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

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Botany</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>cjb-2016-0275.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>21-Apr-2017</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>N/A</td>
</tr>
<tr>
<td>Keyword:</td>
<td>air pollution, tropospheric ozone, intraspecific and interspecific competition, Rhizobium, Triticum aestivum and Trifolium repens</td>
</tr>
</tbody>
</table>
Assessing the impacts of intra and interspecific competition between *Triticum aestivum* and *Trifolium repens* on species responses to ozone.

Analía I. Menéndez*, Pedro E. Gundel, Laura M. Lores, M. Alejandra Martínez-Ghersa

*E-mail address: analiam@agro.uba.ar*

IFEVA, Facultad de Agronomía, Universidad de Buenos Aires, CONICET.

Av. San Martin 4453 C1417DSE. Buenos Aires, Argentina.
Abstract

Tropospheric ozone is considered the most phytotoxic air pollutant due to its oxidizing power. The main objective of this study was to analyze the effect of intra and interspecific competition between *Triticum aestivum* 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 above responses. Monocultures and mixtures of both species in different densities were sown. Pots were transferred to open top chambers with 90-120 ppb and 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 might have a greater role in determining responses to novel air pollutants than species sensitivity to the xenobiotic *per se*.

Keywords: air pollution, tropospheric ozone, intraspecific and interspecific competition, *Rhizobium, Triticum aestivum* and *Trifolium repens*
Introduction

Global background ozone concentration has increased since the pre-industrial era due to anthropogenic emissions of its precursors, pollutants such as nitrogen oxides ($\text{NO}_x$) 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, might 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 of agricultural production occur at ozone exposure above 40 ppb, a threshold level reached in a number of countries worldwide (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 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 ozone might not only affect individual plant growth, but also the pattern
of competitive interactions, through changes in biomass partitioning. In some species above-ground 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 at lower rates (Weiner 1985). Competition itself could also modify the impact of ozone or the species response to the pollutant. Under competition, differences in individual size of the same or different species are exacerbated as large plants usurp resources and grow at the expense of small ones, generating a hierarchy of sizes and exploitation. Reduction in growth rate of smaller plants could result in a decreased ozone uptake, indirectly erasing competitive differences between plants (Fredericksen et al. 1996). Moreover ozone effects on plant species mixtures could depend on the characteristics of the species involved, their mixing ratio, or on environmental conditions (Nussbaum et al. 2000; Bassin et al. 2007). Results based on quality parameters support that legumes are more *O_3*-sensitive than several 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 between species and change the composition of pastures (Nussbaum et al. 1995; Wilbourn et al. 1995; Gimeno 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 the ozone-induced growth reduction was similar to the reduction in monocultures. However, when water availability was restricted, the above mentioned results were reverted (Nussbaum et al. 2000).
Indirect effects of ozone and plant responses to the pollutant may occur due to interactions with symbiotic organisms. Although penetration of ozone into soil supporting plants is believed to be limited essentially 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 roots of the 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 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 if early ozone exposure would have an impact on the establishment of the mutualistic interaction between the legume species and *Rhizobium spp*. We predicted that (1) the effect of ozone on plant growth depends on the species and sowing density, (2) the effect of ozone on biomass production per plot depends on species proportion, and (3) ozone effects on the legume species negatively impact the establishment of rhizobia root nodules interfering with their mutualistic interaction.
Materials and methods

We tested our predictions growing seedlings of *Triticum aestivum* (var. Buck) (wheat) and white clover 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.

Experimental procedures

Seeds of both species were sown in monocultures and mixtures in 1000 cm³ (15 cm diameter) plastic pots filled with a mixture of regular potting substrate. All pots were placed in a greenhouse. Mean temperature during the experiment was 15.2 ± 4 °C. Monocultures consisted in a series of densities obtained by sowing an increasing weight of seeds per pot: <0.003 g, 0.2 g, 1.2 g, 2.4 g, 3.6 g, 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 rate was 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. On the contrary, we did not try to reproduce natural conditions, but instead we aimed to assess the effect 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\(^{-1}\)) 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 by seeds of the other species (replacement series) (de Wit 1960). From the combination of the different weights of seeds sown, five proportions resulted in the mixtures: 100:0, 75:25, 50:50, 25:75 and 0:100 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 and three control chambers. After ozone treatment all pots were returned to the greenhouse (see below).

**Ozone exposure treatments**

Six 8 m\(^3\) “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), 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, CA). Ozone chambers received a daily fumigation that increased ozone concentration to a maximum of 90 to 120 ppb. Mean day temperatures were regulated and monitored at 15º 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 lux with cloud cover. Photoperiod during the experiment was about 14 hrs.

