Forest understory clover populations in enriched CO_2 and O_3 atmospheres: Interspecific, intraspecific, and indirect effects

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Abstract

The effects of CO_2 and tropospheric O_3 on forest trees are increasingly the subject of experimental evaluation. Little is known, however, about the effects of these gases on understory plant taxa. At the Aspen free-air CO_2 and O_3 enrichment (Aspen FACE) site we assessed colonization and establishment of two common forest understory species, red (*Trifolium pratense*) and white (*Trifolium repens*) clover. To better understand these natural patterns in red clover, the more responsive of the two clover species, we also assessed intraspecific variation in growth performance to altered atmospheric conditions. Natural red clover populations were larger in enriched CO_2 atmospheres, whereas white clover populations showed no response to CO_2. Neither species showed beneficial or detrimental responses to enriched O_3 atmospheres. Nine red clover genotypes exhibited similar, but counterintuitive, decreases in shoot and root biomass, and increases in foliar nitrogen concentrations and root nodule numbers, under elevated CO_2. We suggest that in enriched CO_2 habitats, increased shading from rapidly growing trees offsets the positive benefits of CO_2 for understory plant performance. Enriched CO_2 and O_3 atmospheres may have large direct and indirect effects on colonization, establishment, and performance of common understory plants. Such changes may in turn alter forest community and ecosystem dynamics.
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1. Introduction

Rising concentrations of CO_2 and tropospheric O_3, as a result of increasing industrialized activity (IPCC, 2001), alter plant performance in both natural and managed ecosystems. Enriched CO_2 and O_3 atmospheres typically have opposing effects on plant productivity, with CO_2 resulting in increased plant growth and O_3 causing decreased growth (Makino and Mae, 1999; Krupa et al., 2001; Karnosky et al., 2003). In combination, however, elevated CO_2 may partially ameliorate the negative effects of elevated O_3 on plants (Heagle et al., 1998; Dickson et al., 1998; Donnelly et al., 2000; Percy et al., 2002; Karnosky et al., 2003).

Predicting plant population- and resulting community-level responses to such global atmospheric changes is problematical (Poorter and Navas, 2003). First, there may be interspecific changes in community structure, as increasing levels of CO_2 and/or O_3 may alter species growth patterns to different degrees, leading to decreased performance of certain taxa (Hättenschwiler and Körner, 2000; Belote et al., 2004; Karnosky et al., 2005). Second, intraspecific variation in response to elevated CO_2 and O_3 atmospheres is common in plants (Pell et al., 1992; Foy et al., 1995; Dickson et al., 1998; Reichenauer et al., 1998; Van der Kooij et al., 2000; Lindroth et al., 2001; Castells et al., 2002; Goverde et al., 2002). As it is the relative fitness of plant genotypes in enriched CO_2, O_3, and $+CO_2 + O_3$ atmospheres that determines the outcome of intraspecific competition (Curtis et al., 1994, 1996; Bazzaz et al., 1995; Davison and Reiling, 1995; Andalo et al., 2001; McDonald et al., 2002), population and community differences will reflect selection for genotypes that are able to adapt to those changes. Thus, consequent changes in competitive ability both among and within species can significantly alter community composition.

The majority of research on plant responses to enriched CO_2 and/or O_3 atmospheres has focused on dominant agricultural (Mauney et al., 1992; Kimball et al., 1999, 2002) and forest (DeLucia et al., 1999; Karnosky et al., 2003, 2005) species. In comparison, relatively little work has been conducted on understory plant populations. Understory plant colonization (the

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initial occupation of soil by seedlings; Lincoln et al., 1998) and establishment (successful growth and reproduction; Lincoln et al., 1998) may have large impacts on community dynamics. For example, trophic interactions may be altered, as both detrimental and beneficial insects frequent forest understory plants for food and harborage (Hamilton et al., 2004). Understanding how changing atmospheric conditions alter forest understory plant populations, however, presents a unique challenge. As overstory taxa exhibit differential growth rates under enriched CO_2 (DeLucia et al., 1999) and/or O_3 environments (Karnosky et al., 2003, 2005), tree canopy development may alter light levels which, along with atmospheric conditions, may concomitantly influence understory plant growth.

