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Carry-over effects of ozone on root growth and carbohydrate concentrations of ponderosa pine seedlings

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Summary Ozone exposure decreases belowground carbon allocation and root growth of plants; however, the extent to which these effects persist and the cumulative impact of ozone stress on plant growth are poorly understood. To evaluate the potential for plant compensation, we followed the progression of ozone effects, with particular emphasis on the development of new roots. Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) seedlings were exposed to ozone for 2 years. Following removal of the seedlings from ozone, root growth was assessed to characterize the carry-over effects on new root production, and carbohydrate concentrations were measured to determine if allocation strategies differed among ozone treatments.

Four months after removal from ozone, dormant seedlings had significantly lower starch concentrations in stems, coarse roots and fine roots than control seedlings. Following root flushing, starch concentrations in all seedlings decreased, with ozone-treated seedlings containing significantly less starch, sucrose, fructose, glucose and total monosaccharides than control seedlings. There was some evidence that stem starch was mobilized to compensate partially for the lower concentrations of root starch in ozone-treated seedlings; however, there was significantly less new root production in seedlings previously exposed to ozone for 2 years than in control seedlings. Early senescence of older needle age classes, perhaps resulting in inadequate available photosynthate, may be responsible for the reduction in new root production during the year following exposure to ozone. Stored carbohydrate reserves, which were depleted in seedlings previously exposed to ozone, were insufficient to compensate for the ozone-induced reduction in canopy photosynthate. We conclude that there are carry-over effects of ozone exposure on ponderosa pine seedlings, including an enhanced potential for seedling susceptibility to other stresses even in respite years when ozone concentrations are low.

Keywords: carbohydrates, carry-over effects, needle senescence, ozone stress, Pinus ponderosa, resource allocation, root production.

Introduction

Ozone effects on trees have been well documented. There is growing concern that the structure of some ozone-exposed forests is gradually changing from ozone-sensitive to ozone-tolerant species. Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) is a relatively ozone-sensitive species and studies have shown that ozone concentrations in southern California are sufficient to reduce photosynthetic capacity, cause premature senescence, and predispose trees to insect and pathogen attacks (Miller et al. 1982, Duriscoe and Stolte 1989).

In many species, root growth is reduced more than shoot growth in response to ozone (Hogsett et al. 1985*a*). Ozone alters root physiology and reduces belowground growth (Manning et al. 1971, Tingey et al. 1976, McCool and Menge 1983, McLaughlin and McConathy 1983, Cooley and Manning 1987, Gorissen and Van Veen 1988, Greitner and Winner 1989, Andersen et al. 1991). The effects of ozone on root growth appear to be the result of reductions in belowground allocation of carbohydrates (McCool and Menge 1983, Gorissen and Van Veen 1988, Spence et al. 1990, Edwards et al. 1992). A shift in resource allocation belowground resulting from ozone stress has the potential to alter the functioning of rhizosphere organisms, and may impact nutrient cycling (Andersen and Rygiewicz 1995).

The cumulative impact of ozone stress on the ability of seedlings to produce new roots is poorly understood. Previously, we found that ozone exposure during one year resulted in less new root growth in the year following exposure (Andersen et al. 1991). Therefore, we have characterized growth and carbohydrate concentrations throughout the plant to understand better how plants allocate resources in response to ozone stress, and to quantify the carry-over effects of the stress. The hypothesis that ozone exposure during one year alters patterns of growth and carbohydrate utilization during subsequent years was tested. Specifically, we determined the carry-over and cumulative effects of ozone on root and whole-plant growth, and examined the patterns of carbohydrate utilization before and after root flush to evaluate seedling ability to

mobilize stored reserves to meet the carbohydrate demands of growing roots.

Materials and methods

Growth conditions

Six months before the start of the ozone exposure treatments, which were conducted in Corvallis, OR in June 1989, 2-year-old nursery-grown bareroot ponderosa pine seedlings (seed source from 915–1070 m in Sierra Nevada Mountains, CA) were transplanted to PVC pipe pots (10 × 31 cm) containing a 1:1 mixture of Pro-mix BX (Premier Brands Inc., Stamford, CT) and perlite. Seedlings were irrigated with a balanced nutrient solution (Downs and Hellmers 1975) once each week and with tap water as needed throughout the study. Following termination of the ozone exposures, seedlings were placed in a lath house until mid-December of each year.

