that at this period, more energy was allocated to aboveground tissue at the expense of the belowground tissue since the seedlings were replacing the photosynthetic tissue that was removed in June. The energy that was being used to produce the leaves was most probably coming at the expense of that which would normally be delivered to the roots (Okano *et al*, 1984).

By the end of August, some seedling were beginning to show typical symptoms of ozone stress such as small, mottled leaves, that in some cases, were abscised (Heath and Taylor Jr., 1997) and thin roots. This contrasted with the control seedlings that had healthy green leaves and healthy full roots with many root hairs. These differences became more pronounced at the end of the growing season. The defoliated seedlings were able to replace the biomass that had been removed and had dry weights that were not statistically different from those seedlings from the undefoliated treatments.

By the end of September, the difference between the seedlings from the highest and lowest ozone treatment were greatest, producing very sharp slopes, as the cumulative effects of the ozone became most apparent. The treatment that affected seedling growth at this stage was once again, defoliation, which, counter intuitively, increased seedling growth.

By the end of the summer, the seedlings that underwent defoliation seemed to benefit from the treatment. After defoliation, the seedlings produced a new shoot. Although this new shoot may have withdrawn carbohydrate resources from the rest of the seedlings (notably the roots) during the first 2 months, this condition seemed only

temporary. Once the new leaves had reached full expansion, and were able to photosynthesize on their own, the plants recovered biomass. These leaves were formed during exposure to ozone whereas those on control plants had formed before exposure to ozone. The new leaves appeared to be healthier and more resistant to the ozone stress than those leaves that were present from the beginning of the experiment. It is possible that the first-formed leaves were senescing due to the ozone treatment (Reich *et al*, 1986), and the younger leaves were taking over the role of source leaves. Tissue senescence results in a decrease in the amount of physiologically active biomass. An increase in dead or abscising tissue (whether or not it has been abscised), causes a loss of functional carbon. The effects of stress will upset the carbon balance of the whole plant (Amthor and McCree, 1990). The new leaves became an investment for the seedlings exposed to the high ozone treatments. Although it is said by Heath and Taylor (1997) that oxidative stress, such as that which is caused by ozone exposure, tends to lower plant productivity and make the plant more sensitive to other diverse stresses, defoliation seems to be at least one stress which appears to benefit the plants undergoing this oxidative stress. The defoliation treatment seems to make the seedlings more resistant to the ozone stress, making those seedlings appear better able to withstand the oxidative stress.

This increase in leaf production after defoliation may have been the result of overcompensation, whereby, the seedlings achieved greater fitness by having part of their photosynthetic material removed (Paige and Whitham, 1987). As stated by McNaughton (1983), plants that undergo defoliation very rarely have a reduction in final yield proportional to the amount that was removed or destroyed. Defoliation may therefore induce branching which allows the plant to produce more new leaves due to the increased number of branches (Owen, 1980; McNaughton, 1983). Because the roots of

defoliated plants still remain the same size, they can still transfer the same amount of water and nutrients to the remaining intact tissues. As a result, the photosynthetic efficiency of those leaves will increase as well (Bayne *et al*, 1984) The tissue removed may allow for more sunlight to reach the remaining leaf tissue, also allowing for a more efficient photosynthesis (McNaughton, 1983). Improved water relations can also lead to increased stomatal conductance thereby increasing the photosynthetic rate of the plants. Although some might argue that this growth compensation comes at the expense of photosynthate normally allocated to the roots, this does not seem to be the case in our study, or that of Paige and Whitham (1987), since we actually saw an increase in the root biomass at the end of our experiment (compared to the control).

Soluble Sugar Analysis

For both months that the soluble sugars were analyzed (August and September), the concentration of carbohydrates decreased significantly as ozone levels increased. No other treatment (shading, defoliation, shading/defoliation) had any significant effects. These results contrast with those of Tjoelker *et al* (1993) who examined light and ozone stress on the root carbohydrates of sugar maple seedlings and found no significant difference in root carbohydrates (total soluble sugars and starch) for any of the treatments. In our study for the month of August, the 50 ppb ozone level appeared to be the concentration after which carbohydrate levels dropped sharply, and then evened out. Again for September, soluble sugar concentration decreased significantly with increasing ozone treatments, except for the 200 ppb treatment. However, when seedlings were subjected to other stresses, these decreases did not always appear linear. For example, the shaded seedlings only showed decreases in soluble sugar concentrations at ozone levels above 200 ppb , before which soluble sugars were fairly similar. Interestingly, the soluble

sugar concentrations were high at ozone concentrations of 200 ppb, for almost all treatments, (control, shading, and defoliation alone). It is possible that these high levels of soluble sugars may have been the results of solubilization of the starch reserves (Jensen 1981, Renaud and Mauffette, 1991). The plants may have solubilized their starch reserves to release soluble sugars needed for anti-oxidant formation, or to increase respiration. At 300 ppb, the plants may have exhausted their starch reserves, therefore being left with very little carbohydrate (Jensen 1981). However, the relatively high soluble sugar levels at 200 ppb ozone, may have been the result of some unknown chamber effect.

Mycorrhizal Colonization

The abundance of VAM fungi tended to parallel biomass and soluble sugar concentrations in the roots, whereby ozone tended to cause a cumulative decrease on mycorrhizal development throughout the growing season. It also caused a shift to occur between the ratios of mycorrhizal structures that became more apparent as the season progressed. For the month of July, all the different treatments had a significant effect on mycorrhizal colonization. It seems that all three stresses had some effect on the colonization process whereby the seedlings were not becoming infected as intensely as the control seedlings. It is possible that the stressed seedlings may not have had the energy to support the mycorrhizal fungus, so colonization levels were therefore much lower. Most probably at this point, all the carbon stresses had the same effect on the sugar maple seedlings in inhibiting the colonization of the symbiont. Even though all three stresses probably affected the photosynthetic apparatus and carbohydrate production in very different ways for the seedlings, the overall effects on the mycorrhizal fungi were

the same, whereby the fungus was probably receiving some message from the seedling (most probably in the form of root exudates), that the seedling was unable to accept a symbiont.

