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Elevated atmospheric CO₂ impairs the performance of root-feeding vine weevils by modifying root growth and secondary metabolites

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Keywords:	black vine weevil, carbon dioxide, induced responses, phenolics, roots, soils, secondary metabolites
Abstract:	<p>Predicting how insect crop pests will respond to global climate change is an important part of increasing crop production for future food security, and will increasingly rely on empirically-based evidence. The effects of atmospheric composition, especially elevated carbon dioxide (eCO₂), on insect herbivores have been well studied, but this research has focussed almost exclusively on aboveground insects. However, responses of root-feeding insects to eCO₂ are unlikely to mirror these trends because of fundamental differences between aboveground and belowground habitats. Moreover, changes in secondary metabolites and defensive responses to insect attack under eCO₂ conditions are largely unexplored for root-herbivore interactions. This study investigated how eCO₂ (700 μmol mol⁻¹) affected a root-feeding herbivore via changes to plant growth and concentrations of carbon (C), nitrogen (N) and phenolics. This study used the root-feeding vine weevil, <i>Otiorhynchus sulcatus</i>, and the perennial crop, <i>Ribes nigrum</i>. Weevil populations decreased by 33% and body mass decreased by 20% (from 7.2mg to 5.8mg) in eCO₂. Root biomass decreased by 16% in eCO₂, which was strongly correlated with weevil performance. While root N concentrations fell by 8%, there were no significant effects of eCO₂ on root C and N concentrations. Weevils caused a sink in plants, resulting in 8–12% decreases in leaf C</p>

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	concentration following herbivory. There was an interactive effect of CO ₂ and root herbivory on root phenolic concentrations, whereby weevils induced an increase at ambient CO ₂ , suggestive of defensive response, but caused a decrease under eCO ₂ . Contrary to predictions, there was a positive relationship between root phenolics and weevil performance. We conclude that impaired root growth underpinned the negative effects of eCO ₂ on vine weevils and speculate that the plants failure to mount a defensive response at eCO ₂ may have intensified these negative effects.



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Elevated atmospheric CO₂ impairs the performance of root-feeding vine weevils by modifying root growth and secondary metabolites

Running title - elevated CO₂ and belowground herbivory

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Keywords - *black vine weevil, carbon dioxide, induced responses, phenolics, roots, soils, secondary metabolites.*

39 **Abstract**

40 Predicting how insect crop pests will respond to global climate change is an important part of
41 increasing crop production for future food security, and will increasingly rely on empirically-
42 based evidence. The effects of **atmospheric composition**, especially elevated carbon dioxide
43 (eCO₂), on insect herbivores have been well studied, but this research has focussed almost
44 exclusively on aboveground insects. However, responses of root-feeding insects to eCO₂ are
45 unlikely to mirror these trends because of fundamental differences between aboveground and
46 belowground habitats. Moreover, changes in secondary metabolites and defensive responses
47 to insect attack under eCO₂ conditions are largely unexplored for root–herbivore interactions.
48 This study investigated how eCO₂ (700 μmol mol⁻¹) affected a root-feeding herbivore via
49 changes to plant growth and concentrations of carbon (C), nitrogen (N) and phenolics. This
50 study used the root-feeding vine weevil, *Otiorhynchus sulcatus*, and the perennial crop, *Ribes*
51 *nigrum*. Weevil populations decreased by 33% and body mass decreased by 23% (from 7.2mg
52 to 5.4mg) in eCO₂. Root biomass decreased by 16% in eCO₂, which was strongly correlated
53 with weevil performance. While root N concentrations fell by 8%, there were no significant
54 effects of eCO₂ on **root C** and N concentrations. Weevils caused a sink in plants, resulting in
55 8–12% decreases in leaf C concentration following herbivory. There was an interactive effect
56 of CO₂ and root herbivory on root phenolic concentrations, whereby weevils induced an
57 increase at ambient CO₂, suggestive of defensive response, but caused a decrease under eCO₂.
58 Contrary to predictions, there was a positive relationship between root phenolics and weevil
59 performance. We conclude that impaired root growth underpinned the negative effects of
60 eCO₂ on vine weevils and speculate that the plants failure to mount a defensive response at
61 eCO₂ may have intensified these negative effects.

64 Introduction

65 Increasing crop production to achieve food security in the face of global climate change has become a
66 priority as the world's population continues to grow by 1.2% each year (The Royal Society, 2009).

67 Predicting how crop pests will be affected by global climate change is vital for realising such
68 production goals, and will increasingly rely on empirically based research (Gregory *et al.*, 2009). In
69 particular, there is a considerable amount of research describing how insect herbivores may respond to
70 elevated carbon dioxide (eCO₂) concentrations (reviewed by Bezemer & Jones, 1998), yet this
71 information is almost exclusively concerned with shoot-feeding insects. In a recent review, Staley and
72 Johnson (2008) were only able to identify two studies that investigated the effects of eCO₂ on root-
73 feeding insects (Salt *et al.*, 1996; Johnson & McNicol, 2009), despite the considerable pest status of
74 root-feeding insects in many agro-ecosystems (Blackshaw & Kerry, 2008).

