Seedlings of two Acacia species from contrasting habitats show different photoprotective and antioxidative responses to drought and heatwaves

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Annals of Forest Science (2015) 72:403–414
DOI 10.1007/s13595-014-0438-5

ORIGINAL PAPER

Seedlings of two *Acacia* species from contrasting habitats show different photoprotective and antioxidative responses to drought and heatwaves

Agnieszka Wujeska-Klause · Gerd Bossinger · Michael Tausz

Received: 8 July 2014 / Accepted: 4 November 2014 / Published online: 19 November 2014

Abstract

• **Key message Two *Acacia* species adapted to contrasting habitats showed different response of photoprotective and antioxidative defence systems to imposed drought and heatwave**.

• ***Context*** Predicted increases in drought frequency and intense heatwaves are expected to lead to dieback of sensitive tree species. Stomatal closure restricts CO_2 input into the leaf, resulting in imbalances between light energy-driven electron transport rate and electron consumption in the Calvin cycle. Reactive oxygen species formed under these circumstances have to be kept under control by photoprotective and antioxidative defence systems.

• ***Aims*** We hypothesised that these defence systems behave differently in tree species from contrasting habitats.

• ***Methods*** *Acacia aneura* (adapted to arid habitats) and *Acacia melanoxylon* (adapted to humid habitats) were exposed to two water treatments for 50 days including two short heatwave periods. Responses were assessed by gas exchange, chlorophyll fluorescence and concentrations of antioxidants (phyllodes, roots).

• ***Results*** Photosynthesis and quantum yield of photochemistry decreased significantly in both *Acacia* species, especially after water was withheld in combination with the second heatwave episode. In phyllodes, the concentration of antioxidants remained unchanged until exposure to severe drought and heatwave conditions (except for *A. melanoxylon* where changes in glutathione concentration were observed prior to exposure to severe stress), but after water was withheld and the second heatwave occurred, oxidised forms of glutathione increased. After exposure to the second heatwave, well-watered seedlings of *A. melanoxylon* but not *A. aneura* increased ascorbic acid concentration in phyllodes. Under well-watered conditions, *Acacia* species also showed increased concentration of antioxidants in roots following heatwaves.

• ***Conclusions*** Both *Acacia* species showed photodamage to photosystem II (PSII) after water was withheld and the second heatwave imposed, but with more gradual response in *A. aneura*. Total concentration of investigated antioxidants increased in response to the first (*A. melanoxylon*) and second (*A. aneura*) heatwaves rather than drought stress alone.

Keywords *Acacia aneura* · *Acacia melanoxylon* · ASC · GSH · Arid · Humid · Phyllodes · Roots

Handling Editor: Erwin Dreyer

Contribution of the co-authors Agnieszka Wujeska-Klause: designing and conducting the experiment, writing the manuscript and running data analysis
Gerd Bossinger: supervising the work, detailed reading and revision of the manuscript and coordinating the research project
Michael Tausz: supervising the work, detailed reading and revision of the manuscript and coordinating the research project

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1 Introduction

According to global warming predictions, droughts and heatwaves are expected to become more frequent and longer (IPCC 2013). Drought is already a common climate factor in many regions, but if drought periods increase in intensity or frequency along with more intense heatwaves, then tree species can be negatively affected (Hennessy et al. 2008; IPCC 2013; Teskey et al. 2014). Sensitive species may be unable to

acclimate to extreme weather conditions, which can lead to their dieback with serious effects on environmental sustainability (Allen et al. 2010). Strategies of trees to cope with drought and heat periods may be different between species from arid habitats and species from humid habitats, and the effectiveness of coping strategies can depend on exposure time and stress intensity (McDowell et al. 2008; O'Grady et al. 2009). Decrease of rainfall and more intense heatwaves are likely to cause decline among those tree species adapted to more humid habitats, whereas tree species adapted to arid environments may be more tolerant (Kubiske and Abrams 1994; McDowell et al. 2008). In addition, trees at the seedling stage may be more affected than adult trees, with significant repercussions for the regeneration of a species (Johnson et al. 2011; Teskey et al. 2014).

Environmental stress in general, and drought or heat more specifically, commonly causes photooxidative stress in photosynthetic tissues, a situation where an imbalance between absorbed light energy and energy consumption in the photosynthetic apparatus leads to increased formation of potentially harmful reactive oxygen species (Smirnoff 1993; Asada 2006). In the case of drought stress, stomatal closure is a common early response of plants to limit transpiration (Smirnoff 1993; Chaves et al. 2009; Teskey et al. 2014). Stomatal closure also restricts CO_2 influx into the leaf, which reduces the consumption and regeneration of metabolic energy carriers (NADPH, ATP) in carbon fixation (Smirnoff 1993; Chaves et al. 2009). Despite flexible downregulation of photosystem II (PSII) efficiency, the electron transport chain can continue to transport electrons at higher rates than can be used for the production of metabolic energy carriers (Tausz et al. 2004; Asada 2006; Foyer et al. 2012). As a result, reaction centres of photosystems remain closed (they are unable to accept another electron) and excitation energy or electrons can be transferred to other compounds (foremost among them molecular oxygen), leading to increased generation of reactive oxygen species (ROS) (Tausz et al. 2004; Asada 2006; Kim and Apel 2013).

Plants possess several defence systems to avoid and control the formation of ROS in the photosynthetic apparatus. As a 'first line of defence', the quantum efficiency of electron flow is continuously and flexibly adjusted through processes regulated by thylakoid pH and involving protective xanthophylls that mediate dissipation of absorbed light energy as heat (Demmig-Adams et al. 2012). Other mechanisms, such as photorespiration, cyclic electron flow and the Mehler reaction and heat dissipation, serve as alternative electron sinks maintaining controlled electron flow when carbon assimilation is limited (Asada 2006; Foyer et al. 2012; Noctor et al. 2014). These processes can prevent the production of singlet oxygen and help avoid photoinhibition (Kim and Apel 2013; Noctor et al. 2014), but they are also a source of ROS (H_2O_2, O_2^-) that need to be kept under control.

Production of ROS occurs to some extent also under non-stress conditions, but their concentration is kept under control by the antioxidative defence system (Noctor et al. 2014). An elevated concentration of ROS can be harmful for cell membranes (proteins, lipids) and nucleic acids due to the reactive nature of ROS (Smirnoff 1993; Foyer and Noctor 2009). Non-enzymatic antioxidants, such as the ubiquitous low molecular weight compounds ascorbic acid and glutathione, are an important part of plant defence systems. They are also linked to the activity of enzymatic antioxidants and together form a complex network of defence and redox signalling systems (i.e. ascorbate-glutathione cycle) (Smirnoff 1993; Munné-Bosch 2005; Noctor et al. 2014). Ascorbic acid is abundant in plant cells and has important roles in ROS scavenging and in the regeneration of other antioxidants (α-tocopherol or xanthophylls), as well as in the regulation of cell division and elongation (Potters et al. 2002; Foyer and Noctor 2009). Glutathione is a low molecular weight compound with important roles as a ROS scavenger, in the regeneration of oxidised ascorbic acid (glutathione-ascorbate cycle) and in sulphur assimilation (Potters et al. 2002; Noctor et al. 2012). Notwithstanding the importance of many other antioxidative compounds in plant tissues (for example, α-tocopherol, carotenes, xanthophylls, phenolics), this study focused on the central redox regulators ascorbic acid and glutathione (Foyer and Noctor 2011).

