ARTICLE

Assessing the impacts of intra- and interspecific competition between *Triticum aestivum* and *Trifolium repens* on the species' responses to ozone

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Abstract: Tropospheric ozone is considered to be the most phytotoxic air pollutant because of its oxidizing power. The main objective of this study was to analyze the effect of intra- and interspecific competition between *Triticum aestivum* L. and *Trifolium repens* L. on the responses to high concentrations of ozone of both species, and the role of the symbiotic relationship *Rhizobium – T. repens* on the abovementioned responses. Monocultures and mixtures of both species in different densities were sown. Pots were transferred to open top chambers either with 90–120 ppb ozone or without ozone. Ozone had an overall negative impact on leaf area and biomass production per individual plant. These responses were dependent on species and sowing density in monocultures, but were not changed by species proportion in the mixtures. There was a positive relationship between *Rhizobium* nodules and plant biomass, with a tendency for smaller plants to present lower number of nodules under ozone. These results suggest that competitive and mutualistic interactions could have a greater role in determining responses to novel air pollutants than species sensitivity to the xenobiotic, per se.

Key words: air pollution, tropospheric ozone, intraspecific and interspecific competition, *Rhizobium*, *Triticum aestivum*, *Trifolium repens*.

Résumé : L'ozone troposphérique est considéré comme étant le polluant de l'air le plus phytotoxique à cause de son potentiel oxydant. L'objectif principal de cette étude était d'analyser l'effet de la compétition intra et inter-spécifique entre *Triticum aestivum* L. et *Trifolium repens* L. sur la réponse à des concentrations élevées d'ozone chez les deux espèces, et le rôle de la relation symbiotique *Rhizobium – T. repens* sur les réponses précédentes. Des monocultures et des mélanges des deux espèces à différentes densités ont été semés. Les pots ont été transférés dans des chambres à couvercle ouvert en présence de 90–120 ppb d'ozone ou sans ozone. L'ozone avait un impact négatif global sur l'aire foliaire et la production de biomasse par plant individuel. Ces réponses étaient dépendantes des espèces et de la densité de plantation chez les monocultures, mais elles ne changeaient pas en fonction la proportion des espèces dans les mélanges. Il y avait une relation positive entre les nodules de Rhizobium et la biomasse végétale, les plants plus petits ayant tendance à présenter un plus faible nombre de nodules en présence d'ozone. Ces résultats suggèrent que les interactions compétitives et mutualistes peuvent avoir un rôle plus important sur la détermination de la réponse à de nouveaux polluants de l'air que la sensibilité des espèces aux xénobiotiques en soi. [Traduit par la Rédaction]

Mots-clés : pollution de l'air, ozone troposphérique, compétition intraspécifique et interspécifique, *Rhizobium*, *Triticum aestivum*, *Trifolium repens*.

Introduction

Global background ozone concentration has increased since the pre-industrial era because of anthropogenic emissions of its precursors, pollutants such as nitrogen oxide (NO_x), and volatile organic compounds (VOC) (Wang and Jacob 1998). Further increases in ambient ozone concentration are predicted to occur over the next 50 years (Prather et al. 2003; Solberg et al. 2005). In highly contaminated areas, several days of ambient smog exposure involving 1 h ozone concentration peaks in the range of 120–190 ppb can occur throughout the growing season (Booker et al. 2009), which is more than enough to trigger negative effects on vegetation (Tamaoki 2008; Schnell et al. 2009). Nevertheless, very short exposures can result in foliar changes that persist for several days after exposure is terminated (Vollsnes et al. 2009; Futsaether et al. 2015). Significant losses in agricultural

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production occur at ozone exposure above 40 ppb; a threshold level reached in a number of countries (The Royal Society 2008). This has raised increasing concern about the magnitude of ozone impact, not only on global food supply (Long et al. 2005; Schmidhuber and Tubiello 2007), but also on timber production, conservation, and the genetic diversity of natural plant communities (Krupa et al. 2001; Fuhrer and Booker 2003; Ashmore 2005).