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76 The effects of eCO₂ on root herbivores are unlikely to simply mirror those effects seen for shoot
77 herbivores due to the contrasting habitats of the two (Staley & Johnson, 2008). In particular, the
78 effects of eCO₂ are likely to be largely plant-mediated as CO₂ concentrations in the soil are already
79 considerably higher than atmospheric concentrations, making soil invertebrates pre-adapted to eCO₂
80 conditions (Haimi *et al.*, 2005). Moreover, soil-dwelling herbivores are likely to be more buffered
81 from the direct effects of temperature increases (Bale *et al.*, 2002). Changes in patterns of root growth,
82 nutritional status and secondary metabolism are therefore more likely to drive root-herbivore
83 interactions (Staley & Johnson, 2008). The effect of eCO₂ on root traits vary depending on plant taxa,
84 but there is a general trend for roots to increase in mass relative to shoots (Rogers *et al.*, 1994; Rogers
85 *et al.*, 1996) and to have decreased nitrogen concentrations with a corresponding increase in C:N ratios
86 (Luo *et al.*, 1994; Bezemer & Jones, 1998; Cotrufo *et al.*, 1998). Responses of secondary metabolites
87 to eCO₂ are more variable (Hartley *et al.*, 2000). For carbon-based secondary metabolites, such as
88 phenolics, studies have shown both increases (e.g. Lindroth *et al.*, 1993; Roth & Lindroth, 1994) and
89 decreases (e.g. Hartley *et al.*, 2000; Veteli *et al.*, 2002) can occur under eCO₂ conditions. **A more
90 recent meta-analysis concluded that eCO₂ caused increased concentrations of phenolics in green plant
91 parts (at both ambient and elevated air temperatures), but did not affect constitutive concentrations in**

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3 92 woody tissues (Zvereva & Kozlov, 2006). Instead, this study suggested that elevated temperatures
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5 93 caused decreases in phenolic concentrations, irrespective of CO₂ conditions. However, the majority of
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7 94 the research effort has focussed on secondary metabolites in shoots and has not, to our knowledge,
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9 95 addressed how eCO₂ affects induction of secondary compounds by root herbivores. Given recent
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11 96 evidence that roots deploy an array of defences against root herbivores (Rasmann & Agrawal, 2008),
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13 97 this constitutes an important, but overlooked, aspect of how global climate change will affect plant–
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15 98 herbivore interactions (Van Noordwijk *et al.*, 1998).
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20 100 The objective of this study was to determine how eCO₂ affected a root feeding herbivore via
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22 101 changes to plant growth and concentrations of carbon, nitrogen and phenolics. We focussed
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24 102 on phenolics as they are ubiquitous in terrestrial plants, they have a well characterised role in
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26 103 plant defence, and their concentrations are known to respond to many environmental factors
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28 104 (Harborne, 1994; Hartley & Jones, 1997).
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34 106 The study system used for this research was a perennial crop, blackcurrant (*Ribes nigrum* L.
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36 107 Saxifragales: Grossulariaceae) and the vine weevil (*Otiorhynchus sulcatus* L. Coleoptera:
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38 108 Curculionidae). The vine weevil is a polyphagous parthenogenetic herbivore and has a
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40 109 lifecycle that takes place above- and belowground. The adult lives aboveground and lays eggs
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42 110 that fall into the soil. Eggs hatch and give rise to root-feeding larvae, which can cause
43
44 111 substantial economic damage to horticultural and nursery crops (Moorhouse *et al.*, 1992).
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49 112 This study specifically compared the effects of eCO₂ (700 μmol mol⁻¹) and ambient carbon
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51 113 dioxide (aCO₂) concentrations (375 μmol mol⁻¹) on (i) vine weevil larvae performance, (ii)
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53 114 shoot and root biomass, (iii) leaf and root water concentration, (iv) leaf and root C and N
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55 115 concentrations and (v) root phenolic concentrations. The eCO₂ concentration of 700 μmol
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57 116 mol⁻¹ represents current predictions for the late twenty-first century (IPCC, 2007). We
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59 117 hypothesised that eCO₂ would cause vine weevil performance (abundance and mass) to
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3 118 decrease in response to lower nutritional quality of the roots (increased C:N ratio, lower N
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5 119 and higher phenolic compound concentrations).
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120 **Materials and Methods**

