The interaction of CO_2 concentrations and water availability on organic acids in root exudates of native Australian species

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Abstract

Purpose: Root exudation of organic acids (OAs) facilitates plant P uptake from soil, playing a key role in rhizosphere nutrient economy. However, OA exudation responses to elevated CO_2 concentrations (eCO_2) and water availability remain largely untested.

Methods: We examined the interactive effects of CO_2 and water on OA exudates in three Australian woodland species: *Eucalyptus tereticornis*, *Hakea sericea* and *Microlaena stipoides*. Seedlings were grown in a glasshouse in low P soil, exposed to CO_2 (400 ppm [aCO_2] or 540 ppm [eCO_2]) and water treatments (100% water holding capacity [high-watered] or 25–50% water holding capacity [low-watered]). After six weeks, we collected OAs from rhizosphere soil (OA_{rhizo}) and trap solutions in which washed roots were immersed (OA_{exuded}).

Results: For *E. tereticornis*, the treatments changed OA_{rhizo} composition, driven by increased malic acid exposed to eCO_2 and increased oxalic acid in low-watered plants. For *H. sericea*, low-watered plants had higher OA_{exuded} per plant (+116%) and lower OA_{rhizo} per unit root mass (−77%) associated with larger root mass but fewer cluster roots. For *M. stipoides*, eCO_2 increased OA_{exuded} per plant (+107%) and per unit root mass (+160%), while low-watered plants had higher citric and lower malic acids for OA_{rhizo} and OA_{exuded}: changes in OA amounts and composition driven by malic acid were positively associated with soil P availability under eCO_2.

Conclusion: We conclude that eCO_2 and altered water availability shifted OAs in root exudates, modifying plant–soil interactions and the associated carbon and nutrient economy.

Introduction

Understanding how terrestrial ecosystems respond to global changes requires an accurate prediction of plant responses to increases in atmospheric CO_2 concentrations and changes in water availability. While elevated CO_2 concentrations (eCO_2) generally enhance photosynthetic rates and increase plant growth, multiple lines of evidence suggest that the plant response to eCO_2 depends on soil nutrient status: the positive effect of eCO_2 on plant growth weakens under nutrient-limited conditions but strengthens with increased nutrient availability, such as with fertiliser addition (e.g. Jiang et al. 2020a; Reich et al. 2006a, b) or increased turnover rates (Phillips et al. 2011; Terrer et al. 2016, 2021). Thus, soil nutrient economy and plant nutrient uptake strategy likely drive the magnitude and duration of plant responses to eCO_2. However, despite numerous studies in recent decades, little is known about how future atmospheric CO_2 concentrations will affect these factors (Walker et al. 2021).

Plants exhibit various strategies to acquire nutrients under limited soil nutrient availability, for example, by increasing their relative belowground carbon (C) investment (higher root:shoot ratios) or adjusting their root morphology through root elongation, fine root development and increased root branching (e.g. Hill et

al. 2010; Ostonen et al. 2011). Soil nutrient acquisition can also be enhanced via plant investment of photosynthate to support mycorrhizae (e.g. Eissenstat et al. 2015; Helmisaari et al. 2009). Other strategies to increase nutrient availability include secretion of readily available organic C (i.e. sugars) from roots that acts as substrates for rhizosphere microbes, enhancing their decomposition of organic matter—so-called '*priming*' (Allard et al. 2006; Kuzyakov 2010; Phillips et al. 2011; Terrer et al. 2016; van Hees et al. 2005). Finally, plants can exude low molecular weight organic anions (organic acids; OAs) from roots to mobilise P. Phosphate bound to cations (e.g. Mn^{2+}, Al^{3+}, Ca^{2+}) or adsorbed onto soil mineral surfaces are inaccessible to plants. However, OAs compete with phosphate, preventing it from forming complexes with cations and thus liberating P (Bais et al. 2006; Dakora and Phillips 2002; Ryan et al. 2001). Interestingly, P mobilisation efficiency varies among OAs in the order of oxalic > citric > malic acids on a molar concentration basis (Mendes et al. 2020). Thus, changes in the amount and composition of root-exuded OAs likely impact soil P cycling and associated plant growth.

Soils in Australia are typically ancient and have long been exposed to weathering and leaching, consequently becoming impoverished, particularly in P availability (Vitousek et al. 2010; Walker and Syers 1976; Wang et al. 2010). Therefore, native plant species in Australia are considered well-adapted to low P conditions, with a range of P acquisition strategies such as high rates of OA exudation and mycorrhizal symbiosis (Lambers et al. 2012; Ryan et al. 2012). Indeed, some species in the Cyperaceae, Bossiae and Proteaceae families are particularly well-adapted to P-limited conditions and form *cluster roots* that express especially high rates of OA exudation (Lambers et al. 2012; Playsted et al. 2006; Sousa et al. 2007).

Elevated CO_2 likely increases plant nutrient demands due to enhanced growth and/or altered tissue stoichiometry (i.e. increased C:N, C:P or N:P ratios) (Bassirirad et al. 1996; Dijkstra et al. 2012; Johnson et al. 2004). Plants typically invest excess C belowground to tackle increased nutrient demands, enhancing microbial decomposition of organic matter (*priming*) and ectomycorrhizal nutrient transport (Terrer et al. 2016). In Hasegawa et al. (2016), we previously reported that eCO_2 increased soil P availability during the growing seasons in a free-air CO_2 enrichment (FACE) experiment established in a native, mature *Eucalyptus* woodland in southeastern Australia (EucFACE), where limited soil P availability largely suppresses forest responses to eCO_2 (Ellsworth et al. 2017; Jiang et al. 2020b). Hasegawa et al. (2016) and Ochoa-Huseo (2017) speculated that the increased soil P availability resulted from eCO_2-related C investment belowground and consequent enhancement of root exudates or priming. These studies put forward the hypothesis that increased photosynthetic rates under eCO_2 conditions increase root secretion of OAs into the soil, liberating soil P in line with the expectations from Lambers et al. (2008) and Ryan et al. (2012).

In combination with eCO_2 and global warming, substantial changes in precipitation patterns linked to climate change have caused extreme rainfall events in many parts of the world (Sun et al. 2021). In particular, the southeastern parts of Australia have experienced prolonged periods of drought and flooding on an unprecedented scale in recent years (Australian Bureau of Meteorology:

http://www.bom.gov.au), increasing the urgency for investigating the climate's role in modulating plant strategies for nutrient uptake. However, few studies have scrutinised the responses of OAs in root exudates to altered water availability; those that have, reported that severe drought stress substantially increased OA amounts and shifted rhizosphere OA composition for leguminous species, including several native to Australia, despite lower photosynthetic rates (Sharma et al. 2021; Suriyagoda et al. 2010). This contrasts with the hypothesised effects of eCO_2 enhancing root exudation via *increased* photosynthetic rates (Phillips et al. 2011; Terrer et al. 2016, 2021). Thus, CO_2- and water-induced changes in OA exudates are likely regulated by different mechanisms but remain largely unexplored. Furthermore, little is known about the combined effects of CO_2 and water availability on OA exudation. Here, we implement two extreme scenarios for water availability: high-watered conditions (soil held close to field capacity, i.e. water saturated) and low-watered conditions (soil water availability maintained at a similar level to that of dry, warm seasons in local *Eucalyptus* woodlands in southeast Australia). We study how plant root exudation responds to eCO_2 under those two water regimes by examining the amount and composition of OAs secreted from roots of three plant species native to these woodlands: *Eucalyptus tereticornis, Hakea sericea* and *Microlaena stipoides*.

