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N-fixation in Legumes – an assessment of the potential threat posed by ozone pollution

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1 **Abstract**

2 The growth, development and functioning of legumes are often significantly affected by
3 exposure to tropospheric ozone (O₃) pollution. However, surprisingly little is known about
4 how leguminous Nitrogen (N) fixation responds to ozone, with a scarcity of studies
5 addressing this question in detail. In the last decade, ozone impacts on N-fixation in soybean,
6 cowpea, mung bean, peanut and clover have been shown for concentrations which are now
7 commonly recorded in ambient air or are likely to occur in the near future. We provide a
8 synthesis of the existing literature addressing this issue, and also explore the effects that may
9 occur on an agroecosystem scale by predicting reductions in *Trifolium* (clovers) root nodule
10 biomass in United Kingdom (UK) pasture based on ozone concentration data for a “high”
11 (2006) and “average” ozone year (2008). Median 8% and 5% reductions in clover root nodule
12 biomass in pasture across the UK were predicted for 2006 and 2008 respectively. Seasonal
13 exposure to elevated ozone, or short-term acute concentrations >100ppb, are sufficient to
14 reduce N-fixation and/or impact nodulation, in a range of globally-important legumes.
15 However, an increasing global burden of CO₂, the use of artificial fertiliser, and reactive N-
16 pollution may partially mitigate impacts of ozone on N-fixation.

17 **Capsule:** Assessing ozone effects on leguminous N-fixation

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

19

20 **1. Introduction**

21 Concentrations of tropospheric ozone (O₃), a short-lived greenhouse gas produced via the
22 complex photochemical reaction of volatile organic compounds (VOCs), carbon monoxide
23 (CO) and nitrogen oxides (NO_x), have increased around the world since the start of the
24 industrial era (IPCC, 2013). The polluted Northern Hemisphere has the highest surface

25 concentrations, with annual mean values of 30-40 ppb and increases of 0.5-2.0% occurring on
26 an annual basis in the mid-latitudes (IPCC, 2013). The regional production of ozone is
27 highest during periods of warm temperature, high radiation levels and stable pressure. Spring
28 and summertime mean concentrations exceed 40 ppb across Central and Northern Europe,
29 and 50 ppb across the Mediterranean basin, central Asia and the sub-tropics (Royal Society,
30 2008). Enhanced ozone destruction, due to increases in temperature and atmospheric water
31 vapour, is expected to partially decrease surface ozone concentrations across much of the
32 world in this century, though continuing increases in concentration are likely in the most
33 polluted regions due to the increasing production of primary precursors (IPCC, 2013).
34 Warming may also increase the regional or seasonal potential for ozone formation due to
35 changes in the hemispheric transport of ozone precursors and increases in VOC production
36 (IPCC, 2013).

37 Nitrogen (N) is an essential macronutrient for plants, and it is estimated that legumes
38 contribute up to 70 Tg N annually to agricultural systems (Herridge et al. 2008). N-fixation in
39 nodulating legumes, via symbioses with soil-dwelling N-fixing rhizobacteria, is a novel trait
40 that is thought to have evolved during the early history of the legume family (*Fabaceae*)
41 (Sprent, 2007). In exchange for carbohydrates such as malate, legumes are able to obtain a
42 source of fixed atmospheric N from rhizobia partners; an interplay that requires specified
43 chemical signal exchanges and structures (Geurts and Bisseling, 2002; Rees et al. 2005) and
44 developmental plasticity (Ferguson et al. 2010). Recently, legume crops and fodder have
45 occupied >15% of all agricultural utilised area (FAO, 2014) as a component of intensive,
46 organic, low input, and subsistence agriculture, and are a primary source of dietary protein.
47 The increasing expense of artificial fertilisers, resulting from rising energy costs, has
48 highlighted the importance of legume-based agriculture as a source of crop N, particularly for
49 smallholder producers in developing regions (Lynch, 2007; De Schutter, 2013).

50 As a powerful oxidant, tropospheric ozone is considered the most damaging air pollutant
51 to food production (Ashmore, 2005). Like other plant groups, a typical response of legumes
52 to chronic ozone exposure is reduced productivity, which translates to measurable declines
53 in yield and harvest index (e.g. Betzelberger et al. 2012). Global estimates of yield losses are
54 unavailable for most legume crops, though it is estimated, based on concentration-based
55 exposure indices, that losses due to ozone exceed 10% of soybean yield on an annual basis,
56 equating to some \$2.9-4.9 billion (Avnery et al. 2011). In addition to declining yield, ozone
57 impacts on nutritive quality have also been reported in several arable and forage legumes
58 (Morgan et al. 2003; Gonzalez-Fernandez et al. 2008; Iriti et al. 2009). In grassland
59 situations, ozone pollution may also contribute towards a reduction in the legume fraction of
60 the community (e.g. Wilbourn et al. 1995; Volk et al. 2006), with implications for the
61 ecological and economic sustainability of grasslands (Mills et al. 2011b).

62 Numerous previous studies have highlighted the often high sensitivity of agronomically-
63 important legumes, including soybean, clover, beans, and pulses to ground level ozone (e.g.
64 Hayes et al. 2007; Mills et al. 2007). However, the impacts of ozone on the N-fixing capacity
65 of legumes remain surprisingly neglected, especially at current and near-future concentrations
66 of ozone. In this study, we synthesise the existing literature to assess the potential for impacts
67 of ozone on N fixation under realistic ozone concentrations and summarise current
68 knowledge on the potential mechanisms involved. Using experimentally-derived response
69 functions, we also explore the effects of ozone that may occur on an agroecosystem scale by
70 predicting reductions in clover (*Trifolium* spp.) root nodule biomass in United Kingdom (UK)
71 pasture based on ozone concentration data for a “high” (2006) and “average” ozone year
72 (2008).

73

74 **2. Methods**

75 2.1. Literature survey

76 Searches were conducted within Web of Science (September – December, 2014) and Google
77 Scholar to identify experimental studies related to the effects of ozone exposure on N-
78 fixation, including physiological impacts and effects on an agroecosystem scale. The search
79 terms used included: ozone and nodulation; ozone and nitrogen fixation; ozone and
80 legume(s); ozone and nitrogen metabolism; and ozone with the names of commonly grown
81 legume crops. The main effects of ozone are summarised in Table 1 and described in the text.

