Mycorrhizas and sugar maples: the impact of pollutants.

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SUMMARY

Two year old sugar maple seedlings were grown for a single season in open topped chambers supplied with air containing ozone at varying concentrations. Within each chamber, seedlings were divided into four groups, controls, shaded, partially defoliated and both shaded and defoliated. The seedlings were sampled each month during the growing season and the biomass of roots and shoots, carbohydrate levels and mycorrhizal colonization of the roots were measured.

Generally, biomass and mycorrhizal colonization decreased as the level of ozone increased. The soluble carbohydrate concentrations in the roots also decreased, but not as steeply. Shading and herbivory did not have a large effect on the relationship between ozone and biomass, carbohydrate or mycorrhizal colonization. Defoliation did, however, make the negative relationship with biomass and ozone concentration less obvious.

Of the mycorrhizal structures, arbuscules were most affected by ozone levels, followed by coils, then vesicles. As arbuscules are the major site of nutrient transfer to the plant, this relationship implies that as the ozone stress increases, not only is the level of colonization being reduced, but the effectiveness of the mycorrhiza to the plant may also be reduced.

INTRODUCTION

Mycorrhizae are mutualistic symbioses between fungi and plant roots. There are two major types of mycorrhizae, ectomycorrhizae and endomycorrhizae. In the ectomycorrhizal association, the fungus develops at the tips of the fine roots and forms an extensive sheath on the outside of the root, sometimes referred to as the mantle. The fungus also grows between the cortical cells, forming the Hartig net, but it does not penetrate them (Atlas and Bartha, 1993). A fine root colonized by an ectomycorrhizal fungus becomes more deformed and less branched than its uncolonized counterparts (Atlas and Bartha, 1993). This type of association is very important in boreal and temperate deciduous forests (Atlas and Bartha, 1993; Allen, 1991), as the Pinaceae (spruce, pine, and fir), Betulaceae (birch) and Fagaceae (oak and beech) are almost exclusively ectomycorrhizal families, (Smith and Read, 1997.)

Of the endomycorrhizal associations, the vesicular-arbuscular mycorrhizae (VAM) comprise the largest group and are found on 90% of all vascular plants (Hampp and Schaeffer, 1995). Most agricultural plants have VAM, as do most herbaceous plants (Allen, 1991) and many tropical trees (Smith and Read, 1997). In temperate deciduous forests, this association is found on trees in the Aceraceae (maple), Rosaceae (apple, cherry, plum and serviceberry) and Oleaceae (ash) (Smith and Read, 1997).

Plants benefit from mycorrhizal fungi in many ways. Mycorrhizae aid in the acquisition of nutrients, particularly phosphorus, but also nitrogen and trace metals, such as copper and zinc

(Smith and Read, 1997). The fungal hyphae, which are thinner than the fine roots and longer than the root hairs, grow out from the plant root 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 of 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 plants in acid (Klironomos 1995) and saline soils, (van Duin *et al*, 1989).

Mycorrhizal fungi benefit by getting their energy from the plant in the form of soluble sugars. Unlike the ectomycorrhizal fungi, which can often be easily be grown in culture, the VAM fungi cannot be grown outside the host and must, therefore, be considered to be obligate symbionts (Smith and Read, 1997). These fungi cannot break down materials such as decomposing plant matter (dead wood and leaves) to obtain carbohydrates and must rely on the plant to provide them (Smith and Read, 1997). The fungus can represent a considerable carbon drain on the plant, using 4 - 20% of the plant's carbon resources (Smith and Read, 1997).

The term vesicular-arbuscular mycorrhiza 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 to be the most important structure of the whole VAM complex. In sugar maples, intracellular hyphal coils are formed from which the arbuscules develop (Cooke et al, 1992). Vesicles are swellings, which resemble a balloon, that can be formed inter- or intra-cellularly. They appear to be organs for carbon storage, but, may also serve as spores. Because they are carbon rich, vesicles 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 fungal reproduction) (Powell and Bagyaraj, 1986) .