Eucalyptus species commonly occur in the very poor soils of the coastal fringes of Australia (ABARES 2012; Birk and Turner 1992; Cromer et al. 1981). *E. tereticornis* thrives across eastern Australia (Drake et al. 2015) and is the dominant canopy species at the EucFACE field site. In addition, the genus *Eucalyptus* uniquely forms both arbuscular- and ectomycorrhizae, with the former colonising roots first followed by the latter (Boudarga et al. 1990; Chilvers et al. 1987). *H. sericea* is an understorey shrub species in the Proteaceae family, typically nonmycorrhizal but produces cluster roots and hence is considered well-adapted to low P conditions (Dinkelaker et al. 1995). *M. stipoides* is a native, perennial C_3 grass species, typically associated with arbuscular mycorrhizae (Hill et al. 2010). At the EucFACE site, *M. stipoides* is responsible for ~ 99% of the herbaceous understorey vegetation biomass (Collins et al. 2018; Hasegawa et al. 2018; L. Collins, unpublished data), contributing to 24% of the ecosystem gross primary productivity and thus playing a significant role in its C cycling (Jiang et al. 2020b).

Assessing low molecular weight compounds of root exudates in field conditions is extremely challenging due to their high decomposability and rapid sorption to soil particles. The hydroponic technique—the quintessential methodology for collecting exudates—is unlikely to provide realistic insights into root exudate responses (Vives-Peris et al. 2020). Therefore, we grew plants in a glasshouse in soil collected from the EucFACE field site, allowing us to relate results to earlier findings from the field study. We test the hypotheses that: 1) eCO_2 has positive effects on root exudation; 2) plants growing under low-watered conditions exude larger amounts of OAs than those under high water availability; 3) CO_2 and water treatments alter OA composition, and 4) CO_2-/water-induced changes in OA amounts are concomitant with changes in soil nutrient availability, especially P.

Materials And Methods

Experimental design, setup and growth

Three plant species were examined, each representing one stratum within the native *Eucalyptus* woodlands in southeastern Australia: *E. tereticornis* (canopy tree), *H. sericea* (understorey shrub) and *M. stipoides* (woodland floor graminoid). Seeds of *E. tereticornis* were purchased from a commercial supplier (Australian Tree Seed Centre, CSIRO National Research Collections Australia), while those of *H. sericea* and *M. stipoides* were collected locally in a native *Eucalyptus* woodland near Richmond, NSW (adjacent to the EucFACE field site). The seeds were sown and germinated on seed cell trays in growth chambers (30°C for *E. tereticornis*, 25°C for *H. sericea* and *M. stipoides*). Following germination, trays were transferred to a glasshouse bay. Once seedlings were well-established (*ca.* 2–3 weeks after germination), they were transplanted into cylindrical PVC pots (10 cm diameter · 33 cm height): 20 pots for *E. tereticornis* and 32 each for *H. sericea* and *M. stipoides*. One seedling for *E. tereticornis* and *H. sericea* and three seedlings for *M. stipoides* were transplanted into each pot. Each cylindrical pot was filled with 3.6 kg soil collected adjacent to the EucFACE site (*ca.* 1.6 kg cm^{-3} bulk density). The soil was loamy sand Chromosol of the Clarendon Formation (Bannerman and Hazelton 1990; Barton et al. 2010) with soil pH 5.45, 677 mg kg^{-1} total N, 59 mg kg^{-1} total P and 1.8% organic matter content. A more detailed description of the soil is available elsewhere (Crous et al. 2015; Hasegawa et al. 2016). The soil was collected from the top 0–20 cm, air-dried for two days, sieved (4 mm) to remove plant material and rocks, and thoroughly homogenised before use. When transplanted into pots, seedlings were well-watered and allowed to settle for 2–3 days.

Pots were placed in one of four naturally lit glasshouse compartments located at Western Sydney University in Richmond, NSW, Australia, between March and April 2016. Detailed information on the management of glasshouse conditions is available elsewhere (Ghannoum et al. 2010). Briefly, the glasshouse temperature simulated the 30-year average daily temperature of the local region during the growing season (November–May), with average temperatures of 26 and 18°C for day and night, respectively. The average relative humidity was 70%. Of the four compartments, two received ambient CO_2 (400 ppm), and two received elevated CO_2 (ambient + 140 ppm). CO_2 concentrations in the eCO_2 compartments were raised by injecting CO_2 gas (Food grade, Air Liquid, Australia) from pressurised cylinders. The glasshouse temperature and CO_2 concentrations were constantly monitored and regulated by a control system (Lambda T, ADC BioScientific Ltd., Hoddesdon, Herts, UK). The plants were exposed to two levels of water availability to examine the interactive effects between CO_2 and water treatments. Half of the pots were well-watered (hereafter referred to as '*high-watered*'), with the soil water content maintained at 100% water holding capacity (WHC) (*ca.* 15% gravimetric water content [GWC] or 23% volumetric water content [VWC]). The other half of the pots were maintained at 50% WHC (*ca.* 7% GWC) for the first three weeks and then 25% WHC for the next three weeks until harvest (hereafter referred to as '*low-watered*'). The pots were weighed and watered three times a week with deionised water to meet the target soil water contents. Pots were grouped into blocks, each comprising high-watered and low-watered replicates, resulting in ten blocks for *E. tereticornis* and 16 blocks for *H. sericea* and *M. stipoides*. Half of the blocks received aCO_2 and the other half eCO_2. Blocks were randomly allocated to one of four

glasshouse compartments, rotated within the compartments three times a week and between replicate compartments within the same CO_2 treatment once a week to minimise potential heterogeneity of environmental conditions within and between replicate compartments. A pair of pots for each block was always rotated together and placed next to one another.

Harvest and root exudate collection

After six weeks, the plants were harvested. All plants were watered one day before harvest so that the soil water contents were consistent at 50% WHC. Each pot was upended to remove the full plant and soil contents, and the soil was then gently shaken off the plant. The soil that remained on roots was defined as rhizosphere soil. Root exudates of OAs were collected following the approach in Kidd et al. (2018) using two sequential methods. First, roots were immersed in a known volume (*ca.* 50 ml) of 0.2 mM $CaCl_2$ solution and gently shaken for a few minutes to wash off rhizosphere soil. The collected OAs are referred to as rhizosphere OAs (OA_{rhizo}). Second, following removal of rhizosphere soil, roots were thoroughly washed with Milli-Q water, placed in a known volume (*ca.* 60 ml) of 0.2 mM $CaCl_2$ solution, and incubated for 1 hour at room temperature in a laboratory to collect exudates directly from roots, referred to as OA_{exuded}. The OA_{rhizo} and OA_{exuded} solutions were filtered (0.2 µm) before adding a drop of concentrated phosphoric acid to each to minimise microbial decomposition. Solutions were immediately frozen in liquid nitrogen and stored at − 20°C until analysis. The harvested plants were placed in a drying oven at 80°C for 2–3 days, with dry weight (DW) of above- and below-ground parts measured to obtain total biomass and root:shoot ratios (RSR). For *H. sericea*, the number of cluster roots was counted, and cluster root counts per unit root biomass were calculated.

Analysis of OAs in root exudates

Organic acids were analysed using Reverse Phase Liquid Chromatography as described in Cawthray (2003) for the following compounds: malic, acetic, citric, cis-aconitic, succinic, fumaric and trans-aconitic acids. Malonic and maleic acids were also analysed but not detected in the current study. Oxalic acid was measured following Uloth et al. (2015). Total amounts of OA_{rhizo} and OA_{exuded} equated to the sum of all detected OAs on a mass basis for each growing pot. Total OA_{rhizo} amount was expressed as µg plant^{-1}, and total OA_{exuded} amount was expressed as µg plant^{-1} h^{-1}. Root-mass based amounts of OA_{rhizo} and OA_{exuded} were expressed as µg g^{-1} root DW and µg g^{-1} root DW h^{-1}, respectively. Total OA amounts were converted to C mass using molecular weights and the number of C atoms for each OA to estimate the amount of C secreted from roots as OAs.

Soil nutrients

Plant-accessible nutrients were evaluated using ion exchange resin membranes (IEMs) buried in the top 0–6 cm of each pot, after modifying the method outlined in Hovenden et al. (2008). Anion and cation IEMs were cut into 1 cm · 6 cm pieces. Anion membranes were placed on one side of a plastic tag and cation membranes on the other side, collectively representing an IEM-tag. Two IEM-tags were inserted into the soil in each pot at the start of the CO_2 and water treatments. These tags were collected at harvest and

washed with Milli-Q water to remove adhesive soil. Duplicate IEM-tags for each pot were placed in a falcon tube, mechanically shaken with 40 ml of 0.1 M hydrochloric acid for 1 hour to extract nitrate (NO_3^--N), ammonium (NH_4^+-N) and phosphate (PO_4^{3-}-P). This solution was then diluted ten times to determine extracted N and P concentrations using a continuous flow auto-analyser (AQ2 Discrete Analyzer, SEAL Analytical, Mequon, WI, USA).