121 *Plant growth chambers*

122 **In this study, replicating soil temperature gradients seen in the field was of particular**
123 **importance to accurately determine how roots and herbivores would respond to eCO₂**
124 **(Johnson & McNicol, 2009). To this end, this study used specially constructed chambers that**
125 **allowed soil and air temperature patterns to be regulated in a spatially and temporally realistic**
126 **manner (see Gordon *et al.*, 1995 for full details). Experiments were conducted in two plant**
127 **growth chambers in which CO₂ was maintained at either aCO₂ (375 μmol mol⁻¹) or eCO₂ (700**
128 **μmol mol⁻¹). The experiment was therefore repeated three times to overcome the issue of**
129 **pseudo-replication and chamber effects (discussed by Bezemer *et al.*, 1998). Air temperature**
130 **followed a daily sine function with a midday peak of 20°C (± 0.5°C) and nocturnal low of**
131 **10°C (± 0.5°C). Soil temperature at depths of 0.55m and 1.0m remained constant at**
132 **approximately 12°C whereas soil temperature at 0.1m followed a damped lag function of air**
133 **temperature. Plants were grown in long vertical rhizotubes (120 × 2.5 × 5cm) which were**
134 **surrounded by cooling coils and thermal insulation. [CO₂] and temperature measurements**
135 **were monitored with thermocouples (placed in the soil) and an infra red gas analyser (IRGA**
136 **225, ADC Ltd, UK), respectively, and relayed to a data logger (CR21X, Campbell Scientific**
137 **Ltd) at 2 min intervals, which then directed injections of CO₂ and the coolant as appropriate**
138 **(for full details, see Gordon *et al.*, 1995). Each rhizotube was installed with an irrigation tube**
139 **that delivered 28ml of water every 24hr. The chambers were located in a glasshouse and**
140 **received supplemental lighting (16:8 light: dark) from overhead lamps (Philips 400 W SON-T**
141 **AGRO).**

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143 *Experiments*

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3 144 For each run of the experiment, forty similar sized blackcurrant plants (cv. Ben Connan) were
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5 145 established from stem cuttings in sand (Silver sand, J. Arthur Bowers, Lincoln, UK) and
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7 146 acclimatised to the appropriate CO₂ treatment in the chambers after being transplanted to
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10 147 compost (peat–sand–perlite mix containing 17N:10P:15K; William Sinclair Horticulture Ltd,
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12 148 Lincoln, UK). When plants were a further 6 weeks old (6-8 cm in height) they were
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15 149 transferred to the rhizotubes filled with 1.35kg of an air dried cambisol that had been sieved
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17 150 to <2mm and then watered to 20% gravimetric water content. Vine weevil eggs were
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19 151 harvested from an established culture at SCRI that originated from adult vine weevils
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21 152 collected from a field site (56°447'N, 3°012'W) comprising multiple cultivars of raspberry,
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23 153 strawberry and blackcurrant (see Johnson *et al.*, 2009 for full details). One week after
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25 154 introducing plants to tubes, 35 vine weevil eggs were applied to 10 randomly assigned plants
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27 155 in each chamber. Eggs were *ca.* 5 days old and had melanised (i.e. were viable) by the time
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29 156 tubes were inoculated. Each experiment ran for a further five weeks before all plants were
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31 157 harvested. This represents the substantive period that vine weevils are root feeding (i.e. in
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33 158 larval stages), with most generally going on to adulthood after this period of larval
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35 159 development. Larvae were carefully separated from the root system, counted and weighed on
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37 160 a microbalance. In order to attribute individuals to larval instar, two larvae were selected at
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39 161 random from each tube and examined under a microscope at ×40 magnification to ascertain
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41 162 the size of the head capsule using a micrometer (see La Lone & Clarke, 1981). Fresh plant
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43 163 material was separated and weighed, snap frozen in liquid nitrogen and then stored at -18°C.
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45 164 Material was then freeze-dried and weighed to determine dry mass and water content prior to
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47 165 chemical analysis. The experiment was repeated three times, so that each of the factorial
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49 166 treatments (aCO₂ and eCO₂, with and without weevils) was repeated 30 times over the three
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51 167 runs.
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3 169 *Carbon and nitrogen analysis*
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5 170 Freeze-dried leaf material was ball-milled to a fine powder for all extractions and chemical
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8 171 analyses. The C and N concentration of leaves was determined using an Exeter Analytical
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10 172 CE440 Elemental Analyzer (EAI, Coventry, UK). **Combustion of the weighed sample (ca. 3**
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12 173 **mg) occurred in pure oxygen using helium to carry the combustion products through the**
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14 174 **analytical system.** The C and N concentrations of samples were calculated using standards
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17 175 (Acetanilide) with known C and N concentrations. Benzoic acid was also used as a standard
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20 176 and to check the nitrogen blanks (see Anon, 2010 for full details).
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24 178 *Phenolic analysis*
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27 179 Analysis was carried out using the enzymatic method described by Stevanato *et al.* (2004),
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29 180 which is more specific than the commonly used Folin-Ciocalteu procedure (Waterman &
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31 181 Mole, 1994) having the advantage that it is not affected by interfering substances such as
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33 182 ascorbate, citrate and sulphite (Stevanato *et al.*, 2004). In summary, phenolics were extracted
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35 183 in a 10:1 ratio from 50 mg freeze dried root material by incubating in 0.5 ml 50% methanol at
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37 184 80°C for 2.5 hr. The aqueous phase was removed and cleared by centrifugation. A 1 ml
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39 185 enzymatic reaction was set up using 50 µl of the resultant supernatant mixed with 740 µl 100
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41 186 mM potassium phosphate buffer (pH 8.0), 100 µl 30 mM 4-aminophenazone, 100 µl 20mM
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43 187 hydrogen peroxide and 1U horse radish peroxidase dissolved in 10 µl potassium phosphate
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45 188 buffer. The reaction was incubated at room temperature for 15 min and absorbance read at a
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47 189 wavelength of 500 nm. Absorbance data were converted to catechin equivalents using a
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49 190 standard curve produced by serial dilution (0 – 0.10 mg ml⁻¹ catechin). All chemicals were
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51 191 obtained from Sigma-Aldrich (Dorset, UK).
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60 193 *Statistical analysis*