Ozone toxicity in plants causes visible injury to leaves due to oxidative stress (Bergmann et al. 1999), often coupled with reductions in photosynthesis, biomass accumulation (Power and Ashmore 2002; Ramo et al. 2006), and premature senescence (Bermejo et al. 2003; Novak et al. 2003). There is a wide range in sensitivity to ozone among different plant species (Hayes et al. 2007), which could ultimately lead to changes in species dynamics and community composition. Elevated levels of ozone could not only affect the growth of individual plants, but also the pattern of competitive interactions, through changes in biomass partitioning. In some species, aboveground biomass may be maintained at the expense of roots, e.g., *Festuca ovina* ("Barcea") (Cooley and Manning 1987; Grantz et al. 2006), or stolons, e.g., *Trifolium repens* (white clover) (Wilbourn et al. 1995), which may influence the ability of a species to acquire soil nutrients. It is expected that changes in individual plant size distribution that are often driven by competition (i.e., plant inequalities), occur at higher densities than in the control situations without the stress, because plants would grow more slowly (Weiner 1985). Competition itself could also modify the impact of ozone or the species response to the pollutant. Under competitive situations, differences in individual size of the same or different species are exacerbated as larger plants usrp resources and grow at the expense of smaller ones, generating a hierarchy of sizes and competitiveness. Reductions in the growth rates of smaller plants could result in decreased ozone uptake, indirectly erasing competitive differences between plants (Fredericksen et al. 1996). Moreover, the effects of ozone on the plant community could depend on the characteristics of the species involved, the ratio of the different species, or on environmental conditions (Nussbaum et al. 2000; Bassin et al. 2007). Results based on "quality" parameters suggest that legumes are more O₃-sensitive than several of the grass species (Sanz et al. 2014). Studies designed to measure the effects of ozone on *T. repens* growing with different species have shown that this species is more sensitive to ozone, which could affect the competition among species and change the composition of pastures (Nussbaum et al. 1995; Wilbourn et al. 1995; Gimeno et al. 2004a). Nevertheless, Bass et al. (2006) studied the impact of ozone on *T. repens* growing with *Lolium perenne* (perennial ryegrass) in highly crowded established mesocosms, and found that ozone-induced growth reduction in mixed communities was similar to the reduction in monocultures. However, when water availability was restricted, the results were the opposite (Nussbaum et al. 2000).

The indirect effects of ozone and plant responses to ozone may occur through interactions with symbiotic organisms. Although the penetration of ozone into soil supporting plants is believed to be essentially limited to the soil surface (Stella et al. 2011), decreases in the number of rhizosphere bacteria in soil might be associated with increased levels of tropospheric ozone(Li et al. 2013). For example, air enriched with ozone reduced *Rhizobium* root nodule formation in *T. repens* (Awmack et al. 2007). Carbohydrate transport rate to roots decreases in ozone-treated plants (Andersen 2003; Grantz and Farrar 1999, 2000), and might explain, at least in part, this altered root nodulation of plants under ozone. Moreover, Edwards (1991) suggested that decreased respiration rates in the soil surrounding the roots of ozone-exposed plants may reduce the root-derived organic materials available for microbial proliferation as an indirect result of the ozone exposure. As a consequence, an increase in competitive stress is likely to occur, suggesting that responses to ozone may be affected by competition between species through the modification of mutualistic interactions.

The aims of this study were to determine whether ozone exposure during early plant growth stages would induce differential detrimental effects in the performance of two cropping species of contrasting growth habits, a grass and a legume, and how the level of intra- or interspecific plant competition would modulate these effects. We also investigated whether early ozone exposure would have an impact on the establishment of the mutualistic interaction between the legume species and *Rhizobium* spp. We predicted that (*i*) the effect of ozone on plant growth depends on the species and sowing density, (*ii*) the effect of ozone on biomass production per plot depends on species proportion, and (*iii*) the effects of ozone on legume species could negatively impact the establishment of rhizobia root nodules, thus interfering with their mutualistic interaction.

Materials and methods

We tested our predictions by growing seedlings of *Triticum aestivum* (var. 'Buck'; wheat) and white clover both in monocultures and in mixed competitive stands with varying plant densities and ozone regimes. Two replicate experiments were carried out in the experimental field of the School of Agronomy, University of Buenos Aires University, Argentina (34°35.2′S, 58°35.0′W), during consecutive years (2010 and 2011).

Experimental procedures

Seeds of both species (*Triticum aestivum* and *Trifolium repens*) were sown in monoculture and mixtures in 1000 cm³ (15 cm diameter) plastic pots filled with a mixture of regular potting substrate. All of the pots were placed in a greenhouse. The mean temperature during

the experiment was 15.2 ± 4 °C. Monocultures consisted of a series of densities obtained by sowing an increasing mass of seeds per pot: <0.003, 0.2, 1.2, 2.4, 3.6, and 4.8 g of wheat or white clover. In the lowest density pots (<0.003 g), seedlings were thinned to one per pot one week after emergence. Six pots of each density were sown per species. The rest of the pots produced ca. 5, 30, 60, 90, and 120 wheat seedlings or 80, 480, 960, 1400, and 1900 clover seedlings per pot. Seed vitality was simultaneously tested in independent pots under the same experimental conditions in the greenhouse, and 100% germination rates were recorded. Densities used in our study are much higher than those used by other authors assessing the effects of components of global change on pasture yield (e.g., Hebeisen et al. 1997; Sparks et al. 2015). In these studies, and in commercial forage pastures, sowing densities are around or below optimal density to avoid competitive effects. This is required to achieve maximum yields. However, we did not try to reproduce natural conditions, but instead we aimed to assess the effects of competitive interactions on the response to ozone. For this reason we included single plant pots and pots with densities varying from the recommended sowing rate (~0.25 g/pot) to plant densities that were exceptionally high to ensure that competition occurred.