By the month of August, ozone was the only treatment that had a significant effect on mycorrhizal colonization, although shading still had a tendency to decrease mycorrhizal colonization. By this stage, the aboveground and belowground biomass was no longer significantly different from the intact plants. The defoliated seedlings probably had increased growth rates compared to the intact plants. As a result, the increased photosynthetic rates probably required increased amounts of water and nutrients. The plants benefited from the mycorrhizal symbionts which aided in the transfer of nutrients and water to the actively growing areas to help the seedlings reach the same biomass as the intact plants. By the fourth month, ozone was once again, the only treatment to have a significant effect on mycorrhizal development. Trends seem to show that the plants undergoing shading had slightly less arbuscule production while the seedlings undergoing defoliation had slightly higher arbuscule colonization compared to the control. These results differed from studies by Hayman (1974) and Daft and El-Giahmi (1978) who found that a decrease in illumination and/or photoperiod resulted in decreased overall plant biomass and decreased mycorrhizal colonization (with fewer and smaller arbuscules in the Hayman (1974) study) leading to a less efficient symbiosis between the plant and fungus. Although their shaded conditions (52% for the Hayman study and 75% shade for the Daft and El-Giahmi study) was still lower than our study (80% shade), the main difference was because in their study, they examined agricultural plants (onions and

maize, respectively), which normally are grown in high light conditions, whereas our study examined sugar maple seedlings which normally germinate and establish in very shaded conditions.

Overall, mycorrhizal colonization tended to increase in time for the low ozone treatments whereas mycorrhizal colonization for the higher ozone levels tended to remain very low. The colonization rates for the seedlings exposed to the high ozone treatments seemed to be even lower than those of the seedlings sampled at the start of the season. The difference between the low ozone seedlings compared to the high ozone seedlings was the greatest at the end of the growing season, showing that ozone had a cumulative effect on the seedlings. The arbuscule production decreased sharply, producing the steepest slopes from low to high ozone. Hyphal coil numbers were also decreasing, but not as dramatically as the arbuscules. At the higher ozone levels, the ratio of hyphal coils to arbuscules was becoming closer to one. Because arbuscules in sugar maples develop from hyphal coils, this suggests that fewer coils were being converted into arbuscules by the fungus. This demonstrates that the fungus was perceiving some change in the seedling, and therefore, was putting proportionately less energy into the production of transfer organs for the seedling.

Duckmanton and Widden (1994) showed a significant decrease in arbuscules and a significant increase in hyphal coils and vesicles as ozone was increased from 0X to 3X ambient levels. Other studies that have examined the indirect and direct effects of various stresses (UV-b light (Klironomos and Allen, 1995), base cation imbalances,

(Cooke et al, 1993), or acidic soils, (Klironomos, 1995)) on mycorrhizal fungi have all found generally similar results In all the cases, as the stress increased, the numbers of arbuscules decreased, and the number of vesicles and hyphal coils increased. It has been suggested that the fungus will perceive some physiological change in the plant and will react by putting more energy into its own long-term growth and survival by producing more vesicles and less energy into transfer organs such as the arbuscules (Duckmanton and Widden, 1994). One interesting observation in this study, was the relative lack of vesicles present. This leads us to ask whether the VAM fungus was storing energy within the plant root. It has been shown in previous studies (Cooke *et al*, 1992; Cooke *et al*, 1993; Klironomos, 1995) that vesicles are present in large numbers in mature sugar maples, though we found very few in seedlings. It is possible that the hyphal coils may have served the dual role of both transfer and storage organs within the seedlings under these growing conditions. This study shows that there is still much that we do not know about the relationship between VAM fungi and their hosts and more of an understanding is necessary when it comes to determining the role of the hyphal coil in the VAM complex.

Judging from the mycorrhizal colonization, and the ratios of arbuscules to hyphal coils, the symbiosis appeared to become less efficient as the host plant became less able to support a symbiont. One interesting point to note is that although concentrations of soluble carbohydrates decreased significantly in the plants, many residuals deviated quite largely from the slope. For example, there were many samples that had concentration of carbohydrates as high as the control plants at 200 ppb. If the mycorrhizal fungus was

only reacting to the quantity of soluble carbohydrates in the roots, then the arbuscule levels should have been as high as those found in the control plants. This was not the case in our study, where the seedlings exposed to 200 ppb ozone had some of the lowest arbuscule numbers of all the sampled seedlings. These results are in accordance with Hayman (1974) and Daft and El-Giahmi (1978) who found that in most cases, although there was no significant difference in total soluble carbohydrates found in plant roots exposed to different treatments, the mycorrhizal colonizations (or arbuscule numbers) differed significantly. Although Daft and El-Giahmi did not address the issue, Hayman hypothesized that there may be other factors affecting mycorrhizal conditions during stressful conditions. Results from these three studies suggest that it may not be enough to simply examine the concentrations of total soluble carbohydrates, and that we should also study the ratios of the different types of carbohydrates found in the seedling such as starch, sucrose, glucose and fructose. It is also possible that the high concentration of soluble sugars may not be in a form that is usable by the VAM fungus. It is also likely that the VAM fungus relies on the host plant for substances in addition to soluble carbohydrates such as vitamins, or phytohormones. In future studies, an examination of these other factors, in addition to soluble carbohydrates, might help us gain a better understanding of the relationship between the VAM fungi and their host plant.

Conclusion

In this study, defoliation and shading did not have an additive effect on ozone stress of the sugar maple seedlings for either biomass production, soluble sugars in the roots, or mycorrhizal colonization. By the end of the growing season, ozone was the only stress that had a major effect on the seedlings or their mycorrhizal fungi. Shading did not have any apparent effect since sugar maples are very shade tolerant. Defoliation appeared to be beneficial to the seedlings, probably because of the branching that it induced and possibly the production of adapted leaves. This study was only performed for one growing season, and our results suggest the necessity of long-term studies. Had our study lasted only for two months, we would have falsely concluded that ozone had a relatively small effect, that the effects of defoliation were negative, and that all of the stresses had a detrimental effect on mycorrhizal colonization. This work demonstrates the importance of performing long-term studies not only lasting a full growing season, but including a number of growing seasons. They also demonstrate the need to examine the many interacting stresses that might affect a plant at once.