Statistical analysis

Statistical analyses were performed using R 4.1.2 (R Core Team 2021). We report results at the significance level of = 0.05, and marginally significant results ($P \leq 0.1$) as an important indicator of treatment effects. Each of the studied species was analysed separately. Linear mixed-effects models (LMMs) were performed using the 'lme4' package (Bates et al. 2015) to examine CO_2, water and their interactive effects on OA_{rhizo} and OA_{exuded} (total and root-mass based amount), with block as a random factor. LMMs were also used to evaluate treatment effects on plant/soil variables (total biomass, RSR and soil nitrate, ammonium and phosphate availability, and the number of cluster roots per unit root biomass for *H. sericea*). Response variables were transformed ($\log_e$, square root or Box-Cox transformation) as required to ensure homogeneity of variances and normality of errors prior to analysis (Crawley 2012; Fox and Weisberg 2011). *P* values of the fixed factors in the LMMs (i.e. CO_2, water and CO_2·water) were approximated by *F*-test using Type II ANOVA tests with Kenward-Roger Degrees of Freedom, using the 'car' and 'lmerTest' packages (Fox and Weisberg 2011; Kenward and Roger 1997; Kuznetsova et al. 2017). The amounts of each OA in OA_{rhizo} and OA_{exuded} were evaluated individually to examine the response of each OA to CO_2 and water treatments. Since there were frequent zeros for some OAs (or not detectable) and the values were bound by zero, the distribution and error structure of the data were not normal. Therefore, we employed generalised linear mixed-effects models (GLMMs) using the Gaussian distribution with log link, with block as a random factor. As the Gaussian distribution can handle only non-zero positive values, a small value (i.e. the smallest value detected) was added to all OA values that had zeros prior to analysis. *P* values of the fixed factors in the GLMMs were obtained by *Chi*-squared tests.

Multiple regression analysis was performed to evaluate the associations between total OA amounts and plant/soil measurements for OA_{rhizo} and OA_{exuded}. RSR and soil ammonium availability were $\log_e$-transformed prior to the analysis. Explanatory variables were Z-standardised so that values had mean = 0 and standard deviation = 1. Ninety-five percent confidence intervals (CIs) on the coefficients of the multiple regression model were estimated by parametric bootstrap with 999 simulations using the 'lme4' package. Multicollinearity between the explanatory variables was checked using variance inflation factors (≤ 5). This random factor was removed from the LMMs, GLMMs and multiple regression analysis where block did not explain any of the variations in response variables and did not improve the models, and (generalised) linear models were performed instead to avoid a singular fit (Bates et al. 2015).

Redundancy analysis (RDA) was employed to assess CO_2 and water effects on the composition of OA_{rhizo} and OA_{exuded} for each species using the 'vegan' package (Oksanen et al. 2018). Compositional OA

data were Hellinger-transformed prior to analysis. The interactive term of the combined CO_2 and water treatments did not improve adjusted R^2 values for the tested models and hence was not included in the analysis (Legendre and Legendre 2012). RDA models were tested with 4,999 permutations. Additional permutation tests were performed when significant associations between the OA composition and CO_2 and water treatments were indicated at < 0.05 to analyse the marginal effects of each term. Correlations between the resulting RDA axes (RDA1 and RDA2) and plant/soil variables (total biomass, RSR, soil ammonium, nitrate and phosphate, and the number of cluster roots per unit root biomass for *H. sericea*) were also assessed. RSR was $\log_e$-transformed. Non-metric multidimensional scaling (NMDS) was used for OA_{rhizo} and OA_{exuded} to visualise the pattern of OA composition across the studied species, with the relative abundance of each OA computed and Hellinger-transformed for each plant prior to analysis.

Results

Plant and soil measurements

The effects of CO_2 and water treatments on plant/soil measurements were species-specific. Total biomass generally increased in the eCO_2 treatment, by 20% ($P > 0.1$), 29% ($P < 0.05$) and 33% ($P < 0.1$) relative to aCO_2, for *E. tereticornis*, *H. sericea* and *M. stipoides*, respectively (Fig. 1a, Table S1). No eCO_2 effects occurred for C investment in belowground biomass (i.e. RSR and the number of cluster roots per unit root biomass, Fig. 1b, c) or for soil nitrate concentrations in any studied species (Fig. 1d). The eCO_2 treatment increased soil ammonium concentrations for *E. tereticornis*, relative to aCO_2 (+ 139%, $P <$ 0.001), while they did not change for *H. sericea* and decreased for *M. stipoides* (− 49%, $P < 0.001$). The eCO_2 effects on soil ammonium concentrations were particularly large for low-watered *E. tereticornis* and *M. stipoides* compared to high-watered pots, as indicated by a significant interaction for both species (Fig. 1e). Soil phosphate concentrations were unaffected by the eCO_2 treatment for *E. tereticornis* or *H. sericea*, but significantly increased for *M. stipoides* (+ 79%, $P < 0.01$) relative to aCO_2 (Fig. 1f, Table S1).

Low-watered plants had significantly higher total biomass than high-watered *E. tereticornis* (+ 125%, $P <$ 0.001), *H. sericea* (+ 31%, $P < 0.01$) and *M. stipoides* (+ 37%, $P < 0.05$) (Fig. 1a, Table S1). Low-watered *E. tereticornis* had significantly lower RSR (− 18%, $P < 0.05$) than high-watered plants, while it was higher for *H. sericea* (+ 18%, $P < 0.001$) and did not change for *M. stipoides* (Fig. 1b). Low-watered *H. sericea* had significantly fewer cluster roots per unit root biomass (− 44%, $P < 0.001$) than high-watered plants (Fig. 1c). The water treatments did not affect soil nitrate concentrations for *E. tereticornis*, whereas low-watered *H. sericea* and *M. stipoides* had significantly higher soil nitrate concentrations (+ 106%, $P < 0.001$ and + 111%, $P < 0.001$, respectively) than high-watered plants (Fig. 1d). Low-watered plants had significantly higher soil ammonium concentrations for *E. tereticornis* (+ 127%, $P < 0.001$) but lower concentrations for *H. sericea* (− 4%, $P < 0.05$; removing one outlier in eCO_2:High-watered: − 30%, $P < 0.01$) and *M. stipoides* (− 45%, $P < 0.001$) than high-watered plants (Fig. 1e). Low-watered plants had consistently lower soil phosphate concentrations than high-watered plants for all tested species: *E.*

tereticornis (−52%, $P < 0.001$), *H. sericea* (−57%, $P < 0.001$) and *M. stipoides* (−43%, $P < 0.001$) (Fig. 1f, Table S1).

Quantitative responses of organic acids in exudates

The OA_{rhizo} and OA_{exuded} amounts varied among the three species but within the same order of magnitude when expressed per unit root biomass (Fig. 2). On average, OA_{rhizo} amounts were 376 ± 73, 836 ± 368 and 479 ± 84 μg g^{-1} root DW for *E. tereticornis*, *H. sericea* and *M. stipoides*, respectively (mean ± 1. SE, Fig. S1). OA_{exuded} amounts were 319 ± 72, 175 ± 24 and 249 ± 48 μg h^{-1} g^{-1} root DW for *E. tereticornis*, *H. sericea* and *M. stipoides*, respectively.

Effect of CO_2 treatment on exudation

For *E. tereticornis* and *H. sericea*, there was no evidence of eCO_2 effects on the total amounts of OA_{rhizo} or OA_{exuded} regardless of the units of expression (per plant or unit root biomass) (Figs. 2, S1, Table S2). For *M. stipoides*, eCO_2 did not alter OA_{rhizo} amount but more than doubled OA_{exuded} amount per plant ($P < 0.05$, Fig. 2) and per unit root biomass ($P < 0.05$, Fig. S1b, Table S2), increasing the amount of C secreted from roots from 2.6 to 5.6 ug C h^{-1} per plant (Fig. S2b).