Antioxidants also play an important role in the growth and development of fine roots, where antioxidants can be synthetised or supplied from the canopy (Herschbach et al. 2009). Since the root system is exposed directly to decreasing soil water content (Chaves et al. 2009), changes in the concentration of antioxidants may indicate adjustments of the root system to changing growth conditions, such as low soil moisture conditions or elevated temperature (Shvaleva et al. 2005). Although not many studies described the role of antioxidants in defence systems of roots upon exposure to stress conditions, some authors showed an increase in the concentration of ascorbic acid and α-tocopherol in response to low soil water content, whereas glutathione concentrations did not change (Haberer et al. 2008).

Response to stress depends on its duration and intensity, age of plants or adaptation to their habitat (Smirnoff 1993; Rahantaniaina et al. 2013). Reduced forms of antioxidants react with ROS, leading to their transformation into oxidised forms (Foyer and Noctor 2011). Increasing concentrations of the reduced forms of antioxidants upon stress exposure can indicate an active defence response of plants and mark the ability to withstand stress. Elevated concentrations of oxidised forms may indicate that the defence capacity is overwhelmed (Foyer and Noctor 2011; Rahantaniaina et al. 2013). In addition, initial small changes of the redox state (ratio of oxidised to reduced form) of ascorbic acid or glutathione may also be triggers for subsequent metabolic responses and therefore part

of the stress signalling process (Tausz et al. 2004). Most studies seem to suggest that the concentration of non-enzymatic antioxidants increases under initial stages of stress, but progressing stress can lead to decreased concentrations and therefore reduced detoxification capacity, which is in part dependent on the concentration of reduced antioxidants (Smirnoff 1993; Tausz et al. 2004; Wujeska et al. 2013; Noctor et al. 2014). A recent meta-analysis by Wujeska et al. (2013) suggested that tree species from different ecosystems and with putatively different stress tolerance showed differences in their antioxidative responses to drought stress. Because across different species there are potentially many confounding effects, it was proposed in that study to investigate antioxidative defence system responses between ecologically contrasting, but genetically closely related, species (such as species from the same genus).

Australian native tree species offer such an opportunity, as a large proportion of all species belong to the genera *Eucalyptus* and *Acacia*. For instance, species of the genus *Acacia* are widely distributed in Australia, often closely related to each other, and can alternate along rainfall gradients.

To test whether antioxidative responses to stress are different between congenital species from contrasting habitats, we tested two *Acacia* species from contrasting habitats: *A. aneura* F. Muell ex Benth is adapted to arid habitats and *A. melanoxylon* R. Br. to humid habitats. *A. melanoxylon* is a fast-growing and widely occurring tree species in high-rainfall areas of Victoria, New South Wales and Queensland (Costermans 2006), whereas *A. aneura* is a slow-growing tree with a shallow root system and distributed in low-rainfall regions of Central Australia (O'Grady et al. 2009). We hypothesised that *A. aneura*, adapted to arid conditions, will be better equipped to cope with prolonged drought and heatwaves compared to *A. melanoxylon*. The strategy of *A. aneura* may include (1) stomatal responses consistent with anisohydric behaviour (e.g. O'Grady et al. 2009), (2) successful avoidance of photodamage and photooxidative stress, (3) no or smaller changes in the concentration of antioxidants (Wujeska et al. 2013) and (4) increased concentration of antioxidants in roots in response to drought stress and high temperature.

2 Materials and methods

2.1 Plant material and growth conditions

Two *Acacia* species from environments contrasting in their aridity were chosen: *A. aneura* F. Muell ex Benth adapted to areas with lower rainfall (annual rainfall 200–400 mm, exact provenance unknown) and *A. melanoxylon* R. Br. to higher rainfall (annual rainfall 750–1500 mm; provenance Smithton, Tasmania) (SpeciesBank 2014). Eight-month-old seedlings were bought from Meredith Nursery (Meredith, Victoria, Australia), repotted in 1.5-L pots and grown for 5 months in a semi-controlled glasshouse (Creswick Campus of the University of Melbourne, 143° 53″ E, 37° 25″ S). Three units of potting mix for native species including slow-release fertiliser (Native Mix Superior Potting & Planting Mix, Debco, Australia) were mixed with two units of coarse-grade sand (Propagating sand, Brunnings, Australia). For each species, 80 plants were grown, which were then randomly assigned to one of two treatments: well-watered and drought-stressed. For repeated gas exchange and chlorophyll fluorescence measurements, five plants per treatment were used repeatedly each day during the 50 days of the experiment. The remaining plants (35 per treatment) were used for destructive sampling and biochemical analysis (antioxidants). Plants of both drought-stressed and well-watered treatments were positioned randomly and interspersed with each other.

2.2 Water treatments and glasshouse conditions

Before starting the drought treatment, all seedlings were watered with the same amount of water (kept at or close to field capacity—FC). Plants were watered to FC and weighed (with soil and pot) to determine water use. During the experiment, seedlings were watered and weighed every 2–4 days, depending on the species requirements (*A. melanoxylon* received more water more often than *A. aneura*). From day 1 to day 28 of the treatment, plants in the drought-stressed treatment group received 50 % of the water used by well-watered plants. After day 28, drought-stressed plants were left without watering until day 50. The percentages of FC (maximum soil water content (SWC) calculated after watering) and minimum soil water content (min. SWC, calculated from the pot weight recorded immediately before watering) were calculated based on gravimetric measurements through this time.

Plants received natural light in the glasshouse (approx. 70–80 % of outdoor photosynthetic photon flux density (PPFD)), and the glasshouse was tracking outside conditions except for a cooling system, which was set to maintain day temperatures below a maximum of 25 °C. Technical problems with the cooling system from day 28 to day 30 coincided with sunny conditions outdoors exposing all plants to a simulated heatwave, and to a lesser extent also at the beginning of the experiment from day 3 to day 5. Air temperatures during these periods exceeded 30 °C. Spot measurements using the leaf thermocouple of a LI-COR 6400 (LI-COR, Lincoln, NE, USA) indicated that phyllode temperatures reached well above 30 °C between day 28 and day 30, whereas they remained below 30 °C at all other times.