Most studies on the effects of plant stress on mycorrhizae have used fungal colonization rates as a sign of stress (Vogt *et al*, 1993). The general conclusion is that as plant stress increases, mycorrhizal colonization decreases (Vogt *et al*, 1993). This has been interpreted as reflecting a reduction of the amount of sugar produced in the plant as a result of stress, resulting in a reduction in the colonization level by the fungus as a result of carbon limitation (Duckmanton and Widden, 1994). However, recent studies suggest that moderate stress causes subtle changes in the occurrence of VAM fungal structures. Both Duckmanton and Widden (1994) and Klironomos (1995) have shown that, in stressed plants, arbuscules are less abundant whereas vesicles and coils are more abundant. These authors have suggested that this might be a useful early warning of stress in the plant and that the change in morphology might also indicate a breakdown in the mutualistic relationship. For this reason, studies of stress in plants should include documentation of the VAM morphology.

In this study, we examined the combined effects of atmospheric ozone, shading, and defoliation on biomass accumulation, soluble sugars in the roots, and VAM colonization and morphology, for a single growing season, in 2 year-old sugar maple seedlings.

Materials and methods

Sugar maple seedlings were grown in the open top chambers at the Centre Acéricole de M.A.P.A.Q. in Tingwick, Québec. In early May 1996, before bud break, 600 bare-root, green-house grown (from the Berthierville Nursery), two year-old seedlings were potted using soil obtained from the top 10-15 cm of the surrounding sugar maple forest. The seedlings were allowed to grow outdoors in their pots for one month and were then placed in the growth 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 sunlight. Defoliation was achieved by means of removing ½ 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 (Mauffette, Y., pers. com.). Trees were grown for 4 months and samples were taken at the end of June, July, August and September. At each sampling, 24 seedlings were removed from each chamber, (6 per treatment) for a total of 144 trees per sampling.

Plant Biomass

Each seedling was divided into aboveground (leaves and stems) and belowground (root) parts. The fresh weights were measured for aboveground and belowground parts separately, then small samples (~2 g) of the roots were removed and weighed for sugar analysis and VAM quantification (see below). The plant parts were oven-dried for a minimum of 24 h and reweighed to obtain the dry weight. The dry weights of the of the samples removed were then calculated from the water content of each plant, so that total dry weight could be estimated.

Soluble Sugar Analysis

Approximately 1 g of fine roots was removed, freeze-dried and ground to a fine powder. Ten milligrams of this root powder were 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. This methanol extract was centrifuged at 15,000 x g for 10 min and the pellet was discarded. From the supernatant, 100 ml were removed and added to 100 ml of distilled water and 1.5 ml of anthrone. This solution was incubated at 100 C for 8 min. The solutions were then cooled and the concentration of sugars were read colorimetrically with a spectrophotometer, using a standard curve of known glucose values. The results are shown for the month of September.

VAM Morphology

The remaining portion of fine roots was washed and stored in F.A.A. (formalin acetic acid alcohol) 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 in 0.15% chlorazol black E at 90 C for 20 min. The roots were mounted in glycerin jelly on slides and mycorrhizae were quantified using the magnified intersect method (McGonigle et al., 1990). One hundred intersects were observed for each plant, using a differential interference contrast (DIC) microscope, to obtain percent colonization for each of the VAM fungal structures.

Statistics

Multiple regression analysis was performed using Systat version 5.03 (Wilkinson, 1990).

RESULTS

Ozone had a significant effect on plant biomass (Table 1), with the biomass of both roots and shoots generally decreasing with ozone concentration (Fig. 1). Shading and defoliation, alone and in combination, had no significant effect on the relationship between ozone levels and biomass (Table 1).

Table 1. Regression analysis of biomass vs ozone concentration (September)
* $p < 0.001$, ** $p < 0.05$

Treatment	Structure	R2	p
Control	Shoot	0.218	0.000 *
	Root	0.296	0.000 *
+ Shading	Shoot	0.218	0.783
	Root	0.296	0.847
+ Defoliation	Shoot	0.249	0.022 **
	Root	0.323	0.024 **
Shading and Defoliation	Shoot	0.228	0.194
	Root	0.302	0.294

There was a significant decrease in soluble sugars present in the roots as the concentration of ozone increased (Figure 2a). As with biomass, shading and defoliation, either alone or in combination, had no significant effect on this relationship (Table 2).

