Interactive Effects of CO_2 and O_3 on the Growth of *Trisetum flavescens* and *Trifolium pratense* Grown in Monoculture or a Bi-Species Mixture

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SUMMARY. Golden oat grass (*Trisetum flavescens* L.) and red clover (*Trifolium pratense* L.) were grown as monocultures or bi-species mixtures under controlled conditions and exposed to ambient (350 ppm) or elevated (580 ppm) CO₂, with or without addition of O₃ (diel profile with 150 ppb maximum). Shoot biomass measurements after the initial growth and two re-growth periods were used to determine the specific responses of both species, and the difference in the specific response between monocultures and mixtures. *T. pratense* was much more responsive to CO₂, O₃, and their combination, compared to *T. flavescens*. In the case of O₃ but not of CO₂, the difference in sensitivity between species

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was larger in mixture than in monoculture. In contrast to elevated CO_2 , O_3 significantly reduced the root:shoot ratio in the mixture, which could explain the increasing negative effect of O_3 on clover with progressing harvests.

The relative CO_2 stimulation of *T. pratense* and of the cumulative mixture shoot biomass was larger in the presence than in the absence of O_3 , which was due to an almost complete protection from O_3 stress by elevated CO_2 . In the mixture, the fraction of *T. flavescens* was small and increased during the experiment; this increase was most pronounced with O_3 , but any change in mixture biomass was dominated by the response of *T. repens*.

The results confirm that in grass/legume mixtures legumes are most sensitive to elevated CO_2 and O_3 , but the magnitude of specific responses depends on canopy structure and of plant development. Elevated CO_2 minimizes the negative impacts of O_3 stress on above- and below-ground plant growth. [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com> © 2005 by The Haworth Press, Inc. All rights reserved.]

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INTRODUCTION

The concentration of CO_2 in the atmosphere has risen from pre-industrial levels of about 270 ppm to currently 370 ppm, and a further rise is predicted for the twenty-first century (IPCC, 2001). This increase in CO_2 is expected to affect the biosphere in general, and more specifically agricultural production systems. The majority of studies with agricultural crops tend to suggest that effects of elevated CO_2 on crop growth and yield are positive (Bazzaz, 1990, Idso et al. 1994). However, the CO_2 effect may be altered by limiting environmental conditions (e.g., Meier & Fuhrer, 1997); thus, yield stimulations observed in experiments under non-limiting nutrient and water supply may not be attained under realistic field conditions. Tropospheric ozone (O_3) is an additional factor which at elevated concentrations may modify the CO_2 stimulation. Air pollution by O_3 is of prime importance in many regions, particularly in the northern hemisphere (Chameides et al., 1994). Current levels of O_3 in Europe, the USA and elsewhere are sufficiently high to cause crop losses (Fuhrer et al., 1997).

According to current understanding, plant biomass production is largely protected from toxic O_3 effects in a CO_2 -rich atmosphere. This is mainly due

to ozone exclusion (Allen, 1990), but there is some evidence that treatment with elevated CO_2 also increases the capacity for detoxification (Heagle et al. 1999). When applied in combination, elevated CO_2 can compensate at least for part of the negative effects of elevated O_3 , and the positive effect of elevated CO_2 on yield can be larger in an atmosphere containing elevated levels of O_3 relative to the effect without O_3 (Heagle et al., 1998). Results from a number of studies underline the importance of the interaction between the effects of the two gases, but for quantitative risk assessments the possible variability in response patterns between species and cultivars must be considered.

In contrast to arable crops, grasslands have received little attention with respect to interactive effects of elevated CO₂ and O₃. The situation in multi-species grassland communities is more complex than in monocultures of arable crops; the effects of the two gases and their interaction may differ substantially between co-occurring species (Porter, 1993; Volin et al. 1998), which, in turn, may lead to a shift in the floristic composition of the plant community in an changed atmosphere. Shifts in species mixtures have been shown for pastures exposed to elevated O_3 (Fuhrer et al., 1994), or to elevated CO_2 (Hebeisen et al., 1997). A reduction in the clover fraction in pastures may have important negative consequences for forage quality (Muntefering et al., 2000). The issue is further complicated when grasslands are cut or grazed. In the case of cutting, effects of pollutants may differ between the initial growth and the re-growth periods. For clover it has been shown that O₃ effects on growth are larger during re-growth because of reduced storage of resources necessary for re-growth (cf. Fuhrer, 1997). Effects of O₃ may also depend on the cutting regime, for instance because of a change in O₃ penetration (Ashmore & Ainsworth, 1995). Overall, available information suggests that effects of a change in the atmospheric composition on grassland species depend on the specific physiological and morphological characteristics of the competing species, and also on management.