Ozone exposures

Seedlings exposed to one season of ozone were exposed from June 7 through September 26, 1989 (112 days) in open-top chambers modified as described by Hogsett et al. (1985*b*) and Andersen et al. (1991). For seedlings exposed to two seasons of ozone, the exposure period in the second year was from June 5 through October 3, 1990 (120 days). The profiles were computer generated and constructed to reflect air quality from the Midwest and western USA (Hogsett et al. 1985*b*), having a pattern of episodic occurrence that varied in peak concentrations. During both years, the treatments included charcoal-filtered air (control), episodic low O_3 (EP Low) and episodic high O_3 (EP High) treatments (Table 1). Total exposures were calculated by taking the hourly means, 24 h per day, and summing them over the growing season.

Seedling dormancy

Following termination of the ozone exposures in both years, seedlings were placed in a lath house until mid-December. To satisfy dormancy requirements, seedlings were transferred from the lath house to a growth chamber with conditions set at 5 °C, low light (about 200 μmol m^{-2} s^{-1}), 12-h photoperiod and an RH of about 70%. During the first year of the study, 43 seedlings from each of the three treatments were randomly selected and 10 seedlings were harvested after 48 days in the 5 °C chamber for carbohydrate analyses. During the second year of the 2-year study, 15 of the 48 seedlings from each of the three treatments were randomly selected and harvested for carbohydrate analyses after 57 days in the 5 °C chamber.

Table 1. Ozone treatments during the first and second exposure seasons. Values for ozone are ppm h and are calculated by adding hourly means, 24 h a day, over the exposure period. Abbreviations: EP Low O_3 = episodic low ozone treatment; EP High O_3 = episodic high ozone treatment; and *n* = number of open-top chamber replications.

	Control		EP Low O_3		EP High O_3	
	ppm h	*n*	ppm h	*n*	ppm h	*n*
Year 1	5	3	122	3	168	3
Year 2	15	3	131	2	183	3
Total	20		253		351	

New root growth

In both the first and second years, the remaining 33 seedlings from each treatment were moved from the 5 °C chamber to a growth chamber providing day/night temperatures of 25/20 °C, 60–70% RH, and a 16-h photoperiod. After 4 weeks, the seedlings were harvested and new root growth was quantified (Ritchie and Dunlap 1980). At harvest, carbohydrates were analyzed for 20 of the 33 seedlings used for biomass measurements in each treatment.

Harvest

Roots were carefully washed and separated into coarse and fine roots. Coarse roots were defined as primary lateral roots 3–5 mm in diameter originating from the taproot; fine roots were defined as highest order laterals < 1 mm in diameter. New roots were not present in nongrowing seedlings. Subsamples of coarse roots and fine roots were collected and stored at –70 °C until analyzed for carbohydrates. In seedlings used for the root growth test, new roots (white or light brown with uncollapsed cortex) were removed from each seedling and handled separately. For reporting biomass, each root system was separated into two classes, tap and laterals (coarse + fine), and weighed after drying to constant weight at 70 °C. Needles and stems also were harvested separately and weighed after drying.

Carbohydrate analyses

Following harvest, tissues were stored in a –70 °C freezer until lyophilization. The lyophilized tissue was ground in a Wiley mill equipped with a 40-mesh screen and stored in airtight vials at room temperature. Approximately 50-mg samples of tissue were extracted with 16 ml of methanol/chloroform/water (12/5/3 v/v) overnight with gentle shaking. The supernatant was decanted and the chloroform phase was separated with 6 ml of reverse osmosis water and discarded. The residual starch pellet was dried. The aqueous phase was dried at 40 °C in a stream of filtered air. Samples were resuspended in reverse osmosis water and analyzed for carbohydrates by HPLC coupled with a pulsed amperometric detector (HPLC-PAD). Samples were analyzed for soluble sugars as described by Wilson et al. (1995). The peaks were quantified by comparing peak height with a range of external standards (glucose, fructose, sucrose).

Starch in the insoluble residue was alkali-extracted with 16 ml of 0.5 M NaOH for 1 h. The pH of the samples was adjusted to 4.5–5.0 with 8 ml of 2.0 M acetic acid. The tissue was then incubated with 1 ml of amyloglucosidase (40 units ml^{-1}, Sigma, St. Louis, MO) at 55 °C for 2 h to ensure complete hydrolysis to glucose, which was analyzed by HPLC-PAD for glucose.