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3 194 Split-plot analysis of variance (ANOVA), with three complete blocks (runs) was used to
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6 195 examine the effects of eCO₂ on weevil performance (population size and individual mass) and
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8 196 for the effects of eCO₂ and weevils on plant responses (leaf and root dry mass, water, C and N
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10 197 concentrations and root phenolics). The analysis had a hierarchical block structure whereby
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12 198 CO₂ was applied at the main plot level (chamber) and, for plant responses, weevil presence
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15 199 was applied at the sub-plot level (plant). Where necessary, data were transformed prior to
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17 200 analysis as indicated in figure and table legends. Transformations were chosen to give
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19 201 residual diagnostic plots which best fitted a normal distribution and showed least
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22 202 heteroschedasticity (Sokal & Rohlf, 1995). Analysis of covariance (ANCOVA) and
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24 203 Spearman's rank correlation tests were used to ascertain any relationship between plant traits
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27 204 and weevil performance at the two CO₂ conditions. All analysis was conducted in Genstat
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29 205 (version 12, VSN International, UK).
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Results

Vine weevil larvae were significantly less abundant ($F_{1,2} = 21.46, P = 0.044$) on the roots of plants grown in eCO₂ compared with those grown in aCO₂ (Fig. 1a). In terms of the original inoculation with eggs, this represents survival rates of 24% at aCO₂ and 16% at eCO₂. Larvae were attributed to fourth-fifth instar by assessment of head capsule size (La Lone & Clarke, 1981) in both CO₂ environments. However, the average mass of larvae was lower ($F_{1,2} = 17.12, P = 0.054$, which approached statistical significance at the 95% confidence interval) when they had fed on eCO₂ plants compared to aCO₂ plants (Fig. 1b). Larvae were typically recovered from the top 10cm of the soil profile and there were no apparent differences in vertical distribution of larvae between the two CO₂ treatments.

Elevated CO₂ concentrations did not significantly affect shoot mass in blackcurrant (Table 1) but significantly impaired root growth (Fig. 2a). Maximum rooting depth was similar between the treatments (data not shown). Shoot-to-root ratios and leaf water concentration were also unaffected by either eCO₂ or weevils (Table 1), but water concentrations in roots were reduced by root herbivory and to a lesser extent by eCO₂ (Fig. 2b; Table 1). Concentrations of C and N were not significantly affected by CO₂ in blackcurrant foliage or roots (Table 1). There were, however, significant changes in plant minerals following root herbivory by vine weevils under both CO₂ scenarios (Table 1). Weevils significantly reduced leaf C concentrations (Fig. 2c) and reduced the C:N ratio in roots of blackcurrant (Fig. 2d). This increase in root C:N ratio arose through a decrease in root C and increase in root N, although these differences were not statistically significant when considered individually (Table 1). There was a significant interactive effect of CO₂ and weevil herbivory in terms of root phenolic concentrations, with concentrations rising following root herbivory under aCO₂ but

