Highlighting the threat from current and near-future ozone pollution to clover in pasture

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Full research paper

Abstract

Globally, the legume-rhizobia symbiosis, contained within specialised organs called root nodules, is thought to add at least 30 Tg N annually to agricultural land. The growth and functioning of a modern white clover (Trifolium repens cv. Crusader) and red clover (T. pratense cv. Merviot) cultivar were investigated in current and future ozone scenarios in solardomes. Both cultivars developed leaf injury and had significant reductions in root biomass and root nodule number in response to ozone, with Crusader also displaying a reduced size and mass of nodules. In-situ measurements of N-fixation in Crusader by acetylene reduction assay revealed reduced N-fixation rates in a future scenario with an increased background and moderate peaks of ozone. The implications for the sustainability of temperate pasture are discussed.

Capsule: ozone effects on the growth and functioning of clover cultivars

Keywords: clover; nodulation; ozone; nitrogen fixation: pasture; background ozone

Introduction

Nitrogen (N) fixation by legumes (Fabaceae) is of vital agronomic importance. On a global scale, the legume-rhizobia symbiosis, contained within specialised organs called root nodules, is thought to add at least 30 Tg N annually to agricultural land (Herridge
et al. 2008). At present, legume crops account for ~15% of utilised arable land area (FAO, 2013), constituting the primary source of dietary protein for a substantial proportion of the human population. Legumes are also an essential component of many pasture systems; improving the protein content, nutritional value and uptake of forage, as well as providing ancillary benefits to the structure and long-term fertility of soils (Parsons & Chapman, 1999). In temperate regions of Europe, Oceania and the Americas, clovers (Trifolium spp.) are the most important pasture legume. Surprisingly, given the agricultural importance of clover, little attention has been paid in recent decades to the fact that Trifolium spp. are amongst the most sensitive known to ground-level ozone pollution (e.g. Hayes et al. 2007). Worryingly, concentrations of tropospheric ozone have risen in that time over arguably all of the clover-growing regions of the world (The Royal Society, 2008). The potential for losses in quantity and quality of pasture forage, with a concurrent need for increased usage of artificial fertiliser in current and near-future ozone regimes, formed the motivation for this study.

At present, background levels of tropospheric ozone are high enough to damage sensitive crops across the Northern Hemisphere (Mills et al. 2011a), with a mean concentration of 30-40ppb representing a doubling of the pre-industrial background (Vingarzan, 2004). In respect of its threat to agricultural production and food security, tropospheric ozone is the most important air pollutant (Avnery et al. 2011; Mills et al. 2011a; Wilkinson et al. 2011). Ozone damage occurs in plants via the induction of oxidative stress, leading to foliar injury, impacts on gas exchange, photosynthesis, growth and eventual yield (Wilkinson et al. 2011).

Grassland systems and constituent species have been identified as particularly sensitive to ozone pollution (e.g. Hayes et al. 2007; Mills et al. 2007). Indeed, numerous studies have highlighted the complex response of managed grasslands to ozone (for reviews see Bassin et al. 2007 & Fuhrer, 2009), with pasture forage...
susceptible to reductions in quality and yield, as well as shifts in species composition, with uncertain effects upon the carbon (C) sink strength of grassland systems (see Mills et al. 2012). Most previous experiments on ozone effects on clover were conducted in the 1970s and mid-1990s, usually with ozone profiles exhibiting high peaks and a low baseline concentration, no longer representative of current ambient conditions in Europe. Due to the improved control of precursor emissions, local peak concentrations of ozone have decreased in Europe in the last 20 years, whilst the baseline has steadily risen, in part due to the hemispheric transport of ozone precursors from other regions (Parrish et al. 2012). Furthermore, previous studies often used relatively high ozone concentrations, delivering unrealistically acute dosages (e.g. Letchworth & Blum, 1976; Blum et al. 1983). Results from studies with mixed-species swards are highly complex and range from a gradual reduction in yield of the Trifolium fraction to no overall effect on botanical composition (e.g. Blum et al. 1983; Rebbeck et al. 1988; Heagle et al. 1989; Fuhrer et al. 1994; Ashmore & Ainsworth, 1995; Pleijel et al. 1996; Nussbaum et al. 1995; Wilbourn et al. 1995; Gonzalez-Fernandez et al. 2008; Hayes et al. 2009). Differential sensitivity to ozone induced foliar injury within Trifolium spp. lends utility for their use as ozone biomonitors (Mills et al. 2011b).

