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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 (O_3) 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 CO_2, 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 (O_3), a short-lived greenhouse gas produced via the

22   complex photochemical reaction of volatile organic compounds (VOCs), carbon monoxide

23   (CO) and nitrogen oxides (NOx), 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 measureable 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 faction 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**

4

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 <u>Ozone exposure experiments</u>

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 O_3

117   concentration (ppb) at the time of harvest applied as the predictor variable.

118

119   <u>Predicting spatial patterns in nodule biomass loss in the UK</u>

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 O_3 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 $1km^2$ 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   <u>Evidence for effects of ozone on N fixation</u>

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 ≥500ppb 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   <u>Interactions between ozone and other stressors</u>

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 (CO_2) (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 CO_2 (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 NH_4NO_3 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 NO_3^- 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 <u>Consequences for N availability in agroecosystems</u>

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   (Fernàndez-Fernàndez 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 Hejcman, 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 cm^2 soil surface), scaling this chemical data to a landscape scale would require a number of

366 additional measurements and assumptions at this stage.

367

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 CO_2, 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 CO_2 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

References

Abberton, M.T., Marshall, A.H. 2005. Progress in breeding perennial clovers for temperate agriculture. *The Journal of Agricultural Science*, **143**, 117-135.

Agrawal, M., Agrawal, S.B. 1990. Effects of Ozone Exposure on Enzymes and Metabolites of Nitrogen-Metabolism. *Scientia Horticulturae*, **43**, 169-177.

Ashmore, M. 2005. Assessing the future global impacts of ozone on vegetation. *Plant, Cell & Environment*, **28**, 949-964.

Avnery, S., Mauzerall, D.L., Liu, J., Horowitz, L.W. 2011. Global crop yield reductions due to surface ozone exposure: 1. Year 2000 crop production losses and economic damage. *Atmospheric Environment*, **45**, 2284-2296.

Bassin, S., Volk, M., Fuhrer, J. 2013. Species composition of Subalpine Grassland is sensitive to Nitrogen Deposition, but not ozone, after 7 years of treatment. *Ecosystems*, **16**, 1105-1117.

Better returns programme. 2015. *Improving pasture for better returns*. Available at: http://www.eblex.org.uk/returns.

Betzelberger, A.M., Yendrek, C.R., Sun, J., Leisner, C.P., Nelson, R.L., Ort, D.R., Ainsworth, E.R. 2012. Ozone exposure response for U.S. soybean cultivars: linear reductions in photosynthesis potential, biomass, and yield. *Plant Physiology*, **160**, 1827-1839.

Blomster, T., Salojärvi, J., Sipari, N., Broshé, M., Ahlfors, R., Keinänen, M., Overmyer, K., Kangasjärvi, J. 2011. Apoplastic reactive oxygen species transiently decrease auxin signaling and cause stress-induced morphogenic response in *Arabidopsis*. *Plant Physiology*, **157**, 1866-1883.

Blum, U., Heck, W.W. 1980. Effects of Acute Ozone Exposures on Snap Bean at Various Stages of Its Life-Cycle. *Environmental and Experimental Botany*, **20**, 73-85.

Blum, U., Tingey, D.T. 1977. A study of the potential ways in which ozone could reduce root growth and nodulation of soybean. *Atmospheric Environment*, **11**, 737-739.

British Grassland Society. 2014. *Recommended List of Grasses and Clover 2014*. Available at:http://www.britishgrassland.com/document/recommended-grass-and-clover-list-farmer-handbook.

Calvete-Sogo, H., Elvira, S., Sanz, J., Gonzalez-Fernandez, I., Garcia-Gomez, H., Sanchez-Martin, L., Alonso, R., Bermejo-Bermejo, V. 2014. Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response. *Atmospheric Environment*, **95**, 197-206.

433 Cheng, L., Booker, F.L., Burkey, K.O., Tu, C., Shew, H.D., Rufty, T.W., Fiscus, E.L.,
434 Deforest, J.L., Hu, S.J. 2011. Soil Microbial Responses to Elevated CO_2 and O_3 in a
435 Nitrogen-Aggrading Agroecosystem. *Plos One*, **6**.

436 Chmelíková, L., Hejcman, M. 2014. Effect of nitrogen, phosphorus and potassium
437 availability on emergence, nodulation and growth of *Trifolium medium* L. in alkaline soil.
438 *Plant Biology*, **16**, 717-725.

439 Chouhan, S., Chauhan, K., Kataria, S., Guruprasad, K.N. 2008. Enhancement in
440 leghaemoglobin content of root nodules by exposure of solar UV-A and UV-B radiation in
441 soybean. *Journal of Plant Biology*, **51**, 132-138.

442 Cinderby, S., Emberson, L., Owen, A., Ashmore, M. 2007. *CEE Progress Report: LRTAP*
443 *land cover map of Europe*. Available at:
444 http://www.rivm.nl/bibliotheek/digitaaldepot/PBLCCE PR07 PartI 5.pdf.

445 CLRTAP. 2011. *Manual on Methodologies and Criteria for Modelling and Mapping Critical*
446 *Loads and Levels and Air Pollution Effects, Risks and Trends*. Available at:
447 http://www.icpmapping.org/Mapping_Manual

448 Cong, T., Booker, F.L., Burkey, K.O. & Hu, S. 2009. Elevated Atmospheric Carbon dioxide
449 and O_3 differentially alter Nitrogen acquisition in Peanut. *Crop Science*, **49**, 1827-1836.

450 Coyle, M., Smith, R.I., Stedman, J.R., Weston, K.J., Fowler, D. 2002. Quantifying the spatial
451 distribution of surface ozone concentration in the UK. *Atmospheric Environment*, **36**, 1013-
452 1024.