82

83 2.2. Modelling the effects of ozone on clover root nodule biomass on an agroecosystem scale

84 Ozone exposure experiments

85 In our earlier study (Hewitt et al. 2014), a cultivar of white clover (*Trifolium repens* cv.
86 Crusader) and red clover (*Trifolium pratense* cv. Merviot) recommended for general use in
87 grazed pasture (British grassland society, 2014), were exposed to a range of current and near-
88 future ozone scenarios (treatment means: 33, 35, 40, 45, 51, 54, 66ppb) in year 2012 in
89 solardomes (hemispherical glasshouses) at the CEH Solardome facility near Bangor, North
90 Wales. After extraction of root biomass material in selected treatments, the cultivars showed
91 ozone-induced reductions in nodule development, and *in situ* measurements on the white
92 clover revealed that reduced nodule mass was associated with a significantly reduced N-
93 fixation rate determined after 12 weeks ozone exposure by acetylene reduction assay (ARA)
94 (Hewitt et al. 2014). A second, previously unpublished ozone-exposure experiment, using
95 the same experimental set up and treatments, was conducted in year 2013 on clover/ryegrass
96 pasture mesocosms using *T.repens* cv. Crusader and *Lolium perenne* cv. Abermagic. *L.*
97 *perenne* cv. AberMagic was sown at a rate of 0.28g per pot directly into 10 L pots (27.5 cm
98 diameter x 22cm height) filled with compost (John Innes No. 2; J. Arthur Bowers, Lincoln,
99 UK). The total nitrogen content of soils ranged 1-3% (data not shown). *T. repens* cv.

100 Crusader plants were also propagated from seed, grown in the same compost in plug-plant
101 trays in an unheated glasshouse. Seeds were obtained from a commercial seed supplier, and
102 originated from the UK (Wynnstay Seeds; UK). After 4 weeks of growth, 3 clover plants
103 were transferred to each pot containing growing *L. perenne*, with one clover plant in each
104 third of the pot. To introduce a soil microbe population, pots were inoculated with 400ml of a
105 soil slurry mixture made from 5kg of soil from agricultural grassland (Abergwyngregyn,
106 North Wales, UK, 53°14'N, 4°01'W) and 14L water. Mesocosms were grown for a further 4
107 weeks in ventilated greenhouses under optimum watering conditions. On 07/06/2013, 24
108 pots, of equal size and distribution of clover and grass, were transferred to each of 6
109 'solardomes' and exposed to the ozone treatments for 16 weeks. N-fixation was determined
110 on pots every 4 weeks by ARA, and was reduced by ozone up to 8 weeks exposure (data not
111 shown). As part of a final destructive harvest, clover root and nodule biomass was extracted
112 from 6 pots in each ozone treatment as described previously (Hewitt et al. 2014). For more
113 details regarding experimental procedure, including details of the climate conditions and
114 ozone control system, see Hewitt et al. (2014). In both years, root nodule biomass per pot
115 (n=6 per treatment), was analysed by linear regression, with either ozone exposure
116 (accumulated ozone above a threshold of 40 ppb, AOT40 (ppm.h)) or seasonal mean O₃
117 concentration (ppb) at the time of harvest applied as the predictor variable.

118

119 Predicting spatial patterns in nodule biomass loss in the UK

120 The root nodule biomass per pot for *T. repens* and *T. pratense* in year 2012, and *T. repens* in
121 year 2013, were expressed as relative values determined by regression against the AOT40 for
122 each treatment and dividing by the value of the intercept, indicating zero ozone exposure (0
123 AOT40 ppm.h) or 0ppb seasonal mean O₃ concentration. Although *T. repens* and *T. pratense*
124 differed in their response in root nodule biomass in the 2012 study, the slopes for relative root

125 nodule biomass were not significantly different for red and white clover (linear regression;
126 $p=0.99$), nor was there a significant difference between the responses of *T. repens* cv.
127 Crusader to ozone in 2012 and 2013 ($p=0.88$), when interactions between species or year
128 were considered. All data were combined, and linear regression was performed to provide
129 one exposure-response function for effects of ozone on the root nodule biomass of white and
130 red clover. To aid with a comparison to existing studies, the response of nodule biomass to
131 seasonal mean ozone was also presented (Figure 2b).

132 The exposure-response function was then used to predict percentage reductions in UK
133 pasture in year 2006, a hot and dry year with relatively high ozone concentrations, and 2008,
134 a lower ozone year, and more typical of current climate conditions. The ozone concentration
135 data, for the early season (April-June) and late season (July-Sept), were obtained from the
136 UK air quality monitoring stations (<http://uk-air.defra.gov.uk/>) and used to estimate AOT40
137 values calculated across a 1km x 1km grid, based on the Ordnance Survey grid (Coyle et al.
138 2002). As described previously (Mills et al. 2011b), the 1km² grid data was aggregated to
139 10km x 10km squares for comparison with land-cover data obtained from the Convention on
140 Long-Range Transboundary Air Pollution (CLRTAP) harmonised land cover map (Cinderby
141 et al. 2007). Pasture was identified according to the distribution of the European Nature
142 Information System (EUNIS) E2 Mesic grassland habitat (EEA, 2012). Grid squares with
143 <1% (100ha) pasture land-cover were excluded from the analysis. Maps were constructed
144 using ArcGIS (version 10.1).

145 All statistical analyses were conducted in R software (version 3.0.2).

146

147 **3. Results and discussion**

148 *3.1. Literature survey*

149 The survey of literature identified a total of 26 studies related to ozone effects on N-fixation
150 (Table 1). Most studies have been conducted on soybean (*Glycine max*) and clovers
151 (*Trifolium spp.*), followed by beans and pulses (*Phaseolus spp.*; *Vicia spp.*; *Vigna spp.*),
152 chickpea (*Cicer arietinum*) and peanut (*Arachis hypogaea*). There is wide variation in ozone
153 exposure method and total duration, making a generalisation of effects difficult (Table 1).
154 The majority of studies date from the 1970s and 1980s, with the earliest studies having
155 utilised closed chamber controlled environment experiments, often with short exposures of
156 acute concentrations of ozone (>300 ppb) (Table 1). In more recent years, experiments have
157 been conducted in open-top chambers or field-based exposure facilities which more
158 accurately reflect ambient, or likely near-future conditions, under naturally fluctuating
159 climate conditions (Table 1).

160

161 Evidence for effects of ozone on N fixation

162 The most common effect of ozone in the available literature is on the number, size or mass of
163 root nodules, with 17 studies reporting negative impacts on legume nodulation (Table 1).
164 Where a range of ozone concentrations exists from multiple studies, the effective
165 concentration of ozone for impacts on root nodules appears similar for agriculturally
166 important legume species (Figure 1a), with effect size ranging 10-90% in available literature
167 (Table 1). Acute concentrations of ozone ≥ 500 ppb may affect significant reductions of >10%
168 in soybean nodule number or mass after less than 5 hours exposure (e.g. Tingey & Blum,
169 1973; Blum & Tingey, 1977), though impacts of >20% can also occur in legumes after the
170 seasonal exposure to means >60ppb (e.g. Nasim et al. 2007; Hewitt et al. 2014). Several
171 studies also report decreased N-fixation parameters, with the majority of concerned studies
172 having focused on negative effects in clover and soybean (Table 1), although ozone-induced
173 suppression of N-fixation rates have also been reported in peanut, chickpea, faba bean, mung