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3 230 decreasing with herbivory under eCO₂ (Fig. 2e, Table 1). Apart from root phenolics, there
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5 231 were no significant interactive effects between CO₂ and weevil herbivory (Table 1).
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10 233 Relationships between all plant traits listed in Table 1 and weevil performance were
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12 234 examined. There was a strong positive correlation between root mass and vine weevil larval
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14 235 mass (Fig. 3; $F_{1,52} = 14.75$, $P < 0.001$) under both CO₂ conditions, which did not differ in
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16 236 terms of statistical significance ($F_{1,52} = 1.57$, $P = 0.215$). Unexpectedly, phenolic
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18 237 concentration in roots was also positively correlated with larval mass (Fig. 4a; $F_{1,52} = 14.73$, P
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20 238 < 0.001) at both CO₂ concentrations ($F_{1,52} = 0.14$, $P = 0.710$). A similar positive association
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22 239 was seen between root phenolics and weevil population size (Fig. 4b; $F_{1,53} = 6.09$, $P = 0.017$)
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24 240 at aCO₂, but CO₂ significantly affected this relationship ($F_{1,53} = 4.28$, $P = 0.043$) and the
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26 241 correlation was less apparent at eCO₂ (Fig. 4b).
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34 243 Overall, there were no consistent statistically significant relationships between plant traits and
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36 244 plant chemistry, although there was a very weak correlation between root dry mass and root
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38 245 phenolics on control plants ($r_s = 0.222$, $P = 0.088$), which was stronger at aCO₂ ($r_s = 0.459$, P
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40 246 $= 0.006$) than eCO₂ ($r_s = 0.119$, $P = 0.073$).
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3 248 **Discussion**
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5 249 This study set out to investigate how eCO₂ affected a root herbivore via changes to root
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8 250 growth and chemistry and found that vine weevil performance was impaired under eCO₂, both
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10 251 in terms of population size and body mass (being 33% and 20% lower, respectively). In *R.*
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12 252 *nigrum*, eCO₂ impaired root growth by 16%, but had no significant effect on C and N
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15 253 concentrations. Reductions in root growth were strongly associated with lower body mass in
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17 254 weevils, which probably underpinned the indirect effects of eCO₂ on weevil performance.
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20 255 Phenolic concentrations increased in *R. nigrum* roots challenged by root herbivores under
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22 256 aCO₂, consistent with an induced defensive response, but phenolic concentrations did not
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25 257 increase following herbivory under eCO₂ which suggested that plants could not mount this
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27 258 response at eCO₂.

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32 260 The effects of eCO₂ on root growth have been studied for over 150 plant species (Rogers *et*
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34 261 *al.*, 1994), with most responding to eCO₂ by increasing dry root mass (Rogers *et al.*, 1996).
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36 262 Our study suggests that this is unlikely to happen in *R. nigrum* as root biomass significantly
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38 263 decreased in response to eCO₂, which has also been reported for other plant species (Bader *et*
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41 264 *al.*, 2009; Kohler *et al.*, 2009). While root growth is widely assumed to be enhanced due to
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43 265 the fertilising effect of eCO₂, this is often negated by variations in nutrient availability and the
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45 266 response may, in any case, decrease after several generations (Van Noordwijk *et al.*, 1998).
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48 267 Moreover, plants that are 'inherently' slow growing (e.g. woody perennial crops) are
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50 268 sometimes unable to modify growth rates and internal sinks to the same extent as faster
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53 269 growing plants, so CO₂ induced responses are often less pronounced in such plants (Van
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55 270 Noordwijk *et al.*, 1998). Bader *et al.* (2009) reported that woody plants in their study showed
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57 271 *ca.* 30% reduction in fine root biomass in response to eCO₂, and suggested that lower water
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60 272 requirements at eCO₂ may be responsible. It is unclear whether this could account for

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3 273 reductions in root biomass reported in this study, but our findings provide further evidence
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5 274 that eCO₂ does not necessarily promote root growth (Rogers *et al.*, 1996).
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10 276 Plant C and N concentrations were not statistically significantly affected by eCO₂ but root N
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12 277 concentrations decreased by 8% at eCO₂ in control plants, which is similar to the average
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15 278 generic decrease of 9% predicted by Cortrufo *et al.* (1998). Other studies have reported that
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17 279 eCO₂ did not affect C and N concentrations in roots of woody plants (Handa *et al.*, 2008;
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19 280 Bader *et al.*, 2009), so it seems plausible that C and N concentrations will not significantly
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21
22 281 change in *R. nigrum* under eCO₂ scenarios. In any case, it seems unlikely that changes in C
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25 282 and N were related to the reduced performance of weevils at eCO₂.
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29 284 Under both CO₂ conditions, weevil herbivory significantly reduced leaf C and increased root
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31 285 C:N ratios. This most likely arose due to root herbivory causing a sink in the root system,
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34 286 which diverted carbohydrates away from the foliage and impaired C fixation generally
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36 287 (Blossey & Hunt-Joshi, 2003; Johnson *et al.*, 2008). Similar negative effects of root herbivory
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38 288 on C concentration have been reported for both roots (e.g. Hopkins *et al.*, 1999; Katovich *et*
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40 289 *al.*, 1999) and leaves (e.g. Murray *et al.*, 1996; Dawson *et al.*, 2002). These findings contrast
41
42
43 290 with earlier assumptions that root herbivory generally causes increases in foliar C (e.g.
44
45 291 carbohydrates) through reduced water uptake and plant stress (Masters *et al.*, 1993). This
46
47
48 292 difference may be explained by the fact that leaf water concentrations were unaffected,
49
50 293 suggesting that root herbivory did not induce this type of nutritional stress response in leaves.
51
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54
55 295 The concentrations of root phenolics increased in response to vine weevil herbivory at aCO₂,
56
57 296 but the reverse was seen at eCO₂. Increases in phenolic concentrations are a common anti-
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59 297 herbivore defence exploited by many plants (Hartley & Jones, 1997), and have been shown to