Experimental design and statistical analysis

Ozone effects on plant responses were studied by a single-factor nested experiment that randomly allocated three ozone treatment regimes to nine open-top exposure chambers, three chambers per ozone regime. Analysis of variance (ANOVA) included terms for ozone treatment, chambers within treatment, and between plants within chambers. The *F*-ratio (i.e., the ratio of the treatment means square to the chamber-within-treatment means square) was used to test for ozone effects based on a 0.10 level of significance. For the post-ANOVA investigation of decreases in plant response to increasing amounts of ozone we used polynomial models and tested for slopes significantly different from zero.

Results

Biomass

Seedlings exposed to ozone for two growing seasons were significantly smaller than control seedlings when measured in the nongrowing (dormant) condition (Table 2). Total plant biomass was reduced 58% at the highest ozone concentration. Both above- and belowground growth were reduced by ozone, with belowground growth showing the greatest proportional reductions.

Table 3 shows plant biomass fractions of seedlings 4 weeks after transfer from the 5 °C chamber to favorable growing conditions. Needle, stem, root and total plant biomass were significantly less in ozone-treated seedlings than in control seedlings. The new root growth that occurred during the 4 weeks following removal from the 5 °C chamber was also less in plants previously exposed to ozone than in control plants. Root/shoot ratios were only marginally less ($P < 0.10$) in ozone-treated plants than in control seedlings. Compared with nongrowing seedlings (Table 2), seedlings allowed to flush after removal from the 5 °C chamber showed a slight increase in stem biomass that corresponded to shoot elongation.

Figure 1 shows the percent decrease in new root biomass after one and two seasons of ozone exposure compared to new root biomass of control seedlings. New root biomass was approximately 35% of control values after one exposure season at the highest ozone concentration, but after two seasons, new root biomass was only approximately 25% of control values.

Table 2. Needle, stem, total root and total plant dry weights (g) of ponderosa pine seedlings harvested when dormant. Abbreviations: EP Low = episodic low ozone treatment; and EP High = episodic high ozone treatment.

	Control	EP Low	EP High
Needle dry weight	19.6	6.5***[1]	4.8***
Stem dry weight	17.6	13.0***	11.7***
Total root dry weight	18.9	8.5***	7.3***
Total plant dry weight	56.0	28.1***	23.7***
Root/shoot ratio	0.51	0.43**	0.46*

[1] Asterisks denote statistical significance when compared to the control treatment: * = $P < 0.10$; ** = $P < 0.05$; and *** = $P < 0.01$.

Table 3. Needle, stem, total root and total plant dry weights (g) of growing seedlings of ponderosa pine harvested 4 weeks after removal from the 5 °C chamber. Abbreviations: EP Low = episodic low ozone treatment; and EP High = episodic high ozone treatment.

	Control	EP Low	EP High
Needle dry weight	18.9	4.5***[1]	5.2***
Stem dry weight	23.9	14.8**	13.6**
Total root dry weight	18.8	7.7***	7.0***
New root dry weight	0.12	0.03**	0.02**
Total plant dry weight	61.6	27.0***	25.9***
Root/shoot ratio	0.44	0.44	0.40*

[1] Asterisks denote statistical significance when compared to the control treatment: * = $P < 0.10$; ** = $P < 0.05$; and *** = $P < 0.01$.

Carbohydrate concentrations

Starch concentrations in stems, coarse roots and fine roots of nongrowing seedlings were significantly reduced by ozone (Figure 2). Fine root starch was reduced by 72% in seedlings that had been exposed to the highest concentration of ozone when compared to control values. Four weeks after removal from the 5 °C chamber, starch concentrations were still significantly less in stems, coarse roots and fine roots of seedlings exposed to ozone during the two previous growing seasons than in control seedlings (Figure 3). During the 4-week regrowth period, starch concentrations in coarse roots and fine roots decreased, whereas concentrations in needles remained approximately the same as those in needles of nongrowing seedlings. Stem starch concentrations decreased by 65% in seedlings previously exposed to ozone, whereas stem starch

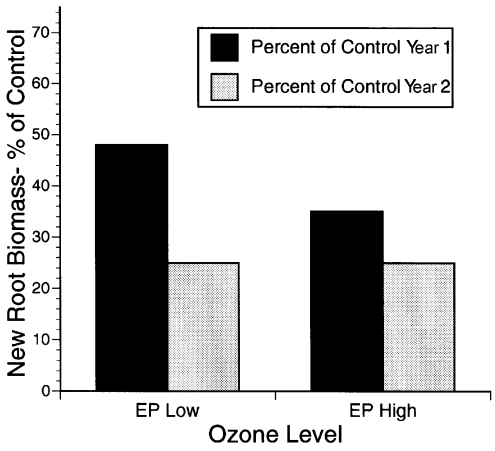

Figure 1. New root biomass after one and two growing seasons, expressed as a percent of the control value. Abbreviations: EP Low = episodic low ozone treatment; and EP High = episodic high ozone treatment.