In order to gain a better insight into some of the possible interactions, the aim of this study was to investigate under controlled conditions the effects of CO_2 and O_3 , singly and in combination, on two species grown in monoculture or in bi-species mixture during three growth periods. Red clover (*Trifolium pratense* L.) and golden oat grass (*Trisetum flavescens* L.) were chosen as typical species of managed permanent grasslands in pre-alpine and alpine regions; they also represent different functional groups, and their shoot architecture differs considerably.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of red clover (*Trifolium pratense* L. cv. Temara) and golden oat grass (*Trisetum flavescens* L. cv. Trisett) were germinated in moist quartz sand. Af-

ter emergence seedlings were transplanted as monocultures or mixtures into 16 cylindrical pots (monoliths with 0.2 m diameter, 0.8 m height) (Meier & Fuhrer, 1997). The lower 50 cm of the pots were filled with washed quartz sand, and the top 30 cm with top soil (60% humus, 23% sand, 10% peat; pH_{H₂O} 6.5-7.0). Monoliths were covered with plastic lids (2 mm) containing 46 holes (12mm diameter) in which the seedlings were planted. Plant density was equivalent to 1500 plants m⁻², and in the mixtures the clover:grass planting ratio was about 1:2. One and 10 days after transplanting the seedling to the pots, rhizobium bacteria were applied to clover roots.

All experiments were carried out in a controlled environment growth chamber with a 14-h photoperiod. The arrangement of the units followed a completely randomized design. Illumination was provided by halogen-metal lamps giving a PPFD of 360 µmol m⁻² sec⁻¹ at the top of the cuvettes. Air temperature followed a diurnal cycle with a maximum of 25°C at noon and a minimum of 15°C at midnight. Each pot was irrigated by means of a peristaltic pump (Ismatec, Glattbrugg, Switzerland) with 100 ml deionized water per day. N-fertilization with ammonium nitrate (NH₄NO₃) was applied three times per growth period, with increasing amounts of N according to the to the estimated biomass. The total N application per growth period was equivalent to 100 kg N ha⁻¹. Solutions for irrigation and fertilization were distributed through a pierced tube placed on the surface of the soil column.

Treatments

Cylindrical plexiglass cuvettes (0.75 m height) were fitted to the top of the pots (Meier & Fuhrer, 1997). Horizontal tubes at the top and at the bottom were used as air inlet and outlet, respectively. Ozone-free air was passed through a CO_2 molecular-sieve, reduced to a pressure of 2 bar and flow-adjusted by a mass-flow controller before being distributed to four separate mixing boxes (DMP, Hegnau-Volketswil, Switzerland) for each of the following treatments:

Control:	$CO_2 = 350 \text{ ppm}$	$O_3 = 0 \text{ ppb}$	
CO ₂ :	CO ₂ = 580 ppm	$O_3 = 0 ppb$	
O ₃ :	CO ₂ = 350 ppm	$O_3 = 150$ ppb, with a diel pattern pass through the maximum at noon a with 0 ppb during the dark period	

 $O_3 \times CO_2$: $CO_2 = 580 \text{ ppm}$ $O_3 = 150 \text{ ppb}$ (same as in the O_3 treatment)

Pure CO_2 from a gas cylinder and O_3 generated from pure oxygen by electrical discharge (Fischer ozone generator 500M, Meckenheim-Bonn, Ger-

many) were injected into the air inside the mixing box of the respective treatment using mass flow controllers. The air from each mixing box was sub-divided to obtain four replicates of each treatment. Air flow was measured with electronic flow meters. The pressure in the cuvettes was kept slightly above atmospheric pressure to reduce possible effects of leaks. To reduce boundary layer resistance inside the cuvettes the air was stirred with propellers (500 rpm) placed at the top of the canopy.

 CO_2 concentration at the inlet of the cuvettes was measured with an IR CO_2 analyzer (Ultramat 5E, Siemens, Germany), and water content with a the dew-point hygrometer (Hygro-M2 monitor, General Eastern, Watertown, MA, USA). Concentrations of O_3 were measured in the inlet tubes with a Dasibi, 1008 RS ozone monitor (Glendale, CA, USA). Gas concentrations in each cuvette were measured using a time-share system with 5-min intervals. O_3 fumigation, monitoring, and data acquisition were computer-controlled (Labview 4, National Instruments, Austin, TX, USA).