2.3 Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence were measured with a LI-COR 6400 (leaf fluorometer chamber 6400-40) on the same phyllodes and seedlings between 10.00 a.m. and 1.00 p.m. On the same day as gas exchange measurements, pre-dawn chlorophyll fluorescence measurements were performed using a portable fluorometer (OS30p$_+$, Opti-Sciences, Inc., Hudson, NH, USA) and the maximum quantum efficiency of PSII (F_v/F_m) was determined. For gas exchange and simultaneous fluorescence measurements, LI-COR conditions were as follows: CO_2 concentration (reference) was set at 400 ppm, flow rate at 500 μmol m^{-2} s^{-1} (*A. melanoxylon*) or 250 μmol m^{-2} s^{-1} (*A. aneura*; to account for the smaller phyllodes) and light intensity at 1200 μmol m^{-2} s^{-1} PPFD (which was found to be saturating for both species). Phyllode temperature during measurements was 20–25 °C and relative humidity in the chamber 50–60 %. The following parameters were recorded: net CO_2 assimilation rate (A), stomatal conductance (g_s) and efficiency of open reaction centres in PSII in the light (F_v'/F_m'). After 50 days, these plants were used for antioxidant analysis. Relative stomatal conductance (rel g_s) was calculated by dividing each g_s value by the maximum g_s obtained for the species and the whole experiment, determined as the 95-percentile of all g_s measurements. At the beginning of the experiment, mean leaf mass area (LMA) was 213.9± 63.7 g m^{-2} (*A. melanoxylon*) and 254.6±94.5 g m^{-2} (*A. aneura*) (p=0.274).

2.4 Glutathione and ascorbic acid determination

Glutathione and ascorbic acid concentration was measured on fully expanded phyllodes and fine roots (<2-mm diameter). Samples were frozen in liquid nitrogen immediately and then freeze-dried for 72 h. Afterwards, samples were weighed (ca. 50–60 mg of plant material) and ground to a fine powder with a pestle, a mortar, equal amounts of polyvinylpolypyrrolidone (PVPP), fine quartz sand and liquid nitrogen. Then, 1.5 mL of 4.5 % (*w*/*v*) metaphosphoric acid was added and mixed by vortex. The samples were centrifuged for 2.5 min at 16,100×g. The supernatants (extracts) were decanted and stored at −20 °C for later analysis.

A 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) assay was used to determine the total (GSH+GSSG) and oxidised glutathione (GSSG). Aliquots of the extracts were neutralised in potassium phosphate buffer (250 mM, pH 7), and to one of the aliquots, 2-vinylpyridine was added to block the reduced form of glutathione and assess GSSG concentration. Excess 2-vinylpyridine was removed by adding triethanolamine to the extract. The other aliquot of the extract did not receive 2-vinylpyridine and was used to determine the total (GSH+ GSSG). A working solution containing DTNB, dimethyl sulphoxide (DMSO) and glutathione reductase was mixed with the extracts and β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) was used to start the reaction. Glutathione concentrations of both forms were determined by recording absorbance at 412 nm at room temperature. Concentrations of the total and oxidised form were calculated according to Sgherri et al. (1994). Reduced GSH was assessed by the difference between total GSH and GSSG. This method is based on Knörzer et al. (1996).

To determine total ascorbic acid (ASC+dehydroascorbic acid (DHA)), the supernatant (extract) was neutralised in sodium phosphate buffer (150 mM, pH 7.4) containing triethanolamine (1.5 M *v*/*v*), and then, DL-Dithiothreitol (DTT, 20 mM) was used to reduce DHA to ASC. After incubation for 15 min, *N*-ethylmaleimide (NEM, 0.5 % (*w*/*v*)) was added to remove excess DTT from the extract. To determine the reduced form of ASC, replicate extracts were treated with de-ionised water. Ascorbic acid in the extracts was determined by treating with trichloroacetic acid (10 % *w*/*v*), orthophosphoric acid (40 % *v*/*v*), 2,2′-dipyridil (4 % in 70 % ethanol) and iron(III) chloride (15 % *w*/*v*). After incubation for 1 h at 37 °C, the concentration of total and reduced ascorbic acid was determined using absorbance at 525 nm at room temperature. This method is based on Knörzer et al. (1996).

2.5 Statistical analysis

Statistical analysis and graphs were performed using RStudio (version 0.98.501; open source software; © 2009–2013 RStudio, Inc.). For the analysis of repeated measurements (gas exchange and chlorophyll fluorescence) on the same individual seedlings for the entire 50 days, a linear mixed effects model was used (‘lme’ from R package ‘nmle’). Day of the experiment was used as the within-subjects factor, and species and treatment were the between-subjects factors. Antioxidants at each sampling day were analysed using a univariate general linear model with the factors day, treatment (well-watered and drought-stressed) and species (*A. aneura* and *A. melanoxylon*) (‘aov’ from R package ‘stats’). Individual differences between well-watered and drought-stressed plants on each experimental day were determined using t test (‘t.test’ from R package ‘stats’). For all analyses, p values of less than 0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.001). In addition, individual differences between the species for each treatment separately and at each sampling day were determined using t tests (‘t.test’ from R package ‘stats’). To determine if data deviated from normality, Levene’s test was used, and when the test was significant (‘leveneTest’ from R package ‘car’), log data transformations were used. Data were checked visually for the correlations between variances and means. To determine the relationships of stomatal responses to the percentage of FC, the three-parameter sigmoid curve was used

and calculated using the equation: $g_s = \frac{a}{1+e^{-\frac{\%FC-c}{b}}}$ (Brodribb and Cochard 2008). The curve was fitted by using quantile regression to estimate the boundary line at the 95 % level for each species separately. Curves were constrained to 0 at low soil water content and rel g_s to reflect the distribution of the points. Quantile regression was performed with the quantreg package (version 5.05) (Koenker et al. 2013).

3 Results

3.1 Gas exchange and chlorophyll fluorescence

Drought stress reduced net CO_2 assimilation rate (A; Fig. 1) and stomatal conductance (g_s; Fig. 2) in both *Acacia* species. In *A. aneura*, A and g_s decreased gradually, whereas they were more variable before declining sharply on the 28th day in *A. melanoxylon* (when high air temperature coincided with the withholding of water). Stressed seedlings showed a significant reduction of A from day 9 and day 4, whereas g_s decreased significantly from day 9 and day 7 (*A. aneura* and *A. melanoxylon*, respectively). There were no significant changes in well-watered seedlings of both species throughout the whole experiment. Soil moisture decreased gradually for drought-stressed seedlings and remained constant in well-watered ones for both *Acacia* species (Fig. 3). In *A. aneura*, relative stomatal conductance (rel g_s) was 1 when soil moisture ranged between 100 and 60 % FC (Fig. 4). In contrast, in *A. melanoxylon*, rel g_s decreased to ~0.8 at 75 % of FC. When soil moisture reached 50 % FC, rel g_s of *A. aneura* still was relatively high (rel g_s=~0.9), whereas in *A. melanoxylon*, it dropped below 0.5 of rel g_s. Both species had rel g_s close to 0 when soil moisture dropped below 10 % FC. Minimum soil water content (min. SWC in %) was constant for well-watered seedlings in pots of both *Acacia* species during the experiment (except for a drop on day 11 in pots of both treatments of *A. melanoxylon*) (Fig. 3). Min. SWC was decreasing gradually in pots of drought-stressed seedlings (both species), reaching around 10 % at the end of the experiment. Min. SWC were comparable among species and ranged between 61 and 83 % for well-watered *A. aneura*, 10–61 % for drought-stressed *A. aneura*, 46–88 % for well-watered *A. melanoxylon* and 16–60 % for drought-stressed *A. melanoxylon*.