Nodulation in legumes is primarily controlled by long distance root and shoot-derived signalling (termed autoregulation of nodulation (AON)) (Mortier et al. 2012). A complete understanding regarding the molecular nature of AON signalling, and more generally, the role of C and N supply in the determination of nodule number, remains obscure (e.g. Ludidi et al. 2007; Mortier et al. 2012). N-fixation is an energy-intensive process, and nodules in legumes are a strong sink for assimilates, such that root and shoot growth may be suppressed in hypernodulating mutants (e.g. Ito et al. 2007; Yoshida et al. 2010). Superfluous nodulation is regulated by a shoot-derived inhibitor (SDI), with the long-distance transport and differential concentration of auxin,
brassinosteroids and jasmonic acid (JA) suggested as likely candidates for the SDI signal (Mortier et al. 2012). Nodulation is also determined by local hormonal regulation, with JA, abscisic acid (ABA) and ethylene together acting as local negative regulators of nodule initiation (Mortier et al. 2012).

Ozone-impacts on nodulation or N-fixation have been shown in several legumes including soybean (Tingey & Blum, 1973; Reinhart & Weber, 1980; Jones et al. 1985; Pausch et al. 1996), peanut (Ensing et al. 1985; Cong et al. 2009) and beans (Manning et al. 1971; Blum & Heck, 1980). Research by Blum & Tingey (1977) does not support a significant direct influence of ozone on legume root nodules, with reduced photosynthate translocation suggested by this, and other studies, as the cause for a reduction in nodule growth (e.g. Tingey & Blum, 1973; Reinhart & Weber, 1980).

Stable isotope studies by Pausch et al. (1996), and Cong et al. (2009), also attribute ozone impacts on N-fixation to a reduced availability of assimilate. However, relatively few studies have directly addressed the impacts of ozone on clover nodulation; still less having explored the mechanistic basis of these effects, and the potential impacts on pasture sustainability caused by the current and near-future concentrations of ozone.

Letchworth & Blum (1976) reported a reduction in nodule growth in T. repens in response to acute exposure in closed chamber studies, although nitrogenase activity per nodule, and per plant, was not significantly altered. In contrast, Ensing et al. (1982), and Montes et al. (1983), in open-top-chamber studies, reported ozone-induced reductions in N-fixation in T. pratense and T. repens respectively. Further, ozone-induced reductions in total N or % N in T. repens biomass are reported by Letchworth & Blum (1976), Blum et al. (1983) and Montes et al. (1983), with some studies reporting some effect upon the crude protein content (e.g. Blum et al. 1983; Fuhrer et al. 1994; Sanz et al. 2005) and digestibility (e.g. Fuhrer et al. 1994; Sanz et al. 2005; Muntifering et al. 2006; Gonzalez-Fernandez et al. 2008) of Trifolium forage. Ozone impacts may occur
in earliest root tip development in Trifolium spp. (Vollnes et al. 2010), whilst infection
by rhizobia may afford some level of protection to ozone impacts on growth relative to
non-inoculated controls (Miller et al. 1997).

Given the considerable agronomic importance of clover, there is a need to update and
expand our understanding of the influence of ozone on nodulation and N-fixation in
current clover cultivars. In this study, the effects of ozone on the injury, stomatal
conductance (gs) and biomass accumulation of T. repens and T. pratense cultivars,
recommended for general use in grazed leys (British Grassland Society, 2013) are
assessed, with ozone exposure profiles representing a realistic range of reduced peak
and increased baseline scenarios. The effect of ozone on the nitrogenase activity of the
T. repens cultivar is also determined in-situ, and potential implications for the
sustainability of temperate pasture are discussed.