Harvests

Plants were cut 4 cm above the soil surface 29, 45, and 61 days after transplanting the seedlings to the exposure units. The shoot material was separated by species. At the end of the experiment, roots were separated from the soil by a washing procedure (Meier & Fuhrer, 1997) to determine total root biomass. All biomass weights was determined after drying at 80°C for 24 h. Data are expressed in units of dry weight per experimental unit.

Statistical Analysis

Treatment mean differences in specific biomass data from individual harvests were tested for significance using a multiple comparison test (Fishers LSD). Normality of the data was tested prior to analysis. Analysis of variance (ANOVA) was used to test for the effects of treatments and their interactions on cumulative shoot and root biomass data. The statistics were calculated using NCSS 2000 statistical software (Kaysville, UT).

RESULTS

The two species produced similar amounts of shoot biomass when grown in monoculture and exposed to the control atmosphere, and the differences between the three harvests were small (Figure 1). The elevated O_3 treatment affected shoot biomass in both species to a different extent. Elevated O_3 caused a reduction in biomass in *T. pratense* as compared to the control which was significant in the case of harvest 2 and harvest 3. In *T. flavescens* the negative effect of O_3 was only significant for harvest 1. Elevated CO_2 had no effect on *T*.





flavescens but caused a stimulation of biomass in *T. pratense* which was significant during the first growth period. In the $O_3 \times CO_2$ treatment, biomass production never differed significantly from the control, except for the last harvest in the $O_3 \times CO_2$ treatment.

In mixture with *T. pratense*, growth of *T. flavescens* was strongly reduced, compared to growth in monoculture, but increased with progressing harvests (Figure 1c). No significant treatment effects were detected. In contrast, *T. pratense* in the control treatment produced more biomass than in monoculture (Figure 1d), and the negative effect of O_3 was always significant and larger than in monoculture. Conversely, the effect of elevated CO_2 was never significant. Also, in the combined gas treatment, *T. pratense* biomass was not different from the control.

Total mixture shoot biomass increased with progressing harvests in all treatments (Figure 2a). Because of the dominance of *T. pratense* in the mixture, treatment effects on total were similar to those observed in *T. pratense*. The biomass ratio of *T. flavescens* to *T. pratense* in the mixture also increased from harvest 1 to harvest 3 (Figure 2b). This increase was largest in the O₃ treatment, thus indicating a stronger increase in *T. flavescence* relative to *T. pratense* with progressing duration of the experiment. This pronounced O₃ effect was absent in the presence of elevated CO₂, which by itself had no significant effect on the species ratio.

Over three growth periods, specific cumulative biomass in the monocultures for *T. flavescens* was similar to the biomass of *T. pratense*, whereas in the mixture, *T. pratense* was stimulated at the expense of *T. flavescens* (Figure 3). In the monocultures, O_3 reduced the biomass of both species, while CO_2 tended to have the opposite effect. In the $O_3 \times CO_2$ treatment, shoot biomass was equal to the control. In the mixture, no effects of CO_2 and O_3 on *T. flavescens* were found, in contrast to *T. pratense* in which O_3 reduced the cumulative biomass by 44%. The ratio of biomass in mixture to biomass in monoculture was unaffected by the treatments in *T. flavescens*, but significantly reduced in *T. pratense*.

Cumulative mixture shoot and root biomass, and their ratio, differed between treatments (Figure 4). The most pronounced effect was observed in the O_3 treatment which significantly reduced shoot and root biomass, and the root:shoot ratio. The effect of CO₂ was not significant for shoot and root biomass, and root:shoot ratio. ANOVA revealed a significant treatment effect for roots and shoots (*P* < 0.001), but no significant interaction of CO₂ and O₃ (Table 1).

