Extreme drought deactivates ABA biosynthesis

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Abstract

The phytohormone abscisic acid (ABA) is synthesized by plants during drought to close stomata and regulate desiccation tolerance pathways. In conifers and a few angiosperms with embolism resistant xylem a peaking-type (p-type) response in ABA levels has been observed, in which ABA levels increase early in drought then decrease as drought progresses, declining to pre-stressed levels. The mechanism behind this dynamic remains unknown. Here we sought to characterize the mechanism driving p-type ABA dynamics in the conifer *Callitris rhomboidea*and the highly drought resistant angiosperm *Umbellularia californica* . We measured leaf water potentials (Ψ_l), stomatal conductance, ABA, conjugates and phaseic acid (PA) levels in potted plants during a prolonged but non-fatal drought. Both species displayed a p-type ABA dynamic during prolonged drought. Measuring ABA levels in bench dried, rehydrated branches collected before and after the peak in ABA levels revealed that in both species ABA biosynthesis is deactivated in leaves that have been dehydrated beyond leaf turgor loss point. Considerable conversion of ABA to conjugates was found to occur during drought, but not catabolism to PA. The mechanism driving the decline in ABA levels in p-type species appears to be conserved across seed plants and is mediated by sustained conjugation of ABA and the deactivation of ABA biosynthesis as Ψ_l becomes more negative than turgor loss.

Introduction

Drought is a leading cause of plant mortality (Brodribb *et al.* , 2020) with the severity and frequency of droughts driving plant evolution and species distributions (Engelbrecht *et al.* , 2007; Bowles *et al.* , 2021). Death caused by drought is largely due to the formation of embolism in the xylem which blocks water transport leading to hydraulic failure and tissue desiccation (Brodribb & Cochard, 2009; Urli *et al.* , 2013; Cardoso *et al.* , 2020; Brodribb *et al.* , 2021). A key adaptation in vascular plants that prevents declines in water potential and thus embolism formation during drought is the closure of stomata (Martin-StPaul *et al.* , 2017; Brodribb *et al.* , 2021). Stomata are dynamic valves on the surface of leaves that open and close in response to environmental and endogenous signals (Raschke, 1975). During drought stomata close to prevent excessive evaporation. The mechanisms that drive stomatal closure during drought have long been debated (Tardieu & Davies, 1993; McAdam & Brodribb, 2014). In seed plants the phytohormone abscisic acid (ABA) plays a critical role in closing stomata during drought stress (Mittelheuser & Van Steveninck, 1969; Jones & Mansfield, 1970; McAdam & Brodribb, 2012). ABA biosynthesis during drought is believed to be triggered by a loss of cell turgor as leaves dehydrate, with peak ABA biosynthesis occurring at a Ψ_l that is close to turgor loss point (Ψ_{tlp}) in herbaceous species (Pierce & Raschke, 1980; Davies *et al.* , 1981; Creelman & Mullet, 1991; McAdam & Brodribb, 2016).

