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

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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.

1. 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 and 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 30–40 ppb 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

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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 and 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 and Ainsworth, 1995; Pleijel et al., 1996; Nussbaum et al., 1995; Wilbourn et al., 1995; González-Fernández 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 and Blum, 1973; Reinhart and 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 and Heck, 1980). Research by Blum and 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 and Blum, 1973; Reinhart and 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 and 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 and Hofstra (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 and 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; Munifering et al., 2006; González-Fernández 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.

2. Materials and methods

2.1. 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 5 L plant pots (22 cm diameter × 19.1 cm 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 200 ml of a soil slurry mixture made from approximately 5 kg of soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14′N, 4°01′W) and 14 L 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; 3 m diameter, 2.1 m high) at the CEH solardome facility near Bangor, North Wales, with 6 pots of each cultivar per solardome.

2.2. 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 (Fig. 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 8 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 min 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.

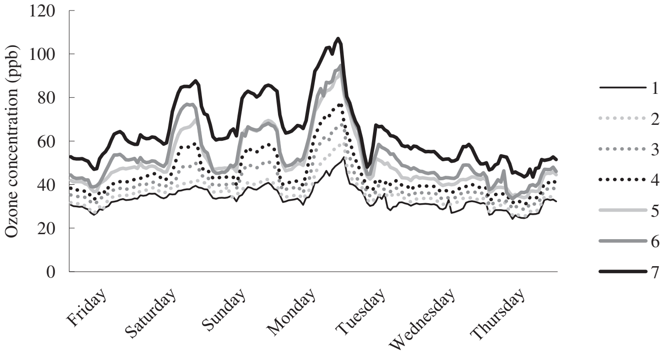

Fig. 1. Average weekly ozone profile for the seven ozone treatments (see Table 1 for treatment details).