Effect of water treatment on exudation

For *E. tereticornis* and *M. stipoides*, the water treatments did not affect the total amount of OA_{rhizo} or OA_{exuded} regardless of expressed units (per plant or unit root biomass) (Figs. 2, S1, Table S2). For *H. sericea*, while the total OA_{rhizo} amount per plant did not change between water treatments (Fig. 2a), the low-watered plants had 77% less total OA_{rhizo} amount per unit root biomass than high-watered plants ($P < 0.05$, Fig. S1a, Table S2), decreasing the amount of C in OA_{rhizo} from 470 to 104 μg C g^{-1} root DW (Fig. S2). In contrast, low-watered *H. sericea* seedlings had greater OA_{exuded} amounts per plant (+ 116%, $P < 0.05$, Fig. 2b) and per unit root biomass (+ 32%, $P > 0.1$, Fig. S1b) than high-watered seedlings, although the latter was not statistically significant (Table S2), increasing the amount of C in OA_{exuded} from 4.7 to 9.4 μg C $plant^{-1}$ h^{-1} (Fig. S2).

Association between exudates and plant/soil measurements

For *E. tereticornis*, total OA_{rhizo} amount had a significant positive association with total biomass, but total OA_{exuded} did not correlate with any tested variable (Table S3). For *H. sericea*, no associations occurred between total OA_{rhizo} amount and any tested variable, but total OA_{exuded} positively correlated with total biomass and RSR (Table S3). For *M. stipoides*, OA_{rhizo} amount positively correlated with total biomass and soil phosphate concentrations, and OA_{exuded} amount positively correlated with total biomass (Table S3).

Qualitative responses of organic acids in exudates

OA compositions in OA_{rhizo} and OA_{exuded} varied among the three species (Fig. 3). Only *H. sericea* secreted cis- and trans-aconitic acids. The exudates from *M. stipoides* mainly comprised malic acid (11–50% and 19–80% in OA_{rhizo} and OA_{exuded}, respectively) and citric acid (7–54% and 0–45%), while those from *E. tereticornis* mainly comprised oxalic acid (27–96% and 51–84%) (Fig. 3a, b, Table S4).

Effect of CO_2 treatment on OA composition

For *E. tereticornis*, the CO_2 treatment significantly altered OA_{rhizo} composition ($P < 0.01$, Fig. 4a, Table S5), likely driven by increased malic acid at eCO_2 relative to aCO_2 (+ 322%, $P < 0.05$, Table S4), but did not affect OA_{exuded} composition ($P > 0.1$, Fig. 4d). For *H. sericea*, the CO_2 treatment did not affect OA_{rhizo} or OA_{exuded} composition (Table S5, Fig. 4b, e). For *M. stipoides*, the CO_2 treatment significantly altered OA_{rhizo} composition ($P < 0.05$, Fig. 4c), despite no main CO_2 effects on any detected OA when analysed individually (Table S4). However, an interaction occurred between the CO_2 and water treatments for citric acid in OA_{rhizo} for *M. stipoides*: eCO_2 significantly increased the amount of citric acid relative to aCO_2 under high-watered but not low-watered conditions (Table S4). A similar pattern occurred for malic acid in OA_{rhizo}, but it was not statistically significant. For *M. stipoides*, the CO_2 treatment weakly influenced OA_{exuded} composition ($P < 0.1$, Fig. 4f, Table S5), possibly due to changes in shikimic acid, as its exudation rate was 7-fold higher under eCO_2 than aCO_2 (Table S4).

Effect of water treatment on OA composition

For *E. tereticornis*, the water treatment significantly altered OA_{rhizo} composition ($P < 0.05$, Fig. 4a, Table S5), likely driven by increased oxalic acid under low-watered compared to high-watered conditions (+ 80%, $P < 0.05$, Table S4), but did not affect OA_{exuded} composition ($P > 0.1$, Fig. 4d, Table S5). For *H. sericea*, the water treatment did not affect OA_{rhizo} or OA_{exuded} composition (Fig. 4b, e, Table S5). For *M. stipoides*, the water treatment significantly altered OA_{rhizo} composition ($P < 0.05$, Fig. 4c, Table S5), likely driven by increased citric acid (+ 100%, $P < 0.05$) and decreased malic (–45%, $P < 0.01$) and shikimic acids (–40%, $P < 0.1$) under low-watered compared to high-watered conditions (Table S4). Changes in oxalic acid may have also contributed to the shift in OA_{rhizo} composition as it was only observed in low-watered and not high-watered *M. stipoides*. For *M. stipoides*, the water treatment also significantly altered OA_{exuded} composition ($P < 0.05$, Fig. 4f, Table S5), likely due to increased citric acid under low-watered compared to high-watered plants (+ 215%, $P < 0.05$, Table S4). Furthermore, malic acid may have contributed to the compositional shift in OA_{exuded} as indicated by its relatively larger negative value for the species score along the first RDA axis accounting for 11.0% of the total variability (Fig. 4f). The amount of malic acid in OA_{exuded} for *M. stipoides* was so low that it was undetectable in some pots. While there was no evidence of CO_2 or water treatment effects on malic acid, this OA was detected more often in high-watered plants at eCO_2 (7 of 8 pots), with higher mean levels, than other conditions (2–3 of 8 pots) (Table S4).

Association between OA composition and plant/soil measurements

As mentioned above, the CO_2 and water treatments significantly altered OA_{rhizo} composition in *E. tereticornis*, with the RDA axes correlated with soil phosphate concentrations ($P < 0.05$) and plant biomass ($P < 0.1$, Fig. 4a). Along the first RDA axis, accounting for 24% of the total variation in data, soil phosphate concentrations negatively correlated with oxalic acid concentrations in low-watered pots under aCO_2 and positively correlated with malic acid in high-watered pots under eCO_2 (also see Fig. 3a). For *M. stipoides*, the CO_2 and water treatments strongly influenced OA_{rhizo} and OA_{exuded} composition, with RDA axes strongly correlated with changes in total biomass and soil phosphate concentrations (Fig. 4c, f); under eCO_2:High-watered conditions with a relatively high proportion of malic acid, OA composition correlated with high soil phosphate concentrations, whereas under eCO_2:Low-watered conditions with a relatively larger proportion of citric acid, OA composition correlated with high total biomass (also see Fig. 3a, b).

Discussion

We performed a unique study investigating the effects of increased CO_2 concentrations and altered water availability on OA amount and composition in root exudates from three plant species representing different strata within native *Eucalyptus* woodlands in southeastern Australia: *E. tereticornis* (canopy tree), *H. sericea* (understorey shrub) and *M. stipoides* (woodland floor graminoid). The CO_2 and water treatments caused quantitative and qualitative changes in root exudates, which were highly species-dependent. As expected, eCO_2 positively affected plant growth, although this was not statistically significant for *E. tereticornis* ($P > 0.1$). To our surprise, plants grown under low-watered conditions outperformed those under high-watered conditions for all three species, and thus low-watered conditions seemed more optimal for these species than the high-watered conditions.

Overall, the total amount of OAs in root exudates (per plant basis) was mainly driven by plant size (i.e. biomass): larger plants had higher exudation rates within the same species. Also, significant shifts in OA composition in response to CO_2 and/or water treatments (i.e. OA_{rhizo} from *E tereticornis* and OA_{rhizo} and OA_{exuded} from *M. stipoides*) were accompanied by changes in plant biomass. Thus, eCO_2 and water availability likely indirectly influence OA amount and composition through altered plant size. This is not surprising as fluxes and compositions of root exudates often depend on plant developmental stage (Aulakh et al. 2001; Baptist et al. 2015; Canarini et al. 2016; Darwent et al. 2003). Indeed, studies have shown that altered fluxes in root exudates under contrasting environmental conditions (e.g. eCO_2, drought or warming) occur alongside changes in plant growth and morphology for various plant growth forms (e.g. crops, grasses or trees) (Calvo et al. 2019; de Vries et al. 2019; Yin et al. 2013).