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3 298 increase in response to root herbivore attack (Kaplan *et al.*, 2008). Indeed, there is growing
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5 299 evidence that roots are highly responsive in terms of inducible defence mechanisms,
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8 300 triggering synthesis of secondary metabolites in both leaves (e.g. phenolics) and the roots
9
10 301 (e.g. nicotine) (Kaplan *et al.*, 2008; Rasmann & Agrawal, 2008). This seems to be consistent
11
12 302 with the optimal defence theory (Stamp, 2003), which suggests that plants invest more into
13
14 303 **constitutive** defences in those tissues that are regularly attacked, whereas they are more reliant
15
16 304 on induced defences when herbivore attack is more intermittent. By their nature, root
17
18 305 herbivores are generally aggregated in the soil and attacks on plant roots tend to be sporadic
19
20 306 and localised (Brown & Gange, 1990) which would clearly make inducible defence a more
21
22 307 profitable strategy against root herbivores according to the optimal defence theory. Why eCO₂
23
24 308 resulted in altered defence responses is beyond the scope of this study, however, previous
25
26 309 work undertaken in *Arabidopsis* demonstrated that eCO₂ had significant effects on expression
27
28 310 of a number of transcripts involved in secondary metabolism, abiotic and biotic stress
29
30 311 responses, and cellular signalling (Li *et al.*, 2008). It is therefore likely that signal
31
32 312 transduction and defence responses may be altered in *R. nigrum* at eCO₂ environment.
33
34 313 **In this study, constitutive levels of phenolic compounds in roots that had not been attacked by**
35
36 314 **weevils were unaffected by eCO₂, which is consistent with the predictions of Zvereva &**
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38 315 **Kozlov (2006) for woody plant tissues.**
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48 317 While it seems likely that vine weevil larvae induced a defensive response in the roots of *R.*
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50 318 *nigrum*, as has been reported elsewhere for spinach (*Spinacia oleracea*) roots (Schmelz *et al.*,
51
52 319 1999), root phenolics were positively correlated with vine weevil population size and body
53
54 320 mass in this system. The defensive response following root damage therefore had no
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56 321 detrimental effects on vine weevils at the concentrations found in this study. The positive
57
58 322 relationship between vine weevil performance and root phenolics may be partly explained by
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1
2
3 323 the weak linkage between phenolic concentrations and root biomass (or another correlated
4
5 324 beneficial root feature) but it is also possible that phenolics could be beneficial for some
6
7
8 325 insects that are able to offset their negative effects. For instance, phenolics have been shown
9
10 326 to be nutritionally beneficial (Bernays & Woodhead, 1982; Bernays *et al.*, 1983) and act as
11
12 327 phagostimulants (Simmonds, 2001) for several insects. In particular, Bernays & Woodhead
13
14
15 328 (1992) suggest that insects could become adapted to using phenolics for cuticle sclerotization
16
17 329 to conserve amino nitrogen when feeding on woody plants, which are generally poorer in
18
19 330 terms of N concentration. A positive relationship between vine weevil performance and
20
21 331 phenolics has also been found in red raspberry (*Rubus idaei* L.) (Clark, 2010), which provides
22
23 332 further support for this observation. Zvereva & Kozlov (2006) suggested that constitutive
24
25 333 concentrations of phenolics in woody tissues are likely to decrease when elevated air
26
27 334 temperatures are combined with eCO₂ conditions, which may exacerbate the negative effects
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29 335 of eCO₂ on vine weevils in terms of reduced phenolic concentrations but also mitigate the
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31 336 effects through increased root growth at higher temperatures.
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338 *Conclusions*

339 Given the strong positive correlation between root biomass and weevil population size ($P <$
340 0.001), it seems reasonable to assume that the impaired root growth seen at eCO₂ was related
341 to reduced weevil performance at eCO₂. This is supported by studies between above- and
342 belowground herbivores, which have identified root biomass as the principal factor driving
343 root herbivore performance (Moran & Whitham, 1990; Masters *et al.*, 1993), but see Soler *et*
344 *al.* (2007). Elevated CO₂ also reduced water concentration in roots, which was also closely
345 related to weevil performance, and may therefore have also contributed to the negative effects
346 of eCO₂ on weevils. Roots challenged by weevils under eCO₂ conditions had the lower
347 phenolic concentrations, a factor which was also associated with poorer performance. If