453 Crush, J.R., Caradus, J.R. 1996. Increasing symbiotic potentials in white clover. *Agronomy*
454 *Society of New Zealand Special Publication*, **11**, 91-94.

455 De Schutter, O. 2013. *End of mission statement by the Special Rapporteur on the right to*
456 *food, Malawi 12 to 22 July 2013*. Available at:
457 http://www.ohchr.org/EN/NewsEvents/Pages/DisplayNews.aspx?NewsID=13567&LangID=
458 E.

459 Dentener, F., Stevenson, D., Ellingsen, K., Van Noije, T., Schultz, M., Amann, M., Atherton,
460 C., Bell, N., Bergmann, D., Bey, I., Bouwman, L., Butler, T., Cofala, J., Collins, B., Drevet,
461 J., Doherty, R., Eickhout, B., Eskes, H., Fiore, A., Gaus, M., Hauglustaine, D., Horowitz, L.,
462 Isaksen, I.S., Josse, B., Lawrence, M., Krol, M., Lamarque, J.F., Montanaro, V., Müller, J.F.,
463 Peuch, V.H., Pitari, G., Pyle, J., Rast, S., Rodriguez, I., Sanderson, M., Savage, N.H.,
464 Shindell, D., Strahan, S., Szopa, S., Sudo, K., Van Dingenen, R., Wild, O., Zeng, G. 2006.
465 The global atmospheric environment for the next generation. *Environmental Science and*
466 *Technology*, **1**, 3586-3594.

467 EEA. 2011. *Air Quality in Europe - 2011 Report Technical Report No. 12/2011*. European
468 Environment Agency, Copenhagen.

469 EEA. 2012. *Mesic Grasslands*. Available at http:// http://eunis.eea.europa.eu/habitats/167.

470 Ensing, J., Hofstra, G., Roy, R.C. 1985. The Impact of Ozone on Peanut Exposed in the
471 Laboratory and Field. *Phytopathology*, **75**, 429-432.

472 Ensing, J., Hofstra., G. 1982. Impact of the air pollutant ozone on acetylene reduction and
473 shoot growth of red clover. *Canadian Journal of Plant Pathology*, **4**, 337-342.

474 Esfahani, M.N., Sulieman, S., Schulze, J., Yamaguchi-Schinozaki, K., Shinozaki,K., Tran,
475 L.P., 2014. Mechanisms of physiological adjustment of N_2 fixation in *Cicer arietinum* L.
476 (chickpea) during early stages of water deficit: single or multifactor controls. *The Plant*
477 *Journal*, **79**, 964-980.

478 FAO. 2014. *Faostat database*. Available at http://faostat.fao.org/.

479 Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.H., Lin, Y.H., Reid, D.E., Gresshoff,
480 P.M. 2010. Molecular analysis of Legume Root Nodule Development and Autoregulation.
481 *Journal of Integrative Plant Biology*, **52**, 61-76.

482 Ferguson, B.J., Mathesius, U. 2004. Signaling interactions during nodule development.
483 *Journal of Plant Growth Regulation*, **22**, 47-72.

484 Fernández-Fernández, M.I., Gallego, M.C., García, J.A., Acero, F.J. 2011. A study of surface
485 ozone variability over the Iberian Peninsula during the last fifty years. *Atmospheric*
486 *Environment*, **45**, 1946-1959.

487 Flagler, R.B., Patterson, R.P., Heagle, A.S., Heck, W.W. 1987. Ozone and Soil-Moisture
488 Deficit Effects on Nitrogen-Metabolism of Soybean. *Crop Science*, **27**, 1177-1184.

489 Flechard, C.R., Ambus, P., Skiba, U., Rees, R.M., Hensen, A., van Amstel, A., van den Pol-
490 van Dasselaar, A., Soussana, J.F., Jones, M., Clifton-Brown, J., Raschi, A., Horvath, L.,
491 Neftel, A., Jocher, M., Ammann, C., Leifeld, J., Fuher, J., Calanca, P., Thalman, E.,
492 Pilegaard, K., Di Marco, C., Campbell, M., Nemitz, E., Hargreaves, K.J., Levy, P.E., Ball
493 B.C., Jones, S.K., van de Bulk, W.CM., Groot, T., Blom, M., Domingues, R., Kasper, G.,
494 Allard, V., Ceshia, E., Cellier, P., Laville, P., Henault, C., Bizouard, F., Abdalla, M.,
495 Williams, M., Baronti, S., Berretti, F. 2007. Effects of climate and management intensity on
496 nitrous oxide emissions in grassland. *Agriculture, Ecosystems and Environment*, **121**, 135-
497 152.

498 Galant, A., Koester, R.P., Ainsworth, E.A., Hicks, L.M., Jex, J.M. 2012. From climate
499 change to molecular response: redox proteomics of ozone-induced responses in soybean. *New*
500 *Phytologist*, **194**, 220-229.

501 Geurts, R., Bisseling, T. 2002. Rhizobium Nod Factor Perception and Signalling. *The Plant*
502 *Cell*, **14 (suppl.)**, S239-S249.

503 Gonzalez-Fernandez, I., Bass, D., Muntifering, R., Mills, G., Barnes, J. 2008. Impacts of
504 ozone pollution on productivity and forage quality of grass/clover swards. *Atmospheric*
505 *Environment*, **42**, 8755-8769.

506 Greitner, C.S., Winner, W.E. 1989. Effects of O_3 on alder photosynthesis and symbiosis with
507 Frankia. *New Phytologist*, **111**, 647-656.