Total OA amount in OA_{rhizo} ranged from 376–836 μg g^{-1} root DW across the three species in this study. When expressed on a molar basis, these figures corresponded to 3.5–6.7 μmol g^{-1} root DW (data not shown). Kidd et al. (2018) collected OAs in root exudates from 10 pasture leguminous species using the same technique as the present study, reporting OA_{rhizo} amounts from 12–191 μmol g^{-1} root DW. Thus, our values were generally smaller, which could be attributed to the species in our study having much thicker roots (or lower specific root lengths) than those in Kidd et al. (2018): when expressed on a root length basis instead of root mass, OA amounts in Kidd et al. (2018) ranged from < 1–14 nmol cm^{-1} root (except *Cicer arietinum* L. (C) with 68 nmol cm^{-1} root), similar to our values (7–13 nmol cm^{-1} root, data not shown). Our results suggest that the studied species do not invest as much C as the pasture legumes reported in Kidd et al. (2016, 2018) for forming root structures that are particularly suitable for root exudation, such as root hairs (Jungk 2001; Yan et al. 2004). It should also be noted that the growth media differed between the two studies: Kidd et al. (2018) grew plants in washed river sand, which minimises the adsorption and microbial decomposition of OAs, likely resulting in greater amounts of rhizosphere OA than those in native soil (Ryan et al. 2012).

Roelofs et al. (2001) evaluated OA exudation from seven Australian Proteaceae (*Banksia*, *Hakea* and *Dryandra*) species hydroponically cultivated in nutrient solutions, reporting OA exudation rates from cluster roots ranging from 1.0–2.5 nmol g^{-1} root fresh weight s^{-1}. If root DW is assumed to be 10% of fresh weight, as assumed by Roelofs et al. (2001), these figures equate to 36–90 μmol g^{-1} root DW h^{-1}, higher than the value for *H. sericea* in this study (6.7 ± 2.9, mean ± 1 standard error, data not shown). This was likely due to our collection of root exudates from the whole root system, including cluster and non-cluster roots, whereas Roelofs et al. (2001) only sampled from cluster roots. The OA_{exuded} from *H. sericea* in our study was predominantly represented by trans-aconitic acid, while citric and malic acids were undetected. This compositional pattern is more similar to non-cluster than cluster roots (Roelofs et al. 2001), suggesting that cluster roots may have played a relatively small role in OAs of root exudates in the present study.

Effect of CO_2 treatment on exudation

For *E. tereticornis*, there was no evidence of a CO_2 effect on OA amounts in root exudates. Also, in contrast to previous studies showing positive effects of high CO_2 exposure on the growth of *Eucalyptus* species (e.g. Atwell et al. 2007; Ghannoum et al. 2010), total biomass of *E. tereticornis* did not change in response to eCO_2 in this study, which could be associated with low P availability constraints on the fertilisation effect of eCO_2 on plant growth (Collins et al. 2018; Crous et al. 2015; Duan et al. 2019; Ellsworth et al. 2017). However, given the lack of CO_2 effect on RSR, these results suggest that *E. tereticornis* did not alter its C investment balance for nutrient uptake in response to eCO_2. In contrast, a shift in OA_{rhizo} composition from *E. tereticornis* occurred in response to eCO_2 (see below).

For *H. sericea*, there was no evidence of CO_2 effects on OA_{exuded} or OA_{rhizo} amount. There was also no evidence of a CO_2 effect on cluster root production, in contrast to a previous study that reported an increased number of cluster roots in white lupin (*Lupinus albus*) in response to eCO_2 using a hydroponic technique (Campbell and Sage 2002). A plethora of hydroponic studies report increased cluster root production with reduced P supply (e.g. Campbell and Sage 2002; Neumann et al. 1999; Shane et al. 2003). As argued above, the OA composition of *H. sericea* in this study was similar to non-cluster roots rather than cluster roots (Roelofs et al. 2001). Combined with the significant increase in biomass of *H. sericea* under eCO_2, this species appeared to have had adequate access to P without the need to invest C into further cluster root production or root exudation.

For *M. stipoides*, increased OA_{exuded} amounts under eCO_2 were likely due to increased biomass and photosynthetic rates. This species did not alter biomass allocation between above- and below-ground parts but had larger amounts of OA_{exuded} per unit root biomass under eCO_2 than aCO_2, suggesting a greater proportional C investment into exudation under eCO_2. In line with this, Pathare et al. (2017) found that eCO_2 increased net CO_2 assimilation rates in *M. stipoides* by 28% in a local *Eucalyptus* woodland where the seeds of this species were collected for our study.

Effect of water treatment on exudation

We did not measure photosynthetic rates; hence, it is not possible to conclude whether plants were water stressed or not. However, unexpectedly, all the studied species expressed higher total plant biomass under low-watered conditions than high-watered conditions, suggesting that the low-watered treatment provided relatively more optimal growth conditions than the high-watered treatment.

For *E. tereticornis*, the water treatment did not change the OA amounts in root exudates, despite higher total biomass and lower RSR for the low-watered plants. This was surprising as drought inhibits the growth of a wide range of *Eucalyptus* species relative to well-watered conditions (Atwell et al. 2007; Ngugi et al. 2003a, b). Plants in this study may have experienced anaerobic soil conditions due to the watering regime adopted to bring pots back to full field capacity every 2–3 days. Together with slightly smaller amounts of root exudates per unit root biomass, these results suggest that *E. tereticornis* seedlings invested less C in OAs or belowground biomass and more C in aboveground growth under low-watered conditions relative to high-watered conditions. This may be related to their survival strategy at an early phase of juvenile seedling development, where this species is typically exposed to intense competition for light with surrounding herbaceous species on the forest floor in natural woodlands (Collins et al. 2018).

For *H. sericea*, the total OA_{exuded} amount per plant increased and the total OA_{rhizo} amount per unit root mass decreased under low-watered conditions relative to high-watered conditions. While this species allocated more biomass belowground under low-water conditions (increased total biomass and RSR), the number of cluster roots per unit root biomass decreased. These results suggest that *H. sericea* invested C into developing non-cluster roots, and its larger root system could maintain similar whole plant OA_{exuded}

levels under both watering regimes. Sharma et al. (2021) reported that a drought treatment suppressed plant photosynthesis and increased OA_{rhizo} amounts in *Cicer arietinum* L. While the mechanisms for these observations remain untested, these authors speculated that increased OA_{rhizo} amounts were an artefact of surplus C (instead of active nutrient mining) resulting from 1) a time lag between drought-induced suppression of plant growth and photosynthesis and 2) hampered microbial decomposition in the rhizosphere. However, in our study, the altered OA_{rhizo} amount per unit root biomass observed in *H. sericea* was probably due to a morphological shift in roots, as indicated by fewer cluster roots per root biomass in low-watered than high-watered plants, not changes in surplus C. We speculate that this may be due to the suboptimal conditions of high-watered plants, which did not invest C for soil resource exploration, compared to low-watered plants. *Hakea* species are evolutionally well-adapted to survive severe drought events and bushfires in Australia (Bradstock et al. 1994). The *H. sericea* seeds used in our study were collected from the local native *Eucalyptus* woodland where soil rarely reaches its full WHC (Hasegawa et al. 2016), and thus the provenance of this species may have been well-adapted to the hot, dry climate with well-drained sandy soil. Climate models predict that southeastern Australia will experience prolonged drought and more intensified rainfall events in the future (Dey et al. 2019; Grose et al. 2020; Guerreiro et al. 2018). Extreme precipitation can expose plants to particularly high water conditions for extended periods; our study suggests that *H. sericea* may struggle, changing root structure and OA (and therefore C) exudation.

Higher plant growth for *M. stipoides* under low-watered than high-watered conditions did not increase the total amount of OAs exudated, as they were counterbalanced by smaller amounts per unit root biomass. Higher plant growth for this species under low-watered conditions was also observed by Piñeiro et al. (2021) using the same soil and seeds collected adjacent to EucFACE. On a similar note, net assimilation rates of *M. stipoides* measured at EucFACE peaked at ~ 7% of soil VWC (the same level as the low-watered conditions in our study) and plateaued (or even slightly declined) as soil VWC further increased (Pathare et al. 2017). Thus, the provenance of *M. stipoides* used in our study did not invest C into exudation under low-watered conditions, despite greater plant growth.