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3 348 phenolics do promote vine weevil performance at the concentrations found here, rather than
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5 349 act as an anti-feedant (Bernays & Woodhead, 1982), then the failure of plants to mount a
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7
8 350 'defensive' response at eCO₂ in this study may have actually been detrimental to weevils.
9

10 351

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Table 1. Summary of statistical analysis for plant responses to elevated CO₂ and weevil herbivory. Statistically significant effects indicated in **bold** where $P < 0.05$. Where appropriate, data was transformed (¹log+1, ²arcsine square-root or ³log, or ⁴Box-Cox) prior to analysis. N = 30 in all cases. For CO₂ DF = 1,2; for weevils and the interaction between CO₂ and weevils DF = 1,112.

Plant response	Factor	CO ₂		Weevils		CO ₂ × weevils	
		$F_{1,2}$	P	$F_{1,112}$	P	$F_{1,112}$	P
Plant growth	Shoot dry mass ¹	0.01	0.939	0.01	0.930	1.08	0.301
	Root dry mass ¹ (Fig. 2a)	54.27	0.018	2.47	0.119	0.89	0.346
	Shoot:root ratio	1.63	0.330	2.26	0.135	0.62	0.434
Water relations	Leaf water content ²	0.54	0.539	0.41	0.524	0.21	0.645
	Root water content (Fig. 2b) ²	15.94	0.050	4.51	0.036	0.64	0.427
Plant minerals	Leaf C (Fig. 2c)	0.16	0.731	6.00	0.016	0.42	0.518
	Leaf N ³	0.08	0.808	0.52	0.472	0.41	0.524
	Leaf C:N	0.40	0.590	0.63	0.429	0.05	0.821
	Root C ³	1.96	0.296	0.61	0.437	1.16	0.283
	Root N	0.04	0.858	1.17	0.283	1.25	0.266
	Root C:N (Fig. 2d)	0.82	0.461	4.12	0.045	0.25	0.618
Root secondary metabolites	Phenolics ⁴ (Fig. 2e)	0.75	0.477	0.11	0.737	4.24	0.042

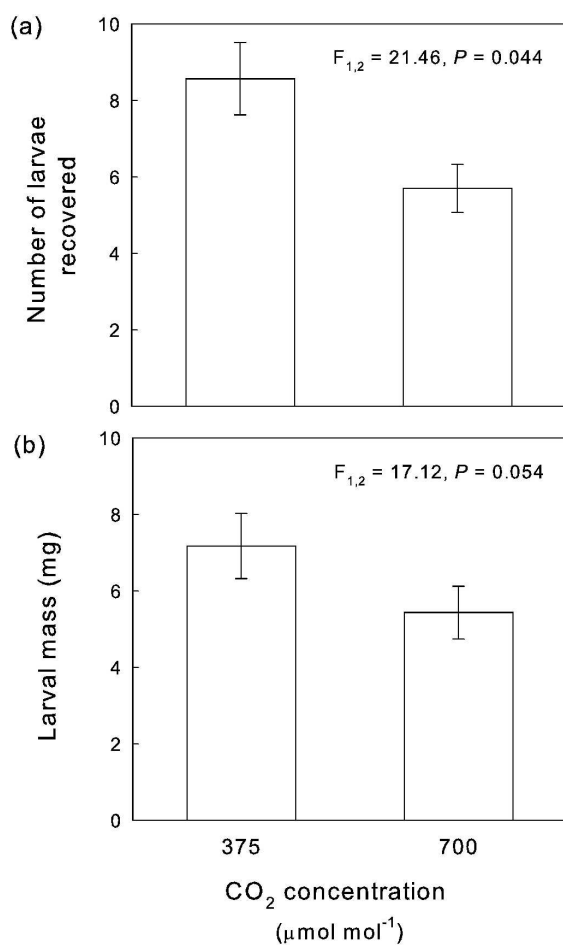


Figure 1. Effects of eCO₂ on vine weevil larval performance; (a) number of larvae recovered per plant and (b) average larval mass. Mean values ± S.E. shown, (a) N = 30 (b) N = 29 as no larvae were recovered from one plant. Larval data log+1 transformed prior to analysis. 215x279mm (600 x 600 DPI)