Experiment 2. Effect of Elevated CO_2 and O_3 on Sugar Maple Seedlings and Mycorrhizal Fungi

Methods and Materials

This experiment took place in Nancy, France at the Université Henri-Poincaré à Nancy 1. Sugar maple seedlings were grown from seed obtained from the Berthierville Nursery in Quebec, Canada. Seeds were soaked in water for 14 days. The seeds were then germinated in clean sand in the dark for about three months. When the roots were between 3 to 10 centimeters long, the seedlings were planted in 512 cm^3 pots containing commercial organic soil mixed with sugar maple forest topsoil (from Experiment 1) containing native mycorrhizal inoculum in the form of chopped up roots and spores. The seedlings were then placed in each of 4 phytotronic chambers and were allowed to acclimate for about one month under artificial light (250 mol m^{-2} s^{-2} PPFD). The temperature was maintained at 24°C during the day and 20° during the night (16 hour photoperiod). The relative humidity was 75%. After a month-long acclimation period, all the seedlings had one pair of true leaves which we will refer to as the first leaves. The four treatments which then started were as follows: control (10 ppb O_3, 350 ppm CO_2), high CO_2 (10 ppb O_3, 650 ppm CO_2), high O_3 - high CO_2, (200 ppb O_3, 650 ppm CO_2) and high O_3 (200 ppb O_3, 350 ppm CO_2). Ozone was only administered during the light period whereas carbon dioxide was administered 24 hours a day.

The first samples were taken at Day 0 (mid-October), at the very beginning of the experiment before the treatments started. This was to ensure that there was no experimental bias. After that, another two samples were taken at Day 30 (mid-

November) and Day 61 (mid-December) after which, the experiment ended. For the first samples, since the treatments had not yet started, we only took 6 seedlings per treatment. For the two subsequent samples, we examined 25 seedlings per treatment for a total of 224 seedlings for this study.

Biomass allocation, soluble sugar analysis and VAM morphology were determined as described in Experiment 1.

Statistics

A two-factor analysis of variance without replication was performed for this experiment. Ozone and CO_2 were the two factors that were measured against biomass and total soluble carbohydrates of the different plant components (Zar, 1996).

Results

Biomass Allocation

At the beginning of the experiment there was no significant difference in biomass of the plants among any of the chambers (Figure 7, Table 7). At this time, the seedlings had developed their first two leaves, which represented the largest biomass compartment.

After 31 days, the first two leaves (first leaves) had grown and expanded. There was still no significant difference in dry weight, though the first leaves from those seedlings exposed to high ozone generally had less biomass (Figure 8, Table 8). Similar results were found for the roots (Figure 8, Table 8). The new leaves exposed to low ozone however, had a significantly higher biomass than those exposed to high ozone. However, the CO_2 treatments had no significant effect on biomass production (Figure 8, Table 8). For the stems, both the O_3 and CO_2 had significant effects on dry weight accumulation (Figure 8, Table 8).

After 61 days there was no significant difference between the dry weights of the first leaves (Figure 9, Table 9) from the various treatments. For the second leaves there was a significant effect of ozone, but not of CO_2 (even though they had the highest biomass). In the case of the stems and roots, there was no significant effect for any of the treatments, however, the seedlings from the high ozone had lower biomass than those from any of the other treatments (Figure 9, Table 9). The biomass of the high CO_2 high O_3 seedlings was intermediate to the control and the high O_3 seedlings, but these differences were not significant (Figure 9, Table 9).

In terms of total biomass across the 3 sample dates, there was no significant difference in dry weight accumulation for any of the sampling periods (Table 9). However, for day 31, the high ozone seedlings had a lower biomass, and the high CO_2 seedlings had a slightly higher biomass than those from the other treatments (Figure 13). For Day 61, the high ozone treatment had a lower biomass, the high CO_2 had higher biomass, and the control and high CO2, high O3 had values in between the other 2 treatments (Figure 13).

Figure 7. Biomass of the different plant parts at Day 0.