In most herbaceous species, as well as tree species with relatively vulnerable xylem, as drought progresses ABA levels increase and continue to do so until embolism forms (Zeevaart, 1980; Brodribb *et al.* , 2014). Brodribb and McAdam (2013) investigating ABA dynamics under long term drought in conifers discovered a divergent strategy in ABA dynamics. In the highly drought tolerant Cupressaceae species *Callitris rhomboidea* R. Br. ex Rich. & A. Rich.(Cupressaceae) native to arid regions of southeastern Australia (Crisp*et al.* , 2019), stomata closed at the onset of drought stress driven by an increase in ABA levels (Brodribb & McAdam, 2013). However, once plants were dehydrated to –4 MPa, ABA levels stopped increasing, and over the subsequent 10 days of soil drought declined to prestress levels (Brodribb & McAdam, 2013). The reduction in ABA levels under long-term drought in *Callitris* meant that stomata transitioned from closure being driven by ABA to closure being the result of a passive reduction in guard cell turgor, similar to the mechanism of stomatal closure under drought in ferns and lycophytes which have stomata that are insensitive to ABA (McAdam & Brodribb, 2012). This dynamic of ABA levels during drought was termed a "peaking-type" (p-type) ABA dynamic and has subsequently been well characterized across the conifer phylogeny, being associated with the evolution of highly resistant xylem, defined as xylem requiring at least –4 MPa of tension to induce embolism in at least 50% of the xylem (Brodribb *et al.* , 2014). Conifer species from both the derived Cupressaceae (including species from both the Southern Hemisphere callitroid and sister Northern Hemisphere cupressoid clades) as well as Taxaceae have evolved a p-type ABA dynamic under long-term drought (Brodribb *et al.* , 2014). P-type ABA dynamics have been observed in the field in*C. columellaris* F.Muell in which six months of no rainfall each year in the dry season in Northern Australia leads to a seasonal p-type ABA dynamic, such that at the end of the dry season stomata are closed yet ABA levels are as low as plants in the middle of the wet season when Ψ_l are highest (McAdam & Brodribb, 2015). Three studies so far have documented a p-type ABA dynamic in angiosperm species (as recently reviewed by Hasan *et al.* (2021)). One study documented this response in the considerably drought tolerant Central Australian native tree *Acacia aptaneura* Maslin and J.E.Reid (Fabaceae) (Nolan *et al.* , 2017) while two studies have documented the response across six species of arid adapted *Caragana* (Fabaceae) native to Inner Mongolia, China (Yao *et al.* , 2021a; Yao *et al.* , 2021b). All of the angiosperm species in which a p-type ABA dynamic during drought has been observed have highly resistant xylem to embolism formation with the Ψ_l of peak ABA occurring between –3.5 and –4 MPa (Nolan *et al.* , 2017; Yao *et al.* , 2021a; Yao *et al.* , 2021b). From these observations we would hypothesize that highly resistant xylem is required for the evolution of a p-type ABA dynamic across seed plants, and not just in gymnosperms (Brodribb *et al.* , 2014). Resolving the mechanistic unknown driving the p-type ABA dynamic during drought remains challenging because, while highly resistant xylem has evolved independently in at least 130 species from 62 genera and 20 orders of seed plants (McAdam & Cardoso, 2018), there still remains no species with resistant xylem that yet has a sequenced genome. This lack of genetic information means that resolving the mechanism driving ABA level decline under long term drought requires a more classical physiological and biochemical approach (Hasan *et al.* , 2021).

There are a number of possible drivers for the p-type dynamic in ABA levels during long term drought. Given that more than 90% of accumulated ABA synthesized under drought is catabolized into the primary catabolite phaseic acid (PA) when plants are rewatered (Milborrow, 1974), one explanation for the decline in ABA levels during long term drought in p-type species could be activated ABA catabolism. ABA is catabolized into PA by two biochemical steps encoded by cytochrome P450 *CYP707A* genes, the expression of these genes is upregulated when plants are rewatered during drought stress, and when plants are exposed to high humidity (Kushiro *et al.* , 2004; Okamoto *et al.* , 2009). ABA can also be reversibly inactivated by conjugation with UPD-glucose to abscisic acid-glucose ester (ABA-GE) (Milborrow, 1970; Lee *et al.* , 2006). ABA-GE can be stored in the vacuole (Burla *et al.* , 2013), as well as exported from the leaf in the phloem (Zeevaart & Boyer, 1984). Conjugation occurs by a single biochemical step, catalysed by two isoforms of β-glucosidase (Lee*et al.* , 2006; Xu *et al.* , 2012). There could be an enhanced rate of conjugation of ABA to inactive forms under long term drought that explains the decline in ABA levels. An additional explanation for the decline in ABA levels under long term drought could be the cessation of *de novo* biosynthesis of ABA. The loss of cell turgor is a well-described trigger for increasing the expression the gene encoding the rate limiting step in ABA biosynthesis in angiosperms, 9-*cis* -epoxycarotenoid deoxygenase (*NCED3* in*Arabidopsis*) (Qin & Zeevaart, 1999; Sussmilch *et al.* , 2017; Bacete *et al.* , 2022), only a relief of low cell turgor, via rehydration, is known to decrease the expression of this rate limiting step gene in the ABA biosynthetic pathway (Qin & Zeevaart, 1999). While never described the cessation of *de novo* ABA biosynthesis at a threshold Ψ_l under drought would lead to a decrease in ABA levels that is independent of changes in the rate of ABA catabolism or conjugation. A final, but least likely explanation, is an increase in the rate of ABA export from the leaf via the phloem, phloem flux from the leaf is a major sink for foliage derived ABA (Jeschke *et al.* , 1997; Castro *et al.* , 2019). Given that the rate of phloem flux is presumably low or non-existent when assimilation has ceased during drought (Sevanto, 2014), a common occurrence when stomata are closed, this seems the least likely driver for a decline in ABA levels under drought.