Plant mixtures were generated by keeping the highest constant sowing rate (4.8 g of seeds) and replacing part of the seeds of one species in a monoculture with seeds of the other species (replacement series) (de Wit 1960). From the combination of the different mass of seeds sown, five proportions resulted in the mixtures: 100:0, 75:25, 50:50, 25:75 and 0:100 w/w of wheat:white clover, respectively. The effects of interference at one total density may be very different from interactions at a different density, and changes in total density are very common and important in nature (Inouye and Schaffer 1981; Weiner 1980). For this reason, we used a modification of the replacement series, varying total plant density together with species proportion (Snaydon 1991). This allowed us to evaluate the effect of species proportion (with one or more species in the pot) on the response to ozone, within a range of densities. Six pots of each mixture were transferred to the three ozone treatment and the three control chambers. After ozone treatment, all of the pots were returned to the greenhouse (see below).

Ozone exposure treatments

Six 8 m³ open-top chambers (OTC) with transparent polyvinyl chloride (PVC) walls mounted on a metal structure were used (Hogsett et al. 1985; Lefohn et al. 1986). Open-top chambers are a proven exposure technology in air pollution field research, developed to provide an environment that closely resembles ambient conditions (Heagle et al. 1973; Rogers et al. 1983). Three chambers were used for the control treatment (ozone free ambient air, Oz–) and three for the ozone treatment (charcoal filtered air with ozone added, Oz +). Ozone was generated by a spark-discharge-type ozone generator (OZ5000; Dobzono SA, Buenos Aires, Argentina), and ambient air was pumped through an activated charcoal filter with an air dryer, which eliminated the risk of nitrogen oxides as a by-product of ozone formation, by removing water vapor and increasing ozone production. Ozone concentration was continuously monitored using Model 450 ozone Monitor API-Teledyne Instrument (Teledyne Advanced Pollution Instrumentation, San Diego, California, USA). The ozone chambers received a daily fumigation that increased ozone concentration to a maximum of 90 to 120 ppb. Mean day-time temperatures were regulated and controlled at 15 °C (similar to mean air temperature measured in the greenhouse) by means of an air conditioning appliance Model Split 3000 (Surrey SA, Buenos Aires) placed in each chamber. Observed mean temperature values did not differ significantly between chambers. Relative humidity in each chamber averaged 50%. Light intensity within the chambers, reduced by a shading compound on the plastic, varied between 2100 and 6500 lx with cloud cover. The photoperiod during the experiment was about 14 h (14 h light – 10 h dark).

Half of the pots from each monoculture and mixture treatment were randomly assigned to the ozone exposure treatment (Oz+) and the other half to the control group (Oz–). Pots corresponding to each group were further separated into three groups and randomly assigned to one of either the Oz+ or control (Oz–) chamber, so that each chamber included one replication of all monoculture densities for each species and one replication of each mixture proportion. The plants getting the Oz+ treatment were exposed for 4 h per day for five days, while the rest of the plants received ozone-free air (Oz–).

Plant growth measurements

Two weeks after ozone exposure, the average area per leaf was estimated for 10 randomly selected leaves per pot for all of the treatments. Clover leaf area per leaf was first calculated using the maximum width (W) and length (L) of each leaf in the equation $W \times L/2$ (Gamper 2005). In the case of wheat plants, different algorithms have been suggested for estimation of leaf area (Rao et al. 1967; Miralles and Slafer 1991). In this study, the maximum leaf length for wheat was chosen as the best estimate of average leaf area for plants under different densities (Cogliatti et al. 2010). Leaf area of all of the leaves was then summed up to calculate the leaf area per plant. Above-ground plant biomass was then harvested in all monoculture and mixture pots. Ten plants from each monoculture pot (except for the individual plant pots) and five plants of each species in the mixture pots were randomly selected and kept separated for individual mass assessment. The rest of the plants in the pot were harvested to determine total biomass per pot, keeping plants of the two species in the mixture separated.

All of the plant material was oven-dried at 70 °C for a minimum of four days before biomass was determined.