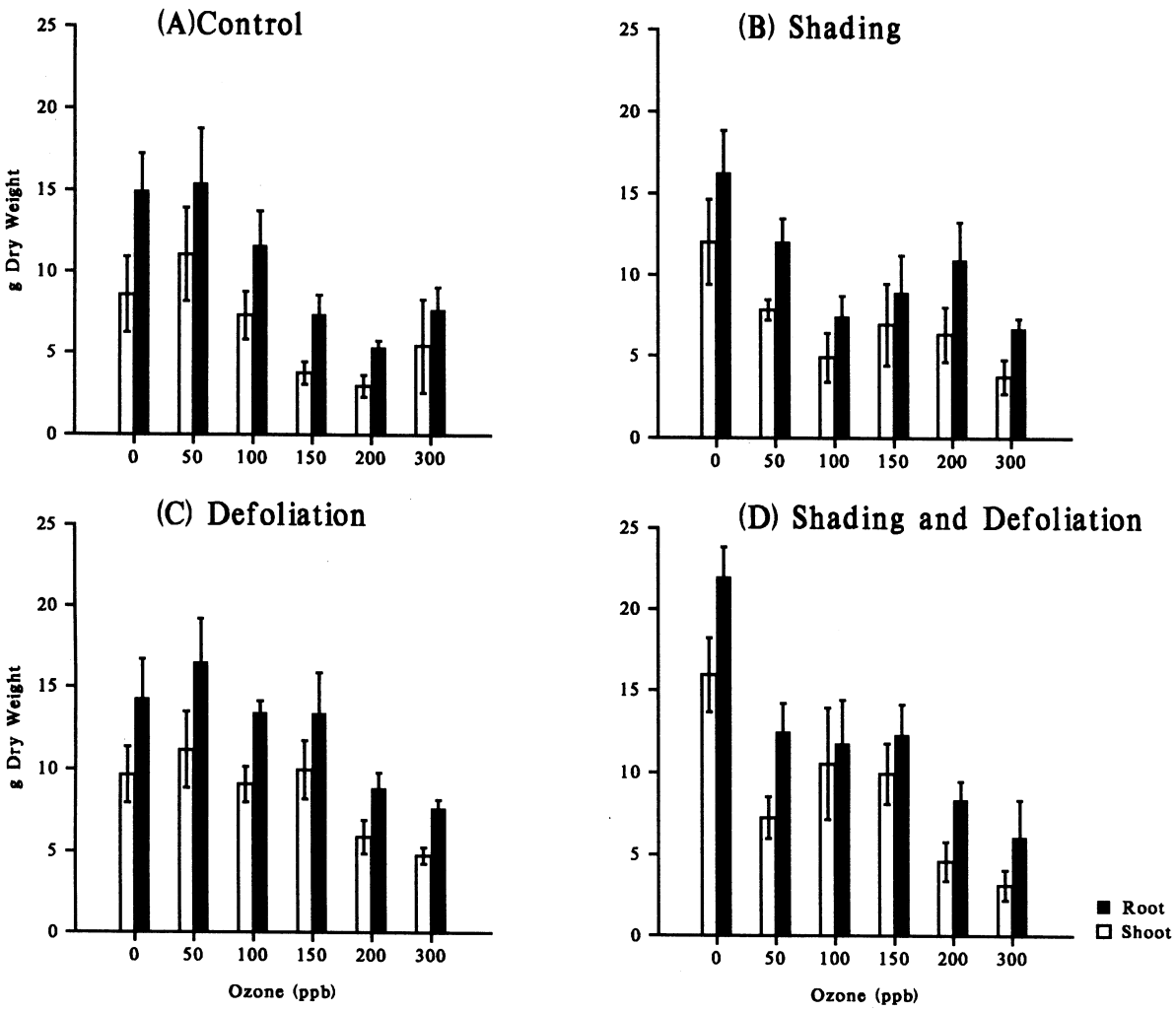

Figure 1. Biomass Production for September

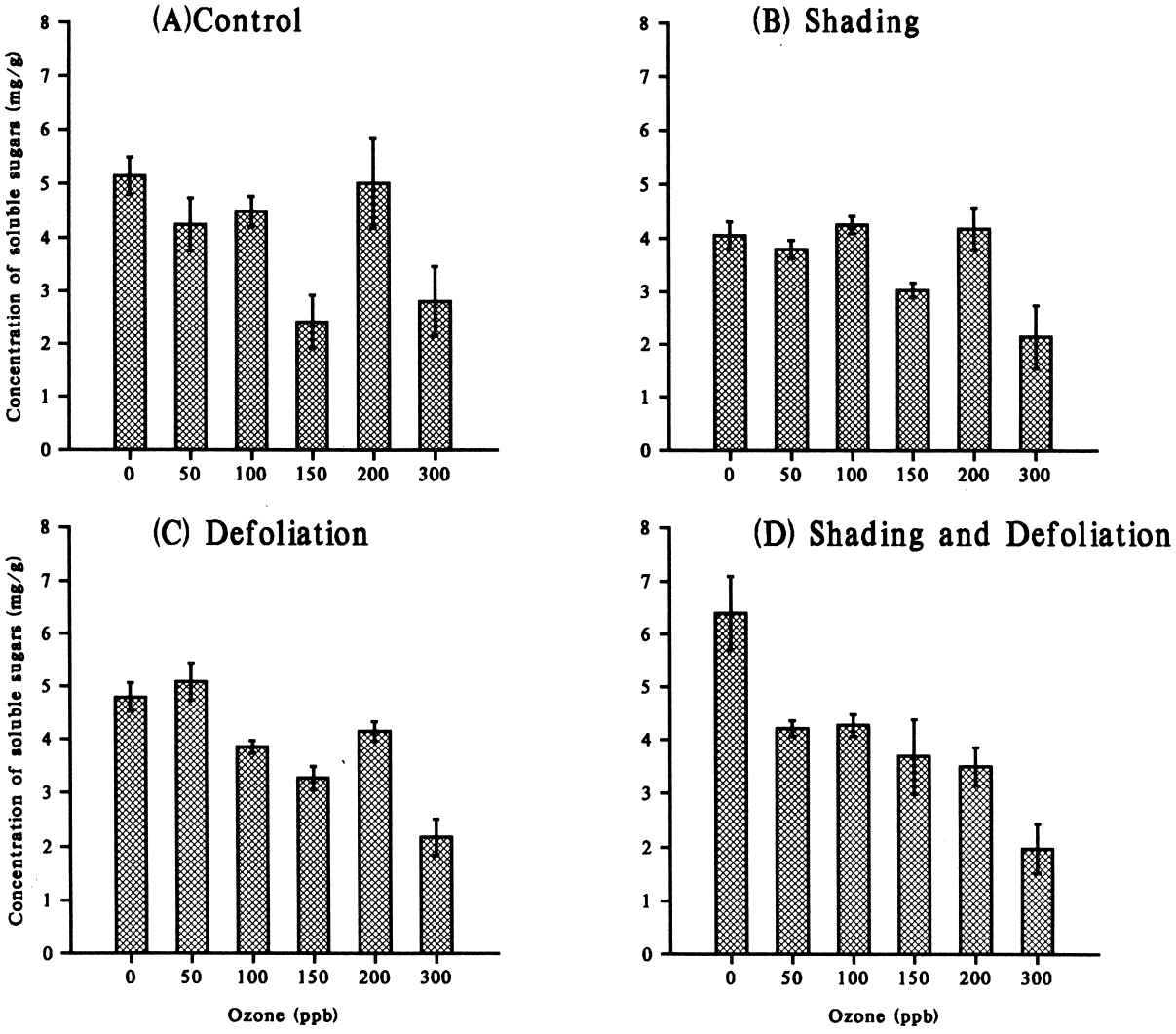

Figure 2. Sugar Concentration in Roots. (September)

Table 2. Regression analysis of sugar concentrations vs ozone levels (September)
* $p < 0.001$.

	R2	p
Control	0.382	0.000 *
+ Shading	0.383	0.655
+ Defoliation	0.387	0.367
+Shading and defoliation	.383	0.307

Table 3. Regression analysis of VA-mycorrhizal structures vs ozone levels for each month.
* $p < 0.001$, ** $p < 0.05$

Month	Structure	m	R^2	p
July		-0.077	0.146	0.000 *
August	**Arbuscules**	-0.117	0.176	0.000 *
September		-0.203	0.478	0.000 *
July		-0.0225	0.126	0.000 *
August	**Coils**	-0.019	0.068	0.002 **
September		-0.0438	0.242	0.000 *
July		-0.0025	0.033	0.0287 **
August	**Vesicles**	-0.003	0.034	0.0277 **
September		-0.0093	0.074	0.00163 **

The level of colonization by mycorrhizae increased steadily throughout the experiment and arbuscule numbers were generally high, numbers of coils were much lower and vesicle numbers were extremely low (Fig. 3). There was a significant negative relationship between the abundance of arbuscules, vesicles and coils and ozone concentrations (Figure 3a; Table 3). In general, the R^2 values increased as the season advanced, and the slope for the regressions was steeper. Thus, at the end the experiment, in September, the effect of ozone was most

obvious (Figure 3c, Table 3). The abundance of coils and vesicles, however, was much lower than the abundance of arbuscules throughout the experiment (Fig. 3.).