Here we sought to characterize the mechanism driving declines in ABA levels under long term drought in p-type seed plant species. We conducted experiments on two species, the model system for characterizing p-type ABA dynamics, the gymnosperm species *C. rhomboidea* (Cupressaceae) native to South Eastern Australia and the highly drought resistant evergreen angiosperm species *Umbellularia californica* (Hook. & Arn.) Nutt. (Lauraceae) native to coastal forests and the foothills of the Sierra Nevada in Western North America (DiLeo*et al.* , 2014). Ψ_l, canopy conductance (g_c), foliage ABA, PA and conjugate levels were measured in potted plants of each species through a prolonged drought treatment until a non-fatal Ψ_l was reached. We tested four mechanistic hypotheses for the p-type ABA dynamic: (1) increased catabolism of ABA into either PA or (2) conjugation, occurs at a threshold Ψ_l, (3) a cessation of ABA biosynthesis, driven by osmotic adjustment or turgor loss, and/or (4) the phloem export of ABA from leaves. The role of export was assessed by girdling branches in drought stressed plants. We developed a novel technique based on bench dehydration to assess the ability of shoots to rapidly synthesize ABA to assess whether ABA biosynthesis was deactivated under long-term drought.

Materials and Methods

Plant material

Individuals of both species were grown from seed in pots in a mix of Indiana Miami topsoil, ground pine bark and sand at a 0.5:1:0.5 ratio. Plants were between three and five years of age at the time of experiment. The plants were grown at Purdue University under controlled glasshouse conditions with approximately 12h natural light supplemented with LEDs (16h day, 8h night; Illumitex Power Harvest I4, TX, USA) providing a minimum photon flux at pot height of 150 µmol quanta m^{-2} s^{-1}. Under well-watered conditions, plants received daily irrigation and complete liquid nutrients (Miracle-Gro® Water-Soluble All Purpose Plant Food, The Scotts Company LLC, OH, USA) once every month. Glasshouse temperatures were set at 28 °C during the day and 22 °C during the night. Air circulation fans in the glasshouse ensured continual air circulation, reducing boundary layer conductance.