Effect of CO_2 and water treatments on OA composition in association with plant/soil measurements

The effect of CO_2 and water treatments on OA composition varied among species, altering OA composition for *E. tereticornis* and *M. stipoides*, mainly via changes in malic acid, with contributions from citric, oxalic and shikimic acids. These are common OAs in root exudates, previously reported in a range of plant species, that facilitate plant growth via, for instance, P mobilisation, Al detoxication and nitrate uptake (Badri and Vivanco 2009; Dakora and Phillips 2002; Vives-Peris et al. 2020). In contrast, there was no evidence of treatment effects on OA composition in *H. sericea*. Exudations from this species were distinct from those of *E. tereticornis* and *M stipoides*, mainly comprising trans-aconitic and oxalic acids with a small proportion of cis-aconitic acids. This was the only species containing cis-/trans-aconitic

acids in this study, aligning with previous observations of trans-aconitic acid in a wide range of Proteaceae species but rarely in other plant species (Roelofs et al. 2001).

Interestingly, soil phosphate concentrations strongly correlated with shifts in OA composition for *E. tereticornis* and *M. stipoides*. The total OA_{rhizo} amount for *M. stipoides* (but not *E. tereticornis*) positively correlated with soil phosphate, with malic acid the primary OA for this species (Fig. 3a). This suggests that *M. stipoides* exposed to eCO_2 (or low-watered conditions) secretes a relatively larger (or smaller) quantity of malic acid in the rhizosphere, facilitating (or suppressing) soil P mobilisation (Bais et al. 2006; Dakora and Phillips 2002; Ryan et al. 2001). In contrast, despite *E. tereticornis* increasing the amount of malic acid exuded in the rhizosphere in response to eCO_2, it did not change the total OA_{rhizo} amount or soil phosphate concentration. Furthermore, despite higher efficiency of P mobilisation by oxalic than malic acids (on a molar concentration basis) (Mendes et al. 2020), the increased amount of oxalic acid in OA_{rhizo} in low-watered *E. tereticornis* plants co-occurred with decreased soil phosphate concentrations. While many hydroponic studies have demonstrated increased rates of root exudation at low P supply to facilitate P uptake (e.g. Khorassani et al. 2011), responses differ among organic compound types. For instance, Wang et al. (2015) reported increased rates of citric and malic acids but decreases in succinic acid under low P supply relative to high P supply in canola, barley and potato plants. In our study, CO_2 and water treatment did not influence the C allocation of *E. tereticornis* to root exudation but shifted OA composition; however, this did not seem to facilitate P mobilisation. The relationship between particular OAs and soil P availability is highly species-specific, especially when using soil from a natural ecosystem.

Estimating C fluxes from root exudates of OAs in a Eucalyptus woodland under eCO_2

Although it varies substantially among plant species, roots secrete approximately 11–40% of assimilated C (Badri and Vivanco 2009; Jones et al. 2009), with OAs accounting for a significant fraction (Farrar et al. 2003; Jones 1998). For instance, OAs are responsible for 16–31% C in root exudates of maize (Gransee and Wittenmayer 2000). Hence, changes in the exudation rates of OAs could significantly change the amount of C secreted from plant roots to soil. Our study provides empirical estimates of the amounts of C secreted from the studied plant species in the form of OAs. Our study was motivated by the EucFACE experiment—established in a local native *Eucalyptus* woodland to investigate ecosystem-scale responses to increased atmospheric CO_2—where *M. stipoides* is responsible for ~ 99% of the understorey vegetation biomass (Hasegawa et al. 2018; L. Collins, unpublished data). Given that the soil and seeds of *M. stipoides* used in this study were collected from this site, the figures obtained from *M. stipoides* may be relatively realistic estimates of C secreted from roots under field conditions, while *E. tereticornis* and *H. sericea* may not be comparable to those in the field as they were juvenile seedlings when harvested. If we focus on the exudation of *M. stipoides*, we can derive estimates of belowground C inputs from the grassy understorey: OA_{rhizo} amounts were 4.9 ± 1.6 (aCO_2) and 9.0 ± 1.7 (eCO_2) μg C $plant^{-1}$ and OA_{exuded} amounts were 2.6 ± 1.2 (aCO_2) and 5.6 ± 1.4 (eCO_2) μg C $plant^{-1}$ h^{-1}, with average aboveground biomass of 0.3 ± 0.03 and 0.4 ± 0.04 g $plant^{-1}$, respectively (mean ± 1 standard error). Collins et al. (2018)

estimated the aboveground biomass of the understorey at EucFACE to be ~ 329 g m^{-2}. Using this value, we can approximate that *M. stipoides* secretes 4.5 ± 1.3 (aCO_2) and 9.8 ± 2.5 (eCO_2) mg C m^{-2} in the rhizosphere and 2.1 ± 0.9 (aCO_2) and 4.8 ± 1.0 (eCO_2) mg C m^{-2} h^{-1} from roots at the EucFACE site. The annual gross primary productivity in the understorey estimated from *M. stipoides* is 497 and 552 g C m^{-2} $year^{-1}$ in aCO_2 and eCO_2 plots, respectively (Jiang et al. 2020b). Thus, *M. stipoides* roots may have transferred a non-negligible fraction of assimilated C into this system's soil in the form of OAs. At EucFACE, low soil P availability constrained the positive CO_2 effects on the overstorey growth of *E. tereticornis* (Ellsworth et al. 2017), whereas *M. stipoides* increased its dominance after three years of CO_2 fumigation (Hasegawa et al. 2018). Given that *M. stipoides* dominated the understorey coverage of this site, it may have contributed to eCO_2-induced increases in soil P (as reported in Hasegawa et al. (2016)) via qualitative and/or quantitative changes in root exudates and further supported its growth stimulation by eCO_2.

Conclusion

We found that CO_2 and water treatments mainly influenced OA amount and composition via increased plant biomass. In general, bigger plants within a species had greater amounts of exudates. When expressed per unit root biomass, OA amounts increased in response to eCO_2 for *M. stipoides*, and low-watered *H. sericea* plants exuded lower amounts of OAs in the rhizosphere than high-watered plants. Compositional changes in OAs driven by altered exudation of malic acid were associated with changes in soil P availability, possibly explaining the decreased P availability under low-watered conditions and increased P at eCO_2 for *M. stipoides* (but not *E. tereticornis*). Together with previous studies reporting eCO_2-induced increases in soil phosphate availability and soil C fluxes at the EucFACE experiment, our study suggests that *M. stipoides* significantly contributes to soil nutrient and C cycling via root exudation of OAs in the local *Eucalyptus* woodland. Thus, increased CO_2 concentrations and drastic changes in precipitation patterns (and resultant soil water availability) will likely influence OA amount and composition of the studied Australian native species in low P soil. Quantitative and qualitative changes in root exudates can alter abiotic and biotic interactions in the rhizosphere (de Vries et al. 2019; Drake et al. 2013) and modify edaphic structure by influencing soil aggregates and organo-mineral compounds (Bronick and Lal 2005; Keiluweit et al. 2015), such that the observed changes in OAs in root exudates in this study have cascading impacts on ecosystem functioning.

Statements & Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. Shun Hasegawa led material preparation, data collection and statistical analysis. Megan H. Ryan led root exudate analysis. The first draft of the manuscript was written by Shun Hasegawa and Sally A. Power, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

We intend to archive the raw data and the R scripts that reproduce the results presented in this manuscript and on Zenodo with a unique DOI and make them publicly available.