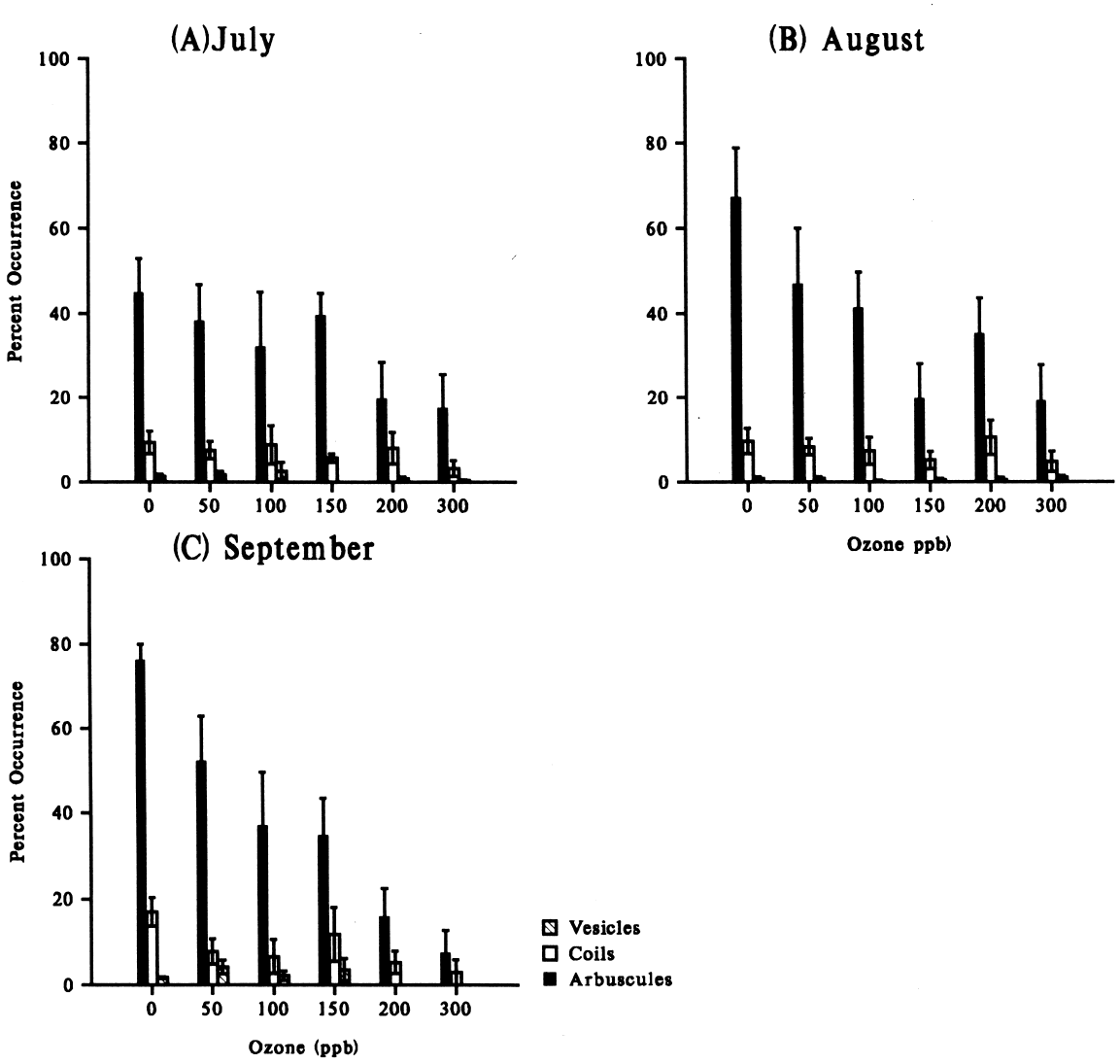

Figure 3. Percent Occurrence of Mycorrhizal Structures

The negative relationship between coils and arbuscules and ozone levels was not as strong as that between ozone and arbuscules (Table 3).