174 bean and cowpea. As above, the effective range of ozone for a suppression of N-fixation rates
175 does not appear to differ between legume species (Figure 1b), with reductions in N-fixation
176 ranging from ~8-99% (Table 1). In peanut, seasonal exposure to treatments of 49-70 ppb is
177 sufficient to reduce tissue N content and N-fixation rates compared to charcoal-filtered
178 controls (Cong et al. 2009). Similarly, mean daytime exposures up to 107 ppb reduced
179 soybean N-fixation rates by up to 58% (Flagler et al. 1987; Pausch et al. 1996b Cheng et al.
180 2011), with a significant decline in the number and dry mass of soybean root nodules also
181 occurring with exposure to concentrations >100 ppb (Zhao et al. 2012). N-fixation rates in
182 white clover were reduced >50% by seasonal means of 60-80 ppb (Montes et al. 1983, Hewitt
183 et al. 2014). A seasonal mean of 66 ppb was also sufficient to reduce the number, size and
184 mass of clover root nodules, and increase the density of small, likely non-fixing
185 pseudonodules (Hewitt et al. 2014). In general, seasonal ozone means of 40-60ppb or more,
186 or short-term exposure to concentrations of >100ppb, appears sufficient to achieve a
187 reduction in N-fixation rates or impact nodulation in the agricultural legumes assessed (Table
188 1; Figure 1). Although root nodule structure can differ substantially, ozone similarly affects
189 legumes with determinate (e.g. soybean, common bean) or indeterminate root nodules (e.g.
190 clover, chickpea) (Table 1). Ozone has also been shown to disrupt N-fixing symbioses in
191 non-legumes, such as Alder (*Alnus* spp., Greitner and Winner, 1989).

192

193 The physiological basis for ozone effects on N-fixation

194 Ozone enters the plant primarily through the stomata, and thus ozone uptake or flux is highly
195 related to the hydrological status of the plant (Wilkinson and Davies, 2010). In addition to
196 directly reducing photosynthetic rates and capacity, ozone exposure may cause the diversion
197 of carbon and other resources to above-ground growth, defence and repair, including the
198 synthesis of antioxidants and structural carbohydrates (Wilkinson et al. 2011). Callous tissue,

199 built up in the phloem as a response to ozone exposure, may also cause a mechanical
200 hindrance to the flow of carbon and resources to the root system (Wilkinson et al. 2011).
201 Thus, although the leaves are the main site of ozone exposure, the early limitation of growth
202 often occurs in below ground organs (e.g. Hewitt et al. 2014). Reduced N-fixation rates in
203 nodulating legumes could result from a decrease in root nodule size or number, or in the
204 specific activity of the nitrogenases, the central group of oxygen-sensitive rhizobial enzymes
205 responsible for the catalysis of atmospheric N into ammonia (Rees et al. 2005). Ozone does
206 not directly affect root nodules as it does not penetrate through the soil surface (Blum and
207 Tingey, 1977), though the leachates of ozonated plants may influence the growth and
208 subsequent colonisation of soil rhizobia and root fungi (Manning et al. 1971; Kochhar et al.
209 1980; Umponistira et al. 2009). A reduced supply of detritus may lead to a suppression of N-
210 fixation in soil microbial communities in legume agroecosystems (He et al. 2014). As
211 determined from stable isotope studies, ozone-induced reductions in N-fixation rates are
212 thought to ultimately arise from a reduced availability and translocation of carbon assimilates
213 to the root system during growth (Pausch et al. 1996a, b, Cong et al. 2009), with a reduced
214 sucrose flow to nodules the most likely cause (Udvardi and Poole, 2013). N-fixation rates
215 may also recover from ozone episodes over time (e.g. Ensing & Hofstra, 1982; Flagler et al.
216 1987). In chronic ozone exposures, reduced N-fixation rates may be associated with
217 reductions in root biomass, though they may occur independently of impacts on above-
218 ground yield (e.g. Hewitt et al. 2014). In red clover (*Trifolium pratense*), the most severe
219 impacts on root nodule number may be avoided due to higher stomatal conductance rate and
220 possible ability to maintain a supply of assimilates to the root system (Hewitt et al. 2014). As
221 ozone impacts photosynthesis at a metabolic level (e.g. Sun et al. 2014), impacting root
222 growth (Hewitt et al. 2014), disrupting flavonoid synthesis (Galant et al. 2012) and reducing
223 the leghaemoglobin content of root nodules (Blum and Heck, 1980), its proximate

224 mechanisms are superficially similar to ultraviolet radiation (UVB) (e.g. Chouhan et al.,
225 2008; Sharma and Guruprasad, 2012; Hectors et al. 2014). This is in contrast to below-
226 ground stressors such as drought and salinity, which initially inhibit nodulation by directly
227 disrupting the redox status of roots or nodules (e.g. Munoz et al. 2012; Esfahani et al. 2014).
228 Although the molecular mechanism that regulates optimal root nodule number has not yet
229 been elucidated (*sensu* Soyano et al. 2014), a role for all the main phytohormones has been
230 implicated in nodule development (e.g. Ferguson et al. 2004; Mortier et al. 2012; Ryu et al.
231 2012). This may suggest some commonality with abiotic defence responses in the mesophyll,
232 and hint at potential mechanisms by which ozone could cause reductions in nodule number,
233 including the long-distance transport of ozone-induced stress hormones (Tamaoki, 2008),
234 Mitogen-Activated Protein Kinase (MAPK) signalling cascades (Samuel et al. 2000), or a
235 reduced flow of Auxin to the root system (Blomster et al. 2011). Ozone can decrease nitrate
236 and nitrite reductase activity (Agrawal and Agrawal, 1990; Smith et al. 1990) and impair the
237 mobilisation or partitioning of N (e.g. Pausch et al. 1996b; Zhang et al. 2014). Altered N or
238 protein content of plant tissues may occur as a consequence of shortfalls in N-fixation (e.g.
239 Agrawal and Agrawal, 1990). However no consistent effect is apparent in the available
240 literature (Table 1), and ozone-induced impacts on N-fixation or N partitioning may increase
241 (Letchworth and Blum, 1977; Blum & Heck, 1980), decrease (Blum and Heck, 1980; Flager
242 et al. 1987; Agrawal and Agrawal, 1990; Cong et al. 2009; Umponistira, 2009) or have no
243 effect (Montes et al. 1983; Pausch et al. 1996b) on the N content of tissues, organs or whole
244 plants depending upon exposure method and duration, and ozone concentrations used (Table
245 1). Given the practical difficulty in observing *in vivo* changes directly, below-ground
246 responses to ozone in general remain poorly characterised and most studies concerned with
247 this question have used destructive techniques (e.g. Letchworth and Blum, 1977). The
248 acetylene reduction assay, the most commonly employed technique in the literature to assess

249 N-fixation, can also be used non-destructively by measuring gas changes in a chamber placed
250 over soil containing roots (Hewitt et al. 2014).