Half of the pots of each monoculture and mixture treatments were randomly assigned to the ozone exposure treatment (Oz+) and the other half to controls (Oz-). Pots corresponding to each group were further separated into three groups and randomly assigned to one of each 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 of the Oz+ treatment were exposed for 4 h per day during five days, while the rest of the plants received ozone-free air (Oz-).
Plant growth measurements

Two weeks after ozone exposure, average leaf area per leaf was estimated in ten randomly selected leaves per pot in the all treatments. Clover leaf area per leaf was first calculated using the maximum width (W) and length (L) of each leaf in the equation WxL/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 maximum wheat leaf length was chosen as the best estimate of average leaf area for plants under different densities (Cogliatti et al. 2010). Leaf area of all 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 weight 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 plant material was oven-dried at 70°C for a minimum of 4 days before biomass was determined.

A separate run of the clover monocultures experiment 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 weights were assessed and on each plant, the number of root nodules containing Rhizobium was counted.

Statistical Analysis
In order to increase replicability, the experiments were done twice, in different years. Data was first analyzed considering year a random factor and ozone and densities as fixed factors (Moore and Dixon 2015). As there was no effect of year and no year x treatment interactions, we pooled data from both years for further analysis and presentation. Two way Analyses of variance (ANOVA) followed by Tukey test for multiple comparisons was used to evaluate differences in leaf area/leaf and leaf area/plant for wheat and clover plant in monocultures, due to ozone exposure (Oz- vs. Oz+) and sowing rate. ANOVA was also used to evaluate differences in plant dry weight/plant and plant dry weight/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. Crowding effect on intraspecific competition for each species and 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, since this is the recommended way of comparing two or more data frequency sets (Chakravati et al. 1967). We tested the cumulative size-class frequency curves (CSCFC) of different individuals under each ozone treatment for normality, using Kolmogorov-Smirnov nonparametric test. We then compared each pair of curves (species x 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 weight (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 applied and models adequacy was assessed graphically (Crawley 2007; Zuur et al. 2009).
Results

Clover plants exposed to ozone had smaller leaves than control plants ($P < 0.001$) while density decreased mean leaf area independently of ozone treatment ($P = 0.056$). Across all sowing densities, average reduction of leaf area for clover was $45\%$. Leaf size of wheat plants was not sensitive to either density or ozone (Table 1). In contrast O$_3$ and density reduced leaf area/plant of 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 ozone condition, plants showed a decrease of individual plant biomass with increasing 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). 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). Ozone effect was much stronger on clover since 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 in the same condition ($P < 0.001$) (Fig. 3). In mixture treatments, wheat always
contributed in a larger proportion to 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 densities was not apparent when the wheat was growing together with clover (Fig. 3).

Number of nodules per plant were highly dependent on 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 exposure treatment ($Z$-value $= 1.364$, $P < 0.172$). Nonetheless, ozone reduced on average, the number of nodules (estimate $= -0.349$) relative to that of not exposed 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 four fold more nodules per g of plant biomass than the average for all plants, whereas 2/3 of exposed plants (20 individuals) had below the average for all plants (data not shown).

**Discussion**

Wheat and clover exhibited differential responses to elevated ozone and these effects were altered by competition. Ozone produced visible injury in both species; however, reduction of leaf area and biomass per plant was larger for clover than for wheat, suggesting that wheat was more tolerant to ozone than clover. This is concurrent with past literature showing higher susceptibility of Fabaceae (legume) species to ozone (Gimeno et al. 2004b; Sanz et al. 2011). In particular, clover 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 weight) and biochemical defense.
capacity (Batty and Ashmore 2003). Wheat leaf area per plant was reduced by ozone, although mean leaf size was not affected. In turn, both mean leaf size and leaf area/plant were reduced by ozone exposure in clover, suggesting that metabolic activities involved in clover leaf expansion were impaired by ozone, while number of total leaves or leaf duration were the parameters affected in wheat. As a result, plants of both species exposed to ozone probably resulted in different changes in SLA determining further 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 photoasimilates (Fuhrer and Booker 2003), which in turn might be affected under competitive stress (Davison and Barnes 1998). In our study both species grew under a different density range, i.e. the same seed weight sown corresponded to a higher seed number for clover than for wheat. As a consequence, available 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 in a different magnitude depending on sowing density. Ozone effect on plant biomass was lower for both species at high plant densities. This was somehow unexpected as competition would jeopardize antioxidant 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 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 et al. 1999). However, environmental factors such as water availability, which changes with plant density, modify ozone sensitivity in the field.
(Wilkinson and Davies 2010). In any case, it is possible that the lower contaminant effect on plants growing at high densities was not related to a higher level of tolerance but to a lower amount of ozone entering the plants due to lower stomatal conductance.