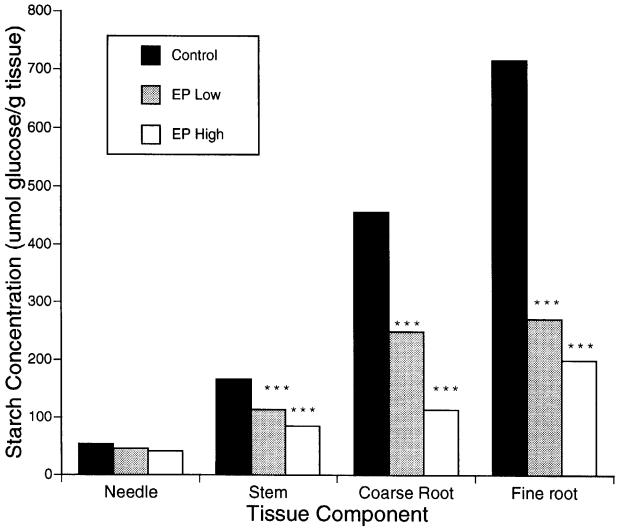

Figure 2. Starch concentration in ponderosa pine needles, stem, coarse and fine roots of seedlings harvested when dormant prior to the root growth test. Abbreviations: EP Low = episodic low ozone treatment; and EP High = episodic high ozone treatment. Asterisks denote statistical significance when compared to the control treatment (* = $P < 0.10$; ** = $P < 0.05$; and *** = $P < 0.01$).

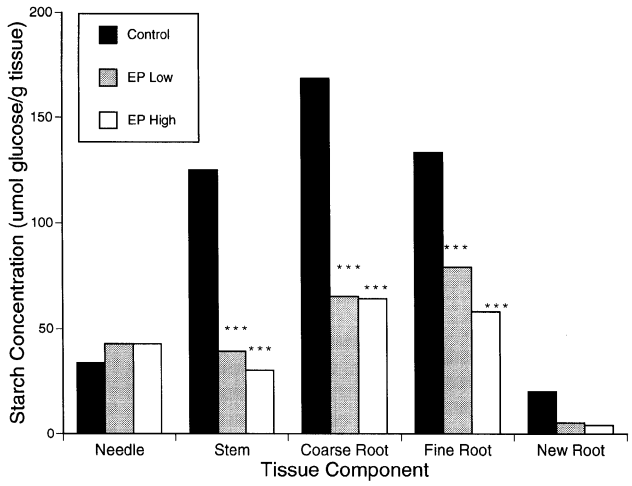

Figure 3. Starch concentration in ponderosa pine needles, stem, coarse and fine roots, and new roots of growing seedlings harvested following the root growth test. Abbreviations: EP Low = episodic low ozone treatment; and EP High = episodic high ozone treatment. Asterisks denote statistical significance when compared to the control treatment (* = $P < 0.10$; ** = $P < 0.05$; and *** = $P < 0.01$).

concentrations of control seedlings were reduced by approximately 25% during the regrowth period.

Glucose concentrations varied little by tissue type and growth status in nongrowing and growing seedlings (Table 4). In nongrowing seedlings, glucose concentrations were significantly higher in stems of seedlings exposed to ozone during the previous two growing seasons than in stems of control seedlings. Glucose concentrations in coarse roots were significantly reduced by ozone, although overall concentrations were relatively low. In growing seedlings, ozone significantly reduced glucose concentrations in coarse, fine and new roots. New roots of seedlings previously exposed to the highest ozone regime had glucose concentrations 21% of those of control seedlings.

Fructose concentrations were higher in needles than in other tissue types (Table 4). Stem fructose concentrations were significantly greater in nongrowing seedlings exposed to ozone during the previous two growing seasons than in control seedlings. Otherwise, fructose concentrations were similar among treatments in each of the tissue types. Four weeks after removal from the 5 °C chamber, fructose concentrations were significantly lower in all root fractions of seedlings exposed to ozone when compared to control seedlings. Fructose concentrations of fine roots and new roots were reduced by 78 and 81%, respectively, in seedlings in the highest ozone regime compared to control seedlings. Needle fructose concentrations decreased during the 4-week growth period following removal from the 5 °C chamber, whereas fructose concentrations in other tissues did not change substantially over the same time period.