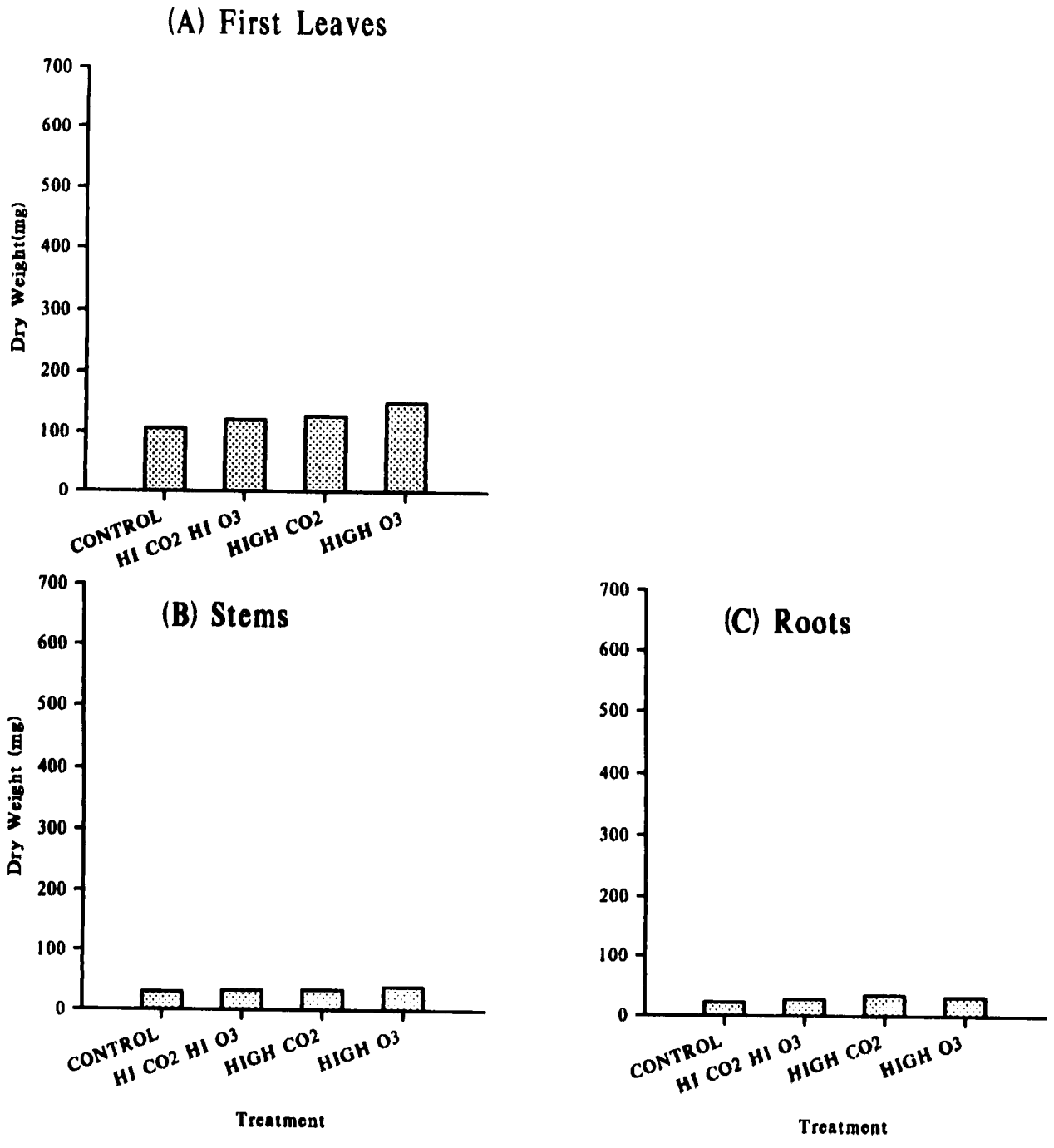

(A) First Leaves
Dry Weight(mg)
700
600
500
400
300
200
100
0
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
(B) Stems
Dry Weight (mg)
700
600
500
400
300
200
100
0
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment
(C) Roots
700
600
500
400
300
200
100
0
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment

Figure 8. Biomass of the different plant parts at Day 31

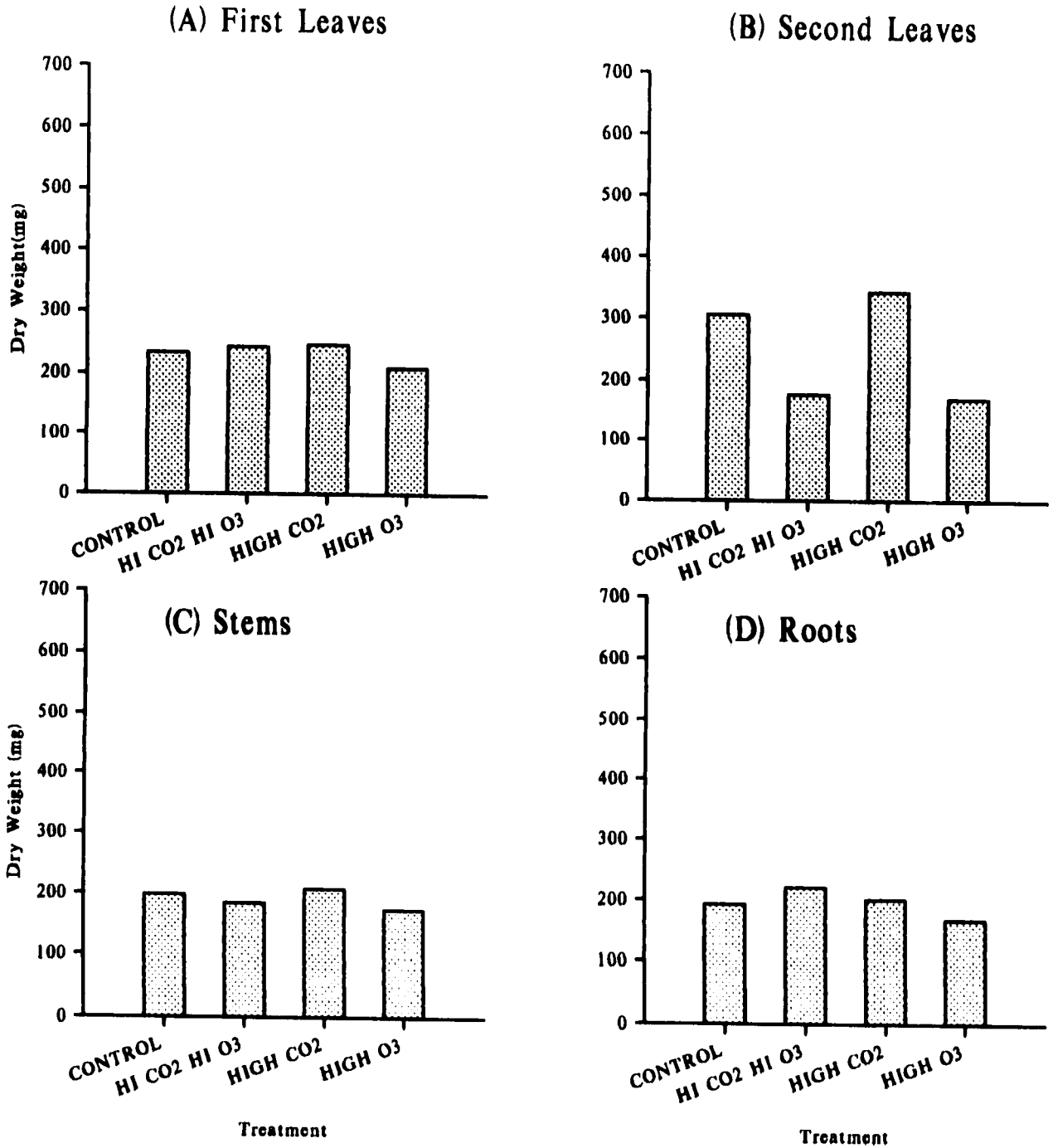

(A) First Leaves
(B) Second Leaves
(C) Stems
(D) Roots
Dry Weight(mg)
Dry Weight (mg)
0
100
200
300
400
500
600
700
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment

Figure 9. Biomass of the different plant parts at Day 61.