Table 4. Regression analysis of VA-mycorrhizal structures vs ozone levels for month of September. * $p < 0.001$, ** $p < 0.05$

Treatment	Structure	R2	p
Control	Arbuscules	0.478	0.000 *
Shading		0.483	0.269
Defoliation		0.492	0.066
Shading and Defoliation		0.478	0.782
Control	Hyphal coils	0.242	0.000 *
Shading		0.242	0.953
Defoliation		0.259	0.090
Shading and Defoliation		0.243	0.720
Control	Vesicles	0.074	0.002**
Shading		0.132	0.004**
Defoliation		0.085	0.208
Shading and Defoliation		0.091	0.119

Figure 4 and Table 4 show, separately, the analyses for VAM morphology among the different treatments for the month of September. No significant effects of the treatments were detected. However, certain trends seem apparent. Shading appears to cause a slight (Figure 4b) decrease in mycorrhizal colonization, whereas defoliation seems to slightly stimulate mycorrhizal colonization (Figure 4c). The combination of shading and defoliation seems to give results in between the two other treatments.

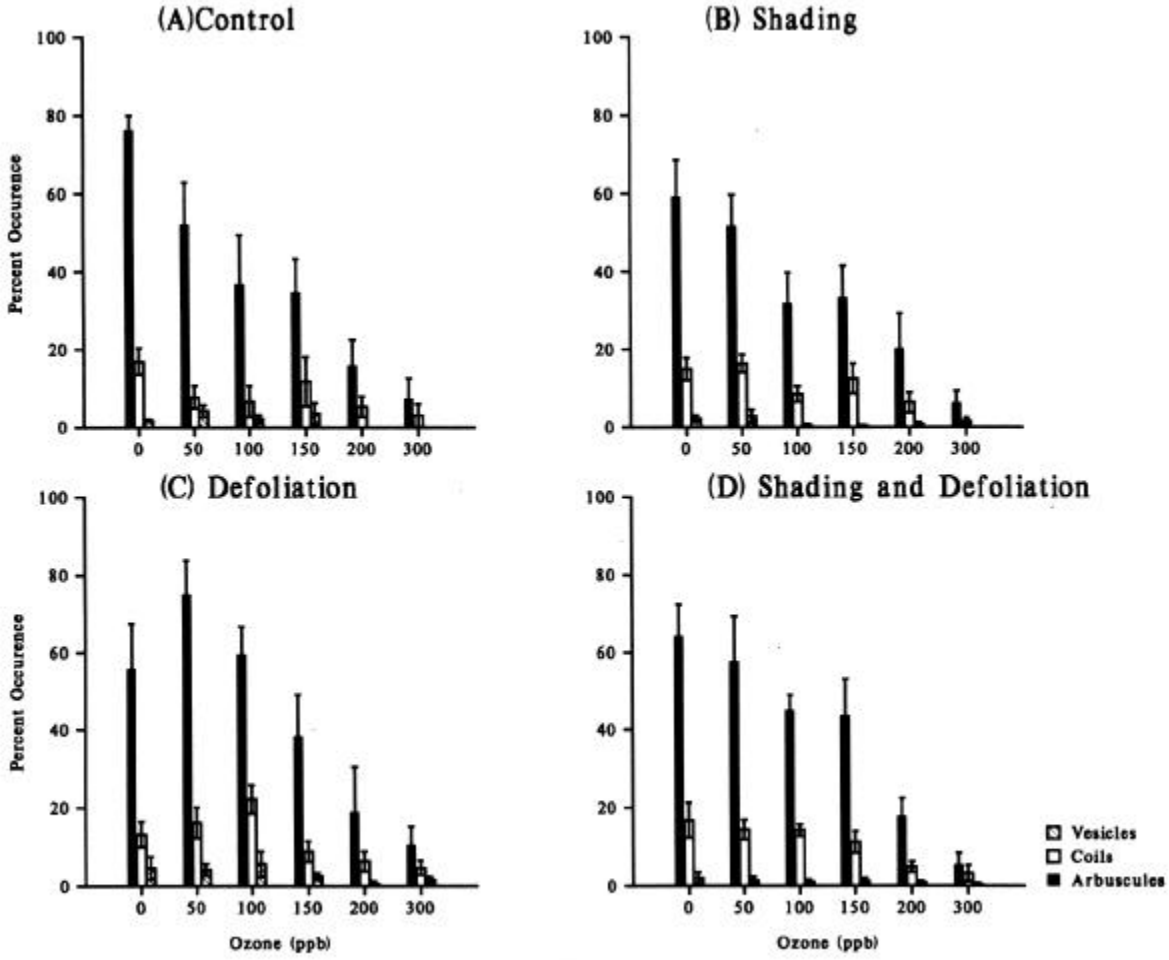

Figure 4. Percent Occurrence of Mycorrhizal Structures (September)

DISCUSSION

Ozone had a significant effect on plant growth and health. Seedlings grown at high ozone concentrations began showing signs of decline as early as the end of July. Those seedlings growing at concentrations of 200 and 300 ppb O_3 were more yellow in appearance than those growing at the lower concentrations. By the end of August some seedlings exposed to the higher concentrations of ozone died. At this stage, seedlings growing at high concentrations of ozone had stunted growth, mottled leaves and senescent leaves that, in some cases, were already abscised. Seedlings at low ozone levels 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 treatments 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 more yellow. These differences were most obvious at the end of the growing season.

Seedlings grown under shaded conditions did not seem to be affected by the treatment, however, the 50% defoliation treatment had a significant effect on plant growth. Those seedlings that underwent defoliation produced a new shoot that the intact seedlings did not produce. These leaves were, therefore, formed during ozone exposure. 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 (those that were formed prior to ozone exposure). It is possible that the first-formed leaves were senescing due to the ozone treatment and that the younger leaves were, therefore, taking over the role of source leaves. The new leaves, therefore, became an investment for the seedlings exposed to the high ozone treatments.

Biomass was also greatly affected by the ozone treatment. Both root and shoot biomass decreased with increasing ozone concentrations. Ozone affects the thylakoid membranes in the chloroplasts (Taiz and Zeiger, 1991) and therefore decreases photosynthesis. This decrease in photosynthetic ability is, therefore, translated into a decrease in plant growth, not only because of a direct reduction in sugars available for growth (Jensen 1981), but also because sugars are probably required for the production of secondary metabolites, such as the anti-oxidant, glutathione, to combat the effects of ozone (Schmeiden et al, 1993).