251

252 Interactions between ozone and other stressors

253 Eleven studies have considered the interactive or additive effects of ozone and other stressors
254 or variables on N-fixation (Table 1), including interspecific competition (Kochhar et al. 1980;
255 Montes et al. 1983), salinity (Welfare et al. 2002), sulphur dioxide and acid rain (Reinhart
256 and Weber, 1980; Jones et al. 1985; Nazzar, 2002), nitrous oxide (Nasim et al. 2007) drought
257 (Flager et al. 1987) and elevated concentrations of carbon dioxide (CO₂) (Cong et al. 2009;
258 Cheng et al. 2011). The effects of these additional environmental stressors are largely
259 additive. Nodulation may be reduced in *T. repens* by leachates of ozone-exposed *Fescue*
260 plants but not non-exposed plants (Kochhar et al. 1980), and competition with companion
261 grasses may exacerbate ozone-induced reductions in N-fixation rate in clover-fescue pasture
262 (Montes et al. 1983). Ozone-induced reductions in N-fixation and other effects may be
263 ameliorated by the stimulatory effect of elevated CO₂ (Cong et al. 2009; Cheng et al. 2011).

264 Only two studies have considered the interactive effects of N-availability and ozone on N-
265 fixation (Montes et al. 1983; Smith et al. 1990). As N-fixation is an energy-intensive process
266 (Rees et al. 2005), excessive inputs of N to agricultural systems can suppress nodule
267 development (e.g. Nanjareddy et al. 2014) or fixation rates (Streeter and Wong, 1988), and
268 lead to reduced growth or yield (e.g. McCurdy et al. 2014). In experiments on clover-fescue
269 (*Festuca arundinacea*) pasture, no interaction between ozone and treatments of NH₄NO₃ was
270 apparent on fixation rate, though the factors may affect N-fixation singly (Montes et al.
271 1983). In soybean, interactions between ozone and NO₃⁻ treatment in nodule activity were
272 unclear (Smith et al. 1990). In addition to artificial fertiliser, high levels of N deposition
273 (>10kg N ha⁻¹ yr⁻¹) co-occur across regions that have also seen large increases in tropospheric

274 ozone (Dentener et al. 2006), making these two factors major drivers of change in polluted
275 regions such as the Mediterranean (EEA, 2011). In Mediterranean pasture legumes, such as
276 subterranean clover, (*T. subterraneum*), striated clover (*T. striatum*) and yellow serradella
277 (*Ornithopus compressus*), N deposition and ozone may interact to drive reductions in
278 nutritive quality (Sanz et al. 2005) and terpene emissions (Llusia et al. 2014), which may
279 result in lower competitiveness (Llusia et al. 2014). However, N-deposition may also
280 stimulate growth, reducing the sensitivity of legumes to impacts on above-ground biomass
281 and ozone-induced foliar injury (Sanz et al. 2007; Calvete-Sogo et al. 2014). Thus, the N-
282 enrichment of agricultural soils, via artificial fertiliser or N-deposition, has the potential to at
283 least partially mitigate ozone impacts on N-fixation. This effect may be particularly important
284 in low-growth rate grassland habitats, where species composition displays more sensitivity to
285 levels of N than to long-term ozone pollution (e.g. Bassin et al. 2013). Adding extra N to
286 compensate for ozone effects on N-fixation in agroecosystems may have important
287 environmental consequences, including a decline in species diversity (Bassin et al. 2013) and
288 increased GHG emissions (e.g. Flechard et al. 2007).

289

290 Consequences for N availability in agroecosystems

291 Few studies have reported decreased plant contributions to soil N as a consequence of
292 reduced N-fixation rates (Table 1). Montes et al. (1983) report a linear decline in soil nitrate
293 (NO_3^-) concentrations in clover/fescue pasture after seasonal exposures to elevated ozone and
294 reduced total N content of soil was found in short-term experiments with Cowpea
295 (Umponstira et al. 2009). Reduced N inputs to soil were also found in 4 year-long
296 experiments with soybean, although total N in the soil surface was unaffected (Cheng et al.
297 2011). Indeed, other than the studies by Cheng et al. (2011) and Flagler et al. (1987), wherein
298 soybean N-fixation rates were annually assessed from low and high ozone plots for multiple

299 growth seasons, there have been no long-term experiments to our knowledge that have
300 investigated the consequences of ozone on N-fixation in any legume species (Table 1). It is
301 therefore unknown whether decreased inputs of organic N due to ozone leads to lasting
302 declines in soil fertility and an increased reliance on artificial fertiliser in agroecosystems.

303

304 *3.2. Modelling the effects of ozone on clover root nodule biomass on an agroecosystem scale*

305 Ozone exposure-response relationships for root nodule biomass

306 When the data for white and red clover and 2012 and 2013 experiments were combined, the
307 response function for relative root nodule biomass displayed a strong negative relationship
308 with accumulated ozone exposure ($r^2=0.72$; $p=0.004$, Figure 2a), with a 10% reduction at an
309 AOT40 of 5 ppm.h, and >50% reduction in relative nodule biomass occurring at AOT40s of
310 > 20 ppm.h (Figure 2a). These results are comparable with the accumulated ozone exposure
311 necessary to achieve a 10% reduction in the shoot biomass of *T. repens*, requiring a 12 week
312 AOT40 of 4.7 ppm.h (Mortensen and Bastrup-Birk, 1996). A significant effect of ozone on
313 relative root nodule biomass did not occur below an AOT40 of 6 ppm.h (Figure 2a). This is
314 above a critical level of 3 ppm.h for the protection of European grasslands (CLRTAP, 2011),
315 although surface concentrations of ozone across Europe frequently exceed this exposure level
316 (Fernández-Fernández et al. 2011; Mills et al. 2011b). For easy comparison with the other
317 studies discussed, a strong negative relationship for relative nodule biomass was also found
318 against seasonal mean ozone concentration ($r^2=0.60$; $p=0.002$; Figure 2b). However, the
319 response against accumulated ozone exposure is preferred due to the higher fit ($r^2=0.72$) and
320 passage through the intercept at the concentrations assessed (Figure 2a).

321

322 Spatial patterns of nodule biomass loss in 2006/2008

323 A total of 1124 10 x 10km grid-squares across the UK contained >1% pasture cover.
324 Predicted reductions in relative nodule biomass were most widespread in the spring of year
325 2008, with >8% reductions predicted across most of Wales, western areas of England and
326 southern Scotland (Figure 3). In the spring of 2006 and 2008, ozone effects were predicted to
327 be greatest for pasture in coastal or upland regions, with pronounced effects in pasture-rich
328 areas of central Wales and the South-West in both years (Figure 3). The mean percentage
329 reductions were similar for both spring periods, but the inter-quartile range for year 2006 was
330 larger at 6.2-13% compared to 9.2-12.6% in the spring of 2008 (Figure 3). In the late season
331 (July-Sept) of both years, it was predicted that ozone impacts on relative root nodule biomass
332 were lower than in the spring (Figures 3 and 4). This effect was most pronounced in the
333 cooler, relatively wet late-season of 2008, with a mean reduction in relative nodule mass of
334 1.4% and an inter-quartile range of 0.9-1.6% (Figure 4). Thus, in a hot, dry, year such as
335 2006, with prolonged periods of high ozone during the spring and summer, sustained impacts
336 on clover nodule biomass may potentially occur across UK pastures, though some impacts
337 may be also be apparent in the spring of cooler, wetter years. The largest predicted losses in
338 root nodule biomass, occurring in the early season (April-June) in Wales and western regions
339 of the UK in both years, are consistent with a broader trend of current and near-future ozone
340 impacts on agriculture in North-West Europe (Mills et al. 2011a).