508 Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M. 2007. Meta-analysis of the relative
509 sensitivity of semi-natural vegetation species to ozone. *Environmental Pollution*, **146**, 754-
510 762

511 Hayes, F., Mills, G., Ashmore. 2010. How much does the presence of a competitor modify
512 the within-canopy distribution of ozone-induced senescence and visible injury? *Water, Air &*
513 *Soil Pollution*, **210**, 265-276.

514 He, Z., Xiong, J., Kent, A.D., Deng, Y., Xue, K., Wang, G., Wu, L., Van Nostrand, J.D.,
515 Zhou, J. 2014. Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a
516 soybean agro-ecosystem. *The ISME Journal*, **8**, 714-726.

517 Hectors, K., Van Oevelen, S., Geuns, S., Guisez, Y., Jansen, M.A.K., Prinsen, E. 2014.
518 Dynamic changes in plant secondary metabolites during UV acclimation in *Arabidopsis*
519 *thaliana*. *Physiologia Plantarum*, **152**, 219-230.

520 Herridge, D.F., Peoples, M.B., Boddey, R.M. 2008. Global inputs of biological nitrogen
521 fixation in agricultural systems. *Plant and Soil*, **311**, 1-18.

522 Hewitt, D.K.L., Mills, G., Hayes, F., Wilkinson, S., Davies, W. 2014. Highlighting the threat
523 from current and near-future ozone pollution to clover in pasture. *Environmental Pollution*,
524 **189**, 111-117.

525 IPCC. 2013. *Climate Change 2013: The Physical Science Basis. Contribution of Working*
526 *Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*
527 [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia,
528 V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom
529 and New York, NY, USA, 1535 pp.

530 Iriti, M., Di Maro, A., Bernasconi, S., Burlini, N., Simonetti, P., Picchi, V., Panigada, C.,
531 Gerosa, G., Parente, A., Faoro, F. 2009. Nutritional Traits of Bean (*Phaseolus vulgaris*)
532 Seeds from Plants Chronically Exposed to Ozone Pollution. *Journal of Agricultural and*
533 *Food Chemistry*, **57**, 201-208.

534 Jones, A.W., Mulchi, C.L., Kenworthy, W.J. 1985. Nodule Activity in Soybean Cultivars
535 Exposed to Ozone and Sulfur-Dioxide. *Journal of Environmental Quality*, **14**, 60-65.

536 Kochhar, M., Blum, U. & Reinart, R.A. 1980. Effects of O_3 and (or) fescue on ladino clover:
537 interactions. *Canadian Journal of Botany*, **58**, 241-249.

538 Letchworth, M.B., Blum, U. 1977. Effects of Acute Ozone Exposure on Growth, Nodulation
539 and Nitrogen-Content of Ladino Clover. *Environmental Pollution*, **14**, 303-312.

540 Llusia, J., Bermejo-Bermejo, V., Calvete-Sogo, H., Penuelas, J. 2014. Decreased rates of
541 terpene emissions in *Ornithopus compressus* L. and *Trifolium striatum* L. by ozone exposure
542 and nitrogen fertilisation. *Environmental Pollution*, **194**, 69-77.

543 Lynch, J. 2007. The Roots of the Second Agricultural Revolution. *Australian Journal of*
544 *Botany*, **55**, 493-512.

545 Manier, N., Deram, A., Broos, K., Denayer, F.O., Van Haluwyn, C. 2009. White clover
546 nodulation index in heavy metal contaminated soils - a potential bioindicator. *Journal of*
547 *Environmental Quality*, **38**, 685-692.

548 Manning, W.J., Feder, W.A., Papia, P.M. & Perkins, I. 1971. Influence of foliar ozone injury
549 on root development and root surface fungi of pinto bean plants. *Environmental Pollution*, **1**,
550 305-312.

551 Manning, W.J., Feder, W.A., Vardaro, P.M. 1973. Benomyl in Soil and Response of Pinto
552 Bean Plants to Repeated Exposures to a Low Level of Ozone. *Phytopathology*, **63**, 1539-
553 1540.

554 Manning, W.J., Papia, P.M., Feder, W.A. 1972. Influence of long term low levels of ozone
555 and benomyl on growth and nodulation of pinto bean plants. *Phytopathology*, **62**, 497.

556 McCurdy, J.D., McElroy, J.S., Guetral, E.A., Wood, C.W. 2014. White Clover inclusion
557 within a Bermudagrass Lawn: Effects of Supplemental Nitrogen on Botanical Composition
558 and Nitrogen Cycling. *Crop Science*, **54**, 1796-1803.

559 Mills, G., Buse, A., Gimeno, B., Bermejo, V., Holland, M., Emberson, L., Pleijel, H. 2007. A
560 synthesis of AOT40-based response functions and critical levels for agricultural and
561 horticultural crops. *Atmospheric Environment*, **41**, 2630-2643.

562 Mills, G., Harmens, H., participants of the ICP Vegetation. 2011a. *Ozone Pollution: A hidden*
563 *threat to Food Security*. CEH, Bangor, UK, available at:
564 http://icpvegetation.ceh.ac.uk/publications/thematic.html

565 Mills, G., Pleijel, H., Braun, S., Buker, P., Bermejo, V., Calvo, E., Danielsson, H., Emberson,
566 L., Fernandez, I.G., Grunhage, L., Harmens, H., Hayes, F., Karlsson, P.E., Simpson, D.
567 2011b. New stomatal flux-based critical levels for ozone effects on vegetation. *Atmospheric*
568 *Environment*, **45**, 5064-5068.