A separate run of the clover monoculture experiments was used for evaluating the impact of ozone on nodule formation. In this case, only single plants and a high sowing rate (3.6 g seeds) were used. Roots from 6-week-old plants were recovered by immersion of the pot in water and rinsing the soil away. Shoot and root dry mass was assessed, and on each plant, the number of root nodules containing *Rhizobium* was counted.

Statistical analysis

To increase replicability, the experiments were done twice, in different years (2010 and 2011). Data was first analyzed considering year as a random factor, and ozone and densities as fixed factors (Moore and Dixon 2015). As there was no effect of year and no year × treatment interactions, we pooled data from both years for further analysis and presentation. Two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was used to evaluate differences in leaf area/leaf, and leaf area/plant, resulting from ozone exposure (Oz– vs. Oz+) and sowing rate, for wheat and clover plants in monocultures. ANOVA was also used to evaluate differences in plant dry mass/plant and plant dry mass/pot in monocultures among sowing rates and ozone treatments, and differences in total biomass per pot among mixtures and ozone treatments. All analyses were performed with Infostat Professional version 2008 (Argentina) and GraphPad Prism 5. The effects of crowding on intraspecific competition for each species, and the impact on response to ozone exposure was assessed comparing the population's size structure for each species under ozone and ambient treatments. We used individual plant biomass to construct cumulative relative frequency polygons of plant size for each species, because this is the recommended way of comparing two or more data frequency sets (Chakravarti et al. 1967). We tested the cumulative size-class frequency curves (CSCFC) for different individuals under each ozone treatment for normality, using the Kolmogorov–Smirnov nonparametric test. We then compared each pair of curves (species × sowing rate under each ozone treatment), reporting the maximum vertical deviation between them as the statistic *D*.

The ozone effect (categorical variable with two levels) on the relationship between *Rhizobium* nodule number per plant (count data) and individual plant mass (continuous variable) was analyzed with generalized mixed linear model (glmer, package 'lme4'; Bates et al. 2014) in R (R Development Core Team 2008). Model selection was then applied and the models' adequacy was assessed graphically (Crawley 2007; Zuur et al. 2009).

Results

Clover plants exposed to ozone had smaller leaves than the control plants ($P < 0.001$), while increased density decreased mean leaf area independently of ozone

Table 1. Leaf area production of plants growing in monocultures under control (Oz–) or O_3 (Oz+) treatments. Only some density levels were chosen for comparison.

Sowing rate (g)	Wheat		White clover	
	Oz–	Oz+	Oz–	Oz+
Leaf area/leaf (mm²)				
0.003	264.7	259.2	271.3	147.1*
0.2	234.3	229.4	213.9	78.2*
1.2	241.2	234.3	217.2	84.3*
2.4	246.1	212.5	152.1	40.9*
3.6	250.7	224.7	146.1	48.5*
Leaf area/plant (cm²)				
0.003	44.17	29.12*	14.58	6.34*
0.2	23.13	21.21*	4.68	3.59*
1.2	10.31	9.87	4.32	2.88*
4.8	7.00	6.94	3.31	3.20
	Wheat		White clover	
	F	P	F	P
Leaf area/leaf				
Sowing rate	19.06	0.27	20.28	**0.056**
O_3	8.41	0.13	42.42	**<0.000**
Sowing rate × O_3	5.13	0.82	0.59	0.987
Leaf area/plant				
Sowing rate	83.31	**<0.000**	53.54	**<0.0001**
O_3	2.91	**0.0273**	11.33	**0.0073**
Sowing rate × O_3	5.88	**0.0272**	15.90	**0.0193**

*, $P < 0.05$ comparing Oz– and Oz+ within each species and sowing rate; P values in bold font are statistically significant.

treatment ($P = 0.056$). Across all of the sowing densities, average reduction in leaf area for clover was 45%. The leaf size of wheat plants was not sensitive to either sowing density or ozone (Table 1). In contrast, ozone treatment and increased sowing density reduced leaf area/plant for both species ($P < 0.001$) (Table 1).

The negative impact of ozone on biomass per plant was, on average, also larger for clover than for wheat (Fig. 1). Plant biomass reduction due to ozone was 36% for clover, and 2% for wheat. Regardless of the ozone treatment, plants showed a decrease in individual plant biomass with increasing sowing density (Fig. 1). Interestingly, ozone affected the total biomass produced per pot in wheat, but not in clover. However, the negative effect of ozone on wheat biomass per pot depended on sowing density ($P = 0.03$).