Materials and methods

Clover cultivars

T. repens cv. Crusader, a medium-leaved cultivar used for frequent cutting and grazing,
and T. pratense cv. Merviot, used for cutting and finishing autumn stock, (hereafter
referred to as Crusader and Merviot) were sown as seeds into cell trays in compost
(John Innes No. 2; J. Arthur Bowers, Lincoln, UK) in late spring 2012. Seeds were
obtained from a commercial seed supplier, and originated from the UK (Wynnstay
Seeds; UK). Plants were propagated in plug-plant trays in an unheated glass-house,
watered by hand as necessary and thinned when appropriate to one seedling per cell.

After 3 weeks of growth, seedlings of each cultivar were transferred into 5L plant pots
(22cm diameter x 19.1cm depth), filled with sterile topsoil (Gravelmaster, UK), with 4
seedlings arranged evenly in each pot. To introduce a soil microbe population, pots
were inoculated with 200ml of a soil slurry mixture made from approximately 5kg of
soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14′N, 4°01′W) and 14L water. Seedlings were grown for a further 4 weeks. On 06/07/2012, 42 pots per cultivar, selected for consistent size, were then transferred to a series of 7 ‘solardomes’ (hemispherical glasshouses; 3m diameter, 2.1m high) at the CEH solardome facility near Bangor, North Wales, with 6 pots of each cultivar per solardome.

Ozone system and treatments.

Plants were then exposed to a range of ozone treatments based on an episodic profile recorded at a rural ozone monitoring site (Aston Hill, Wales, UK, 52°50′N, 3°03′W) with a unique treatment in each solardome. Treatments were designed to reflect future ozone scenarios, with peak concentrations reduced by more than the background (Figure 1). Treatments were applied to the solardomes randomly. Plants were exposed to the ozone treatments for a three-month period, starting 11/07/2012 and finishing 03/10/2012.

Ozone was provided to the solardomes by a G11 ozone generator and a workhouse oxygen generator (Dryden Aqua, UK), with ozone added to charcoal-filtered air, and with concentration determined by a computer-controlled ozone injection system (LabVIEW version 8.6; National Instruments, Texas, US). Ozone was distributed to each solardome via PTFE tubing, with the concentration inside each solardome measured for 5 min every 30 minutes using two ozone analysers (400a, Enviro Technology Services, Stroud, UK) of matched calibration. In one solardome, ambient air temperature, photosynthetically active radiation (PAR) and vapour pressure deficit (VPD) were continuously monitored by an automatic weather station (Skye Instruments Ltd, Llandridod Wells, UK). Plants were rotated within each dome weekly and watered twice-weekly, with additional watering when necessary to maintain soil moisture content at or near field capacity.
After 3 weeks exposure, visible ozone injury and senescence was scored for each cultivar across each ozone treatment. The number of injured leaves (ozone injury >25% leaflet area) in a representative quarter of each pot was recorded and expressed as a percentage of the total number of leaves.

Stomatal conductance (gs)

Stomatal conductance (gs) of both cultivars was determined at intervals throughout the growth season across all ozone treatments in naturally fluctuating climatic conditions. All measurements were made using a porometer (AP4, Delta T Devices, Cambridge, UK), between 10:00-16:00h, on the abaxial surface of leaves displaying <10% ozone injury and senescence. Solardomes were visited in random order, and measurements were made in the presence of ozone. Soil moisture content was determined after every measurement with a hand-held soil moisture probe and sensor (ML2x ThetaProbe, HH2 Moisture Meter; Delta T Devices, Cambridge, UK).

Biomass harvest

After 12 weeks of growth, the shoot, root and nodule mass of the plants from each cultivar was harvested. Shoot biomass was harvested for the entire pot in October. For rapidly-growing Merviot, a mid-season harvest of shoot biomass was also performed in late August after 7 weeks exposure by cutting back to 7cm. Below-ground biomass was determined from a representative quarter of each pot, due only to the extensiveness of the root system. Furthermore, below-ground biomass was determined in treatments 1, 4 and 7 only, as harvest of the roots took almost 3 weeks; even with cold storage, it was considered inappropriate to store soil samples for longer than this due to the re-growth
or decomposition of root material. Nodules were excised from the root system, counted
and weighed. Shoots and roots were dried for a minimum of 48 hours at 60˚C or until
constant mass was achieved. Nodule biomass was air dried and sized into two
categories based on maximum length (<0.1-0.7mm; 0.7->1.5mm). Root biomass, nodule
biomass and nodule numbers per pot were calculated as follows:

Root biomass pot⁻¹ = (root biomass quarter⁻¹/soil mass quarter⁻¹)*soil mass pot⁻¹

Nodule biomass pot⁻¹ = nodule biomass g root⁻¹*root biomass pot⁻¹

Nodules pot⁻¹ = nodules g root⁻¹*root mass pot⁻¹

Mass-per-nodule, root:shoot, total biomass and root:total biomass were also determined.
To allow comparison with previously published data, and to facilitate analysis of ozone
effects on a UK scale, biomass variables were expressed to accumulated exposures
above a threshold of 40ppb during daylight hours at canopy height (AOT40, units
ppm h⁻¹ (after Fuhrer (1994)).

Acetylene reduction assays (ARA)
Assessments of system nitrogenase activity were performed on Crusader in treatments 1
and 7, using a method adapted from Lindstrom (1984). Two weeks prior to the assay,
two sealable 400ml plastic bottles, with the bottom removed and fitted with a gas
septum, were inserted to a depth of 2cm into the centre of each pot. For the assay, a
10% acetylene atmosphere was generated inside one bottle by removing 10% of the air
and immediately replacing it with acetylene gas (BOC, Guildford, UK). The second
bottle acted as a control to determined baseline ethylene generated from the soil.
Acetylene was stored and transported to the solardome facility in inert gas bags
(SUPELCO, Bellefonte, US), which were vented to the atmosphere and flushed through
with N₂ after use. 15 ml gas samples were taken from the bottles at 0, 4 and 8 and 24 hours, with a 1ml sub-sample analysed for ethylene content using a mass-selective detector (Model 6890, Agilent Technologies, Santa Clara, US). Ethylene peak area was determined using G17O1DA analytical software (version D.00.00.38; Agilent Technologies, Santa Clara, US). Two assays were performed, in similar climate conditions, in the 10th and 11th weeks of exposure.

Statistical analyses

The precise ozone control system used in the solardomes allowed small changes in ozone profile to be simulated, facilitating dose-response analyses. We note that the lack of treatment replication may raise concerns about pseudo-replication. However, we believe the benefit of using more treatments outweighs this limitation, as published previously by Mills et al. 2009, Hayes et al. 2012 and others. Air flow rates are matched between solardomes, and where recorded, climatic conditions did not vary significantly from solardome to solardome (e.g. leaf temperature, see supplementary information). For consistency with existing literature, injury and gs, variables were each analysed by general linear regression, with the 3 week (for injury data) or 12 week AOT40 value for each treatment applied as the predictor variable. For biomass and ARA variables, parameters were analysed via one-way analysis of variance (ANOVA) with 12 week AOT40 values in the former and 10 and 11 week AOT40 values in the latter applied as a factor. For nodule size, each size category was analysed separately against the 12 week AOT40 value for each treatment. To exclude outliers due to very high or low PAR, a cohort of gs data for Crusader (n=133) and Merviot (n=104) was selected for analysis using the 25-75% quartile range of all recorded ambient PAR data for each cultivar respectively. Post hoc Tukey’s honest significant difference tests were applied to assess pairwise differences between means where ANOVA revealed a significant
effect of ozone. Insufficient gs data was collected for the modelling of ozone flux-effect relationships. All analyses were conducted using R software version 2.15.2 (R Core Development Team, 2012).

Results

Ozone concentrations and climate conditions

During the course of the experiment, the seven ozone treatments generated seasonal 24 hr means of 33, 36, 40, 45, 51, 54 & 66ppb and AOT40 values of 0.4, 1.0, 2.7, 5.2, 8.6, 11.5 and 18.5ppm h\(^{-1}\) (Figure 1; Table 1). Ozone concentrations increased in each treatment during the weekend reaching a maximum peak on Mondays, and a minimum on Thursdays (Figure 1). The ozone treatments successfully simulated decreasing peak and background concentrations, with greater reductions in peak than background ozone. Mean daylight (when PAR >50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) air temperature and VPD were 21.3°C and 0.84 kPa for the study period, with maxima of 24.6°C and 1.14 kPa. Mean daytime PAR was 521 \(\mu\)mol m\(^{-2}\)s\(^{-1}\), with an average daily maximum of 814 \(\mu\)mol m\(^{-2}\) s\(^{-1}\).