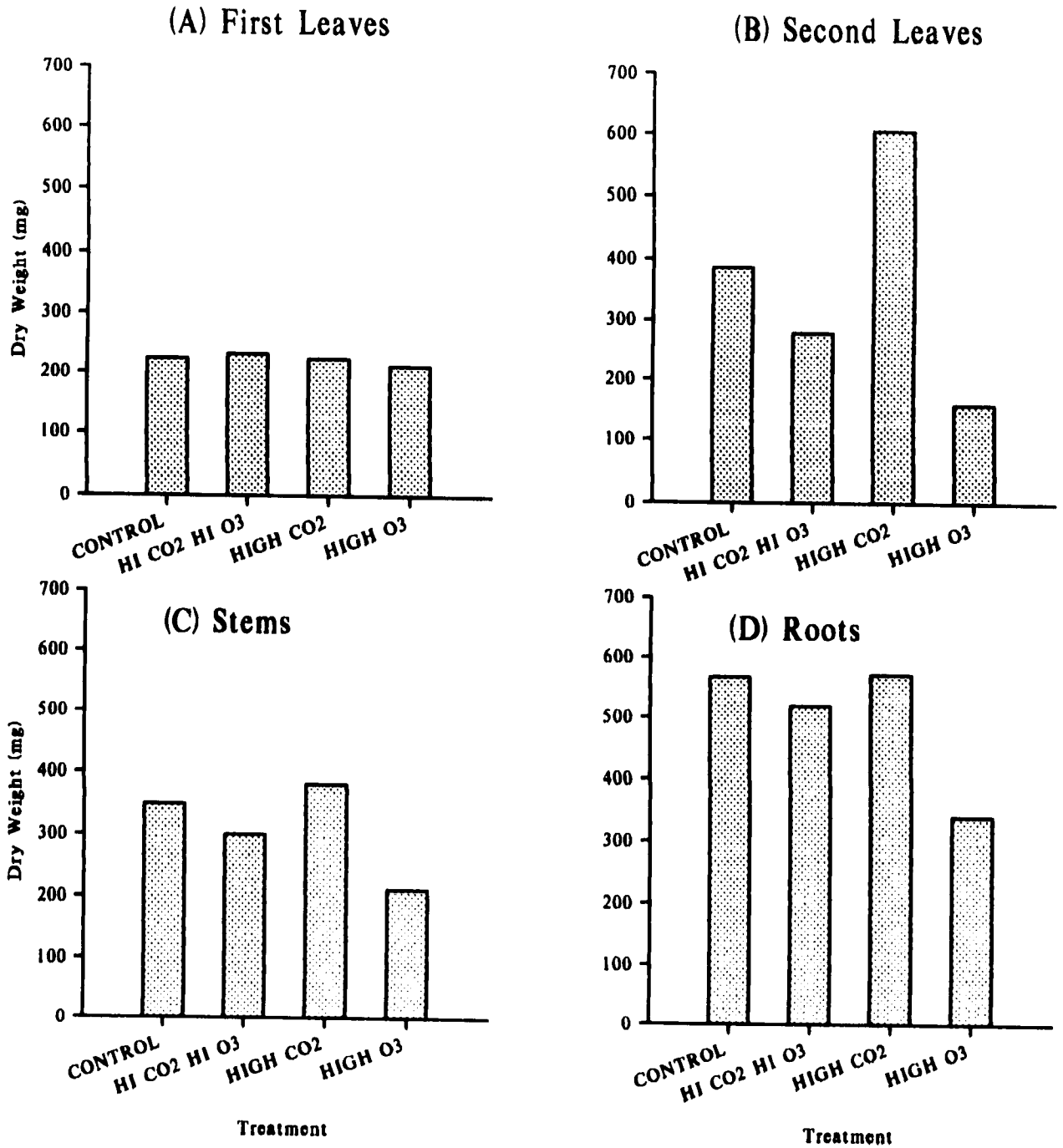

(A) First Leaves
(B) Second Leaves
(C) Stems
(D) Roots
Dry Weight (mg)
0
100
200
300
400
500
600
700
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment

Figure 10. Total biomass production throughout experiment.