Intraspecific competition was poor at the lowest sowing density for wheat, thus the cumulative size-class frequency curves (CSCFC) presented a normal distribution pattern (Fig. 2). The consequences of competition among individuals became apparent as plant crowding was greater, showing a skewed CSCFC (Fig. 2). At the two lower density levels of wheat, ozone did not alter the patterns of CSCFC, but it had a strong impact at the two higher densities. In these latter treatments, ozone erased the consequences of competition on size-class distribution frequencies, mainly due to the elimination of the larger sized individuals (Fig. 2). The ozone effect was

Fig. 1. Biomass accumulation at final harvest for wheat and clover plants growing in monocultures at different sowing densities in the control and ozone (Oz– and Oz+) treatment groups. Values are the mean ± SE.
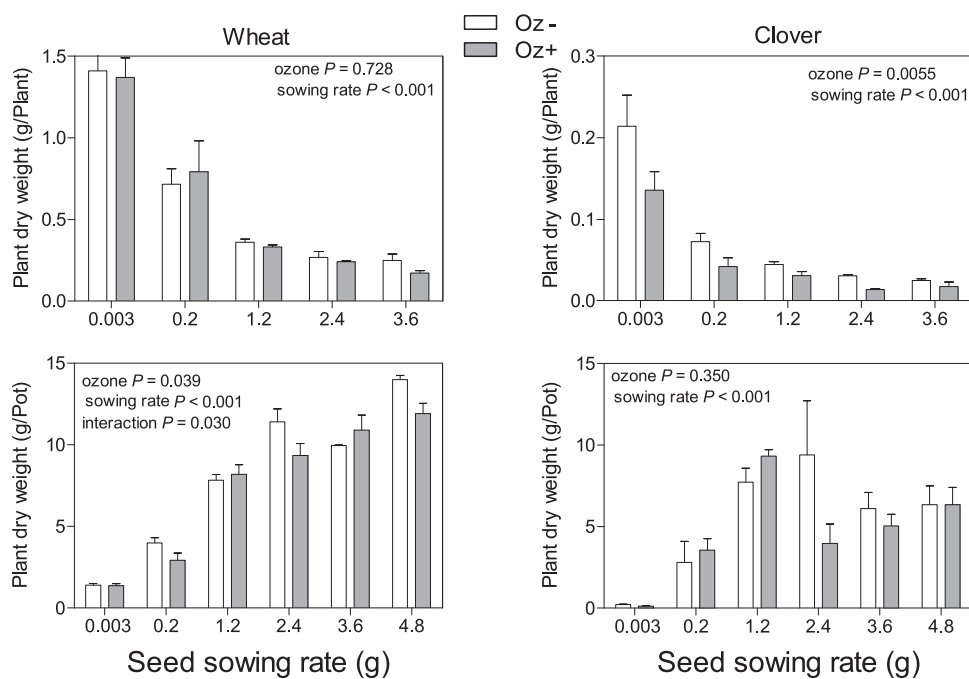
much stronger on clover because the patterns of CSCFC were changed at all sowing densities, especially by reducing the higher-sized individuals (Fig. 2).

Total biomass per pot produced by wheat in monoculture was significantly higher than that of clover under the same conditions ($P < 0.001$) (Fig. 3). In the "mixture" treatments, wheat always contributed a larger proportion of the total biomass per pot than clover ($P < 0.05$) (Fig. 3). The negative effect of ozone on total biomass per pot observed for the wheat monocultures across sowing densities was not apparent when the wheat was growing together with clover (Fig. 3).

The number of nodules per plant was highly dependent on the sowing density ($F_{[1,9]} = 16.756$, $P = 0.003$) (Fig. 4). There was a significant positive relationship between *Rhizobium* nodules and plant biomass (Z value = 13.060, $P < 0.001$) independently of the ozone treatment (Z value = 1.364, $P < 0.172$). Nonetheless, ozone, on average, reduced the number of nodules (estimate = −0.349) relative to that of the control plants (Z value = −3.229, $P = 0.001$) (Fig. 4, inset). About 1/3 (10 individuals) of the sampled plants not exposed to ozone had almost fourfold more nodules per gram of plant biomass than the average for all plants, whereas 2/3 of exposed plants (20 individuals) had fewer than average nodules for all plants (data not shown).