Sucrose concentrations varied widely in nongrowing seedlings in all ozone treatments, and only marginally significant ozone effects were observed in seedlings in the highest ozone regime (Table 4). Four weeks after removal from the 5 °C chamber, sucrose concentrations were significantly lower in stems, and coarse and fine roots of ozone-treated seedlings compared to controls. Sucrose concentration was lower in new roots of ozone-treated seedlings than in new roots of control seedlings, however variability was high and results were only marginally significant in the highest ozone treatment ($P < 0.10$).

Monosaccharides in roots of growing seedlings were significantly reduced by prior exposure to ozone (Table 4). In nongrowing seedlings, stem concentrations were significantly higher in ozone-treated plants than in control plants; however, after 4 weeks of regrowth, stem concentrations were similar among treatments. The greatest ozone-induced reduction in monosaccharides was observed in new roots of growing seedlings, where concentrations were reduced by 80% compared to control seedlings.

Discussion

The results after one and two seasons of ozone exposure suggest that previous year exposure alters patterns of growth and carbohydrate utilization during subsequent years, and the greatest apparent cumulative impact was on the roots. The reduction in new root growth was not simply the result of less total root biomass in ozone-treated seedlings because new root biomass per g total root was reduced by more than 50% in seedlings exposed to the highest concentration of ozone compared to control seedlings (6.4 versus 2.9 mg g^{-1}). The carryover effects of ozone on the ability of ponderosa pine seedlings to produce new roots may increase seedling susceptibility to nutrient and water stress during establishment.

Table 4. Concentrations of glucose, fructose, sucrose, and monosaccharides (μmol g_{DW}^{-1}) in needles, stem, coarse roots, and fine roots of ponderosa pine seedlings harvested when dormant (Dormant) and 4 weeks after removal from the 5 °C chamber (Growing). Abbreviations: EP Low= episodic low ozone; and EP High = episodic high ozone.

	Glucose			Fructose			Sucrose			Monosaccharide		
	Control	EP Low	EP High	Control	EP Low	EP High	Control	EP Low	EP High	Control	EP Low	EP High
Needles												
Dormant	142.1	142.3	123.8	140.9	161.4	132.9	9.5	55.8	17.6	283.0	303.8	256.7
Growing	111.3	83.7	72.1*	91.9	73.7	62.6	24.7	39.6	31.5	207.6	157.3	133.7*
Stem												
Dormant	54.1	68.0***[1]	73.6***	40.4	63.4***	68.7***	110.9	95.9	78.6*	92.6	116.6**	144.3***
Growing	32.7	30.3	38.7	30.7	25.2	31.9	94.9	48.5***	33.3***	63.4	55.5	70.6
Coarse roots												
Dormant	34.7	27.1*	22.1***	32.3	31.6	26.1	70.3	61.6	53.9	67.0	58.7	48.2**
Growing	38.0	23.6**	19.3***	43.5	25.6***	18.5***	70.2	35.9***	21.8***	81.3	41.3***	34.3***
Fine roots												
Dormant	23.7	19.6	22.4	19.8	22.4	24.1	67.9	55.0	46.3*	43.4	42.2	43.0
Growing	26.3	13.4***	8.8***	29.2	9.5***	6.4***	41.9	18.9***	14.4***	54.1	22.8***	16.3***
New roots												
Growing	54.3	18.7**	11.2**	61.9	19.7**	11.8**	86.0	25.7	16.3*	115.6	38.4**	23.0**

[1] Asterisks denote statistical significance when compared to the control treatment: * = $P < 0.10$; ** = $P < 0.05$; and *** = $P < 0.01$.

Reductions in total plant growth resulting from ozone exposure were cumulative but were not fully additive (Figure 1). In nongrowing seedlings, total seedling biomass in the high ozone treatment was 55.6 and 42.3% of control seedling biomass after 1 and 2 years of exposure, respectively. To determine whether the reductions in growth of ozone-treated plants during the second growing season were simply the result of the smaller size of ozone-treated plants as a result of ozone-induced growth reductions in the first year, relative growth rates were calculated to correct for size differences at the beginning of the second growing season caused by previous exposure to ozone ((lnDW2 – lnDW1)/(T2 – T1)). The relative growth rate of seedlings during the second exposure season was 0.65 g g^{-1} $season^{-1}$ for plants in the highest ozone regime compared to 0.92 g g^{-1} $season^{-1}$ for controls, suggesting that the reductions in growth were not simply the result of the smaller size of ozone-treated seedlings at the start of the second growing season. Therefore, although some physiological or growth compensation may have occurred in ozone-exposed seedlings during the second year, there was still a substantial impact of ozone on growth during the second year.