341 We appreciate that our predicted impacts on root nodule biomass assume non-limited
342 nodulation, and do not consider other important factors such as N-availability (Chmelíková
343 and Hejzman, 2014), soil quality (Manier et al. 2009), hydrological status (Wilkinson and
344 Davies, 2010), or the antioxidant capacity of plants (Scebba et al. 2003). In addition, our
345 predicted impacts are based on modern commercial cultivars of clover, which may differ in
346 sensitivity to established clover in M2 grassland habitat, although clover from this habitat
347 does respond strongly to ozone (e.g. Hayes et al. 2010). Similarly, the abundance of clover

348 within pasture grid-squares, which could not be controlled for, would substantially influence
349 N-fixation rates (Better returns programme, 2015), and could itself be negatively influenced
350 by ozone pollution (e.g. Wilbourn et al. 1995). Ozone-effect data also has a closer fit to
351 stomatal-flux based indices (Mills et al. 2011, a, b), leading exposure-based indices to
352 overestimate losses. In general, mixed and established grassland communities may display a
353 large degree of inertia to ozone stress, and the effects of rising ozone on European grasslands
354 are uncertain (e.g. Stampfli and Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014). These
355 limitations are key sources of uncertainty to this assessment, and the potential for exaggerated
356 ozone-impacts on clover nodulation are recognised by the authors. Nevertheless, the
357 modelling exercise presented here provides the first indication of the potential effects ozone
358 pollution may have on legume nodulation on an agroecosystem scale, based on data from
359 realistic experiments. It is unknown how well direct reductions in root nodule biomass would
360 translate to N-fixation rates in pasture, though clover N-fixation rates are often associated
361 with nodule weight or size (Crush and Caradus, 1996; Selge and Higuchi, 2000; Hewitt et al.
362 2014). Using averaged N-fixation data from the 2012 and 2013 experiments, root nodule
363 biomass explained 50% of the variation in N-fixation rate ($r^2=0.50$; $p=0.04$; data not shown).
364 However, as N-fixation rates were determined from these studies by ARA (units: nL ethylene
365 cm² soil surface), scaling this chemical data to a landscape scale would require a number of
366 additional measurements and assumptions at this stage.

367

368 **4. Conclusion**

369 For the first time, this study has considered the potential impacts of ozone on leguminous N-
370 fixation in a systematic way. The paucity of studies concerned with ozone effects on N-
371 fixation is noteworthy considering the vast number that have highlighted the impacts of
372 ozone on legume growth and functioning. As determined from several ozone-exposure

373 experiments, seasonal exposure to mean ozone concentrations of 40-60 ppb, or to short-term
374 acute concentrations of >100ppb, is sufficient to reduce N-fixation rates or impact nodulation
375 in important agricultural legumes. Effects of ozone on N-fixation and nodulation ultimately
376 result from a reduced availability of C assimilates. However, the possible proximate effects
377 of ozone, such as the action of long-distance stress hormones, and disruptions in the flow of
378 auxin to developing nodules, have not been investigated and are worthy of further study.
379 Based on ozone exposure (AOT40), annual impacts on clover root nodule biomass may occur
380 in UK and temperate pastures, with sustained impacts occurring in years with higher ozone
381 concentrations. Potential short-falls in N-fixation, and economic and environmental
382 consequences, have not yet been considered on an agroecosystem basis and also present an
383 important subject for further investigation. From the few interactive studies conducted, an
384 increasing global burden of CO₂, the use of artificial fertiliser, and reactive N pollution, may
385 provide some mitigation of the impacts of ozone on leguminous N-fixation.

386 The use of legumes for agronomic purposes will likely increase in the future, stimulated
387 by their potential to reduce agricultural greenhouse gas emissions (Smith et al. 2007), the
388 high price of artificial fertilisers (Lynch, 2007) and by advances in the selection of legume
389 germplasm for stress tolerance and improved performance (e.g. Abberton and Marshall,
390 2005). Multiple interacting environment stressors, such as ozone, drought, elevated CO₂ and
391 N deposition will be increasingly important in determining the sustainability of legume-based
392 agriculture and food production, particularly in parts of the world where severe issues with
393 food security already exist (e.g. De Schutter, 2013).