Discussion

Wheat and clover exhibited differential responses to elevated ozone levels and these effects were affected by interspecies competition. Ozone produced visible injury in both species; however, the reductions in leaf area and biomass per plant were greater for clover than for wheat, suggesting that wheat was more tolerant to ozone than clover. This is in agreement with published literature showing higher susceptibility of Fabaceae (legume) species to ozone (Gimeno et al. 2004*b*; Sanz et al. 2011). Clover, in particular, has been reported as a highly ozone-sensitive species (Hewitt et al. 2014). The bulk of results from experiments with single plants suggest that three traits can be identified as important controls of ozone sensitivity: stomatal control (Torsethaugen et al. 1999), specific leaf area (SLA, i.e., unit leaf area per unit of mass), and biochemical defense capacity (Batty and Ashmore 2003). Wheat leaf area per plant was reduced by ozone, although the mean leaf size was not affected. In turn, both mean leaf size and leaf area/plant were reduced in clover because of ozone exposure, suggesting that metabolic activities involved in clover leaf expansion were impaired by ozone, whereas in wheat, the total number of leaves or leaf duration were the parameters affected. Thus, plants of both species exposed to ozone produced different changes in SLA, further indicating differential susceptibility to the pollutant. On the other hand, chemical defense is based on the production and transport of energy-expensive antioxidant molecules. Hence the ability to tolerate ozone might be related to available photoassimilates (Fuhrer and Booker 2003), which in turn might be affected by competitive stress (Davison and Barnes 1998). In our study, both species grew under a different density range, i.e., the same seed mass sown corresponded to a higher seed number for clover than for wheat. As a consequence, the available

Fig. 2. Cumulative frequency of individual plant mass (g/Plant) in the control (Oz–, solid line) and ozone (Oz+, broken line) treatment groups for wheat and clover monocultures. Values are presented for the *D* statistic and *P* value comparing both data sets using the Kolmogorov–Smirnov (KS) test. Asterisks (*) indicate that values for the mass of plants in a given treatment group have a normal distribution.
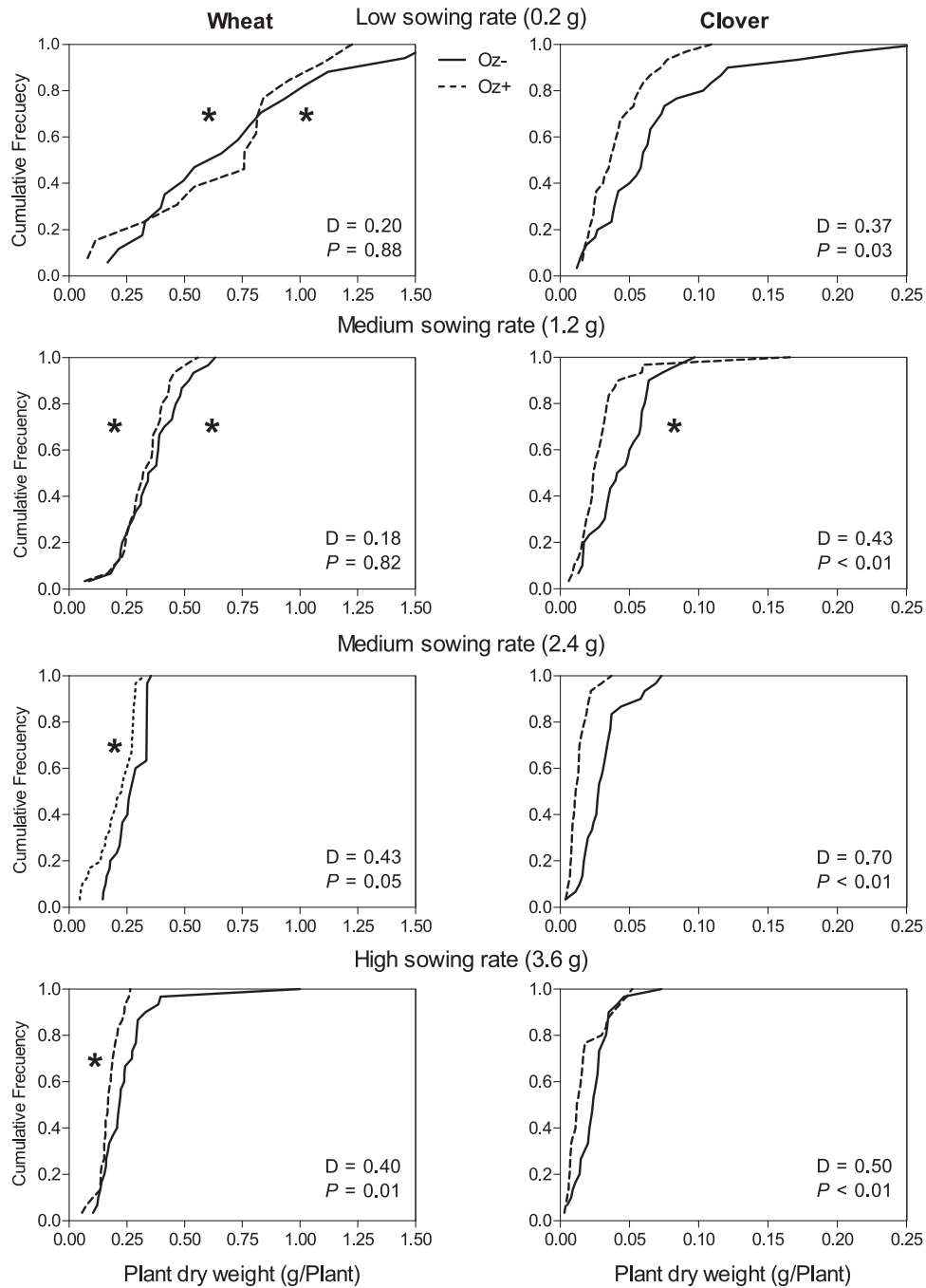
ground area for growth of each individual plant was more affected in clover, and therefore, competition stress was probably more severe for this species (Mithen et al. 1984).