Maximum quantum efficiency of PSII in the dark (F_v/F_m) and in the light (F_v'/F_m') decreased in drought-stressed seedlings after day 28 (when water was withheld in coincidence with high temperatures), whereas it remained high in well-watered seedlings (Fig. 5). F_v/F_m did not vary between treatments in both species until the 28th day. F_v'/F_m' was reduced in drought-stressed *A. melanoxylon* seedlings on day 14 and day 21. It decreased significantly in drought-stressed trees of both species after day 28.

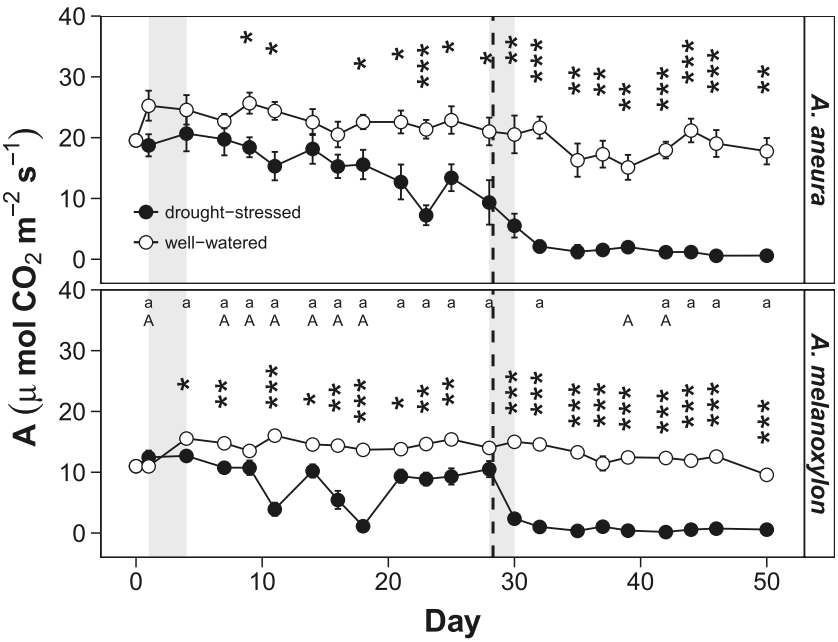

Fig. 1 Net CO_2 assimilation rate (A) of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. The *vertical line* indicates the start of a period where water was withheld completely. The *shaded areas* indicate the time of heatwaves when air temperatures exceeded 30 °C. Values are means (±SE) of n=5. Effects of sampling day, species and treatment as well as their interaction were statistically significant (p<0.01), and *asterisks* indicate significant differences between water treatments at each sample time (*p<0.05; **p<0.01; ***p<0.001). *Letters* indicate significant differences between the species under drought-stressed (*capital letters*) and well-watered treatment (*small letters*)

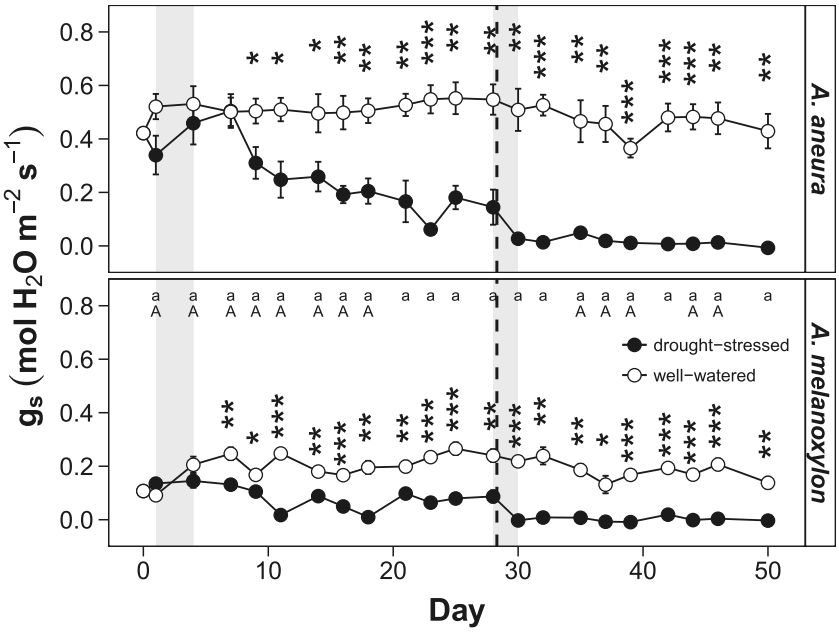

Fig. 2 Stomatal conductance (g_s) of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of $n=5$. Effects on all levels and their interactions were statistically significant ($p<0.001$)

3.2 Concentration of non-enzymatic antioxidants in phyllodes and roots

3.2.1 Glutathione

In *A. melanoxylon*, total glutathione concentration (GSH+GSSG; Fig. 6) was lower in phyllodes of drought-stressed seedlings than in well-watered ones (already significant on day 14), whereas total glutathione in *A. aneura* did not change between treatments until the last day. In *A. melanoxylon*, GSH+GSSG did not show an increase in the concentration after day 28 (when trees were exposed to heatwave conditions). Phyllode GSH+GSSG seemed to respond to some extent to heat events with more rapid response after the first heatwave. Both species showed a peak in phyllode GSH+GSSG concentration following the earlier heat period, and *A. aneura* but not *A. melanoxylon* also showed such a peak a few days after the second heatwave, irrespective of drought-stressed treatments (Fig. 6). The ratio of oxidised to total glutathione (GSSG%; Fig. 6) increased in drought-stressed seedlings of both *Acacia* species after day 28. *A. melanoxylon* seedlings also showed a peak of GSSG% during the first heat period, and also well-watered seedlings of *A. melanoxylon* (but not *A. aneura*) showed increased proportion of GSSG after the second heatwave.