569 Montes, R.A., Blum, U., Heagle, A.S., Volk, R.J. 1983. The Effects of Ozone and Nitrogen-
570 Fertilizer on Tall Fescue, Ladino Clover, and a Fescue Clover Mixture.2. Nitrogen-Content
571 and Nitrogen-Fixation. *Canadian Journal of Botany*, **61**, 2159-2168.

572 Morgan, P.B., Ainsworth, E.A., Long, S.P. 2003. How does elevated ozone impact soybean?
573 A meta-analysis of photosynthesis, growth and yield. *Plant, Cell and Environment*, **26**, 1317-
574 1328.

Mortensen, L., Bastrup-Birk, A. 1996. Critical levels for biomass production in white clover (*Trifolium repens* L.) exposed in open-top chambers, in; *Critical levels for Ozone - experiments with crops, wild plants and forest tree species in the Nordic Countries* (Skärby, L., Pleijel, H (eds)), Nordic Council, Copenhagen, Denmark.

Mortier, V., Holsters, M., Goormachtig, S. 2012. Never too many? How legumes control nodule numbers. *Plant, Cell and Environment*, **35**, 245-258.

Munoz, N., Robert, G., Melchiorre, M., Racca,R., Lascano, R. 2012. Saline and osmotic stress differentially affects apoplastic and intraceullar reactive oxygen species production, curling and death of root hair during *Glycine max* L.-*Bradyrhizobium japonicum* interaction. *Environmental and Experimental Botany*, **78**, 76-83.

Nasim, G., Bajwa, R., Hakeem, A. 2007. Response of arbuscular mycorrhizal mungbean plants to ambient air pollution. *International Journal of Environment Science and Technology*, **4**, 295-310.

Nanjareddy, K., Blanco, L., Arthikala, M.K., Affrantrange, X.A., Sanchez, F., Lara, M. 2014. Nitrate regulates rhizobial and mycorrhizal symbiosis in common bean (*Phaseolus vulgaris* L.). *Journal of Integrative Plant Biology*, **56**, 281-298.

Nazzar, L.E.A.A. 2002. Effect of Ozone and simulated acid rain on growth, nitrogen fixation and peroxidase activity in Faba Bean (*Vicia faba* L.) Plants. *Asian Journal of Plant Sciences*, **1**, 456-461.

Pausch, R.C., Mulchi, C.L., Lee, E.H., Forseth, I.N., Slaughter, L.H. 1996a. Use of ^{13}C and ^{15}N isotopes to investigate O_3 effects on C and N metabolism in soybeans .1. C fixation and translocation. *Agriculture Ecosystems & Environment*, **59**, 69-80.

Pausch, R.C., Mulchi, C.L., Lee, E.H., Meisinger, J.J. 1996b. Use of ^{13}C and ^{15}N isotopes to investigate O_3 effects on C and N metabolism in soybeans .2. Nitrogen uptake, fixation, and partitioning. *Agriculture Ecosystems & Environment*, **60**, 61-69.

Rees, D.C., Tezcan, F.A., Haynes, C.A., Walton, M.Y., Andrade, S., Einsle, O., Howard, J.B. 2005. Structural basis of biological nitrogen fixation. *Philosophical Transactions of the Royal Society A*, **363**, 971-984.

Reinert, R.A., Weber, D.E. 1980. Ozone and Sulfur Dioxide-Induced Changes in Soybean Growth. *Phytopathology*, **70**, 914-916.

Royal Society. 2008. *Ground level ozone in the 21st century: future trends, impacts and policy implications. RS policy document, 15.08.* Available online at https://royalsociety.org/policy/publications/2008/ground-level-ozone.

Ryu, H., Cho, H., Choi, D., Hwang, I. 2012. Plant hormonal regulation of nitrogen-fixing nodule organogenesis. *Molecules and Cells*, **34**, 117-126.

610   Samuel, M.A., Miles, G.P., Ellis, B.E. 2000. Ozone treatment rapidly activates MAP kinase
611   signalling in plants. *The Plant Journal*, **22**, 367-76.

612   Sanz, J., Bermejo, V., Gimeno, B.S., Elvira, S., Alonso, R. 2007. Ozone sensitivity of the
613   Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content.
614   *Atmospheric Environment*, **41**, 8952-8962.

615   Sanz, J., Muntifering, R.B., Bermejo, V., Gimeno, B.S., Elvira, S., 2005. Ozone and
616   increased nitrogen supply effects on the yield and nutritive quality of *Trifolium*
617   *subterraneum. Atmospheric Environment*, **39**, 5899-5907.

618   Scebba, F., Soldatini, G., Ranieri, A. 2003. Ozone differentially affects physiological and
619   biochemical responses of two clover species; *Trifolium repens* and *Trifolium pratense.*
620   *Environmental Pollution*, **123**, 209-216.

621   Selge, A., Higuchi, S. 2000. Increasing the forage legumes symbiotic N_2 fixation activity.
622   *Proceedings of the 18th General Meeting of the European Grassland Federation*, 44-56.

623   Sharma, S., Guruprasad, K.N. 2012. Enhancement of root growth and nitrogen fixation in
624   Trigonella by UV-exclusion from solar radiation. *Plant Physiology and Biochemistry*, **61**, 97-
625   102.

626   Smith, H., Neyra, C., Brennan, E. 1990. The Relationship between Foliar Injury, Nitrogen
627   Metabolism, and Growth Parameters in Ozonated Soybeans. *Environmental Pollution*, **63**,
628   79-93.

629   Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S.,
630   O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., Howden, M., McAllister, T., Pan, G.,
631   Romanenkov, V., Scheider, U., Towprayoon, S., Wattenbach, M., Smith, J. 2007.
632   Greenhouse gas mitigation in agriculture. *Philosophical Transactions of the Royal Society B*,
633   **363**, 789-813.