The only treatment, other than ozone, to have a significant effect in biomass production, was defoliation (Table 1), which, counter- intuitively, increased biomass. This increase in growth was due to the production of a second shoot that the intact seedlings did not produce.

Soluble sugar concentrations decreased significantly with increasing ozone treatment, except for the 200 ppb treatment. However, when seedlings were subjected to the other stresses, these decreases did not always appear to be linear. For example, the shaded seedlings only showed decreases in soluble sugar concentrations at ozone levels above 200 ppb, before which soluble sugar concentration were fairly similar. Interestingly, the soluble sugar concentrations are 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 result 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, thus being left with very little carbohydrate, (Jensen 1981). However, it is also possible that the relatively high soluble sugar levels at 200 ppb ozone could be the result of an unknown chamber effect.

The increasing negative relationship between arbuscular colonization and ozone over time (Figures 3 a-c) may reflect a cumulative effect of ozone on mycorrhizal development as the season progressed. This cumulative effect of ozone on the plants may have caused the plants to become less able to support the mycorrhizal symbiont as the summer progressed.

Duckmanton and Widden (1994) showed that as ozone levels increased from 0X to 3X ambient levels, there was a significant decrease in arbuscule production and a significant increase in coil and vesicle production. Other studies that have examined the effects of stress on mycorrhizal fungi in sugar maples have shown that, for various stresses, such as acid soils (Klironomos 1995), UV-B light exposure (Klironomos and Allen 1995) or base cation imbalances (Cooke et al, 1993) the trends in VAM morphology were generally similar, with arbuscule numbers decreasing and vesicle and hyphal coil numbers increasing with increasing stress. It has been suggested that the mycorrhizal fungus reacts to physiological changes in the plant by

allocating more energy to long-term survival, such as the production of vesicles, and less energy into transfer organs, such as arbuscules (Duckmanton and Widden, 1994). As the arbuscules in maple develop from hyphal coils, (Cooke et al, 1993) this implies that the fungus was not converting as many coils to arbuscules at the higher ozone levels. Duckmanton and Widden (1994) speculated that the hyphal coil may function like an arbuscule, that is, in nutrient transfer from the mycorrhizal fungus to the plant, but that it is less efficient, and less expensive to produce than the arbuscule. During times of stress, the fungus should, therefore, produce less arbuscules from the coils.