Here, we describe two experiments from the Aspen free-air CO_2 and O_3 enrichment (Aspen FACE) site, in which we sought to better understand patterns of forest understory colonization and establishment. First, we conducted a multiple-year survey of the natural abundance of red (*Trifolium pratense*) and white (*Trifolium repens*) clover colonizing FACE rings, to determine whether two closely related understory species show similar population-level responses to enriched CO_2 and/or O_3 atmospheres. Second, as red clover was found to be more responsive than white clover to altered atmospheric conditions, we assessed intraspecific variation in performance of nine red clover cultivars to enriched CO_2 and/or O_3 atmospheres, to better understand natural patterns of colonization and establishment.

2. Materials and methods

2.1. The Aspen FACE site

The Aspen FACE site, located near Rhinelander, WI, USA (45.7°N, 89.7°W), consists of aggrading forest stands exposed to realistic future concentrations of CO_2 and/or O_3 (Dickson et al., 2000). Each FACE ring is divided into three sections: mixed trembling aspen (*Populus tremuloides*) genotypes, aspen and paper birch (*Betula papyrifera*), and aspen and sugar maple (*Acer saccharum*) (Dickson et al., 2000). Site preparation for each ring included disking of the soil in summer 1996. Seedling trees were planted in 1997 and fumigation commenced in 1998. Roundup® herbicide was applied to the soil surface twice per year in 1998 and 1999 to control emergent vegetation while trees became established. Beginning in 2000, a diverse ground flora, derived from the soil seed bank and from natural seed dispersal, developed in each ring.

The Aspen FACE site consists of 12, 30 m diameter rings arranged in a 2×2 factorial randomized block design (Dickson et al., 2000). Four treatments comprise each block: (1) control, (2) elevated CO_2 (+CO_2), (3) elevated O_3 (+O_3), and (4) elevated CO_2 and O_3 (+CO_2 + O_3). Elevated CO_2 levels represent concentrations predicted for the year 2060 (Dickson et al., 2000). O_3 levels are elevated in a diurnal profile ca. 1.5-fold that of background concentrations, replicating levels of a moderately polluted city in the Great Lakes region of North America (Dickson et al., 2000). For each ring, a computer-controlled trace gas monitoring system adjusts the concentrations of both CO_2 and O_3 (Dickson et al., 2000).

Trace gas levels for May–August 2000 and 2002 were: (1) control (350 and 360 $\mu l l^{-1}$ CO_2; 36 and 33 $nl l^{-1}$ O_3), (2) elevated CO_2 (545 and 542 $\mu l l^{-1}$ CO_2; 36 and 33 $nl l^{-1}$ O_3), (3) elevated O_3 (350 and 360 $\mu l l^{-1}$ CO_2; 49 and 49 $nl l^{-1}$ O_3), and (4) elevated CO_2 and O_3 (545 and 542 $\mu l l^{-1}$; 49 and 49 $nl l^{-1}$), respectively. Average temperature and relative humidity for the same time periods were: (1) control (23.5 and 24.6 °C; 84 and 85% rh), (2) elevated CO_2 (23.9 and 23.2 °C; 82 and 83% rh), (3) elevated O_3 (23.3 and 24.2 °C; 84 and 84% rh), and (4) elevated CO_2 and O_3 (23.2 and 24.0 °C; 84 and 84% rh). Average precipitation levels for May–August 2000 and 2002 were 124 and 98 mm month^{-1}, respectively.

Due to the differential growth responses of overstory tree species to the trace gasses, light levels reaching the forest understory differed in response to gas treatments. Average maximum photosynthetic active radiation (PAR) levels, measured 2 m from the forest floor, were for 2000 and 2002: (1) control (1.97 and 1.83), (2) elevated CO_2 (1.42 and 1.04), (3) elevated O_3 (1.83 and 1.78), and (4) elevated CO_2 and O_3 (2.12 and 1.84), in mmol m^{-2} s^{-1}.