Canopy conductance and hormone levels

To measure midday whole plant transpiration pots were enclosed in a black plastic bag and covered in aluminium foil secured around the stem with a reversible cable tie to eliminate evaporation from the soil medium. During drought pots were weighted (Mettler Toledo, OH, USA) 30 minutes before and after solar midday. Drought was initiated by withholding water. Samples for Ψ_l determination and foliage hormone analysis were collected 30 minutes after solar midday after final masses were taken. Samples were wrapped in damp paper towel, then aluminium foil, placed inside a ziplock bag and then an insulated box, for transportation to the lab. Ψ_l was measured using a Scholander pressure chamber (PMS Instrument Company, OR, USA) and microscope for accurate determination of balance pressure, by slowly pressurizing and depressurizing the chamber. After measuring Ψ_l, a subsample of tissue was then taken for hormone analysis (see below). Water was withheld from plants until they reached -6 MPa (at this Ψ_l there was not signs of leaf death or damage). Once this threshold Ψ_l was reached (at least 10 d after a peak in ABA levels was measured), plants were rewatered to soil field capacity. Measurements were made on rehydrated plants until whole plant transpiration approached the levels measured prior to drought (4 d). At the end of the experiment total plant foliage area was determined. In *U. californica* leaf area was calculated by scanning leaves (Epson Perfection V39 Scanner, Epson America, Inc., CA, US) and quantifying leaf area using ImageJ (NIH). A mean leaf area for an individual leaf was determined from these images (12.68 cm^2) which was used to adjust total plant leaf area during the experiment to account for leaves periodically harvested for Ψ_l and foliage hormone analysis. In *C. rhomboidea*leaf area was calculated from the ratio of leaf dry weight to leaf area. Whole plant leafy branch area was harvested and dried to completeness at 70°C for 48h, after which dry mass was taken. Total leaf area was calculated from the ratio of dry mass to leaf area (70.80 cm^2 g^{-1}) determined from sub-samples prepared in the same way. In *C. rhomboidea* a mean area of sample collected for Ψ_l and hormone analysis was determined from 10 random samples (2.86 cm^2). This mean sample area was used to correct whole plant leaf area for declines caused by sampling. Temperature and relative humidity measurements were recorded every 10 min using a HOBO MX2301A Data Logger (Onset Computer Corporation, MA USA), suspended at plant height in the glasshouse. The gravimetric determination of whole plant water loss was then used to calculate canopy conductance conductance (g_c) by calculating whole plant transpiration (E, mol m^{-2} s^{-1}) using equation 1 and the leaf area determined above.

$$\mathrm{E} = \frac{\text{moles of water lost}}{area \quad s} \quad (1)$$

Mean vapor pressure deficit of the atmosphere (VPD) for the hour during which E was measured using equation 2.

$$\mathrm{VPD} = (610.7 * 10^{\frac{7,5*T}{237.3+T}}) * \frac{(100-RH)}{100} (2)$$

Where T is mean air temperature and RH is relative humidity for the duration of the transpiration measurements.

g_c was then calculated from E and VPD using equation 3:

$$\mathrm{g_c} = \frac{E}{VPD \times P_{\mathrm{atm}}} \quad (3)$$

Where P_{atm} is atmospheric pressure. We assumed negligible boundary layer conductance due to the constant air circulation in the glasshouse, and that leaf temperature approximated air temperature.

Girdling experiments

To test for an effect of reduced phloem transport during drought on foliage hormone levels, prior to drought a single, large branch on three individuals was girdled. Branches were girdled by carefully removing two to three cm of periderm just above the intersection of a side branch and the main, leading stem. Once periderm was removed a hydrogel was applied to the xylem (Tensive, Parker Labs, Fairfield, NJ, USA). Samples from girdled and non-girdled branches were harvested concurrently to determine Ψ_l using a Scholander pressure chamber and to collect a subsample of tissue for hormone quantification.

Rapid bench dehydration assay to assess ABA biosynthetic capacity

A method was developed to test for the capacity of leaves to rapidly synthesize ABA, relying on the well described effect of bench dehydration being able to induce ABA biosynthesis in leaves (Wright & Hiron, 1969). Branches were excised from individuals of each species at cardinal timepoints prior to water stress imposition and 10 d after peak ABA levels. To test whether tissues were capable of ABA biosynthesis branches were excised under water and rehydrated overnight, the following morning branches were dehydrated on a bench for 12 h in*C. rhomboidea* and 7 h in *U. californica* . Samples from the drying branches were collected periodically in *U. californica* or every 3 h in *C. rhomboidea* , to determine Ψ_l and foliage ABA and ABA-GE levels. Laboratory environmental data was logged to ensure a constant VPD (2.1 kPa) was maintained during the experiment.