634   Soyano, T., Hirakawa, H., Sato, S., Hayashi, M., Kawaguchi, M. 2014. Nodule inception
635   creates a long-distance feedback loop involved in homeostatic regulation of nodule organ
636   production. *Proceedings of the National Academy of Sciences*, **111**, 14607-14612.

637   Sprent, J. 2007. Evolving ideas of legume evolution and diversity; a taxonomic perspective
638   on the occurrence of nodulation. *New Phytologist*, **174**, 11-25.

639   Stampfli A., Fuhrer J. 2010. Spatial heterogeneity confounded ozone-exposure experiment in
640   semi-natural grassland. *Oecologia*, **162**, 515-522.

641   Streeter, J., Wong, P.P. 1988. Inhibition of nodule development and N_2 fixation by nitrate.
642   *Critical Reviews in Plant Sciences*, **7**, 1-23.

643   Sun, J.D., Feng, Z.Z., Ort, D.R. 2014. Impacts of rising tropospheric ozone on photosynthesis
644   and metabolite levels on field grown soybean. *Plant Science*, **226**, 147-161.

645 Tamaoki, M. 2008. The role of phytohormone signaling in ozone induced cell-death in plants. *Plant Signaling and Behaviour*, **3**, 166-174.

647 Tingey, D.T., Blum U. 1973. The effects of ozone on soybean nodules. *Journal of Environmental Quality*, **2**, 341-342.

649 Udvardi, M., Poole, P.S. 2013. Transport and Metabolism in Legume-Rhizobia symbiosis. *Annual Review of Plant Biology*, **64**, 781-805.

651 Umponstira, C., Kawayaskul, S., Chuchaung, S. & Homhaul, W. 2009. Effect of Ozone on Nitrogen Fixation, Nitrogenase Activity and Rhizobium of Cowpea (*Vigna unguiculata* (L.) Walp). *Naresuan University Journal*, **17**, 213-220.

654 Volk, M., Bungener, P., Contat, F., Montani, M., Fuhrer, J. 2006. Grassland yield declined by a quarter in 5 years of free-air ozone fumigation. *Global Change Biology*, **12**, 74-83.

656 Volk, M., Wolff, V., Bassin, S., Ammann, C., Fuhrer. 2014. High tolerance of subalpine grassland to long-term ozone is independent of N input and climatic drivers. *Environmental Pollution*, **189**, 161-168.

659 Welfare, K., Yeo, A., Flowers, T.J. 2002. Effects of salinity and ozone, individually and in combination, on the growth and ion contents of two chickpea (*Cicer arietinium* L.) varieties. *Environmental Pollution*, **120**, 397-403.

662 Wilbourn, S., Davison, A.W., Ollerenshaw, J.H. 1995. The use of an unenclosed field fumigation system to determine the effects of elevated ozone on a grass-clover. *New Phytologist,* **129**, 23-32.

665 Wilkinson, S., Davies, W.J. 2010. Drought, ozone, ABA and ethylene. *Plant, Cell and Environment*, **33**, 510-525.

667 Wilkinson, S., Mills, G., Illidge, R., Davies, W.J. 2011. How is ozone pollution reducing our food supply? *Journal of Experimental Botany*, **63**, 527-536.

669 Zhang, W., Wang, G., Liu, X., Feng, Z. 2014. Effects of O_3 exposure on seed yield, N concentration and photosynthesis of nine soybean cultivars (*Glycine max* (L.) Merr.) in Northeast China. *Plant Science*, **226**, 172-181.