2.2. Effects of elevated CO_2 and/or O_3 on natural clover colonization and establishment

To determine whether atmospheric composition alters natural colonization and establishment patterns of forest understory plants, numbers of red and white clover plants were surveyed in the aspen–maple section of each FACE ring in 2000 and 2002. Red and white clovers are appropriate representative species, as they are some of the most abundant dicotyledenous plants in the Aspen FACE understory (C.S. Awmack and R.L. Lindroth, unpublished data). In 2000, we recorded the number of red and white clover plants growing in each of four randomly placed 1 m × 1 m quadrats in the aspen–maple quadrant of each ring. In 2002, clover and other understory plant densities were much higher (C.S. Awmack, unpublished data) and we used only three randomly placed quadrats per ring.

2.3. Effects of elevated CO_2 and/or O_3 on red clover intraspecific performance

To better understand the natural colonization and establishment patterns of red clover in the FACE rings, in 2002 we investigated the performance of nine red clover cultivars commonly grown in the northern plains of the United States (Marathon, Arlington, Juliet, BY 222, HC 82, C 328, Reddy, Cinnamon, and Red Star) to enriched CO_2 and/or O_3 atmospheres. Red clover plants were initially grown from seed in standard potting medium (Scotts Metromix) in a greenhouse at ambient CO_2 and O_3 with daily watering. After 23 days, seedlings were individually transplanted into 1 l pots containing local topsoil (Mussons Brothers, Rhinelander, WI, USA). Plants were maintained in the greenhouse for an additional 20 days, until they were ca. 10 cm tall, whereupon they were distributed to the aspen–maple section of each FACE ring. Upon placement in the FACE rings, plants were only exposed to natural precipitation. Four plants of each cultivar were randomly allocated to each ring ($n = 36$ total
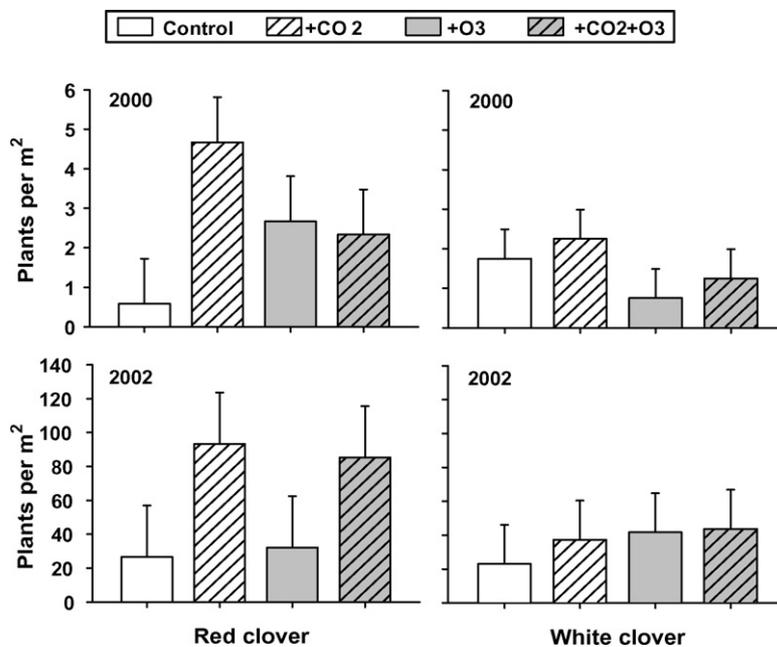
Fig. 1. Effects of CO_2 and/or O_3 on naturally occurring red and white clover plant populations, in the aspen–maple section of FACE rings. Data are means (+1S.E.) of three replicate FACE rings (year 2000, four samples per plot; year 2002, three samples per plot; *n* = 12 and 9, respectively). Note the difference in the scale of the *y*-axis between 2000 and 2002.

plants per ring). Plants were individually covered with a fine mesh sleeve, to prevent insect colonization. Three weeks later, after plants had grown substantially since initial out-planting, above- and below-ground plant biomass was harvested, root tissues were washed, and all plant material was freeze-dried. Shoot and root dry weights were assessed, the numbers of *Rhizobium* spp. containing root nodules on each plant were counted, and nitrogen concentrations of above-ground plant tissues were measured using a LECO FP528 nitrogen analyzer.