Quantification of ABA, PA and conjugates

Samples for hormone analysis were processed according to McAdam (2015). 15 ng of [$^2H_6$]ABA and [$^2H_3$]PA (OlChemim Ltd, Czech Republic) were added to each sample as an internal standard. To measure ABA-GE levels, an additional aliquot was taken and an alkaline hydrolysis method was used (Hansen & Dörffling, 1999). Hormone samples were resuspended in 200 µl of 2% acetic acid and 10% acetonitrile in H_2O (v v^{-1}). Each sample was then centrifuged at 14800 RPM for 3 minutes and a 100 µl aliquot was taken for analysis. Hormone levels were quantified using an Agilent 6460 series triple quadrupole LC/MS (Agilent, CA, USA) fitted with an xBridge HPLC column (C18, 2.1 x 100 mm, 3.5 µm, Waters Corporation, MA, USA). Solvents used were 2% acetic acid in H_2O (v v^{-1}, Solvent A) and acetonitrile (Solvent B) at a flow of 0.3mL min^{-1}. The running gradient went from 90% Solvent A and 10% Solvent B to 5% Solvent A and 95% Solvent B at 5 minutes and then back to initial values. An aliquot of 10 µL of sample was injected. The LC/MS was operated in negative ion electrospray mode with the needle running at 3.5 kV. To detect each metabolite and respective internal standard we used selected reaction monitoring. We used an ion source temperature of 325 ^oC and nitrogen as the desolvation gas flowing at 8 l min^{-1}. The tandem transitions were m/z 263.1 to 153, 204 and 219, for ABA; for [$^2H_6$]ABA the transitions monitored were m/z 269.1 to 159, 207 and 225. For PA, the tandem transitions were m/z 279.3 to 139.1 and 205; and m/z 282.3 to 142.1 and 208, for [$^2H_3$]PA. The cone voltage was 100V. For all transitions, the collision energy was 5V. Dwell time was set to 50 ms for each channel. Hormone levels were analyzed in the Agilent Quantitative Analysis software. Quantification was done using the m/z 263.1 to 153 and corresponding labelled channel for ABA, and m/z 279.3 to 139.1 and corresponding labelled channel for PA. Hormone levels in terms were calculated as the ratio of endogenous to labelled hormone peak areas, multiplied by the amount labelled ABA added to the sample (in all cases 15 ng), divided by the fresh weight of the sample collected. ABA-GE levels were determined as the difference between ABA levels in a quantified unhydrolyzed sample and the hydrolysed sample (Hansen & Dörffling, 1999).

Pressure-volume curves

Pressure-volume curves were undertaken to determine the degree of osmotic adjustment in response to long term drought prior to drought and in leaves collected from the same plants that had experienced long-term drought sufficient to reduce ABA levels to initial levels measured prior to the drought (Tyree & Hammel, 1972). Five individual leaves or leafy shoots from the same individual were collected and rehydrated overnight to a hydrated state (full hydration was considered when initial Ψ_l was greater than -0.05 MPa). Tissue was scanned to obtain hydrated area then leaves or shoots were dehydrated on the bench, and measurements of weight and water potential were periodically recorded as water potential progressively declined (Tyree & Hammel, 1972). Ψ_{tlp} was calculated from the P-V curves as the Ψ_l at which the relationship between the inverse of relative water content and Ψ_l deviates from a linear regression.

Data analysis

Generalized additive models and standard errors were fitted for ABA level, PA level, and g_s using the gam() function in the MGCV package (Wood, 2011) of R software (v.4.0.5, R Core Team, 2018). ANOVA was perfomed for all comparisons using the aov() function, and significant interactions were determined using the TukeysHSD() funtion of the multcomp package (Hothorn *et al.* , 2008) in R software. Correlation analysis

for ABA and PA levels was performed using the cor.test() funtion in R software. Graphs were generated using the Sigmaplot software (v.10, Systat Software, Chicago, IL, USA).