Growth parameters of individual plants were modified by ozone to a different magnitude, depending on sowing density. The effect of ozone on plant biomass was lower for both species at high plant densities. This was somehow unexpected, as competition would jeopardize anti-

oxidant production (Davies et al. 2003). The cumulative dose of ozone taken up by leaves, which is determined by both stomatal conductance and ozone concentration at the leaf level, is assumed to be a key factor influencing ozone damage to plants (Pleijel et al. 2004). Consequently, differences in ozone sensitivity between species and cultivars have often been positively related to differences in stomatal conductance measured under identical, usually non-limiting environmental conditions (Bungener

Fig. 3. Species' contribution to total biomass in pots seeded with different proportions of wheat and clover, for plants growing in the control (Oz–, open bars) and ozone (Oz+, filled bars) treatment groups. The numbers on the bars are the proportions for plant dry mass of wheat and clover.
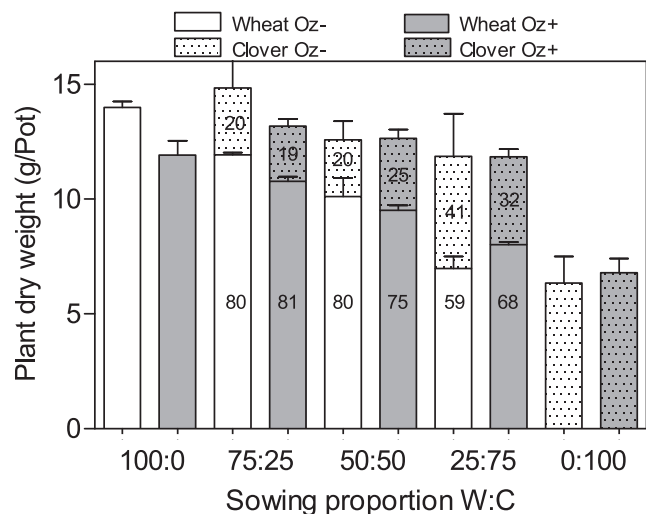
Fig. 4. Number of *Rhizobium* spp. nodules per *Trifolium repens* plant in relation to seed sowing rate in the control (Oz–, open bars) and ozone (Oz+, filled bars) treatment groups. Values are the mean ± SE ($n = 3$ pots; 1 plant in sowing rate 0.003, and 10 randomly selected plants in 3.6). Inset: Correlation between nodule number for *Rhizobium* bacteria and plant mass for *T. repens* in the control (Oz–, solid line) and ozone (Oz+, broken line) treatment groups, corresponding to models obtained through the fitted function from glm.
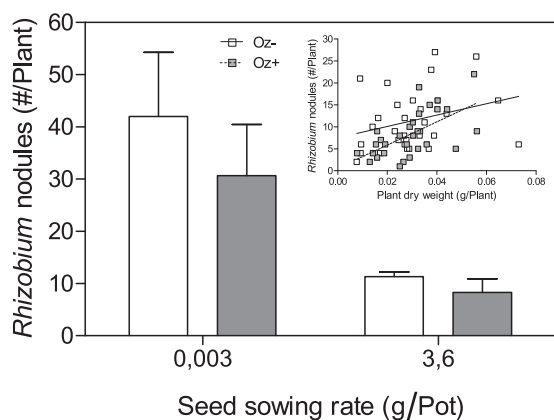
et al. 1999). However, environmental factors such as water availability, which changes with plant density, affect ozone sensitivity in the field (Wilkinson and Davies 2010). In any case, it is possible that the lower contaminant effect of ozone on plants growing at high densities was not related to a higher level of tolerance, but to a reduction in the amount of ozone entering the plants, owing to lower stomatal conductance.