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Figures

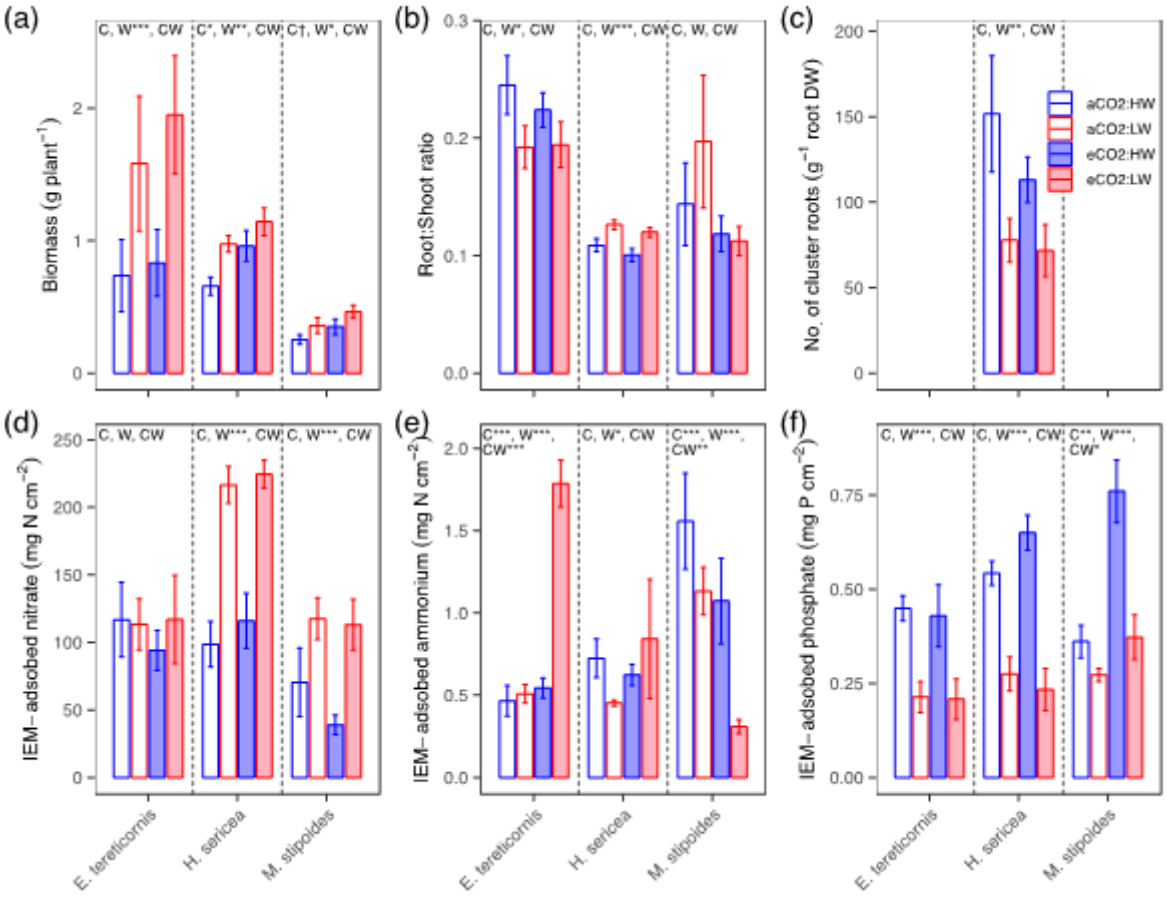

Figure 1

Plant and soil measurements for *Eucalyptus tereticornis*, *Hakea sericea* and *Microlaena stipoides* (mean ± 1 standard error, *n* = 5 for *E. tereticornis* and *n* = 8 for others): a) total biomass, b) root:shoot ratios (RSR), c) number of cluster roots per g root dry weight (DW), and concentrations of d) soil nitrate, e)

ammonium and f) phosphate assessed using ion exchange resin membranes. Ambient (aCO_2) and elevated (eCO_2) CO_2 treatments are depicted with open and filled boxes, respectively. High-watered (HW) and low-watered (LW) treatments are indicated by blue and red colours, respectively. The results of statistical analyses are shown for CO_2 (C), water (W) and their interaction (CW) at the top of corresponding panels for each species, with significance codes: *P* <0.1 (†), <0.05 (*), <0.01 (**) and <0.001 (***).

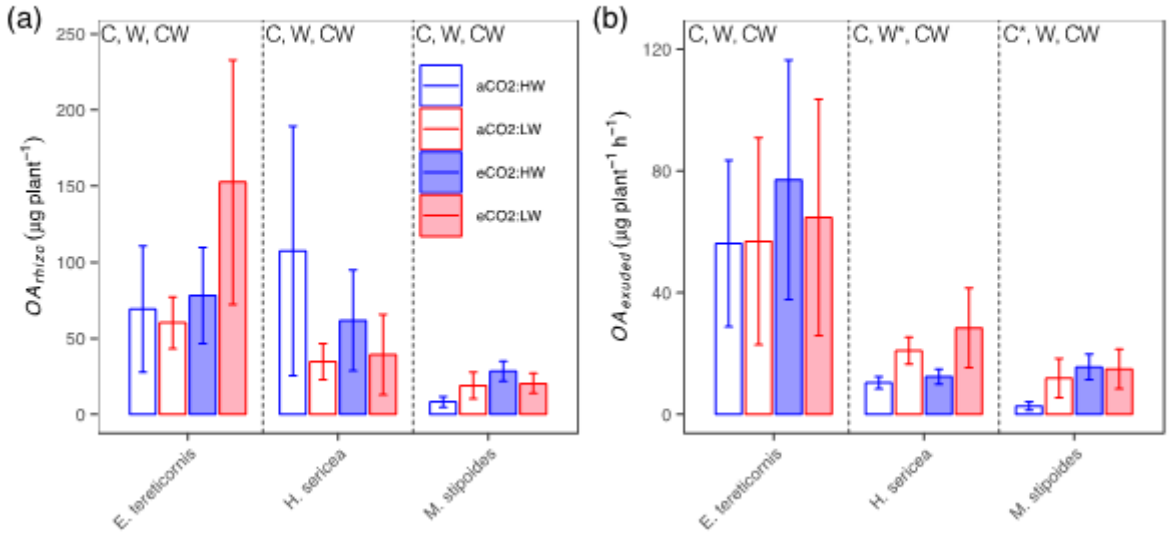

Figure 2

Total amounts of organic acids per plant a) in the rhizosphere soil (OA_{rhizo}) and b) exuded from roots (OA_{exuded}) for *Eucalyptus tereticornis*, *Hakea sericea* and *Microlaena stipoides* (mean ± 1 standard error, *n* = 5 for *E. tereticornis* and *n* = 8 for others). Ambient (aCO_2) and elevated (eCO_2) CO_2 treatments are depicted with open and filled boxes, respectively. High-watered (HW) and low-watered (LW) treatments are indicated by blue and red colours, respectively. Statistical significance is indicated for CO_2 (C), water (W) and their interaction (CW) at the top of corresponding figures for each species: *P* <0.1 (†), <0.05 (*), <0.01 (**) and <0.001 (***).