394

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398

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400

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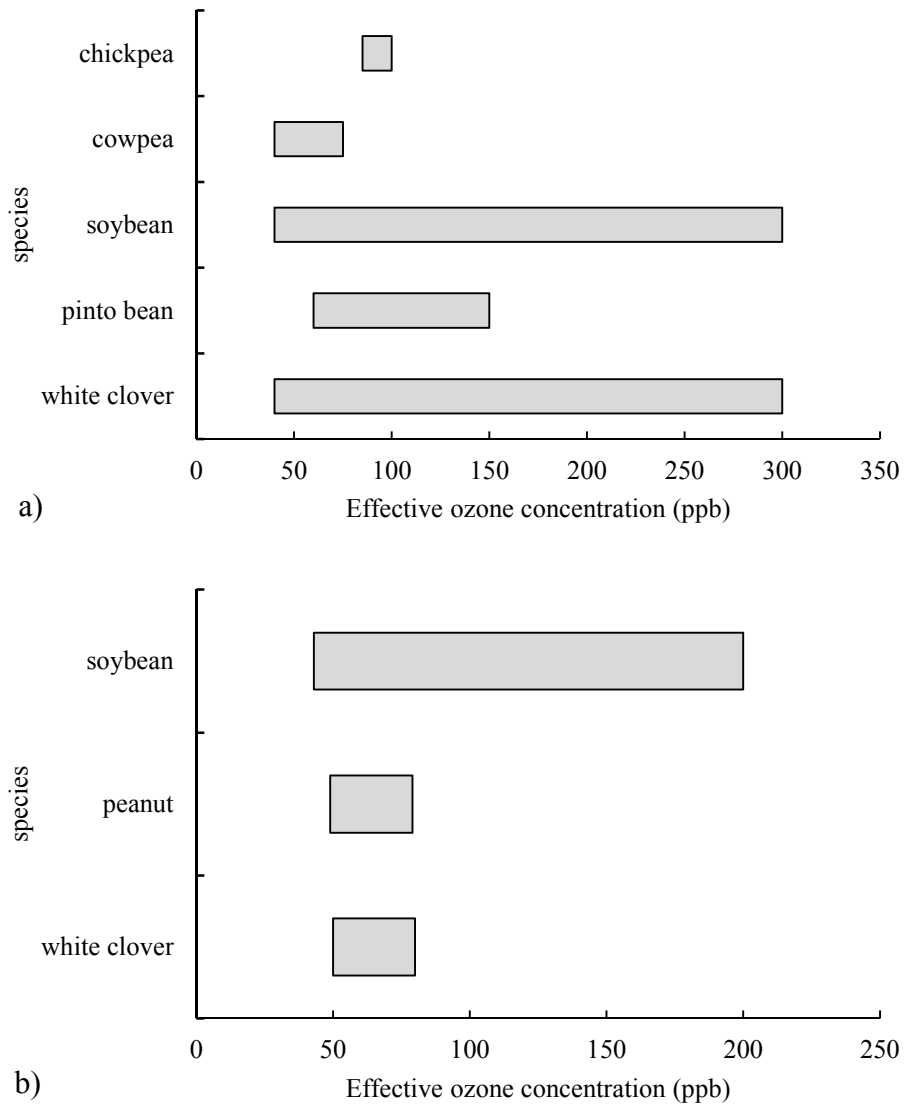
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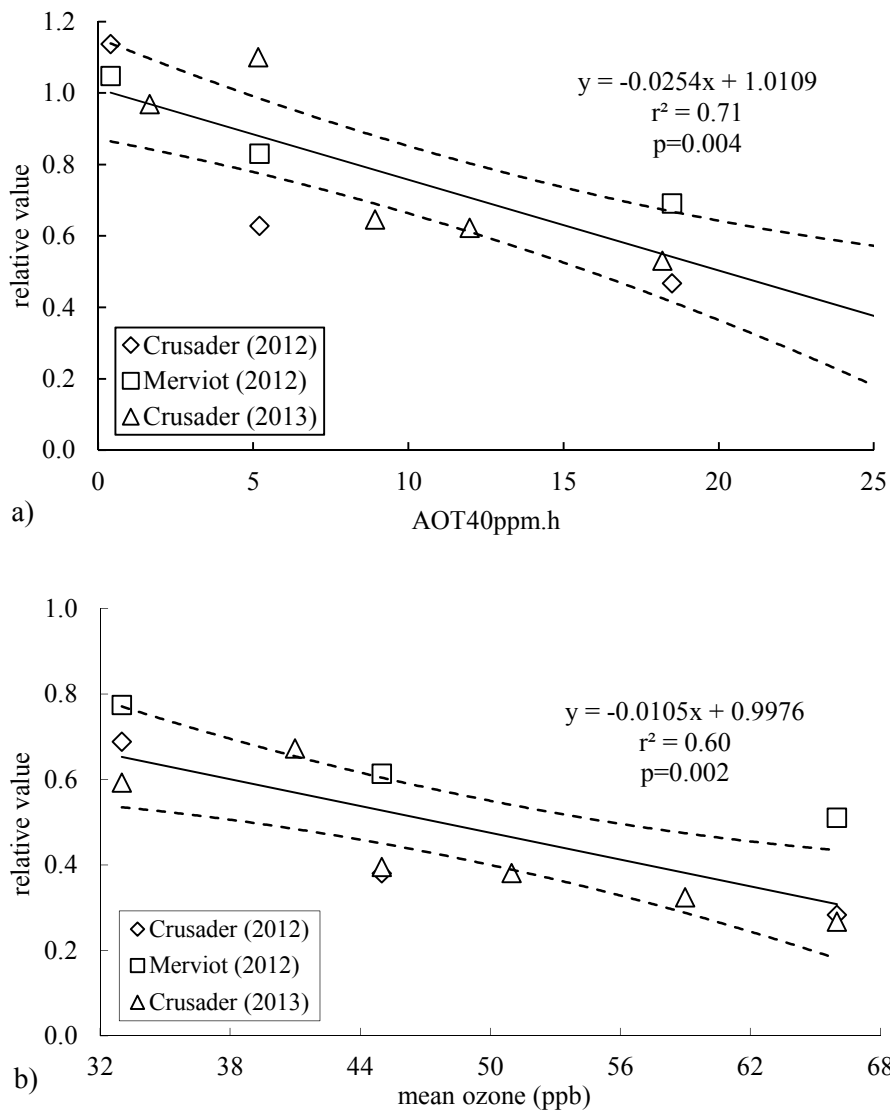
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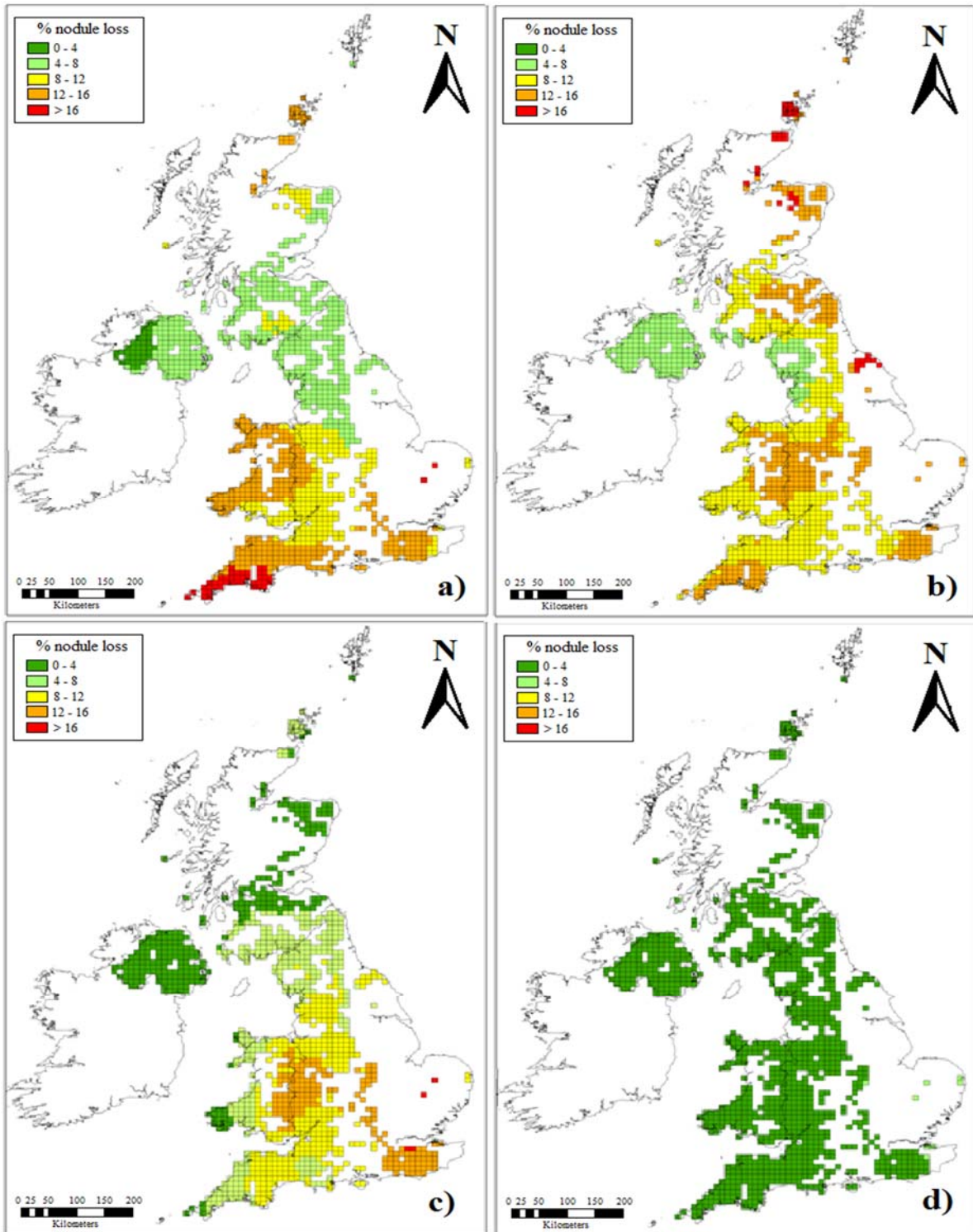
Figure 1: The approximate range of ozone concentrations reported as impacting on (a) the number, size or mass of root nodules, or (b) suppressing N-fixation rates, in agronomically-important legume species (concentrations above 300ppb are excluded).