Ozone-treated seedlings had less needle biomass than control seedlings (Table 3). Reductions in needle biomass resulted primarily from premature senescence of older foliage rather than from reduced growth of the new foliage. Premature senescence resulting from ozone exposure has been reported both in field and laboratory studies (Miller et al. 1982, Duriscoe and Stolte 1989, Plocher et al. 1994, Karnosky et al. 1996). Although photosynthetic compensation of newer foliage has been observed in some species exhibiting premature senescence of older foliage, whole-plant carbon gain is generally reduced in response to ozone (Greitner et al. 1994).

The source of carbohydrates for new root growth in ponderosa pine is unknown; however, in many conifers and some hardwoods it appears that current photosynthate is essential for new root production (van den Driessche 1978, Marshall 1985, van den Driessche 1991, Horwath et al. 1994). Van den Driessche (1991) found that girdling, low CO_2 and dark treatments all resulted in significant reductions in new root growth in Douglas-fir. New root growth did occur in the dark, but new root production was less than 20% of controls. Therefore, in the absence of current photosynthate, Douglas-fir can utilize starch from other plant sources to grow new roots, but stored reserves are insufficient to compensate for the loss in current photosynthate. Sitka spruce (*Picea sitchensis* (Bong.) Carr.) produced new roots even in bark-ringed seedlings, although the number of new roots was reduced by bark-ringing (Philipson 1988). In Sitka spruce, root carbohydrate concentrations were depleted during the root growth test and apparently were used for new root production. In our study, ozone exposure caused premature senescence of older needles of ponderosa pine by the end of the second exposure season, which would reduce photosynthate availability to roots at the beginning of the following year. Karnosky et al. (1996) suggested that senescence of older leaves of *Populus* would have a significant impact on root growth because of their role in transporting photosynthate belowground. We conclude that the combination of reduced availability of photosynthate, coupled with decreased stored reserves in stems, coarse roots and fine roots, contributed to the reductions in new root growth observed during the year following exposure to ozone.

New roots of seedlings developed following ozone exposure had less carbohydrate available, and therefore may have had reduced longevity (Figure 3, Table 4). This was observed after both one and two exposure seasons and suggests a possible

mechanism of reduced plant growth over time (Andersen et al. 1991). Reduced root growth combined with reduced carbohydrate availability in new roots may accelerate root turnover (Marshall and Waring 1985), which could predispose the seedlings to nutrient and water stress. Marshall and Waring (1985) suggested that fine roots receive starch during their development, and if secondary growth does not occur, fine root death occurs when their starch pool is depleted.

Starch concentrations in stems, coarse roots and fine roots were depleted during the 4-week root growth test, suggesting that these stored reserves were utilized during new root production (Figures 2 and 3). In addition, fine root glucose, sucrose, fructose and total monosaccharide concentrations in ozone-treated seedlings were significantly reduced following the 4-week root growth test. For example, stem concentrations of starch were depleted approximately 65% in seedlings in the two ozone treatments, whereas control seedlings showed a 25% reduction in stem starch. The greater loss of stem starch in ozone-treated plants than in control plants may indicate partial compensation for the lower availability of coarse root starch in ozone-treated plants at the start of the root-growth test (Figure 3).

We conclude that the effect of ozone on growth of ponderosa pine is not limited to the year of exposure. Reductions in root growth and carbohydrate concentrations in ozone-exposed trees probably result in less root growth in subsequent years—even when ozone concentrations are low—and may account for the cumulative nature of the ozone response. Root impairment is likely to affect plant response to other stresses; however, because there is often a temporal separation in the occurrence of ozone and other stresses, the response to ozone may go unnoticed. McLaughlin and Downing (1995) reported that ambient ozone reduced radial growth in loblolly pine in the field, and they proposed a possible mechanism linked to belowground effects, including water-stress interactions. Even small ozone-induced reductions in growth, particularly in belowground components, could affect multiple-stress interactions and result in substantial growth reductions over time.

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