On the other hand, plant responses to ozone may be dependent on average individual mass and on the pattern of individual plant size classes resulting from competition and environmental conditions. Testing whether and how subordinate individuals differ from dominants in the response to polluted atmospheres is important for understanding future stand and community structure. We predicted that subordinate and dominant plants growing in dense stands would not equally respond to ozone, and that the resulting reduction in growth would reduce size inequalities in competing stands. In this study, ozone differentially affected the size of individual plants of wheat and clover at different sowing densities. In general, when intraspecific competition was low (i.e., small asymmetry among individuals and normal pattern for frequency of individual sizes), ozone similarly affected all individual-size classes; but when competition was strong, ozone reduced the asymmetry among individuals, mainly by reducing the number of the largest individuals in the population. These results concur with our previous findings for another annual grass species, *L. multiflorum*, in which ozone differentially affected large and small size individuals, thereby modifying distribution frequencies (Martínez-Ghersa and Radosevich 2009). These changes in size inequalities may have profound importance for the stability of production per unit area, resource allocation to reproductive structures, and evolutionary responses to selection (Pan et al. 2003).

The effect of ozone in highly crowded mixtures of the two species was not altered by the proportion of individuals in the mixture, and could not be predicted by the results of the monocultures at several densities. Previous studies with mixtures of *T. repens* – *L. perenne* (Nussbaum et al. 1995) and *T. repens* – *Festuca arundinacea* (Heagle et al. 1989) reported decreases in the proportion of *T. repens* due to exposure to increased ozone levels. However, in this study, the reduction in the contribution of clover to total biomass per pot exposed to ozone was less than expected, given its higher susceptibility to the pollutant. Moreover, ozone affected total biomass per pot in the wheat monocultures, but total biomass remained unaffected in clover monocultures and in the mixtures. This trend suggests that a change in relative plant size caused by ozone may be differentially compensated by the output of the CO_2 assimilatory apparatus of both species, producing no net change in biomass accumulation. Clover's high sensitivity to ozone evidenced in the pure stands was not expressed when it was growing mixed in with wheat because of the canopy's increased complexity (Jäggi et al. 2006). The grass leaves occupy a larger proportion of the above-ground volume, especially at the top layers of the canopy, and the relative difference in leaf density per unit of air volume and in the growth rate between the species in the mixture resulted in greater ozone exposure for wheat than for clover. However, the reduction in interspecies competition that may have resulted from the negative impact of the pollutant on the growth of wheat was not expressed in the ability of the

clover plants to capture more resources and produce more biomass. This inability of the clover plants to respond to the reduction in interspecies competition is probably due to the high level of intraspecific competition among the clover plants, which was always important, even under the effects of ozone.

The susceptibility of clover to ozone is, at least in part, due to the indirect negative effect on its symbiotic interaction with nitrogen-fixing bacteria. Despite the large variability in the number of nodules per plant biomass, the highest values were registered for plants not exposed to ozone. Ozone affected clover plant growth and, to some extent, the ability of individuals to have high number of nodules per plant. The negative impact of ozone on the symbiotic interaction between Fabaceae plants and nitrogen-fixing bacteria may be caused by two non-exclusive mechanisms. Ozone had a greater impact on small, suppressed, individuals that would have fewer assimilates to support the symbiotic relationship. This assertion is in accordance with previous findings showing that air enriched with ozone reduced *Rhizobium* root nodule formation in white clover, probably due to a reduction in carbohydrate transport rate to the roots in ozone-treated plants (Andersen and Rygiewicz 1999; Awmack et al. 2007). The relative cost of sustaining the symbiont could be increased under the stress imposed by ozone, and be expressed in the ability of the individuals to grow (Partida-Martínez and Heil 2011). Finally, ozone can activate plant hormonal defense mechanisms (e.g., salicylic acid) (Ogawa et al. 2005; Tamaoki 2008) and impair the association of the plant with beneficial microorganisms (Walters 2010); in particular, the process of *Rhizobium* nodule formation in *Fabaceae* (Lian et al. 2000). The mechanisms behind the impact of ozone on symbiosis and the greater susceptibility of clover to this pollutant remains to be elucidated.

In summary, our findings show that ozone had a generally negative impact on biomass and leaf area production per individual and per unit area of both species. Clearly, the impact of ozone was higher for clover than wheat, and was altered by density effects. In part, the higher sensitivity to ozone showed by clover could be explained by the negative effect on the establishment of rhizobia root nodules in the leguminous plants, interfering with the mutualistic interaction between them. Nevertheless, the species sensitivity to ozone was not evidenced when plants grew in a mixture. The response to ozone in the mixtures was not affected by species proportion. These results suggest that competitive and mutualistic interactions might have a greater role in determining responses to novel atmospheric pollutants than species sensitivity to the pollutant per se.

Acknowledgements

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