Ozone injury and gs

Both Crusader and Merviot displayed highly significant increases in visible leaf injury with increasing ozone concentrations \((p<0.001\) and \(p=0.01\) respectively) (Figure 2a), with Crusader displaying a significantly higher injury rate with increasing ozone exposure \((p<0.001)\). Baseline injury and senescence were detectable in both cultivars in the lowest exposure treatment (24hr mean of 33 ppb, AOT40 of 0.4ppm h\(^{-1}\)). There was no relationship between mean gs and increasing ozone in Crusader \((r^2<0.10; p=0.54)\) or in Merviot \((r^2=0.21; p=0.09)\) (Figure 2b). However, there was a pronounced cultivar effect, with Merviot displaying significantly higher mean gs rates than Crusader \((p<0.001)\).
Both cultivars had highly significant ozone-induced reductions in root biomass per pot, with a decrease of 61% in Crusader \((p=0.01)\) and 63% in Merviot \((p=0.01)\) in the highest ozone treatment 7 relative to the lowest treatment 1 (Figure 3a). End-of season shoot biomass for each cultivar, and shoot biomass of two individual harvests of Merviot, did not display any significant responses to ozone (Figure 3b). The reduction in root biomass also manifested as proportional declines in root:shoot and root:total biomass ratios for both cultivars (Figure 3c; Table 2). Each cultivar also had reductions in nodule number per pot, with a significant decrease of 36% in Crusader \((p=0.02)\) and reduction of 32% in Merviot \((p=0.09)\) (Figure 3d) in treatment 7 compared to treatment 1. In Crusader, a decreased number of nodules per pot was accompanied by a 40% reduction in the proportion of larger nodules with a maximum length > 0.7mm \((p=0.01)\) (Figure 4a). Consequently, Crusader pots had a 36% reduction in mass-per-nodule \((p=0.04)\) (Figure 3e) and a 60% reduction in nodule mass per pot \((p=0.002)\) (Figure 3f) relative to treatment 1. In contrast, nodule size, mass-per-nodule and nodule-mass-per-pot in Merviot were unaffected by increasing ozone (Figure 3e, f, Figure 4b). However, Merviot displayed increases of 128% in nodule number \((p=0.01)\) and 133% in nodule mass, per gramme of root material \((p=0.02)\), in the high ozone treatment 7 compared to treatment 1 (Table 2). Both Crusader and Merviot experienced a decline in total biomass, with a 13% reduction in the former \((p=0.08)\) and a significant 25% reduction in the latter \((p=0.01)\).

In both assays, a small amount of ethylene was detected after 0 hours, less than 1% of the amount present at the end of the incubation (not shown). In the week 10 assay, mean

**Biomass harvest**

ARA
ethylene evolution per cm$^2$ of soil surface showed a trend for a reduction in treatment 7 after 4 hours incubation compared to treatment 1 ($p=0.06$, Figure 5a). In week 11, ethylene evolution per cm$^2$ was significantly reduced in treatment 7 after 8 hours ($p=0.05$, Figure 5b). No ethylene was detected in either assay after 24 hours.

**Discussion**

This study has updated existing knowledge of the effects of ozone on the growth and functioning of current clover cultivars in present and near-future ozone. We report increased foliar injury and decreased biomass of a white clover (Crusader) and red clover (Merviot) cultivar, with Crusader also displaying a consistent reduction in N-fixation in high ozone. The implications of these effects are discussed below in relation to options for reduction in peak and background atmospheric ozone concentrations.