On the other hand plant response to ozone may be dependent on average individual weight 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 the present study ozone impact modified the frequency of individual size at different densities for wheat and clover. 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 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. 1988) reported
decreases in *T. repens* proportion due to exposure to elevated ozone. However in this study, the reduction of clover contribution 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 it 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 pure stand was not expressed when it was growing in mixture with wheat due to 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 growth rate between the species in the mixture resulted in a greater ozone exposure for wheat than for clover. However the relaxation of interspecific competition that may have resulted from the negative impact of the pollutant on the growth of the grass plants was not expressed in the ability of the clover plants to capture more resources and produce biomass. This inability of the clover plants to respond to relaxation of the interspecific competition is probably due to the high level of intraspecific competition among the clover plants which was always important even under the effect of the air pollutant.

Susceptibility of clover to ozone was, at least in part, due to its indirect negative effect on its symbiotic interaction with the 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. Negative impact of ozone on the symbiotic interaction of Fabaceae plants and nitrogen-fixing bacteria may be caused by two non-exclusive mechanisms. Ozone had greater impact on small suppressed individuals that would have few
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 root 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 the pollutant and expressed in the ability of the individuals to grow (Partida-Martínez and Heil 2011). Finally, ozone can activate the plant hormonal defense mechanisms (*e.g.* salicylic acid) (Ogawa et al. 2005; Tamaoki 2008) and impair the association of plant with beneficial microorganisms (Walters 2010); in particular, the process of *Rhizobium* nodule formation in *Fabaceae* (Lian et al. 2000). The mechanism behind the impact of ozone on the symbiosis and the greater susceptibility of the clover to the pollutant remains to be disentangled.

In summary, our findings show that ozone had generally a 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 legume plants interfering with the mutualistic interaction between them. Nevertheless the species sensitivity to the pollutant was not evidenced when plants grew in mixture. Response to ozone in 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

This study was financially supported by grants of University of Buenos Aires and Foncyt (Argentina). The authors are thankful to Jennifer Landesmann and Laura Ventura for help with field work.

References


Sparks, B., Mundsaw, G., Williams, D., and Barrett, M. 2015. Preplant cultivation techniques and planting date effects on white clover establishment into an existing cool season turfgrass sward. Hort. Sci. **50**: 615-620.


Table 1: Leaf area production of plants growing in monocultures under control (Oz-) or O₃ (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     |
| Sowing rate  | 19.06 | 0.27            | 20.28              | 0.056 |
| O₃           | 8.41  | 0.13            | 42.42              | < 0.000 |
| Sowing rate x O₃ | 5.13  | 0.82            | 0.59               | 0.987 |

* Significant difference between Oz- and Oz+ within each species and sowing rate (P<0.05)
**Figure captions**

**Figure 1**: Biomass accumulation at final harvest for wheat and clover plants growing in monocultures at different sowing densities in control and ozone (Oz- and Oz+) treatments. Bars are means ± standard errors.

**Figure 2**: Cumulative frequency of individual plant weight (g/Plant) in control (Oz-; solid line) and ozone (Oz+, dashed line) treatments for wheat and clover monocultures. D statistic and $P$ value comparing both data sets according to Kolmogorov-Smirnov (KS) test. Asterisks indicate weights of plants under that treatment are consistent with a normal distribution.

**Figure 3**: Species contribution to total biomass in pots sowed with different proportions of wheat and clover seeds, for plants growing in control (Oz-, white bars) and ozone (Oz+, grey bars) treatments. The numbers on bars are the plants dry weight proportions of wheat and clover.

**Figure 4**: Number of *Rhizobium* spp. nodules per *Trifolium repens* plant in relation to seed sowing rate in control (Oz-, white bars) and ozone (Oz+, grey bars) treatments. Values are mean (n = 3 pots; 1 plant in sowing rate 0.003, and 10 randomly selected plants in 3.6) ± standard error. Inset: Correlation between nodule number of *Rhizobium* bacteria and *T. repens* plant weight in control (Oz-, solid line) and ozone (Oz+, dashed line) treatments, corresponding to models obtained through the fitted function from glm.
Figure 1

Wheat

Clover

Seed sowing rate (g)

Seed sowing rate (g)
Figure 2
Figure 3

The bar chart shows the plant dry weight (g/Pot) for different sowing proportions of wheat (W) to clover (C) in both Oz-negative (Oz-) and Oz-positive (Oz+) conditions. The sowing proportions range from 100:0 to 0:100. The chart indicates that the plant dry weight varies depending on the sowing proportion and whether the conditions are Oz-negative or Oz-positive.
Figure 4