2.4. Statistical analyses

To determine whether atmospheric composition influenced the natural patterns of red and white clover colonization and establishment, data were analyzed using randomized block, two-way ANOVAs. As the number of quadrats differed between years, a separate analysis was conducted for each clover species in each year (SAS; Littell et al., 1996). As the Aspen FACE experiment is set up as a 2×2 factorial, main effects were: CO_2 (ambient versus elevated) and O_3 (ambient versus elevated). FACE ring block (1–3) was incorporated into the analysis as a random effect. The dependent variable, number of clover plants m^{-2}, was transformed ($x' = \log_{10}[x]$) to normalize the variance prior to analysis (Zar, 1984).

Intraspecific differences in red clover performance were evaluated with split-plot ANOVAs, using Proc Mixed (SAS; Littell et al., 1996). Whole-plot fixed effects were CO_2 (ambient versus elevated) and O_3 (ambient versus elevated), while ring block (1–3) was incorporated as a random effect. Sub-plot effects consisted of clover cultivar (1–9) and all second and third order interactions between CO_2, O_3, and clover cultivar. Dependent variables were shoot and root dry weights, numbers of root nod-

ules, and shoot nitrogen concentrations. Nitrogen concentrations were transformed ($x' = \arcsin \sqrt{x}$) to achieve normality prior to analysis (Zar, 1984).

3. Results

3.1. Effects of elevated CO_2 and/or O_3 on natural clover colonization and establishment

Red and white clover populations exhibited differential responses to elevated CO_2 (Fig. 1 and Table 1). In 2000, red clover populations were larger in enriched CO_2 atmospheres, whereas white clover populations showed no response. By 2002, the abundance of red and white clover plants in all the treatments had substantially increased, as had the magnitude of the positive

Table 1

Analyses of variance showing the effects of CO_2 and/or O_3 on the natural abundance of red and white clover in FACE rings

	Red clover		White clover	
	$F_{1,6}$[*]	P	$F_{1,6}$	P
Year 2000				
CO_2	6.63	**0.05**	0.37	0.57
O_3	0.37	0.57	0.30	0.56
$CO_2 \times O_3$	2.39	0.18	0.48	0.52
Year 2002				
CO_2	8.37	**0.03**	1.21	0.31
O_3	0.21	0.66	0.04	0.86
$CO_2 \times O_3$	2.12	0.20	0.21	0.67