672 Zhao, T.H., Cao, Y.H., Wang, Y., Dai, Z., Liu, Y.O., Liu, B. 2012. Effects of Ozone Stress on Root Morphology and Reactive Oxygen Species Metabolism in Soybean Roots. *Soybean Science*, **12**,1.
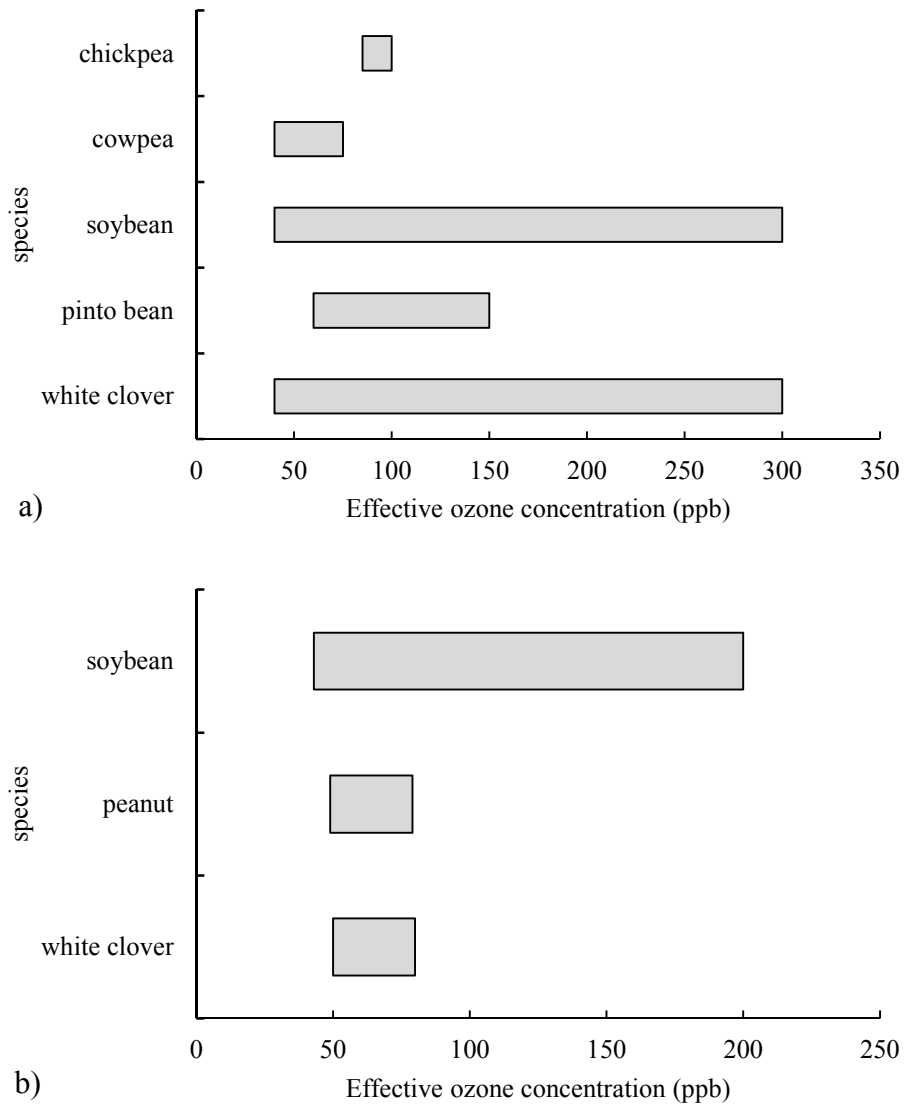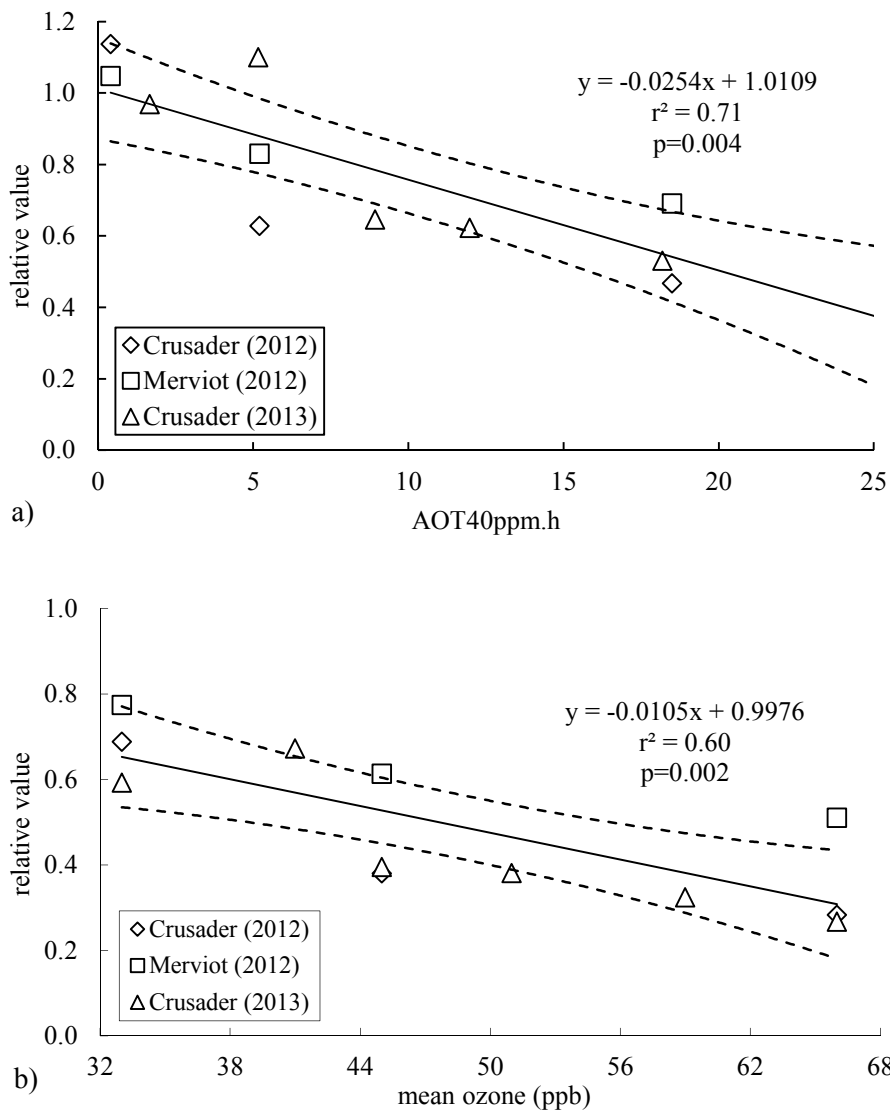
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a)

b)

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

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a) AOT40ppm.h

Figure with equation: $y = -0.0254x + 1.0109$; $r^2 = 0.71$; $p=0.004$

b) mean ozone (ppb)

Figure with equation: $y = -0.0105x + 0.9976$; $r^2 = 0.60$; $p=0.002$

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699 Figure 2: Response relationships for relative root nodule biomass in *Trifolium* spp. combined

700 from experiments with white clover (*T. repens* cv. Crusader) and Red clover (*T. pratense* cv.

701 Merviot) in years 2012 and 2013 with (a) accumulated ozone exposure (AOT40ppm.h) (y=-

702 0.0254x + 1.0109; r^2=0.72; p=0.004; n=6) (b) seasonal mean ozone concentration (ppb)(y=-

703 0.0105x+0.9976; r^2=0.60; p=0.002; n=6).

