METHODS

We investigated the interactive effects of atmospheric carbon dioxide partial pressure and soil waterlogging on three riparian tree species from south eastern Australia. *Casuarina cunninghamiana* and *Eucalyptus camaldulensis* dominate many riparian environments in south eastern Australia; *Acacia floribunda* is also common in this region. These three species are dissimilar phylogenetically and morphologically.

*Experimental Procedure*

We used a fully factorial design comprising using two CO2 treatments: ambient and elevated CO2, and three waterlogging treatments: non-waterlogged control, waterlogged and waterlogged then recovered (n= 8 per treatment combination, per species). We measured plant growth physiology (photosynthetic rate and instantaneous water use efficiency) as well as biomass and biomass allocation, and tissue density traits indicative of ecological strategy and position along economic spectra (Reich et al. 2014).

Pots were constructed from 90 mm by 700 mm (4.3 L capacity) sections of PVC pipe with drilled endcaps, and contained a commercially sourced 80/20 mixture of river sand and soil (Australian Native Landscapes, North Ryde, NSW, Australia). The bottom 2 cm of each pot was filled with gravel (~1 cm particle size) to promote free drainage. 2.5 g / L of time-release fertiliser granules (NPK 19.1, 0, 11.9, Yates Australia, Padstow, NSW, Australia) was mixed evenly through the soil medium.

Seeds were obtained from a commercial supplier (Nindethana Seed Service, Albany, WA, Australia). Seeds of *A. floribunda* were pretreated by scalding in 100 oC water and soaking overnight. All seeds were germinated on moist tissue paper in trays at ~20 oC. Following cotyledon emergence, four seedlings were transplanted into each growing pot; germination was staggered by species to ensure all seedlings were transplanted within 48 hours. After two weeks of growth, plants were thinned to retain a single, medium sized individual.

Plants were grown in glasshouses at Macquarie University, in Sydney, Australia, between June and November. Pots were supported by wire mesh on trolleys; pot positioning on trolleys was randomised with respect to species, and trolleys were rotated weekly to offset potential microclimatic effects. Two levels of CO2 treatment (380-400 ppm and 530-570ppm) were used in two replicate glasshouses per level. These CO2 ranges were monitored and maintained using an automated gas delivery system (Canary Company Pty Ltd, Lane Cove, NSW, Australia). The lower range corresponds to the ambient atmospheric CO2 concentration, while the higher range reflects the predicted atmospheric CO2 concentration in 2050. IPCC (2013) Climate change 2013: A physical science basis. Temperature was maintained between 16 and 28 oC. Plants were watered by a misting sprinkler system three times daily and supplementary hand watering every 3-4 days. Trolleys were swapped between replicate glasshouses monthly.

Waterlogging was initiated after 90 days of plant growth, and lasted 24 days. Plants randomly assigned to “waterlogged” and “recovered” treatments were waterlogged by immersion to within 10cm of the soil surface in 450L plastic tubs. The black tubs were covered with white polythene sheeting to reduce heat absorption. Photosynthetic rate and transpiration rate of plants assigned to the “waterlogged” treatment were measured at the end of the waterlogging period, after which they were harvested. Tubs were drained following the waterlogging period, and “control” and “recovered” treatment plants were grown for 23 days before measurement and harvesting.

Photosynthetic rate (CO2 assimilation rate) and transpiration rate of the newest fully developed leaf were measured for four plants per treatment, between 9am and 12:30pm using a LiCor 6400XT infrared gas analyser (Li-Cor Inc., Lincoln, NE, USA). Photon flux was set to 1500 µmol m-2 s-1 and temperature was held at 28 oC. For leaves which did not completely fill the cuvette, leaf area was measured by digital analysis (ImageJ 1.48 for Windows) of a photograph of the leaf taken against a 2x3 cm2 plastic backdrop, which corresponded to the area of the cuvette. Photosynthetic rate and transpiration rate were determined by correcting values according to the measured area. Instantaneous water use efficiency (WUE) was calculated as the ratio of CO2 assimilation to transpiration.

Upon harvesting, roots were washed free of soil, and the plant was separated into fine (< 1 mm diameter) and coarse roots (excluding dead root biomass), and above ground biomass. Five mature (but not senescing) leaves of each individual were selected for determination of specific leaf area (SLA). Fresh leaf area was determined using a LI-3100C Area Meter (Li-Cor Inc., Lincoln, NE, USA); SLA was calculated as the ratio of fresh area to dry mass. A 5 cm section of stem was cut from 1 cm above the root-stem junction for analysis of stem density. The fresh volume of the stem section was measured using the water displacement method and stem wood density was calculated as the ratio of oven dry mass to green volume. Dry matter content of fine roots was calculated as the ratio of oven dry mass to fresh mass. Samples were dried in an oven at 70 oC for 72 hours and a microbalance (Mettler-Toledo, Greifensee, Switzerland) was used to determine the resulting mass. Fine root proportion was calculated as the ratio of dry fine root biomass to total dry root biomass, and root mass fraction was calculated as the ratio of root dry biomass to whole plant dry biomass. Stunted plants with a shoot length of < 5 cm were not included in the study.

Data analysis

All statistical analyses were performed using the R statistical programming environment (R Core Team 2013). We used two-way analysis of variance (ANOVA) to test for main effects of and interactions between waterlogging and CO2 treatments on physiology (photosynthetic rate, WUE), biomass allocation (root mass fraction, fine root proportion) and functional traits (fine root dry matter content, stem density, SLA). Metrics of biomass (total, root biomass, aboveground biomass) were compared between “control” and “recovered” treatment plants, as plants which received the “waterlogged” treatment were younger at harvest. Post-hoc comparison (Tukey’s HSD) was used to determine which combination of treatments were responsible for interaction effects and waterlogging treatment main effects. Type II sums of squares were used where unbalanced analyses resulted from removal of stunted plants from the study, following Lansgrud (2003). Data were log10 or square root transformed where appropriate to satisfy assumptions of normality inherent in ANOVA. Statistical significance was thresholded at alpha = 0.1 for photosynthetic rate and WUE measurements (n = 4) and 0.05 for all other measurements (n = 8).