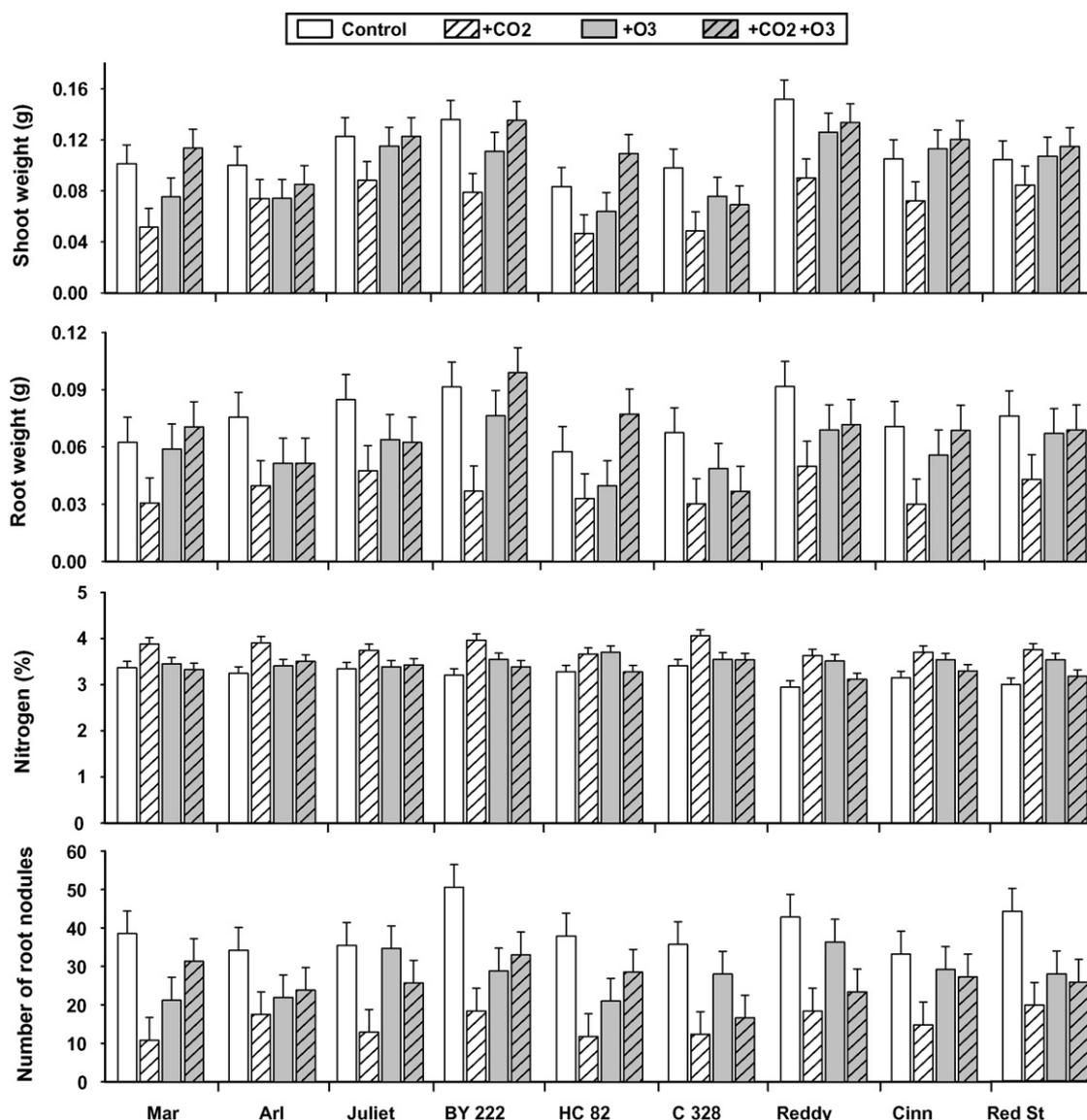[*] Subscripts indicate degrees of freedom for each analysis.

Fig. 2. Effects of CO_2 and/or O_3 on shoot biomass (dry weight), root biomass (dry weight), nitrogen (percent dry weight), and number of root nodules of nine red clover cultivars grown in the aspen–maple section of FACE rings. Data are means (+1S.E.) of three replicate FACE rings (four plants of each of nine cultivars per ring; $n = 432$ total). For clover varieties: Mar, Marathon; Arl, Arlington; Cinn, Cinnamon; Red St, Red Star.

effects of elevated CO_2 on red clover abundance. Enriched O_3 atmospheres had no effect on red or white clover abundance in either year, and the lack of any significant interaction between CO_2 and O_3 suggests that elevated CO_2 did not modify plant responses to elevated O_3. These results demonstrate that changing atmospheric conditions have the potential to alter natural colonization and establishment patterns of common understory plant populations.

Table 2
Analyses of variance showing the effects of CO_2, O_3, and/or plant cultivar on red clover plant performance in FACE rings

Treatment	Shoot dry weight		Root dry weight		Shoot nitrogen		Root nodules	
	F	P	F	P	F	P	F	P
$CO_2{}_{(1,6)}$[*]	2.62	0.16	2.51	0.16	5.76	**0.05**	14.43	**<0.01**
$O_3{}_{(1,6)}$	2.61	0.16	0.52	0.50	0.99	0.36	0.01	0.94
$CO_2 \times O_3{}_{(1,6)}$	13.24	**0.01**	6.28	**0.05**	19.70	**<0.01**	11.19	**0.02**
Cultivar $_{(8,376)}$	8.60	**<0.001**	4.40	**<0.001**	3.08	**<0.01**	1.81	0.07
$CO_2 \times$ cultivar $_{(8,376)}$	0.66	0.75	0.94	0.48	1.09	0.37	0.75	0.65
$O_3 \times$ cultivar $_{(8,376)}$	0.74	0.65	1.44	0.18	0.71	0.68	0.61	0.77
$CO_2 \times O_3 \times$ cultivar $_{(8,376)}$	0.83	0.57	0.78	0.62	1.18	0.31	1.32	0.23

[*] Subscripts indicate degrees of freedom for each analysis.