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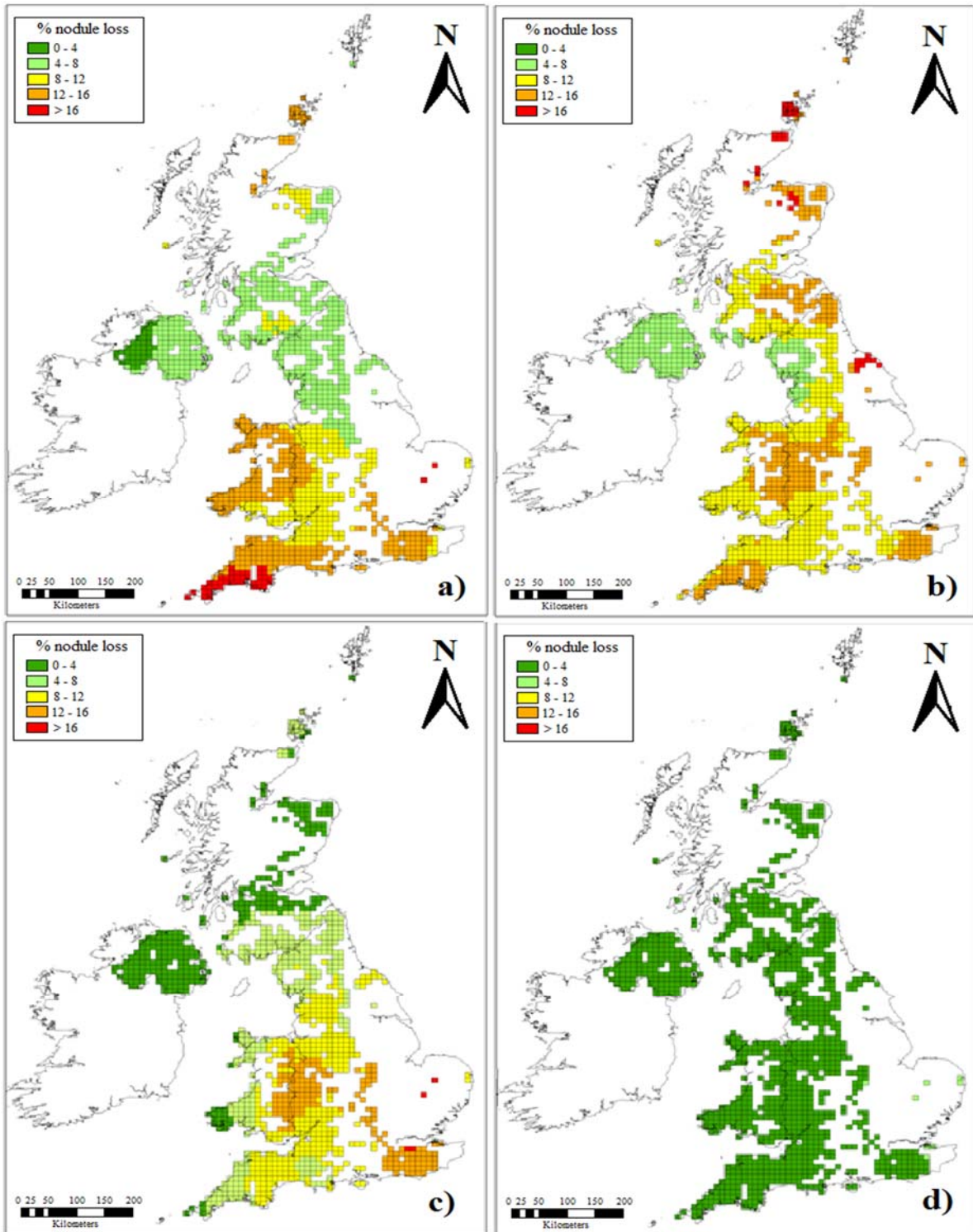
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708 Figure 3: AOT40-based assessments of predicted percent reductions in clover root nodule