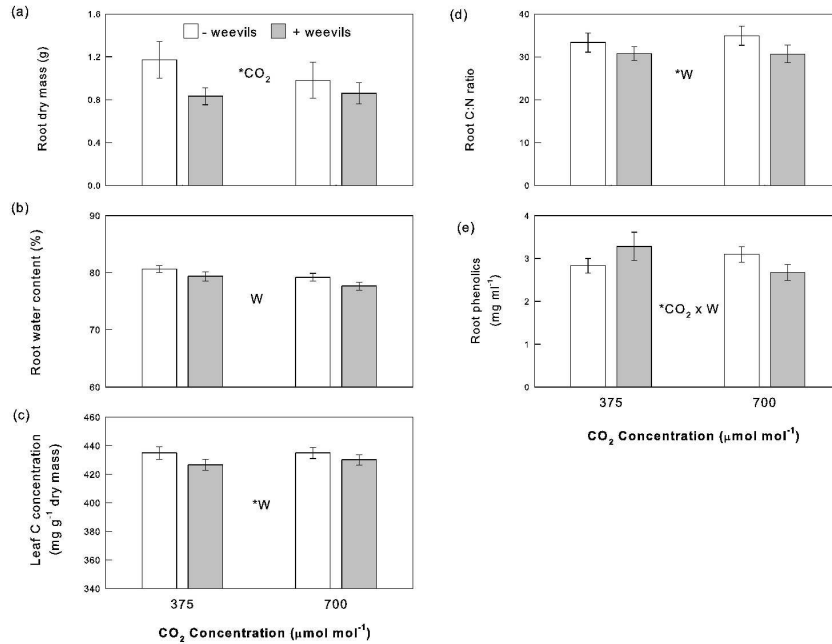


Figure 2. Statistically significant effects of eCO₂ and vine weevils on plant responses; (a) root dry mass, (b) root water concentration, (c) leaf C concentration, (d) root C:N ratio and (e) root phenolic compound concentration expressed as catechin equivalents. Statistically significant effects (see also Table 1) indicated * ($P < 0.05$) followed by CO₂ or W (weevil). Mean values \pm S.E. shown, $N = 30$ in all cases.

296x209mm (600 x 600 DPI)

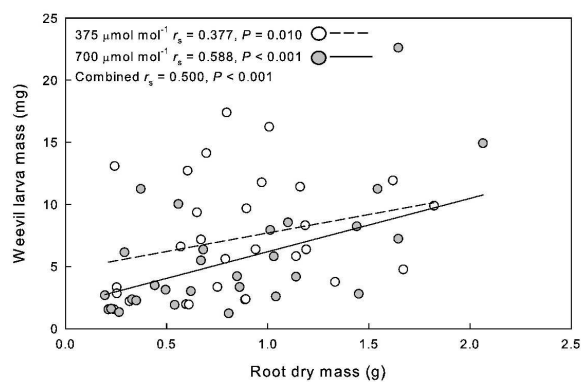


Figure 3. Relationship between root dry mass and weevil mass. Mean values shown. $N = 59$ as there were no larvae on one plant. Correlation analyses shown for each CO_2 concentration and collectively. Line of best fit shown where $P < 0.05$.

209x296mm (600 x 600 DPI)

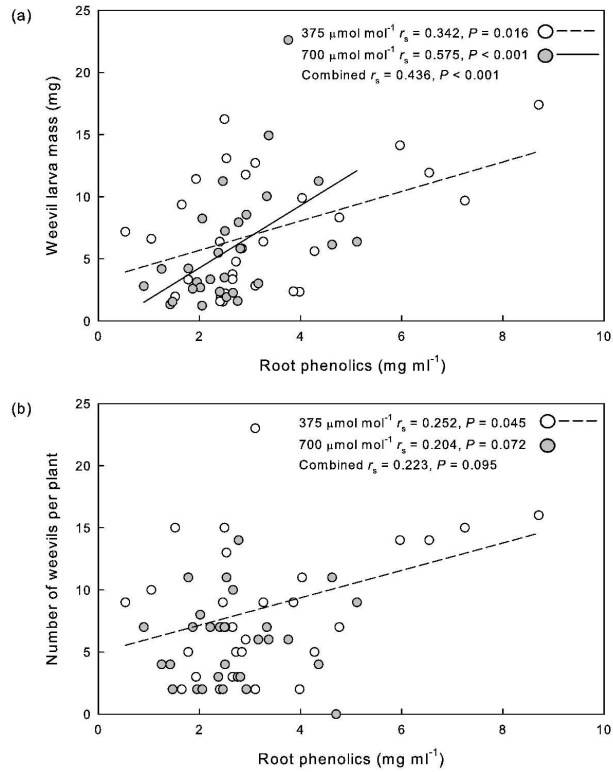


Figure 4. Relationship between root phenolic concentrations and (a) weevil mass and (b) weevil population size. Mean values shown. (a) $N = 59$ and (b) $N = 60$. Correlation analyses shown for each CO_2 concentration and collectively. Line of best fit shown where $P < 0.05$.

209x296mm (600 x 600 DPI)