Results

Peaking-type ABA dynamics in Umbellularia and Callitris

During a soil drought, the evergreen angiosperm species *Umbellularia californica* , like the conifer species *Callitris rhomboidea* , displayed a peaking-type dynamic in foliage ABA levels (Figure 1). Prior to drought at a water potential of -0.7 MPa, mean foliage ABA levels in *U. californica* were 0.714 ± 0.61 μg g^{-1} FW (± SE, Figure 1A). As leaf water potential (Ψ_l) declines, ABA levels in *U. californica* rose to a mean peak of 0.4924 ± 0.4 μg g^{-1} FW (± SE) once Ψ_l had reached to -3.1 MPa (Figure 1A). As *U. californica* experienced more negative Ψ_l ABA levels gradually declined, decreasing back to a mean of 0.109 ± 0.6 μg g at a Ψ_l of -6.02 MPa (Figure 1A). ABA levels in *U. californica* decreased at an average rate of 0.15 μg g^{-1} FW MPa^{-1} during this time. In *C. rhomboidea* mean initial ABA level prior to drought was 0.236 ± 0.11 μg g^{-1} FW (±SE), this increased to a mean peak ABA level of 0.845 ± 0.8 μg g^{-1} FW (±SE) at -2.7 MPa (Figure 1B). After ABA levels peaked they declined by an average of 0.26 μg g^{-1} FW MPa^{-1} to 0.275 ± 0.13 μg g^{-1} FW (±SE) at -5 MPa (Figure 1B). Evidence of leaf death and a lack of recovery in maximum photosynthetic rate following rewatering for both species occurred once Ψ_l declined less than -7 MPa (data not shown). In both species, canopy conductance (g_c) declined exponentially as $\Psi_l$declined, with an 83% to 99% reduction in $g_c$once Ψ_l had declined to the Ψ_l at which peak levels of foliage ABA occurred in *U. californica* and *C. rhomboidea* , respectively (Figure 1C and D). In *U. californica* mean (± SE) maximum g_c prior to drought was 40.9 ± 0.2 mmol m^{-2} s^{-1}, this exponentially declined to a minimum of 0.42 ± 0.2 mmol m^{-2} s^{-1} by -6.02 MPa (Figure 5). In *C. rhomboidea,* mean (± SE) maximum $g_c$prior to drought was 17 ± 0.1 mmol $m^{-2}s^{-1}$, this declined to a minimum of 0.026 ± 0.13 mmol m^{-2} s^{-1} by -6.02 MPa (Figure 6). Stomata remained closed for the duration of the drought in both species.

Catabolism of ABA to phaseic acid cannot explain declining ABA levels during drought

Foliage phaseic acid (PA) levels displayed a peaking-type dynamic as Ψ_l declined during drought in the angiosperm species *U. californica* (Figure 2). PA levels were found to be significantly correlated with ABA levels in *U. californica*(*Pearson's* $r_{57} = 0.74$, $p < 0.0001$) while no significant correlation between PA and ABA levels was observed in the conifer species *C. rhomboidea* (*Pearson's* $r_{23} = 34$, $p = 0.09234$). In *U. californica* mean foliage PA levels were 0.292 ± 0.095 μg g^{-1} FW (± SE) at -0.7 MPa prior to the drought, then as Ψ_l decreased to -3.3 MPa foliage PA levels increased reaching a maximum of 0.969 ± 0.063 μg g^{-1} FW (±SE) (Figure 2). In *U. californica* as drought progressed foliage PA levels declined to a minimum of 0.545 ng g^{-1} FW at -6.02 MPa, at a rate of 0.167 μg g^{-1} FW MPa^{-1} (Figure 2). In the conifer species *C. rhomboidea* , there was very little change in PA levels as drought progressed (Figure 2). PA levels prior to drought were low at a mean of 0.107 ± 0.1 μg g^{-1} FW at -0.65 MPa, there was one period during drought where there was a wide variation in PA levels to a maximum of 0.504 ± 0.08 μg g^{-1} FW around -3.51 MPa (Figure 2). However, PA levels remained at 0.15 ± 0.013 μg g^{-1} FW (± SE) once Ψ_l had declined to -5 MPa.