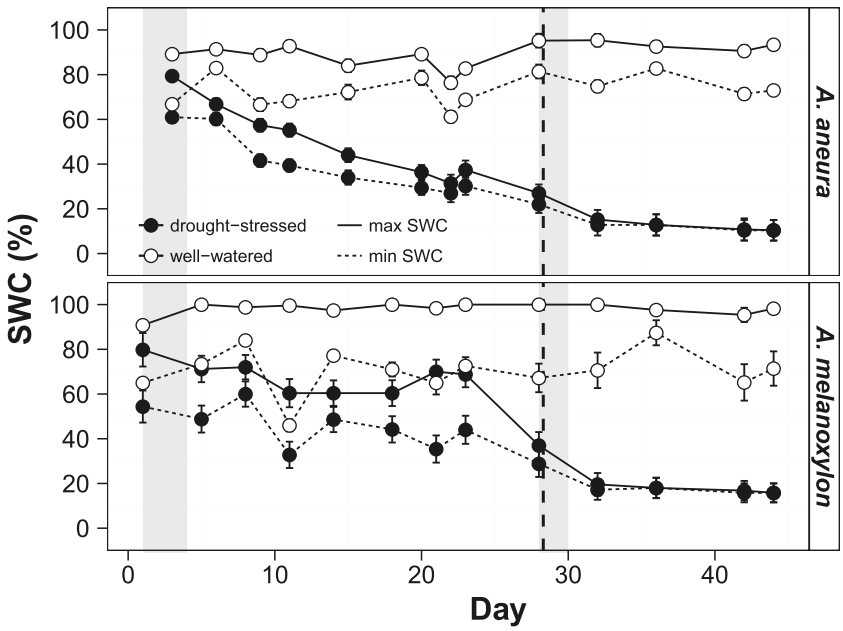

Fig. 3 Minimum (*dashed line*) and maximum soil water contents (*solid line*, SWC) expressed as percentage of FC in pots of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line* and *shaded areas* described as in Fig. 1. Values are means (±SE) of $n=5$. *min. SWC*: Effects of sampling day and treatment as well as their interaction (species by treatment, treatment by day) were statistically significant ($p<0.01$). *max. SWC*: Effects of sampling day, species and treatment as well as interactions (treatment by day) were statistically significant ($p<0.001$)

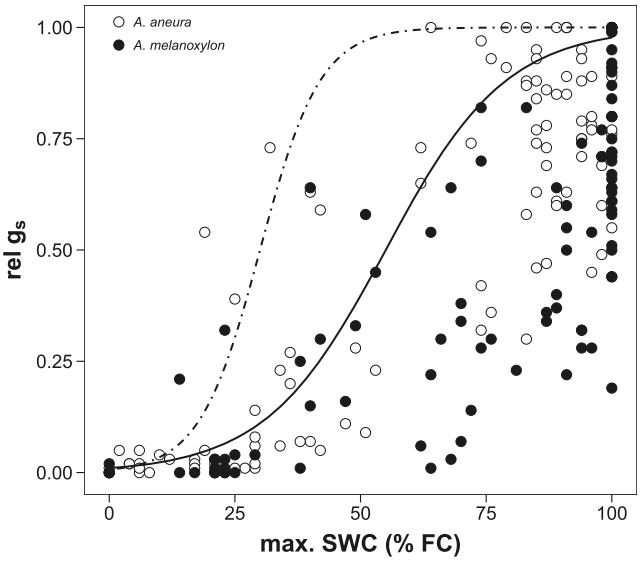

Fig. 4 Response of stomatal conductance of *A. aneura* (*dot-dashed line*; *open circle*) and *A. melanoxylon* (*solid line*; *closed circle*) expressed as relative stomatal conductance (rel g_s) under decreasing maximum soil water content (max. SWC expressed in %FC). *Boundary lines* (95 %) were plotted using three sigmoid function rel $g_s = a/(1+e(-(\%FC - c)/b))$ for each species separately

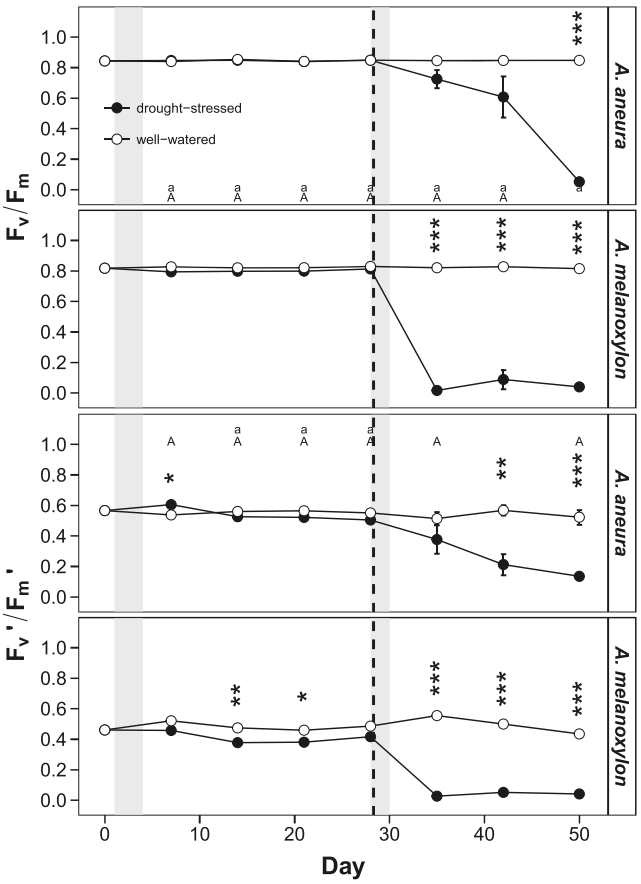

Fig. 5 Maximum quantum efficiency of PSII in the dark (F_v/F_m) and maximum quantum efficiency of PSII in the light (F_v'/F_m') of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of $n=5$. F_v/F_m: Effects of sampling day, species and treatment as well as their interactions were statistically significant ($p<0.01$). F_v'/F_m': Effects of sampling day, species and treatment as well as their interactions (species by treatment, treatment by day) were statistically significant ($p<0.01$)

The concentration of GSH+GSSG in roots was not significantly different between the water treatments in *A. melanoxylon* during the whole experiment (Fig. 7). In line with phyllode glutathione concentrations, root GSH+GSSG concentrations of well-watered and drought-stressed *A. melanoxylon* and well-watered *A. aneura* peaked around day 40, after the second heatwave. This increase was not observed in drought-stressed *A. aneura*, leading to a statistically significant difference between water treatments for this species on day 42. The proportion of oxidised glutathione did not vary significantly between treatments of both *Acacia* species, except for a marginal but significant increase in *A. melanoxylon* at day 50 (Fig. 7). The GSSG% increased slightly after the 28th day in both species and treatments. This species also showed an increase in GSSG% following the first heat period, similar to observations in phyllodes.

3.2.2 Ascorbic acid

Total phyllode ascorbic acid (ASC+DHA; Fig. 8) became significantly lower in drought-stressed than well-watered seedlings during later stages of the experiment. In *A. aneura*, this difference was only significant at the last day, whereas in *A. melanoxylon*, differences were more pronounced and became apparent from day 28 onwards. In *A. melanoxylon*, phyllode ASC+DHA concentrations of well-watered but not drought-stressed seedlings increased after the heatwave following day 28, which resulted in the difference between water treatments. Ascorbic acid (ASC%; Fig. 8) was in a highly reduced state (ca. 80 %) in both species throughout the experiment, but even though changes were small, the ascorbate pool in drought-stressed seedlings was slightly, but significantly, more oxidised towards the end of the experiment. In *A. aneura*, this became only evident on day 50, whereas in *A. melanoxylon*, this oxidation followed shortly after day 28.