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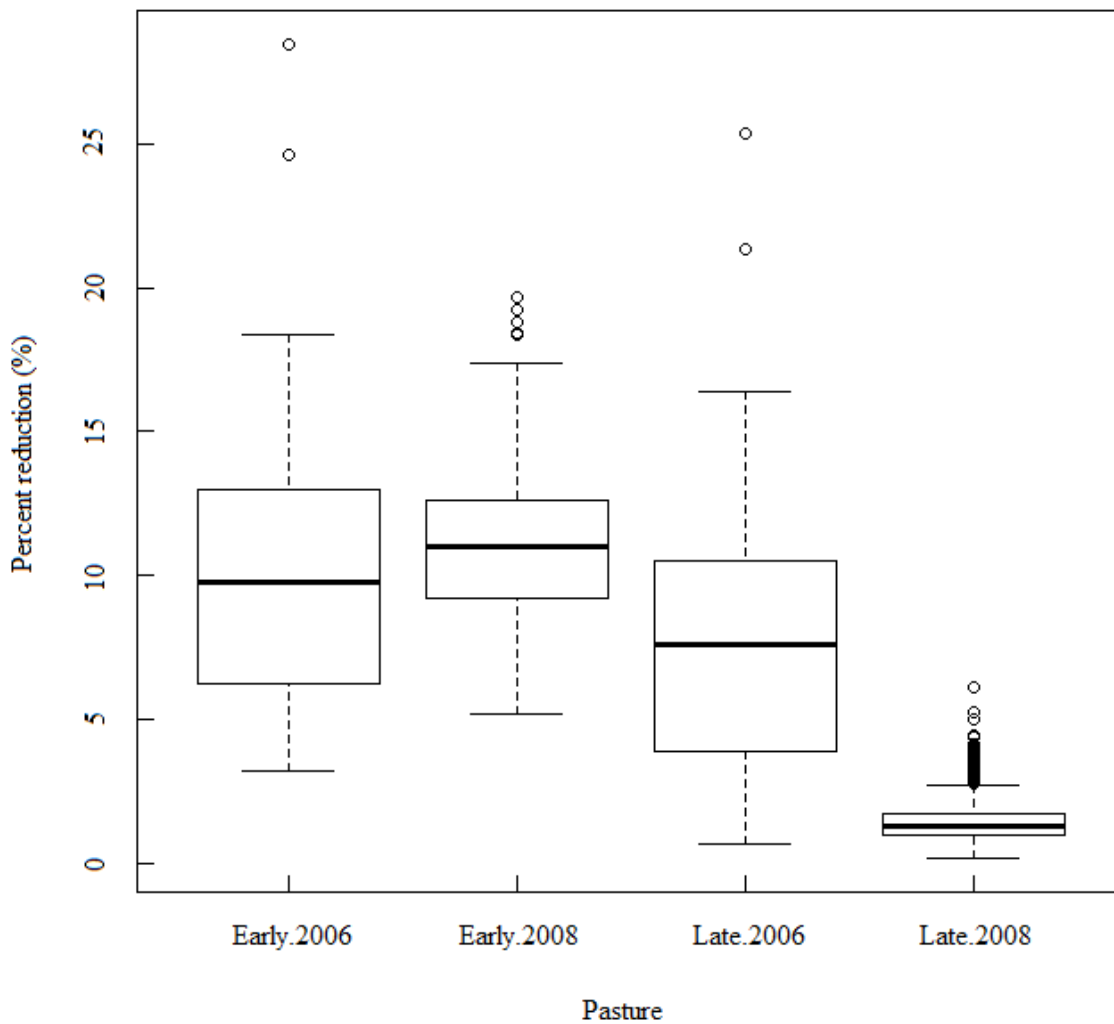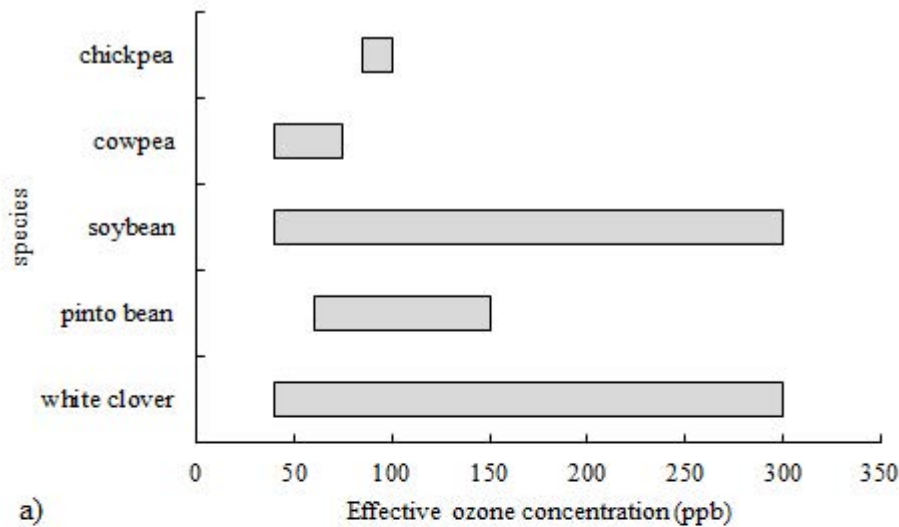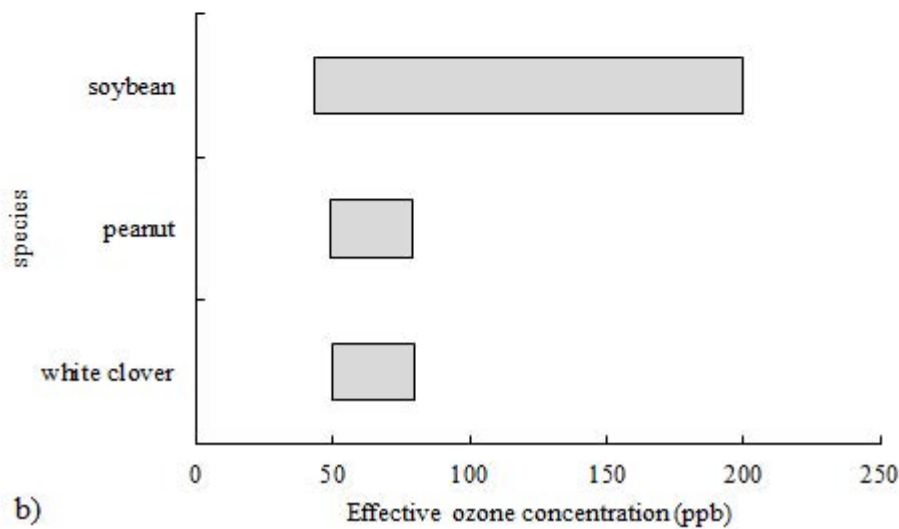
Figure 4: Quantification of ozone-induced reductions in clover root nodule mass for early
season (April-June) and late season (July-Sept) pasture in years 2006 and 2008 using the
grid-square values in Figure 4. Boxes display minimum, median, lower and upper quartiles
and maximum values for grid squares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

a)

$y = -0.0254x + 1.0109$
$r^2 = 0.71$
$p = 0.004$

Crusader (2012)
Merviot (2012)
Crusader (2013)

AOT40ppm.h

b)

$y = -0.0105x + 0.9976$
$r^2 = 0.60$
$p = 0.002$

Crusader (2012)
Merviot (2012)
Crusader (2013)

mean ozone (ppb)