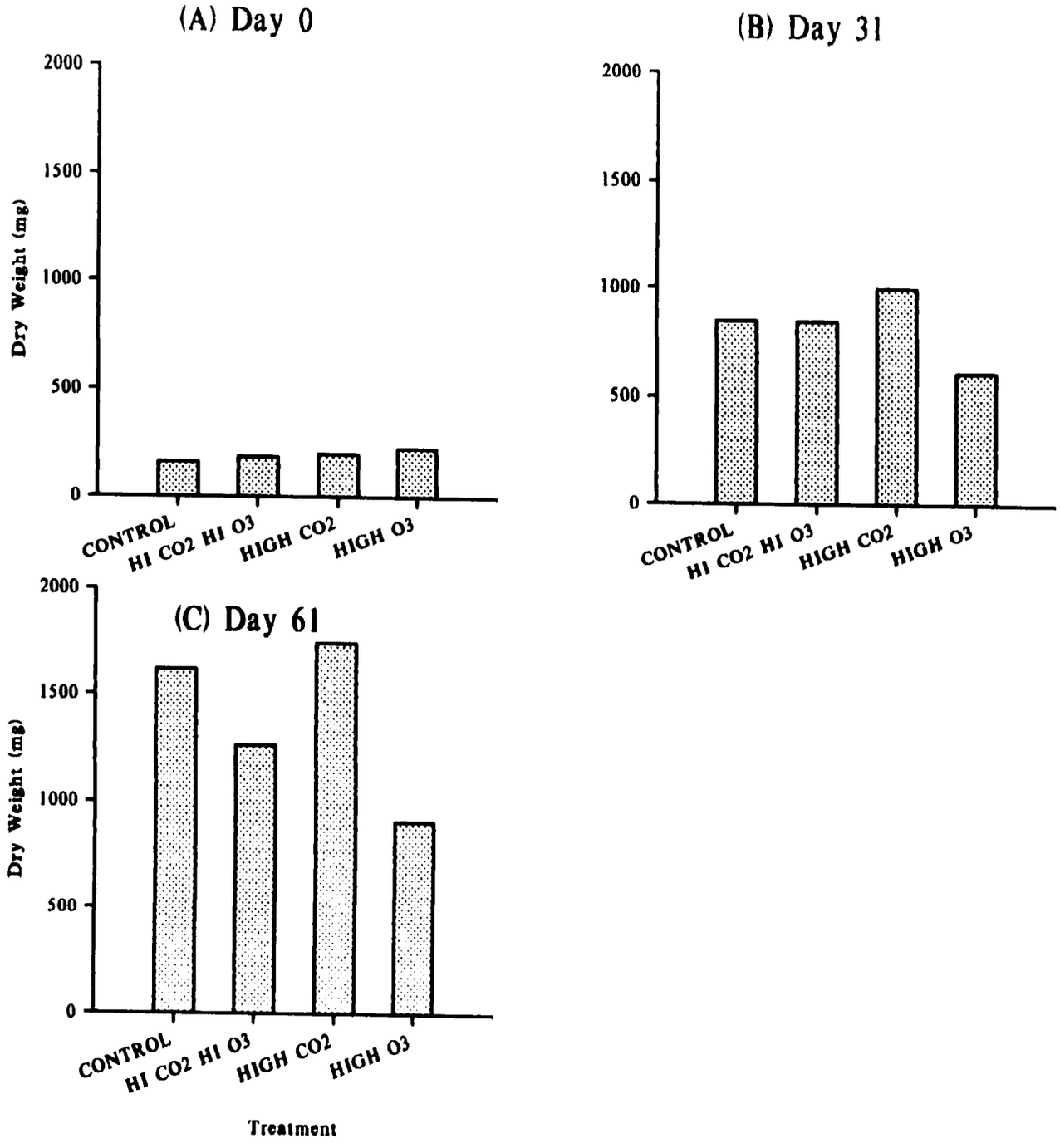

(A) Day 0
(B) Day 31
(C) Day 61
Dry Weight (mg)
2000
1500
1000
500
0
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment

Table 7. F-statistic for Biomass of plant parts.
$\alpha = 0.05$, Critical F = 161

		First leaves	Second leaves	Stems	Roots	Total
Day 0	FO3	0.575	N/A	0.502	0.0416	0.420
	FCO2	0.0217	N/A	0.499	0.290	0.00195
Day 31	FO3	1.732	953.43 *	2094.798 *	0.00242	33.525
	FCO2	5.113	5.434	361.27*	1.942	32.637
Day 61	FO3	0.198	232.601 *	15.979	2.092	35.906
	FCO2	0.453	9.247	4.858	1.007	6.478

Carbohydrate Analysis

The carbohydrate concentrations in none of the plant components were significantly affected by the treatments (Figure 11, Table 11). However, for both the first leaves, and the stems, the lowest carbohydrate concentrations were found in the high ozone/low CO_2 treatments (Figure 11 a and c).

Mycorrhizal Colonization

There was no mycorrhizal colonization for any of the seedlings in any of the treatments.