The concentration of ASC+DHA in roots did not change between treatments for *A. aneura*, except for very small, but significant, decreases in drought-stressed seedlings on day 42 and day 50 (Fig. 9). In *A. melanoxylon*, the difference between treatments was significant on day 42. There was a significant increase in the ASC+DHA concentration in well-watered *A. melanoxylon* on day 14. Ascorbic acid (ASC%; Fig. 9) was in a highly reduced state (80 %) in roots of both species and in both treatments during the first week of the drought experiment. Roots of drought-stressed seedlings of both species showed an increase in the proportion of reduced ascorbate

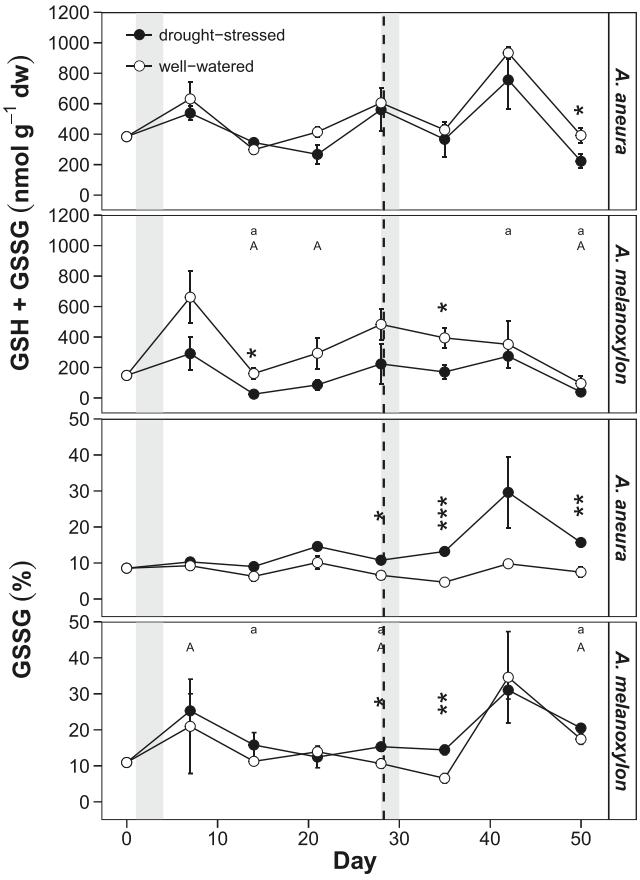

Fig. 6 Total glutathione concentration (GSH+GSSG) and ratio of oxidised to total glutathione (GSSG%) in the phyllodes of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of $n=5$. *GSH+GSSG*: Effects of sampling day, species and treatment as well as their interaction (day by species) were statistically significant ($p<0.05$). *GSSG%*: Effects of sampling day, species and treatment were statistically significant ($p<0.01$)

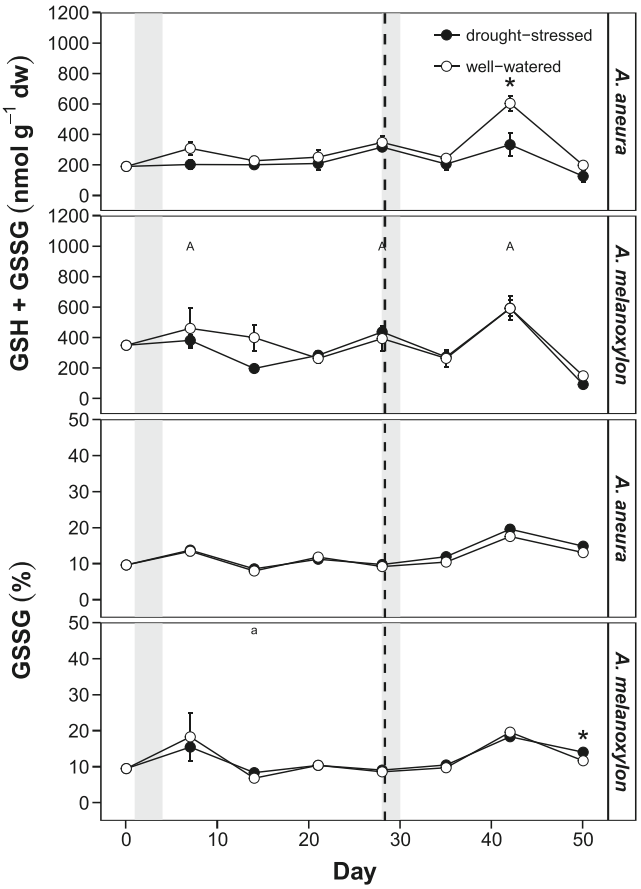

Fig. 7 Total glutathione concentration (GSH+GSSG) and ratio of oxidised to total glutathione (GSSG%) in the roots of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of $n=5$. *GSH+GSSG*: Effects of sampling day, species and treatment as well as their interaction (day by species) were statistically significant ($p<0.05$). *GSSG%*: Effect of sampling day was statistically significant ($p<0.001$)

(i.e. a more reduced, less oxidised state) during the final stages of the drought-stressed treatment.

4 Discussion

Stomatal closure is an important immediate response of plants exposed to drought stress. While reducing transpiration, stomatal closure also limits carbon assimilation and increases the risk of photooxidative stress (Smirnoff 1993; Chaves et al. 2009). In our experiment, the response of g_s to soil moisture was different between the investigated *Acacia* species. Stomata of *A. aneura*, the arid species, were fully open until 50 % of FC which closed sharply upon further soil drying, whereas in *A. melanoxylon*, stomata were closing more gradually and already in response to moderate soil water deficits. *A. aneura* has been described as an anisohydric species, which implies a capacity to tolerate or adjust to strongly negative phyllode water potentials (McDowell et al. 2008; O'Grady et al. 2009). The anisohydric behaviour of *A. aneura* may have allowed stomata to remain open at lower SWC, a strategy that has a positive effect on carbon balance, especially during prolonged drought of medium intensity, but can also bring a risk of hydraulic failure when drought stress becomes more intense (McDowell et al. 2008; O'Grady et al. 2009). This strategy is part of *A. aneura* adaptation to arid and semi-arid habitats (O'Grady et al. 2009; ANBG 2010) and reflected in the differences in stomatal response compared to *A. melanoxylon* adapted to humid habitats.