DISCUSSION

The two species selected for this study differed in their sensitivity to elevated CO₂. In *T. pratense*, cumulative shoot biomass in monoculture inFIGURE 2. Total shoot biomass of mixtures of *T. flavescens* and *T. pratense* (a), and species biomass ratio (b) for each harvest (mean \pm SE, n = 4). Different letters indicate significant differences between treatments for each harvest separately (Fisher LSD contrasts, P < 0.05).



creased by about 14% at elevated CO_2 , compared to the control treatment, while in *T. flavescens* the effect was only 4% and non-significant (Figure 3). It has been demonstrated that legumes generally respond more strongly to elevated CO_2 than grasses (Hebeisen et al., 1997; Lüscher et al., 1998). In the short run, this can be attributed to increased symbiotic fixation of N₂ in legumes which provides the extra N necessary to cope with additional C supply under elevated CO_2 (Zanetti et al., 1998). In contrast, grasses are restricted by N, and only in mixtures with legumes the necessary extra soil N becomes available through the N input by symbiotic N₂ fixation by the legumes. (Zanetti et al., 1997; Lüscher et al., 2000). In the mixture, biomass production in *T. pratense* was less stimulated by elevated CO_2 than in monoculture, al-

FIGURE 3. Cumulative shoot biomass of *T. flavescens* and *T. pratense* grown in monoculture or mixture (a), the ratio between the cumulative biomass produced in mixture and monoculture for each species (b) (mean \pm SE, n = 4). Different letters indicate significant differences between treatments for each harvest separately (Fisher LSD contrasts, P < 0.05).



though the mixture response corresponded to the response of the dominating *T. pratense*, in agreement with data presented by Navas et al. (1999). It has been well been recognized that competition can alter the response of plant species to elevated CO_2 (Navas, 1998). Some species are more responsive to CO_2 when grown in monculture, whereas the reverse is true for other species (cf. Navas et al., 1999). Here, *T. pratense* in monoculture produces a denser canopy than in mixture with the tall-stature grass species. It could be expected that this would favor the CO_2 responsiveness of *T. pratense*. However, the opposite was found in the present experiment. Reduced competition for light in the less dense canopy of the mixture could have reduced the CO_2 responsiveness in *T. pratense*. This interpretation would agree with the notion that the relative

 CO_2 stimulation is less under favorable conditions, than in situations with limiting resources (Idso & Idso, 1994).

In terms of the specific cumulative shoot biomass, T. pratense was more affected by elevated O_3 than T. flavescens (Figure 3). This difference in O_3 sensitivity between the two species confirms earlier findings. Bungener et al. (1999) reported that the growth response to O_3 was much stronger in T. pratense than in T. flavescence, and Nussbaum et al. (2001) related the inter-specific difference in sensitivity to differences in stomatal O_3 uptake. It is well known that the amount of O₃ absorbed by leaves determines the effects of O₃ on plants, and that species with lower O₃ uptake rates may be partially protected from O_3 . The sensitivity to O_3 of T. flavescens observed here was even lower in the mixture than in monoculture, which could be attributed to the stronger shading or better protection from O_3 penetration in the presence of T. pratense plants. In agreement, T. flavescens responded more strongly in mixture with a low stature species (*Centaurea jacea*) than in mixture with T. pratense (Nussbaum et al., 2000). In contrast, the response of T. pratense to O_3 was larger in the mixture than in monoculture, which could be related to reduced self-shading and/or increased penetration of O₃ into the less dense canopy. However, in agreement with Nussbaum et al. (2000), T. flavescens could not benefit from the reduction in T. pratense biomass under O3 stress. These observations underline that effects of CO_2 and O_3 on species mixtures not only depend on the characteristics of the individual species involved, but also on the canopy structure of the mixture. When extrapolating these results to real plant communities it could thus be concluded that the CO_2 response of T. pratense is stronger in a dense canopy, in contrast to the O₃ response. The latter would be favored by less self-shading and less restricted O₃ penetration into the canopy, and that effects of CO₂ and/or O₃ on grassland mixtures may be dominated by the response of the legumes.

The absence of a significant effect of CO_2 on the root:shoot ratio is in agreement with observations made earlier for grass/clover mixtures under non-limiting water and nutrient availability (Meier & Fuhrer, 1997), or native herbs (Ferris & Taylor, 1993), and it suggests that roots respond proportionally, and that biomass partitioning is not altered (Figure 4). However, the effect of elevated CO_2 on root:shoot ratio is highly variable among species and study conditions (Rogers et al., 1996). In one study with 27 herbaceous species, root:shoot ratio decreased in 14 species, increased in 6 species, and remained unchanged in 7 species under elevated CO_2 (Hunt et al., 1991). Thus, the results obtained here should not be generalized and extrapolated beyond the conditions of the experiment. In contrast to CO_2 , O_3 caused a strong reduction in root:shoot ratio, suggesting preferential partitioning of biomass to shoots rather than roots. This is a common effect of long-term O_3 exposure (Cooley & Manning, 1987), and in functional terms it may reflect the shift in partitioning