3.2. Effects of elevated CO_2 and/or O_3 on red clover intraspecific performance

Potted red clover plants grew ca. three-fold while in the FACE rings, sufficient to detect any but the most subtle effects of CO_2 or O_3 on plant performance. Red clover cultivars exhibited few significant responses to the main effects of CO_2 and O_3, but responded strongly to the interaction of the two fumigation treatments (Fig. 2 and Table 2). Elevated CO_2 atmospheres markedly reduced both shoot and root weights under ambient O_3, but increased weights when O_3 was also elevated (Fig. 2). Cultivars exhibited similar responses to CO_2 and O_3 in terms of plant quality (shoot nitrogen content) and root nodule formation. Enriched CO_2 atmospheres increased nitrogen concentrations and reduced root nodulation under ambient O_3, but had opposite or no effects under $+CO_2 + O_3$. While the nine cultivars exhibited a large degree of intraspecific variation in the magnitude of shoot growth responses, root growth responses, and nitrogen concentrations, they all responded similarly to altered atmospheric conditions (i.e., no significant $CO_2 \times$ cultivar, $O_3 \times$ cultivar, or $CO_2 \times O_3 \times$ cultivar interactions). Thus, enriched CO_2 and O_3 atmospheres had modest to large effects on red clover growth patterns and plant quality, although little intraspecific variation was observed.

4. Discussion

Altered atmospheric conditions can influence community composition, such as the relative abundances of invasive C_3 and C_4 understory plants (Belote et al., 2004). Here, we have shown that atmospheric conditions can differentially influence the colonization and establishment of even closely related, naturalized understory plant taxa. In our assessment of red and white clover populations at Aspen FACE, interspecific variation in response to atmospheric composition was readily apparent. Natural populations of red clover were much more abundant in enriched CO_2 atmospheres and became even more plentiful over time. A sympatric species, white clover, however, did not exhibit changes in colonization and establishment in response to altered atmospheric conditions. Thus, elevated CO_2 atmospheres have the potential to alter the composition of forest understory plant communities.

Colonization and establishment patterns of red and white clover likely express differences in competitive ability between the two species. Greater red clover population sizes in $+CO_2$ rings at Aspen FACE may reflect changes in vegetative (clonal) growth patterns or increased survivorship. As red clover flower production generally decreases in elevated CO_2 atmospheres (Rusterholz and Erhardt, 1998), the increased abundance in enriched CO_2 conditions is likely not due to increased seed production. White clover, on the other hand, may not exhibit population increases in response to elevated CO_2 atmospheres because of nutrient constraints (Kemp and Blair, 1991; Niklaus et al., 1998). Nutrient limitation may render white clover unable to assimilate increased levels of CO_2 for increased vegetative growth. White clover seed production and tiller number are also generally unaffected by elevated CO_2 (Clark et al., 1997; Wagner et al., 2001), correlating well with our results.

Augmented levels of tropospheric O_3 did not affect the abundance of naturally colonizing red or white clover plants. This result is surprising, as both species may be adversely affected by elevated O_3 (Leuthy-Krause et al., 1989; Heagle et al., 1991). Heagle et al. (1991) showed that exposure of natural clover populations to elevated O_3 atmospheres resulted in a rapid (within 2 years) increase in the proportion of O_3-tolerant genotypes. Thus, elevated O_3 atmospheres at the Aspen FACE site may have already eliminated O_3-sensitive genotypes by the time this study was conducted. Analogous changes in the relative fitness of O_3-sensitive and O_3-tolerant aspen genotypes were observed within the first several years at Aspen FACE (McDonald et al., 2002).