The decrease in arbuscule numbers seems to show that, as the ozone stress increases, the mycorrhizal fungi have put less energy into forming the energetically expensive arbuscules. There was also a significant decrease in coil production throughout the growing season, yet this decrease was not as dramatic as the decrease in arbuscule numbers (Figures 3a-c). In this study, the shift in mycorrhizal fungal structures production caused the arbuscule to coil ratio to gradually become closer to one. At very high ozone concentrations, (200 and 300 ppb (Figure 1c)), there seems to be equal numbers of arbuscules and coils These results seem to show that a less efficient mycorrhizal symbiosis developed as the plant was less able to support the fungus at the higher ozone levels.

Although there was a significant decrease in vesicle numbers, the numbers were very low. Had this study continued for another growing season, it is possible that the numbers of vesicles would have been higher as the mycorrhizal fungi became more established within the plant roots.

The other stresses, shading and defoliation, alone and in combination had no significant effect on mycorrhizal colonization in the seedlings (Figure 4a-d, Table 4). However, shading appears to slightly decreases the arbuscular colonization at most ozone concentrations, whereas defoliation seems to slightly increase mycorrhizal colonization rates at most ozone concentrations. The combination of shading plus defoliation seems to give values in between those of the other two other treatments (Figures 4 a-d).

These results show that, although sugar maple seedlings are extremely shade tolerant, (Klironomos and Allen 1995) they actually perform well when exposed to higher light intensities. They also maintain high levels of arbuscular colonization at high light levels. This is consistent with the fact that, when a gap forms, maple seedlings grow very rapidly into the gaps to reach canopy heights (Klironomos and Allen 1995). The defoliation treatment, however, seemed to stimulate arbuscule formation at intermediate ozone levels. It is possible that those seedlings that underwent defoliation produced new, more resistant leaves, leading to healthier plants that were better able to support the mycorrhizal fungi.

Although evidence generally shows that arbuscule numbers decrease almost linearly with increasing ozone concentrations, these results could not be totally explained by soluble sugar concentrations in the roots. Although there was a significant decrease in soluble sugar concentrations in the roots, there was no significant difference with the different treatments (Figures 2 a-d, Table 2). The decrease in soluble sugar concentrations in relation to ozone was not always linear, even though the decrease in arbuscule colonizations appeared to be. Finally, the high soluble sugar concentrations at very high ozone levels (200 ppb) did not appear to be correlated with high biomass or mycorrhizal colonization rates. Soluble sugar concentrations may, therefore, not be the only physiological cue to which the mycorrhizal fungi were responding. It is very possible that mycorrhizal fungi not only react to sugar concentrations, but also to the quality of sugars found in the roots, such as the ratios of sucrose to reducing sugars (glucose and fructose). It is also possible that, even though there were high concentrations of sugars in the plant roots, they may not have been available to the mycorrhizal fungus. Finally, it is possible that the mycorrhizal fungus is dependent on the plant, not only for sugars, but for phytohormones, proteins, vitamins, or some other factor that has not been measured. These results show that the mycorrhizal symbiosis may be more complex than originally thought, and that the fungus may be depending on the plant for more than carbohydrate nutrition.

In the study of Duckmanton and Widden (1994), the seedlings were considered to be only moderately stressed by ozone. As a result, there was no significant difference in mycorrhizal colonization rates among the different ozone concentrations. However, the mycorrhizal fungi were very sensitive to apparent physiological changes in the seedlings, and therefore, they reacted by shifting their energy to the production of more coils and vesicles at the expense of arbuscule production. In our study, colonization rates, which were determined by arbuscule numbers, decreased at every ozone concentration (even at the lower ozone levels). The mycorrhizae in this study were sensitive to even apparently small physiological changes occurring within the plant root cells. This experiment, therefore, further stresses the need to study mycorrhizal colonization rates and structures in relation to plant characteristics in order to use them as bio-indicators of plant stress.

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