Figure 11. Total soluble carbohydrates for various plant parts at Day 61

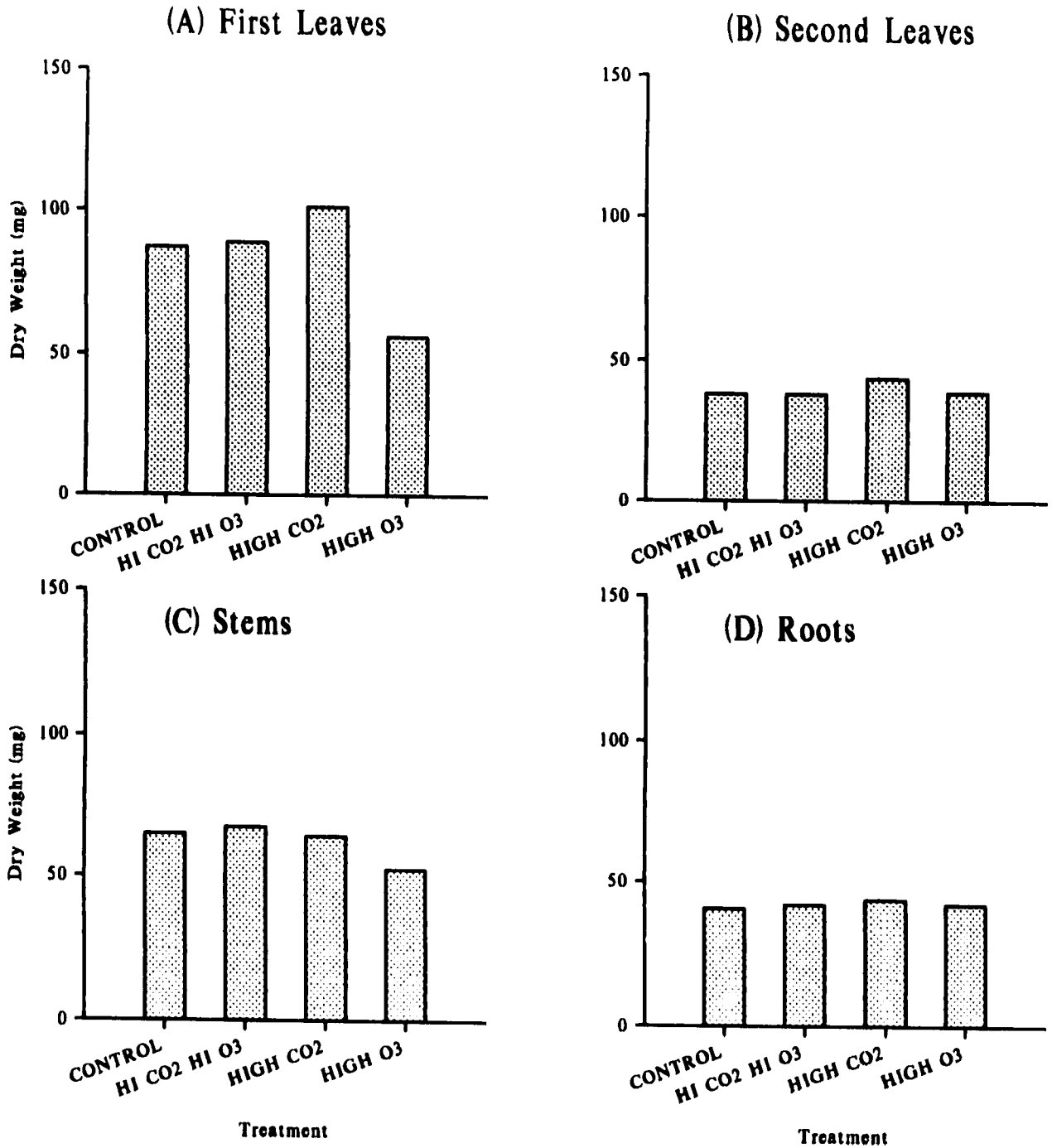

(A) First Leaves
(B) Second Leaves
(C) Stems
(D) Roots
Dry Weight (mg)
150
100
50
0
CONTROL
HI CO2 HI O3
HIGH CO2
HIGH O3
Treatment

Table 8. Total soluble carbohydrates for various plant parts at Day 61.
$\alpha = 0.05$, Critical F = 161

	First leaves	Second leaves	Stems	Roots
FO3	3.169	1.897	0.349	0.000918
FCO2	2.916	0.304	0.858	0.669

Discussion

Biomass Allocation

Ozone was the only pollutant to have a significant effect on the biomass of the sugar maple seedlings. Ozone decreased biomass accumulation for the first leaves and stems by Day 31 and the second leaves by Day 61. Carbon dioxide significantly affected the stems by Day 31, by increasing their biomass. By Day 61 however, these effects were no longer significant. As in the study by Rao *et al* (1995) we found an increase in shoot growth for plants that were grown under high CO_2 however, unlike their study, our results were not statistically significant. Their study did not specify what was meant by shoot growth, but in our study the increased shoot growth experienced by the sugar maple seedlings was mainly second leaf growth. In their study, plants that were grown under high O_3, ambient CO_2 had significantly less biomass than the other plants. In our study, seedlings exposed to high O_3, ambient CO_2 also had smaller biomass, yet these results were not statistically significant. However, one large difference between their results, and ours, was that in their study, there was no significant difference between those plants grown under high CO_2- high ozone compared to those that were grown at high CO_2 only. On the contrary, in the study by Noble *et al*, (1992), seedlings grown at high O_3, high CO_2 were significantly larger than all other treatments (including the control (low O_3, low CO_2) and high CO_2 (high CO_2, low O_3) treatment). In that study, the high O_3 seedlings had the smallest biomass, but the result was not statistically significant. In our study, seedlings exposed to high O_3 with the high CO_2 were smaller than the seedlings exposed to the high CO_2 alone, yet these results were not statistically significant. It seemed that in the studies by Noble *et al*, (1992), Barnes and Pfirmann (1992) and Rao *et al*, (1995),

this ozone tolerance under high CO2 was the result of a more efficient photosynthetic apparatus that increased the formation of antioxidants such as glutathione reductase. In our study, there was no significant compensatory effect of elevated CO_2 on plant growth (although those seedlings had slightly larger biomass than the those from the other treatments). In this sense, our results are similar to those obtained by Mortensen (1995) who also found no interactive effect of CO_2 (560 ppm) and O_3 (62 ppb). In his study, the small birch seedlings exposed to the high ozone/high CO_2 did as poorly as those seedlings that were exposed to the high ozone/ambient CO_2 levels. The differences among all our results may be attributed to the different tolerance levels of the plants to the ozone stress, the concentration of the pollutants, and their exposure (24 hours a day/ 7 days a week compared to peak traffic hours) as well as duration and design (replication) of the experiment.

The biomass allocation patterns for the different treatments that were obtained in this study produced some unexpected results. In the case of the high CO_2 treatment, it was predicted that as the aboveground organs continued to grow, more carbohydrates would accumulate, so that more energy would be transported to the roots, leading to a larger root biomass (Rogers et al, 1993). Although there was no significant effect on the seedlings exposed to the high CO2 treatment, they did have larger dry weights than seedlings exposed to the other treatments. There was no significant difference in the root biomass for any of the treatments, yet the high ozone treatment had lower dry weights than the other treatments. On the other hand, the biomass for the second leaves gave many different results depending on the treatment. In the case of the elevated CO_2

treatment, the new leaves had higher biomass than any of the other treatments. It would appear then, that those seedlings concentrated most of their growth in the second leaves. Comparing the roots with the shoots of the high CO_2 treatments, both organs had equal biomass. These results were contrary to the results of other studies where it was predicted that higher CO_2 concentrations would have led to higher root biomass (Rogers *et al*, 1993). This does not seem to be the case in our study where three of the four treatments (control, high CO_2 (with and without high ozone)) had equal root biomass. In this experiment, only the elevated CO_2 had equal ratios for both the new leaves and the roots, whereas all the other treatments tended to favour root growth over new leaf growth.

These results are particularly interesting when examining the ratios of new leaves to roots for the high ozone treatment. Our results once again, contradict those from other studies where it was found that high ozone would cause the root to shoot ratios to change in favour of the leaves. In our study, the reverse seemed to happen instead. More biomass accumulated in the roots as opposed to the new leaves although the lower dry weights of the high ozone-treated seedlings were not significant.

It is possible that in the case of the high CO_2 treatment, the seedlings may have put more energy into their leaves in an attempt to reach the canopy first. On the other hand, in the case of the high ozone treatment, it is possible that those ozone concentrations were too high to combat, so that the strategy of the seedlings may have been to invest more growth in their roots in an attempt to survive until conditions were again favourable for aboveground growth (Okano et al, 1984).