In the present study, Crusader and Merviot both displayed a partitioning of ozone effects, with systemic reductions in below-ground and total biomass, and an absence of ozone impacts on shoot biomass despite the occurrence of ozone-induced foliar injury and senescence. The maintenance of growth in the shoots at the expense of root biomass has been demonstrated previously in *Trifolium* spp. (e.g. Letchworth & Blum, 1977; Miller et al. 1997), and is otherwise extensively reported as a common response to ozone-induced oxidative stress. Foliar injury may similarly occur in chronic ozone exposures without an effect on above-ground biomass (e.g. in potato; Temmerman et al. 2002). While foliar injury in *Trifolium* spp. may display closer correlations with ozone flux in pasture vegetation than when related to accumulated exposure indices (Mills et al. 2011b; 2011c), clear linear relationships were found with AOT40 values in the non-water limiting conditions of this study.

The overall reduction in nodules-per-pot observed in both cultivars may have arisen from a general reduction in the translocation of photoassimilates to the root system, but
more specifically due to an enhanced regulation of nodulation via downstream AON (Mortier et al. 2012). A reduction in nodule growth in Crusader, manifesting in a reduced mass-per-nodule and an increased proportion of small, likely non-fixing, pseudonodules (Figure 4), would also suggest a reduced availability of assimilate in the root system. This also explains consistent differences in nodule activity (measured by in-situ ARA) in Crusader between treatments 1 and 7. In Merviot, higher gs rates may hint at a greater capacity to supply root nodules with assimilates during ozone-induced oxidative stress (Figure 2), explaining why the growth of individual root nodules was unaffected (Figure 3e; 4b).

The role of phytohormones in moderating above-ground stress responses to ozone is well established, (e.g. Rao & Davies, 2001; Wilkinson & Davies, 2009; Cho et al. 2011), though the influence of ozone on their below-ground action and accumulation remains poorly characterised. In Merviot, the significant increase in nodule density per gramme of root biomass may suggest a decrease in ethylene sensitivity localised within the root vasculature to maintain plant growth (Lohar et al. 2009; Mortier et al. 2012; Chan et al. 2013). Ozone-induced stress ethylene is hypothesised as a general antagonist for ABA signalling (Wilkinson & Davies, 2009). We therefore speculate that an increase in nodule density may also have arisen due to a down-regulation in ABA synthesis and/or signalling, mediated by ozone-induced increases in below-ground ethylene. The results presented, here support the synthesis of published data by Hayes et al. 2007, which indicated a lower ozone sensitivity in *T. pratense* compared to *T. repens*, perhaps due to differences in photosynthetic capacity and resilience, and/or in the production and action of endogenous defence compounds (e.g. Francini et al. 2007).

In subterranean clover (*T. subterranean*), exposure to comparable mean concentrations of ozone affect forage quality in as little as 30 days (Sanz et al. 2005), with impacts in *T. repens* readily apparent after a 3 month period (González-Fernández...
et al. 2008). However, ozone impacts on forage quality, and, in particular, the N and crude protein content of Trifolium forage, do vary, depending on exposure method and community composition, and it is unclear from the available literature how well these parameters lend to assessments of ozone sensitivity within and between Trifolium spp. (Letchworth & Blum, 1976; Blum et al. 1983; Montes et al. 1983, Fuhrer et al. 1994; Sanz et al. 2005). To some degree, ozone impacts on leguminous N-fixation can be compensated by an increased supply and uptake of soil N in short term exposures (e.g. Pausch et al. 1996; Cong et al. 2009). The effect of ozone on forage quality and the soil N pool, through leaf chemical composition and indirectly via reduced fixation, were not determined in this study, but are worthy of further study.

The most severe impacts of ozone on clover biomass, nodulation and N-fixing activity observed in this study occurred in a weekly repeated present-day ozone profile (treatment 7). Further investigations are needed to determine whether these effects presently occur on a landscape-scale basis, and indeed, whether such impacts translate to measurable declines in the productivity, and hence the profitability, of pasture. Nevertheless, on the basis of this study, average reductions in N-fixation, determined after an 8 hour ARA incubation, may potentially lead to an increased fertiliser usage in the highest ozone scenario with additional costs to producers, and potentially detrimental environmental impacts.