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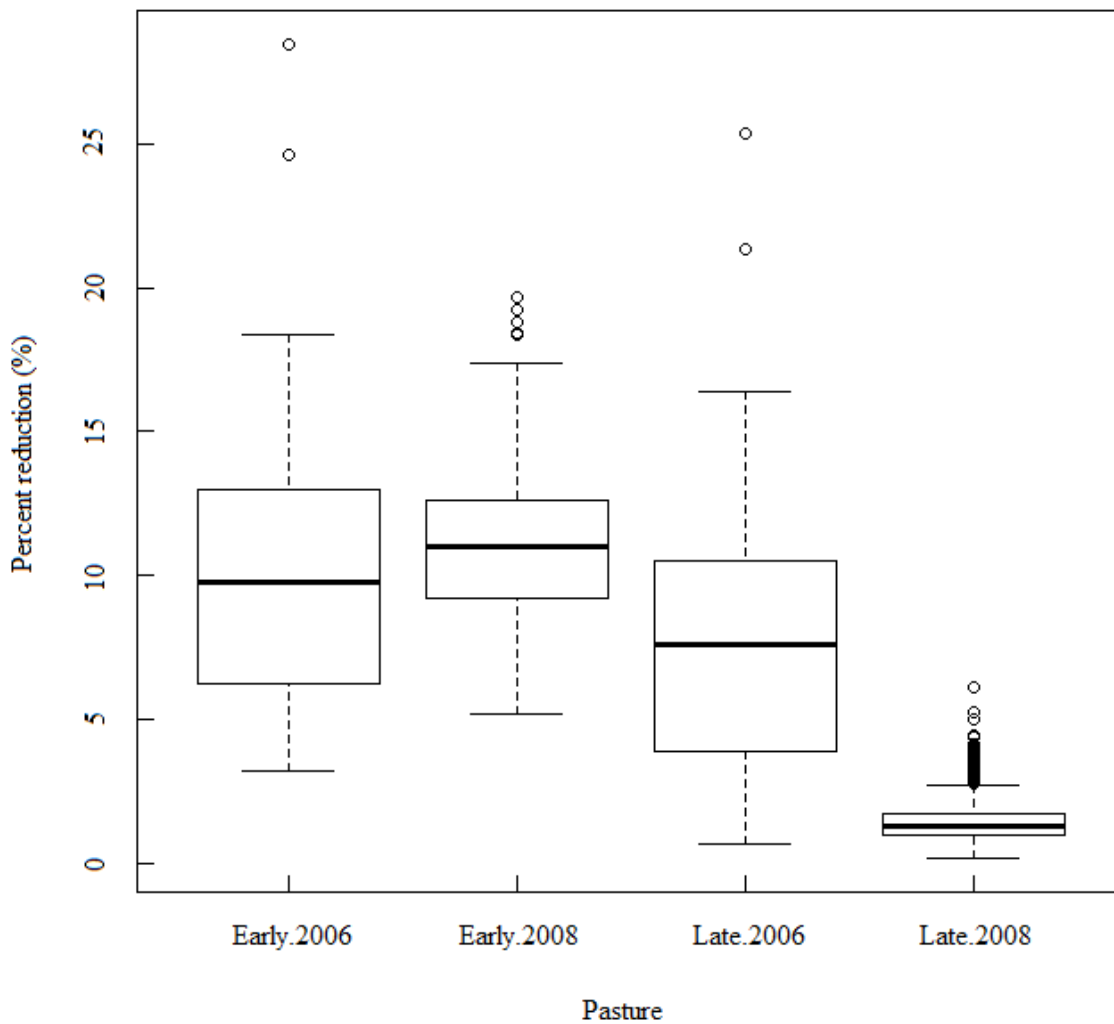
Figure 2: Response relationships for relative root nodule biomass in *Trifolium* spp. combined from experiments with white clover (*T. repens* cv. Crusader) and Red clover (*T. pratense* cv. Merviot) in years 2012 and 2013 with (a) accumulated ozone exposure (AOT40ppm.h) ($y = -0.0254x + 1.0109$; $r^2 = 0.72$; $p = 0.004$; $n = 6$) (b) seasonal mean ozone concentration (ppb) ($y = -0.0105x + 0.9976$; $r^2 = 0.60$; $p = 0.002$; $n = 6$).



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708 Figure 3: AOT40-based assessments of predicted percent reductions in clover root nodule
 709 biomass in the UK scaled for 10 x 10km grid squares, where pasture is present in >1% of the
 710 grid-square, and based on the combined response function of two ozone-exposure
 711 experiments in Figure 2: (a) Early-season (April-June) in year 2006; (b) Early-season in year
 712 2008; (c) Late-season (July-Sept) in year 2006; (d) Late-season in year 2008.