Soluble Sugar Analysis

There was no significant effect of any of the treatments on total soluble carbohydrate concentrations found in the different plant components. Yet certain unexpected trends were apparent. In the case of the first leaves, seedlings from the high ozone treatment had lower total soluble carbohydrate concentrations than those from the other treatments. The reason may have been that the ozone had damaging effects on those leaves, thereby causing their premature senescence. As a result, the first leaves from the ozone treatment may not have produced as much carbohydrate as those from seedlings that were grown in the less stressful conditions. Since less energy was found in the source leaves of the ozone treatment, there were probably fewer carbohydrate to transport to the rest of the organs. As a result, there would have been fewer soluble carbohydrates in the stems which act as the transport organs. However, it is interesting to note that there was no significant difference in the soluble carbohydrate concentrations found in the second leaves, or roots for the different treatments.

For the second leaves, this similarity may have been because the concentrations of soluble carbohydrates may have been the minimal for maintenance of plant survival. Comparing the biomass obtained for the seedlings for Day 31 and 61, the new leaves of the high ozone seedlings did not significantly grow from the previous month. As a result, it would appear that those second leaves were undeveloped compared to those of seedlings from the other treatments, and probably still required carbohydrates from the source leaves (first leaves). The fact that the new leaves from the ozone treatments were still acting as sink leaves might further explain the lower concentration of carbohydrates

found in the source leaves. Since the second leaves could not photosynthesize on their own, they therefore required more energy from the first leaves, compared to the second leaves from the other treatments. As a result, there were fewer carbohydrates available in the source leaves of the ozone-treated plants for their own processes.

As with the second leaves, the roots also had equal concentrations of carbohydrates among all the different treatments. The roots are sink organs that must rely on the source organs for their carbohydrate requirements. For the ozone-treated plants, they were most probably getting their energy from the first leaves since the second leaves were too undeveloped to act as a source for the carbohydrates. As was the case for the first leaves, the fact that all the treatments had the same concentration of carbohydrates in the roots probably shows that there was again, some minimum requirement necessary for actual root survival.

Another interesting point to note was that both the second leaves and the roots had the same concentration of soluble carbohydrate per gram of dry weight. It could therefore be said that they had equal energy demands. This is an interesting result since it was expected that for the low ozone seedlings, since there was no photosynthetic stress, more energy could have been diverted to the roots. As the leaves continued to grow and develop a greater photosynthetic capacity, their needs for more water and nutrients would have required a larger, more developed root system, thereby transferring more carbohydrates to the roots (Rogers et al, 1994). However, the concentration of carbohydrates for the high CO_2 roots was the same for all the treatments. These results

tend to show that in terms of soluble carbohydrates, there was no need to invest more energy into the root system. The reason was probably because the root systems supplied the plants with adequate nutrients and water without having to allocate more energy into the belowground biomass than was necessary (Eamus and Jarvis, 1989) and it may have been an artifact of a pot experiment. On the contrary, we expected that more carbohydrates would have been invested into the new leaves instead of the roots to combat the photosynthetic stress for the high ozone treatments (with and without elevated CO_2).

Mycorrhizal Colonization

It was not known why the mycorrhizal fungi never colonized the seedlings. It is possible that the commercial potting soil contained enough available nutrients that the mycorrhizal symbiosis was unnecessary for the plants to obtain their mineral requirements. It is also possible that the roots were already too developed when the seedlings were transferred from the sand, and potted in the soil. The optimum period for the fungus to colonize the seedlings may have passed at that point. If the seedlings had been germinated directly into the soil, mycorrhizal colonization may have been possible.

Conclusion

Since many of our results run contrary to our predictions, it is probably a sign that we should have run this experiment for a longer period of time, and examined other variables as well. In future experiments, it would be advisable to replicate the experiment so that the treatment effects may appear significant. In the case of the biomass results,

running the experiment for a longer period of time may give results more similar to what is found in the literature where the high photosynthetic stresses favour higher shoot to root ratios, and the lower photosynthetic stresses favour lower shoot to root ratios.

In order to obtain a more comprehensive picture of the actual plant processes, future experiments should also include a complete carbohydrate analysis which examines the ratios of insoluble carbohydrates (starch) to soluble carbohydrates. These studies would help determine how much energy is utilized compared to how much energy is stored by the plant and to which organs the carbohydrates are transported. The quality of those sugars in terms of the ratios of sucrose to reducing sugars (glucose and fructose) should also be examined. It was found that as a plant endures more stressful environments, more of its reserves are converted to reducing sugars to handle the higher metabolic processes (Renaud and Mauffette, 1991). Examining these additional factors may give us a better idea of how a plant truly reacts to the different growing conditions.

Since there was no significant difference in carbohydrate concentrations of the seedling roots for any of the treatments, it is impossible to predict how the mycorrhizal fungi would have indirectly responded to the air pollutants. It is possible that if the mycorrhizal fungi were present, they may have further increased the sink strength of the roots, increasing the amount of carbohydrates allocated belowground. However, it is also possible that the total soluble carbohydrates may be unreliable indicators of how the mycorrhizal fungi would colonize the seedlings. It might be further proof that the mycorrhizal fungi react to something in addition to soluble carbohydrates found in the root.

General Conclusion and Future Directions

From these two studies, the importance of examining multiple stresses on seedlings was shown. It was demonstrated that interacting stresses may sometimes give different results from those predicted. In both studies, ozone had a major effect on plant biomass production.

For Experiment 1, this was the first time that ozone levels were pushed to such sub-lethal concentrations as to start killing some plants. This study demonstrated how the mycorrhizal complex deteriorated and appeared to be less efficient as the ozone levels increased. In our study, defoliation appeared to improve seedling health, and render the seedlings more tolerant of the ozone stress. For future studies, it would be interesting to try different experiments with defoliation, such as different degrees of defoliation, and combine defoliation with other stresses (both photosynthetic and root) to determine how the plant and the mycorrhizal system will respond. Also, it would be a good idea to extend the experiment beyond a single growing season.

In both experiments, root carbohydrates did not always give the expected results, that is to say, decreasing with increasing stress (at all levels of stress). In the case of the mycorrhizal fungi, it may now be the time to start examining other factors that may also affect the symbiosis since it appears that the mycorrhizal fungi may be reacting to substances in addition to the root soluble sugars.

Mycorrhizal fungi were the most sensitive indicator of the ozone stress, more so than biomass allocation, or root carbohydrates, suggesting that as Vogt *et al* (1993) had predicted, they may be a more sensitive early indicator of stress.

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