FIGURE 4. Cumulative shoot biomass, final root biomass, and the root:shoot ratio in mixtures of *T. flavescens* and *T. pratense* after three growth periods (mean \pm SE, n = 4). Different letters indicate significant differences between treatments for each harvest separately (Fisher LSD contrasts, P < 0.05).



in response to reduced C supply due to O_3 impacts on photosynthesis. In the present study, the effect is dominated by the response of *T. pratense* because of the larger fractional biomass. A reduction in partitioning towards roots in this species is detrimental because of the reduction in stored resources (cf. Fuhrer, 1997). The negative impact of O_3 on *T. pratense* was small for harvest 1, and increased with progressing harvests (Figure 1). This confirms the notion that under O_3 stress reduced remobilization of reserves after a cut negatively affects re-growth and is the main mechanism leading to long-term changes in the grass: clover ratio in pastures (Fuhrer et al., 1994). In mixtures, this effect could favor less sensitive species, as indicated here by the stronger increase in the fractional biomass of *T. flavescens* during the re-growth periods as compared to the control. In the long run this could lead to shifts in species dominance (Fuhrer et al., 1994). In the $O_3 \times CO_2$ treatment, the negative effect of O_3 on root:shoot ratio was partly reversed, reflecting the protective effect of elevated CO₂ from O_3 effect.

The effect of elevated CO_2 on plant growth is modified in the presence of O_3 . For instance, in the absence of O_3 , cumulative shoot biomass in *T*. *pratense* was increased by 14% under elevated CO_2 relative to the control, but by some 20% in the $O_3 \times CO_2$ treatment relative to O_3 alone (Figure 3). In the mixture, stimulation of *T. pratense* and cumulative mixture biomass in the $O_3 \times CO_2$ treatment was 64% and 71%, respectively, relative to the treatment with O_3 alone. These results are in agreement with findings for wheat, cotton and soybean (Heagle et al., 1998, 1999, 2000). They clearly demonstrate that

TABLE 1. Statistical analysis^a of the effects of CO_2 and O_3 treatments on cumulative shoot and root biomass of *T. flavescens*/*T. pratense* mixtures.

Factor	Shoot	Root	Root:Shoot
O ₃	***	***	**
CO ₂	*	n.s.	n.s.
$O_3 imes CO_2$	n.s.	n.s.	n.s.

^a ANOVA: n.s. = not significant; * *P* < 0.05; ** *P* < 0.01; *P* < 0.001

in a sensitive species, such as *T. pratense*, effects of elevated CO_2 on growth and productivity depend on the level of O_3 . Relative to the control with no O_3 and with ambient CO_2 , cumulative mixture biomass was reduced by 38% by O_3 alone, but only by 12% in the $O_3 \times CO_2$ treatment, thus indicating that elevated CO_2 provides protection from O_3 stress (Figure 4). It is well known that elevated CO_2 may reduce O_3 sensitivity in a range of species by reducing stomatal conductance, and hence O_3 uptake (Fiscus et al., 1997; McKee et al., 2000). This was confirmed in the present experiment by canopy gas exchange measurements (data not shown). Although the possibility exists that elevated CO_2 increases the potential for cellular detoxification of absorbed O_3 (Polle & Pell, 1999), direct evidence is largely lacking. The results of the present study support the notion that reduced O_3 uptake in elevated CO_2 atmospheres is the more important mechanism.

In summary, the results of this study lead to the following conclusions:

- Effects of elevated CO₂ are modified by O₃ stress; the relative CO₂ stimulation of shoot and root biomass is larger in the presence of O₃, and the negative effects of O₃ are absent in the presence of elevated CO₂.
- Effects of CO_2 and O_3 on shoot biomass production are more pronounced in *T. pratense* than in *T. flavescens*; the low O_3 sensitivity of the latter species coincides with a lower specific responsiveness to elevated CO_2 , particularly when grown in mixture with *T. pratense*. The mixture biomass response is determined by the response of the dominating species (*T. pratense*).
- O₃ stress strongly reduces the root:shoot ratio in the mixture, with possible consequences for re-growth of *T. pratense*, whereas elevated CO₂ has no effect.
- The results indicate that grassland species differ in their sensitivity to CO₂ and O₃, and the specific responses differ between monocultures and mixtures, and between harvests. CO₂ effects on both species tend to be larger in monocultural stands, whereas the opposite applies to effects of O₃.

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