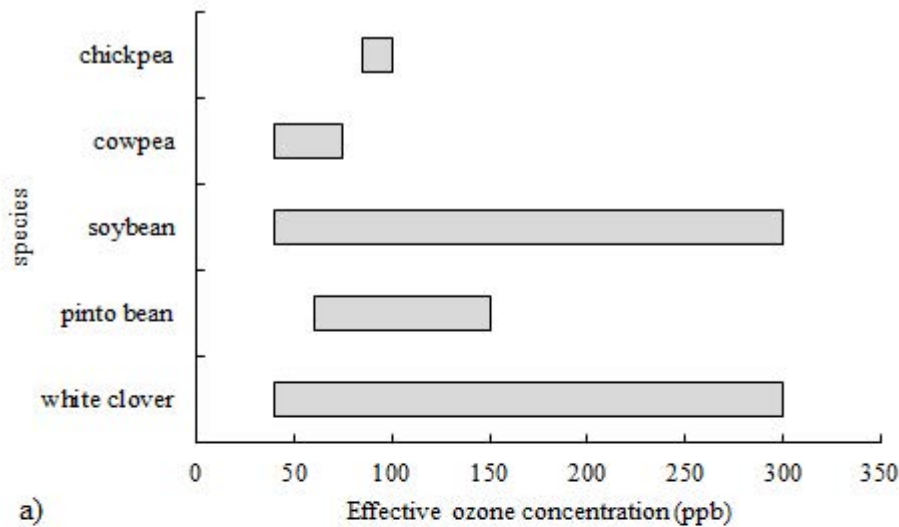


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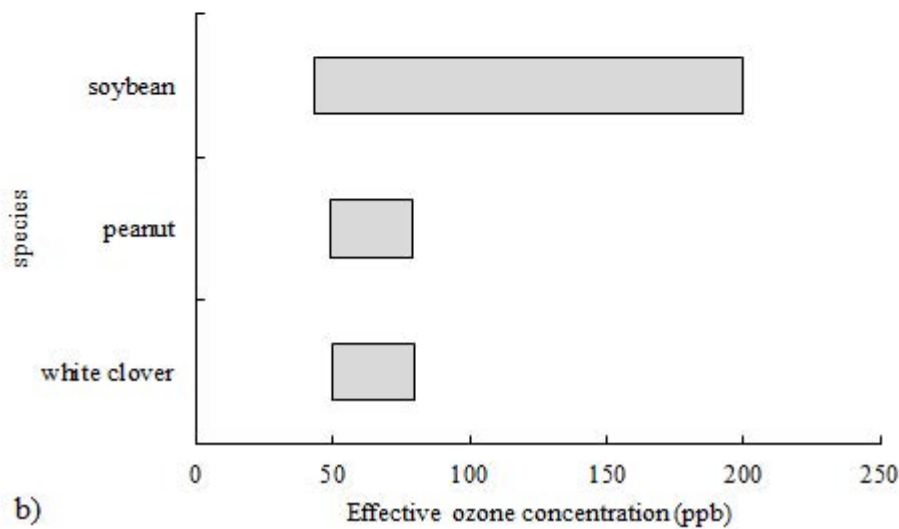
714 Figure 4: Quantification of ozone-induced reductions in clover root nodule mass for early
 715 season (April-June) and late season (July-Sept) pasture in years 2006 and 2008 using the
 716 grid-square values in Figure 4. Boxes display minimum, median, lower and upper quartiles
 717 and maximum values for grid squares (n=1124). Open circles indicate potential outlier
 718 values.

Table 1: Summary of published literature concerning the effects of ozone on leguminous N-fixation, including reported effect-size relative to low or zero ozone controls, for impacts on Nodulation (nodule number, mass etc) and N-fixation parameters (e.g. nitrogenase activity, g N fixed, nodule activity). Key: CC=closed chambers; G=fumigated greenhouse; OTC=open top chambers; A=ambient; S=solardomes; minus and plus signs (-, +) indicate a negative or positive ozone effect respectively; n/a = not applicable; single asterisks (*) denote a single experiment with multiple growth seasons; reported effects are significant at $p \leq 0.05$ unless otherwise stated.

Reference	Year	Species	Exposure method	Effective ozone range/mean	Total exposure duration (per growth season)	Main ozone impacts	Effect size (Nodulation/N fixation).	Additional variables
Manning et al.	1971, 1972, 1973	Pinto bean	CC/G	~60-150 ppb	20-60 days	Nodulation (-)	34%	
Tingey & Blum	1973	Soybean	CC	750 ppb	1 hour	Nodulation (-)	10-14%	
Blum & Tingey	1977	Soybean	CC	500 ppb	4 hours	Nodulation (-)	60%	
Letchworth & Blum	1977	White clover	CC	300-600 ppb	4 hours	Nodulation (-), tissue N content (+)	34-36%	
Blum & Heck	1980	Common bean	CC	300-600 ppb	3 hours	Nodulation (-), tissue N content (+) total N content (-)	12-29%	
Reinhardt & Weber	1980	Soybean	CC	250 ppb	~5 days	Nodulation (-)	33-72%	Sulphur dioxide
Kochhar et al.	1980	White clover	OTC/G	300 ppb	2 hours	Nodulation (-)	47%	Competition/inte raction, leachates
Ensing & Hofstra	1982	Red clover	OTC	A-200ppb	24-48 hours	N fixation (-)	24-50%	
Montes et al.	1983	White clover	OTC	~50-80 ppb	~4 months	N fixation (-), soil N inputs (-)	44-79%	Competition, N availability
Ensing et al.	1985	Peanut	A	A	~3 months	Nodulation (-), N fixation (-)	45-48%/57%	
Jones et al.	1985	Soybean	OTC/CC	~60 ppb	5 days	N fixation (-) ($p < 0.10$)	~8-16%	Sulphur dioxide
Flagler et al.	1987	Soybean	OTC	58-107 ppb	~6 months*	N fixation (-) ($p < 0.10$)	27%	Drought
Agrawal & Agrawal	1990	Chickpea, Faba bean	OTC	~100 ppb	30 days	Nodulation (-), N fixation (-), tissue N content (-); N metabolism (-)	12-34%/ 3-11%	
Smith et al.	1990	Soybean	CC	200 ppb	n/a	N fixation (-), N metabolism (-)	54-68%	N availability
Pausch et al.	1996a, b	Soybean	OTC	43-80 ppb	n/a	N fixation (-), total N content (-)	5-73%	
Nazzar	2002	Faba bean	CC	100 ppb	~3 days	Nodulation (-), N fixation (-)	34-40%/48-99%	Acid rain
Welfare et al.	2002	Chickpea	CC	85 ppb	~6 days	Nodulation (-)	18-21%	Salinity
Nasim et al.	2007	Mung bean	OTC/A	~40-80ppb	~4 months	Nodulation (-), N fixation (-)	21-70%/57-70%	Nitrous oxide
Umponstira et al.	2009	Cowpea	CC	40-70 ppb	7-74 days	Nodulation (-), N fixation (-), tissue N content (-), soil N inputs (-)	31-90%/30-33%	
Cong et al.	2009	Peanut	OTC	49-79 ppb	~4 months	N-fixation (-) (not directly determined), total N content (-)	n/a	Carbon dioxide
Cheng et al.	2011	Soybean	OTC	65 ppb	~6 months*	N-fixation/soil N inputs (-)	37-40%	Carbon dioxide
Zhao et al.	2012	Soybean	OTC	40-110 ppb	n/a	Nodulation (-)	Abstract only	
Hewitt et al.	2014	White clover/Red clover	S	45-66 ppb	3 months	Nodulation (-), N fixation (-)	33-60%/68-83%	



a)



b)