Photosynthesis or more specifically Rubisco activity and regeneration of 1,5-bisphosphate (RuBP) can be negatively affected by high temperatures (Sharkey 2005; Rennenberg et al. 2006). At moderate temperatures, Rubisco can be reversibly deactivated while photorespiration is promoted as an alternative electron sink (Sharkey and Zhang 2010). Because

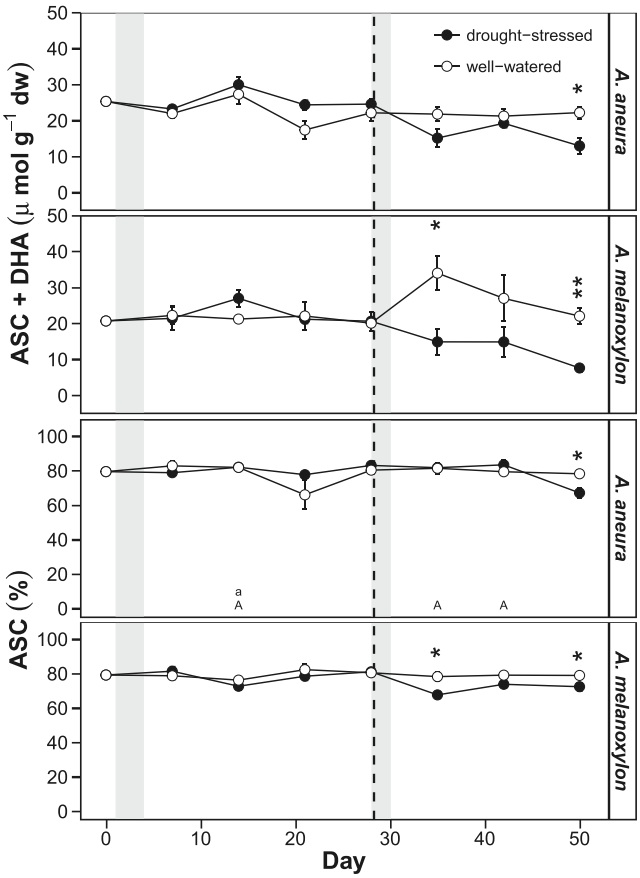

Fig. 8 Total ascorbic acid concentration (ASC+DHA) and ratio of reduced to total ascorbic acid (ASC%) in phyllodes of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of n= 5. *ASC+DHA*: Effects of sampling day, treatment as well as their interactions (day by treatment, species by treatment) were statistically significant (p<0.05). *ASC%*: Effects of sampling day as well as interactions were statistically significant (p<0.05)

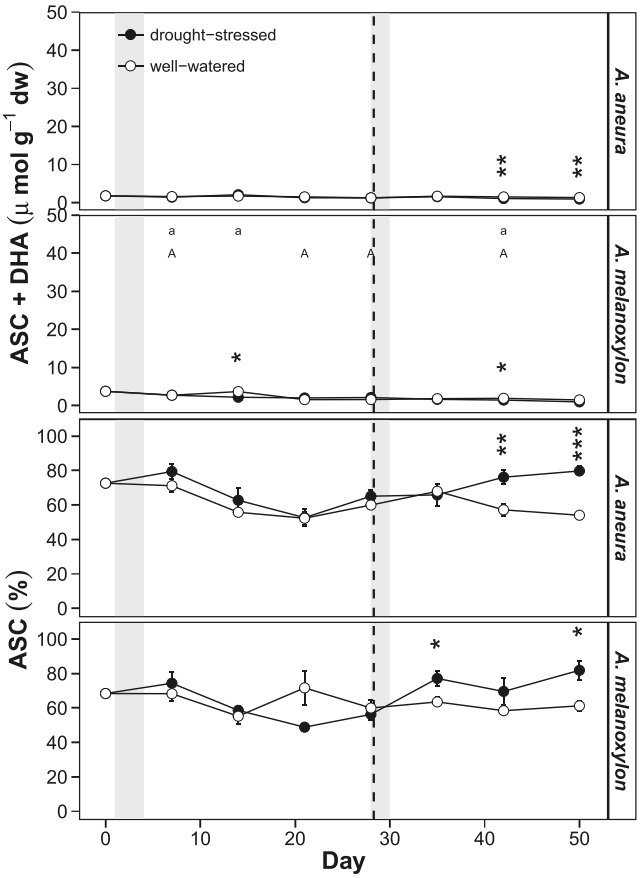

Fig. 9 Total ascorbic acid concentration (ASC+DHA) and ratio of reduced to total ascorbic acid (ASC%) in roots of two *Acacia* species (*A. aneura* and *A. melanoxylon*) under well-watered (*open circle*) and drought-stressed (*closed circle*) treatments. *Vertical line*, *shaded areas*, *letters* and *asterisks* described as in Fig. 1. Values are means (±SE) of n= 5. *ASC+DHA*: Effects of sampling day, species and treatment as well as their interactions (day by species, day by species by treatment, day by treatment) were statistically significant (p<0.05). *ASC%*: Effects of sampling day and treatment as well as their interaction were statistically significant (p<0.001)

well-watered trees of both *Acacia* species had constant A and g_s throughout the experiment, it seems that the heatwave episodes alone did not have a strong effect on photosynthesis. When drought-stressed trees experienced a heatwave, A and g_s responded strongly, suggesting that a combination of drought and heat elicited stronger responses than just decreasing water content (Teskey et al. 2014).

Upon stomatal closure, which also limits access of CO_2 to the leaves and therefore carbon assimilation, plants need to efficiently regulate the dissipation of absorbed light energy as heat and use electrons in alternative pathways to avoid (photo)oxidative stress, processes that form 'first lines of defence' against oxidative stress (Demmig-Adams et al. 2012; Foyer et al. 2012; Noctor et al. 2014). The extent of heat dissipation can be inferred from the quantum efficiency of PSII as measured by chlorophyll fluorescence parameters (efficiency of open reaction centres in PSII in the light (F_v'/F_m') and maximum quantum efficiency of PSII (F_v/F_m)), which are especially useful under stress, i.e. drought or heat (Maxwell and Johnson 2000; Demmig-Adams et al. 2012). In our study, increased heat dissipation, which is associated with a reduction of the efficiency of open reaction centres in PSII in the light (Demmig-Adams et al. 2012), was indicated for *A. melanoxylon* already during early stages of the experiment. Maximum quantum efficiency of PSII values (i.e. the optimum quantum efficiency of PSII after a recovery phase), however, remained around 0.83, which corresponds to undamaged PSII (Maxwell and Johnson 2000; Demmig-Adams et al. 2012). This suggests that the engagement of a flexible and easily reversible heat dissipation mechanism was sufficient to avoid damages to PSII in *A. melanoxylon* under reduced water availability. *A. aneura* did not reduce either efficiency of open reaction centres in PSII in the light or maximum quantum efficiency of PSII during the earlier, deficit irrigation phase of the experiment. After water was

withheld completely, and following a period of high temperatures in the glasshouse, efficiency of open reaction centres in PSII in the light decreased further along with a decrease in maximum quantum efficiency of PSII. Response of maximum quantum efficiency of PSII, indicative of photodamages to PSII (slowly reversible and requiring repair processes), was more gradual in *A. aneura* than in *A. melanoxylon*, where it developed immediately after watering was stopped and high temperatures occurred.