With respect to intraspecific variation, red clover cultivars expressed similar, but counterintuitive, growth responses to CO_2 and O_3. Red clover, like other plants, generally increases in biomass under elevated CO_2 conditions (Heagle et al., 1993; Saebo and Mortensen, 1995) although not invariably so (Leadley et al., 1999). Surprisingly, red clover grown at Aspen FACE in the $+CO_2$ treatment had lower root and shoot biomasses than plants grown under ambient conditions. This is not a unique response, however, as other plants growing in the understory of Aspen FACE show similar reductions in biomass under elevated CO_2 (E.B. Mondor, unpublished data). Elevated CO_2 atmospheres, at ambient ozone, were also associated with an increase in tissue nitrogen concentrations and a decrease in root nodule numbers. The tissue nitrogen response was also contrary to expectation, as enriched CO_2 atmospheres reduce shoot nitrogen concentrations in most plant species (Curtis, 1996; Bezemer and Jones, 1998). Plant growth trajectories under enriched O_3 atmospheres were similarly enigmatic. In general, increased O_3 levels adversely affect red clover performance (Leuthy-Krause et al., 1989; Mortensen, 1992). Conversely, we found little direct effect of O_3 on red clover growth.

Why, then, did our experimental red clover populations exhibit decreased shoot and root biomasses, increased nitrogen concentrations, and decreased *Rhizobium* root nodules in response to enriched CO_2 atmospheres? As even small reductions in light can substantially reduce, or even reverse, the benefits of increased CO_2 obtained by some understory tree seedlings (Hättenschwiler and Körner, 2000), we suggest that the atypical growth responses of our experimental clover populations also resulted from altered light levels. Maple, and especially aspen, trees at Aspen FACE have exhibited remarkable growth increases since project initiation in 1998 (Isebrands et al., 2001; Percy et al., 2002; Karnosky et al., 2003). These growth responses have been substantially influenced by atmospheric composition, as trees in the $+CO_2$ treatments are taller, have a larger volume index (D^2H), and leaf area index (LAI) relative to both ambient, and $+O_3$ conditions (Isebrands et al., 2001; Percy et al., 2002; Karnosky et al., 2003, 2005). As a result of increased LAI under $+CO_2$ conditions, levels of photosynthetic active radiation reaching the understory are much less than those of the control, $+O_3$, or $+CO_2 + O_3$ treatments.

Thus, rapid growth of overstory tree species has created different selective pressures on understory plant populations, over time. For instance, natural clover populations were first assessed in 2000 when aspen and maple tree canopies were more open, allowing higher light levels to reach the forest understory. Two years later, during the second census and also when our experimental (potted) clover was grown, trees were much larger and canopies were more fully developed. Consequently, natural red clover populations may not have been adversely affected by the decreasing light levels in $+CO_2$ conditions, as the plants had already colonized the area and become well-established. Furthermore, as marked changes in light availability occurred over several years, selection for red clover genotypes that exhibit optimal growth responses under these conditions may have occurred (e.g., Heagle et al., 1991). Performance of our experimental populations, however, was assessed when light levels were already greatly reduced. With only nine genotypes and no time for selection to occur, these red clover plants may have been more strongly influenced by the reduced light levels than by the growth-enhancing effects of CO_2. The indirect effect of CO_2, mediated through forest overstory growth, is the most parsimonious explanation as to why plants under $+CO_2$ atmospheres were smaller than, while plants growing under $+O_3$ atmospheres were in many instances as large as, those growing under ambient conditions. Decreased light levels would also explain why clover plants in $+CO_2$ environments, compared to ambient conditions, had higher foliar nitrogen concentrations, i.e., a typical shade response (McDonald et al., 1999). This does not preclude, however, the likely possibility of complex interactions between gas treatments and light levels.

In conclusion, this research demonstrates that forest understory communities are likely to change in response to both direct and indirect effects of atmospheric composition. Such changes in community composition may modify a wide range of ecological processes (e.g., trophic interactions and litter decomposition), in turn altering forest ecosystem dynamics.

Acknowledgements

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