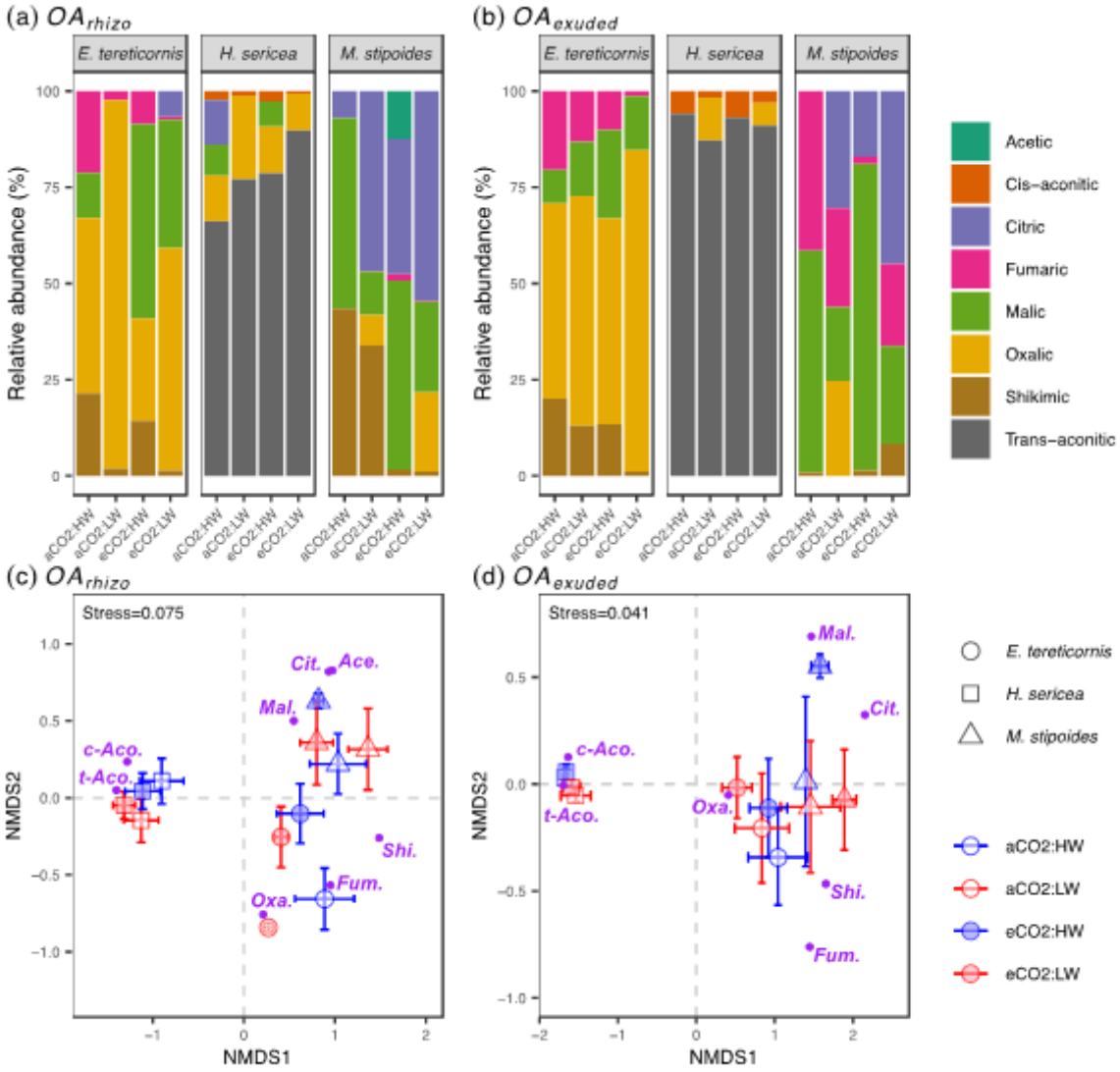

Figure 3

Organic acid (OA) composition in root exudates in response to CO_2 and water treatments. Relative abundance is shown in the upper panels for OAs a) in the rhizosphere soil (OA_{rhizo}) and b) exuded from roots (OA_{exuded}). The lower panels depict ordination plots obtained from non-metric multidimensional scaling (NMDS) analysis for (c) OA_{rhizo} and (d) OA_{exuded}, with centroids and associated standard errors for CO_2 (ambient [aCO_2] and elevated [eCO_2] CO_2) and water treatments (high-watered [HW] and low-watered [LW] conditions) for each species: *Eucalyptus tereticornis*, *Hakea sericea* and *Microlaena stipoides*. Organic acids are indicated in purple: acetic (*Ace.*), cis-aconitic (*c-Aco.*), citric (*Cit.*), fumaric (*Fum.*), malic (*Mal.*), oxalic (*Oxa.*), shikimic (*Shi.*) and trans-aconitic (*t-Aco.*) acids.

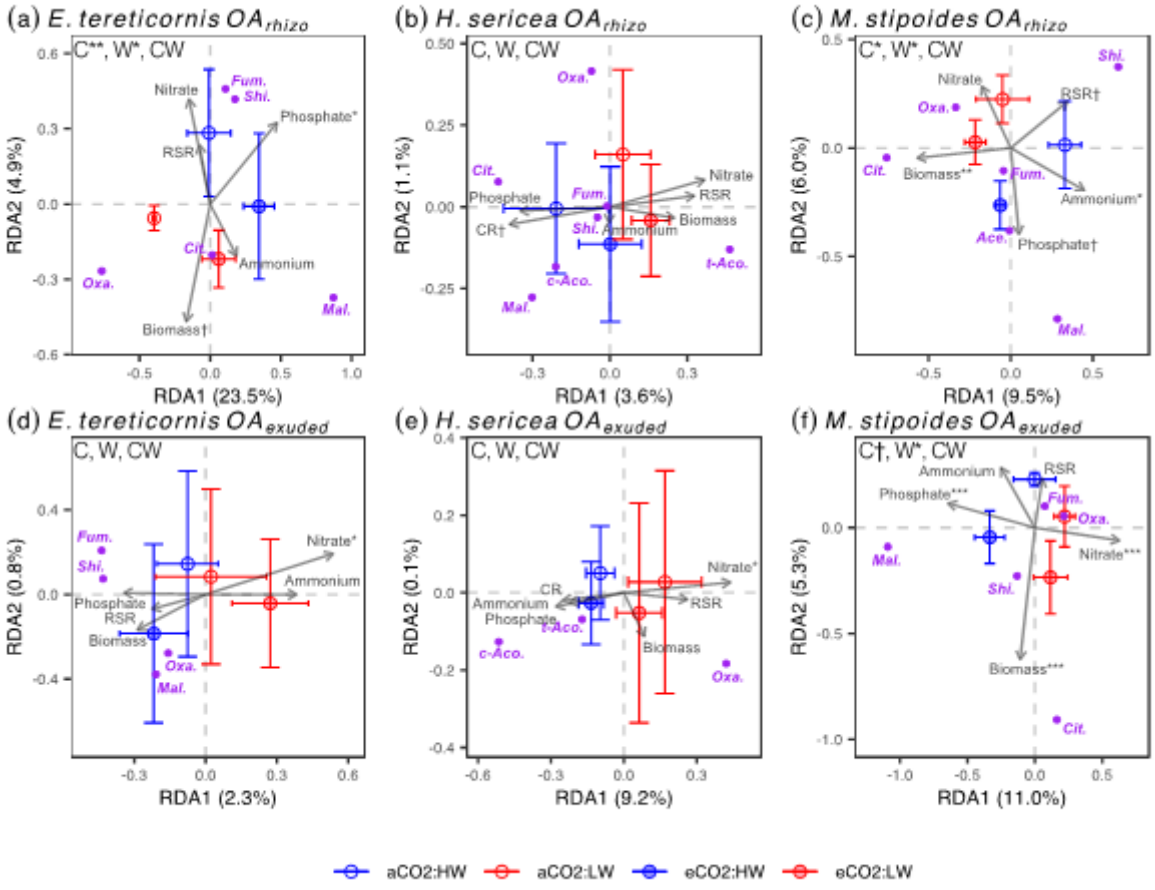

Figure 4

Redundancy analysis (RDA) results of the effect of CO_2 and water treatments on organic acid (OA) composition in the rhizosphere soil (OA_{rhizo}, upper panel) and exuded from roots (OA_{exuded}, lower panel) for *Eucalyptus tereticornis* (a, d), *Hakea sericea* (b, e) and *Microlaena stipoides* (c, f). The results of permutation tests for the significance of constraints are shown for CO_2 (C), water (W) and their interaction (CW) at the top of each panel, with significance codes. Proportion of variability in OA composition attributed to RDA axes is shown within parentheses. The correlation test results for the RDA axes and plant and environmental measurements are shown with arrows: root:shoot ratios (RS), total biomass (Biom), soil nitrate, ammonium and phosphate, and number of cluster roots per g root dry weight (CR; only for *H. sericea*), with significance codes. The significance codes are P <0.1 (†), <0.05 (*), <0.01 (**) and <0.001 (***). OAs are indicated in purple: acetic (*Ace.*), cis-aconitic (*c-Aco.*), citric (*Cit.*), fumaric (*Fum.*), malic (*Mal.*), oxalic (*Oxa.*), shikimic (*Shi.*) and trans-aconitic (*t-Aco.*) acids.

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