If adjustment of electron flow through flexible heat dissipation is insufficient to address potential imbalances between light-driven electrons and electron consumption in carbon fixation, the formation of ROS can increase (Asada 2006; Demmig-Adams et al. 2012; Foyer et al. 2012). Under relatively mild stress, ROS concentration can be regulated by the antioxidative defence system, but under more severe stress, the imbalance between ROS production and scavenging results in (photo)oxidative stress (Smirnoff 1993; Tausz et al. 2004; Noctor et al. 2014). It was suggested that responses of antioxidants to stress may depend on stress severity and stress progression: The concentration of non-enzymatic antioxidants can increase or be unresponsive at early or mild stages of drought while more severe stress can lead to the breakdown of the defence system with decreased antioxidant concentrations and increasingly oxidised antioxidant pools (Smirnoff 1993; Tausz et al. 2004; Wujeska et al. 2013; Noctor et al. 2014). In our study, concentrations of GSH+GSSG remained stable during early stages of the drought-stressed treatment in *A. aneura* phyllodes. However, progressing drought in combination with heat led to increased proportions of oxidised glutathione in a decreased total pool of glutathione in the phyllodes of drought-stressed *A. aneura* seedlings. *A. melanoxylon* showed similar changes in the concentration of GSH+GSSG but with an earlier and more rapid response following the first heatwave than *A. aneura*. These results are consistent with the notion that severe oxidative stress depletes the glutathione pool through oxidative processes (Smirnoff 1993; Tausz et al. 2004; Wujeska et al. 2013; Noctor et al. 2014). Ascorbic acid (ASC+DHA, ASC%) showed a similar response to the treatments to glutathione. In both *Acacia* species, the concentration of ascorbic acid was unresponsive during early stages of the treatment, but severe drought in combination with heat led to lower ASC+DHA concentrations in phyllodes. This response was delayed for *A. aneura* in comparison to *A. melanoxylon*. Such a decrease, especially under severe stress, could be a result of a depleted ASC+DHA pool due to oxidative degradation (Smirnoff 1993; Tausz et al. 2004; Noctor et al. 2014). However, because the ascorbic acid pool remained in a highly reduced state (indicated by ASC%) normally associated with healthy tissues, the reduction of ASC+DHA in this experiment may also have been the result of an adjustment or regulation of the ascorbic acid pool, perhaps related to changes in synthesis or turnover rates of ascorbic acid (Foyer and Noctor 2011; Smirnoff 2011).

The response of the antioxidative defence systems coincided with chlorophyll fluorescence parameters indicating strong engagement of flexible heat dissipation (efficiency of open reaction centres in PSII in the light) and also damages to PSII (indicated by decreased maximum quantum efficiency of PSII). In agreement with results from an earlier meta-analysis addressing antioxidative responses of trees to drought (Wujeska et al. 2013), the responses of both ascorbic acid and glutathione in this present experiment seem to be dependent on stress intensity: Concentrations of antioxidants increase upon exposure to mild or moderate stress, but this is followed by breakdown of the foliar antioxidative defence system when stress became more severe.

In *A. melanoxylon* but not *A. aneura*, the heatwave exposure alone seemed to have a greater effect on the defence systems than drought, as even well-watered seedlings showed an oxidation of the glutathione pool after heatwaves. During the first week of the present experiment, the presence of juvenile leaves along with phyllodes in *A. melanoxylon* may have made drought-stressed seedlings more vulnerable especially to combined stress factors, which may explain the increased oxidation of glutathione in the absence of any putatively adaptive increase in GSH+GSSG (or ASC+DHA) concentrations in deficit-irrigated seedlings after the first heatwave. Well-watered seedlings indicated an acclimatory response to elevated temperature by increased ASC+DHA concentrations (Smirnoff 1993; Tausz et al. 2004). In contrast, *A. aneura* phyllodes seem to have a higher threshold for any such heat response, as indicated by the absence of any indication of oxidative stress and an increase of GSH+GSSG in well-watered seedlings during heat.

There are few investigations of ascorbic acid and glutathione in tree roots exposed to drought: A field study on European beech (*Fagus sylvatica*) concluded that root ascorbate concentrations increased in response to soil drying, but the response was overlaid by seasonal changes. Responses of root glutathione were seemingly unrelated to soil water content in that study (Haberer et al. 2008). In the present study, root glutathione concentrations of both *Acacia* species remained unchanged in response to drought treatments, but increased after heat in well-watered seedlings of *A. aneura* and irrespective of drought treatment in *A. melanoxylon*. The relationship of root antioxidative systems with environmental factors is not straightforward, as both ascorbic acid and glutathione in roots can be supplied from above-ground parts (Herschbach et al. 2009; Smirnoff 2011). In addition, both glutathione and ascorbic acid in roots are also associated with growth activities (Herschbach et al. 2009) and have been implied in whole-plant stress signalling, e.g. in relation to abscisic acid production (Zhao et al. 2005). It is possible that after the heatwave, roots responded with increased growth (or repair) activity (Herschbach et al. 2009), but it remains unclear why such a response was seen in glutathione but not in ascorbic acid. In one study, root glutathione responded to above-ground exposure to ozone (but not to soil drying), a

result that can only be explained by shoot to root translocation of either glutathione or a signal eliciting root glutathione responses (Haberer et al. 2008). In any case, our data provide no clear evidence that changes in root antioxidants precede above-ground responses, nor that root antioxidant systems responded directly to decreasing SWC.

5 Conclusions

- Response of *A. aneura* was consistent with anisohydric behaviour which allowed stomata to remain open and continue to fix carbon at earlier stages of the experiment.
- Photodamage to PSII (as judged by decreases in maximum quantum efficiency of PSII) developed in both *Acacia* species only right after water was withheld and the second heatwave imposed, and this response was more gradual for *A. aneura*.
- In phyllodes, total glutathione and ascorbic acid pools increased in response to heatwave rather than drought stress alone, for *A. melanoxylon* already after the first heat episode and *A. aneura* after the second heatwave. In *A. melanoxylon*, but not *A. aneura*, ascorbic acid concentrations increased in response to the second heatwave in well-watered trees, and decreases in efficiency of open reaction centres in PSII in the light (but not maximum quantum efficiency of PSII) indicated that flexible heat dissipation processes were engaged in *A. melanoxylon*, but not *A. aneura*, at earlier stages of the experiment.
- In roots, non-enzymatic antioxidants were unresponsive to drought stress but increased glutathione concentrations in response to the second heatwave in both *Acacia* species. The response of total ascorbic acid was weaker, and significant changes were observed only in the ratio of reduced to total ascorbic acid.

Acknowledgments AW-K would like to acknowledge staff and students at the Department of Forest and Ecosystem Science of the University of Melbourne. Special thanks to Raymond Dempsey, Markus Loew, Sabine Tausz-Posch and Nina Hinko-Najera for advice during work. We thank reviewers and the editor for many constructive suggestions that improved the manuscript.

Funding AW-K is supported by a Melbourne International Research Scholarship and a Melbourne International Fee Remission Scholarship.

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