Conclusions

This study has provided for the first time some insight into beneficial effects of progressive controls on ozone precursors. On the evidence, controls leading to decreases in peak ozone concentrations by ~30ppb and baseline concentrations by ~10ppb may increase root nodule biomass of white clover by as much as 45%. Controls on the emission of ozone precursors have been included in recent multi-model predictions,
suggesting a globally reduced tropospheric ozone burden by year 2030 in most relative concentration pathways (RCPs) (Young et al. 2013), with regional concentrations displaying an increased sensitivity to climate change (Langner et al. 2013). The potential impacts of ozone on the biomass, nodulation and N-fixation of clover described in this study thus provide a continuing economic and environmental incentive for controls on the emission of trans-boundary ozone precursors.

Acknowledgements

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References


List of figures

Figure 1: Average weekly ozone profile for the seven ozone treatments (see Table 1 for treatment details).

Figure 2: Effects of ozone exposure on (a) mean ozone-induced injury after 3 weeks exposure; (b) mean gs, from measurements made in weeks 4, 5, 8 & 9 where PAR was 317-849 µmol m⁻² s⁻¹ (where white points = Crusader; filled points = Merviot; bars are standard errors).
Figure 3: Change in (a) root biomass pot\(^{-1}\) (b) shoot biomass pot\(^{-1}\) (c) root:shoot (d) nodules pot\(^{-1}\) (e) mass nodule\(^{-1}\) & (f) nodule mass pot\(^{-1}\) in relation to 3 month AOT40 (where white bars = Crusader; grey bars= Merviot; asterixes (*) denote a difference at the \(p=0.05\) level after post-hoc Tukey tests).
Figure 4: Effects of ozone on nodule size in (a) Crusader; (b) Merviot (where white bars = number of nodules between 0.1mm-0.7mm maximum length; grey bars = number of nodules >0.7mm long; asterixes (*) denote a difference at the $p=0.05$ level after post-hoc Tukey tests).
Figure 5: Ethylene evolution in (a) week 10 and (b) week 11 ARAs (where white bars = low ozone treatment 1; grey bars = high ozone treatment 7; asterixes (*) denote a difference at the $p=0.05$ level after post-hoc Tukey tests).
Table 1: Summary of ozone treatments, including minimum and maximum, and climate conditions for the duration of the experiment.

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<th>Treatment</th>
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<td>45</td>
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<td>30</td>
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<td>32</td>
<td>34</td>
<td>33</td>
<td>44</td>
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<td>Season max. conc. (ppb)</td>
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<th>VPD (kPa)</th>
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<td>Max.</td>
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<td>21.3</td>
<td>24.6</td>
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PAR, photosynthetically active radiation; VPD, vapour pressure deficit
Table 2: Summary of additional biomass data. Values are means and standard errors. Significant p values are highlighted in bold.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Crusader</th>
<th></th>
<th>Merviot</th>
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<td>4</td>
<td>7</td>
<td>p</td>
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<td>7</td>
<td>p</td>
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<td>Nodule number (g⁻¹ root biomass⁻¹)</td>
<td>23±6.5</td>
<td>14±3.0</td>
<td>33±4.0</td>
<td>0.11</td>
<td>14±1.5</td>
<td>19±2.5</td>
<td>32±8.5</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Nodule biomass (mg g⁻¹ root biomass⁻¹)</td>
<td>11±2.0</td>
<td>6±1.0</td>
<td>12±2.5</td>
<td>0.56</td>
<td>3.0±0.5</td>
<td>3.0±0.5</td>
<td>7.0±2.0</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Root: total biomass</td>
<td>0.43±0.02</td>
<td>0.37±0.05</td>
<td>0.20±0.009</td>
<td><strong>0.001</strong></td>
<td>0.26±0.02</td>
<td>0.23±0.04</td>
<td>0.13±0.02</td>
<td><strong>0.008</strong></td>
<td></td>
</tr>
<tr>
<td>Total biomass (g pot⁻¹)</td>
<td>51±1.0</td>
<td>57±2.0</td>
<td>44±0.5</td>
<td>0.08</td>
<td>71±5.0</td>
<td>76±4.0</td>
<td>53±2.0</td>
<td><strong>0.01</strong></td>
<td></td>
</tr>
</tbody>
</table>