ABA catabolism to phaseic acid requires rehydration for activation

The rewatering of drought stressed plants activated the catabolism of ABA to PA (Figure 3). In the angiosperm species *U. californica*rewatering whole plants from a Ψ_l of -6 MPa, when foliage ABA levels were the lowest of the entire drought period, resulted in a reduction in foliage ABA levels over four days from 1.62 ±0.35 μg g^{-1} FW to 0.297 ±0.025 μg g^{-1} FW (Figure 3). This reduction in foliage ABA levels in *U. californica* corresponded to an increase in foliage PA levels from 0.42 ±0.08 μg g^{-1} FW to 1.08 ±0.076 μg g^{-1} FW in the 24 h after rewatering (Figure 3). In the conifer species *C. rhomboidea* rewatering whole plants from a similar initial Ψ_l at the angiosperm *U. californica* restored water status and also drove a decline in foliage ABA levels from 0.54 ±0.15 μg g^{-1} FW to 0.16 ±0.03 μg g^{-1} FW in 48 h (Figure 3). Like in the angiosperm species, PA levels in *C. rhomboidea* increased from 0.057 ±0.03 μg g^{-1} FW to 0.4 ±0.13 μg g^{-1} FW (Figure 3).

Accumulation of conjugated forms of ABA during occurred drought and was not influenced by inhibiting phloem transport

In both species foliage ABA conjugate levels increased as drought progressed to a maximum 10 d after peak ABA levels were measured in leaves and when foliage ABA levels were a minimum (Figure 4). In the angiosperm species *U. californica* mean foliage ABA conjugate levels increased in both intact and girdled branches similarly from 0.2 to 2.63 μg g^{-1} FW prior to drought to when ABA levels were the lowest at the end of the drought period (Figure 1 and Figure 4). Similar patterns in foliage ABA conjugate level during drought were observed in the conifer species *C. rhomboidea* , increasing to a much higher level than *U. californica* , from 1.5 to 23 μg g^{-1} FW (Figure 4). Girdling branches and ceasing phloem transport had no effect on the average foliage ABA level dynamics during drought in either species (Figure 5), or on foliage ABA conjugate levels (Figure 4). In the angiosperm species *U. californica* the mean generalized additive models fitted through foliage ABA data as Ψ_l declined was slightly higher over the Ψ_l period at which there was a peak in foliage ABA levels, however foliage ABA levels did decline to levels measured in unstressed leaves once plants had reached a Ψ_l of -6 MPa (Figure 5A). Mean ABA levels in girdled branches of *C. rhomboidea*increased from 0.2 ± 0.1 μg g^{-1} FW at -0.59 MPa to 0.775 ± 0.084 μg g^{-1} FW at -3.16 MPa (Figure 5), this was similar to levels of ABA in un-girdled branches (t-test, $p = 0.3937$).

Δεηψδρατιον τριγγερεδ ABA βιοσψντηεσις ις τεμποραριλψ δεαστιατεδ ιν πλαντς συσταινινγ λοω Ψ_λ

In the angiosperm species *U. californica,* we found that rapid bench dehydration of never-before stressed branches triggered a fast and considerable accumulation of foliage ABA levels (Figure 6). In *U. californica* , foliage ABA levels increased by 0.345 μg g^{-1} FW h^{-1} as branches were dehydrated on the bench (Figure 6). In branches collected from plants under long-term drought at least 10 d after peak foliage ABA levels, Ψ_l decreased rapidly when bench dried while ABA levels remained low and unchanged (less than 0.767 ± 0.092 μg g^{-1} FW, mean ± SE, Figure 6). Ψ_l in never-before stressed branches of *U. californica* which accumulated considerable levels of ABA on rapid dehydration decreased at a slower rate than for branches that were collected from plants that had experienced long-term drought, with WP at 3.25 h after the initiation of dehydration being -0.50 MPa in never-before stressed branches and -2.47 MPa in branches collected from plants experiencing long-term drought that were not accumulating foliage ABA levels (Figure 6). Similarly, in the conifer species *C. rhomboidea* foliage ABA levels increased in unstressed branches from 0.153 ± 0.1 μg g^{-1} FW to 1.743 ± 0.3 μg g^{-1} FW at 6 h while in branches collected from plants under long-term drought at least 10 d after peak foliage ABA levels were measured displayed relative low levels of foliage ABA increasing from 0.090 ± 0.02 μg g^{-1} FW to just 0.225 ± 0.024 μg g^{-1} FW at 6 h (Figure 6). Ψ_l in never-before stressed branches of the conifer*C. rhomboidea* , like those of *U. californica* , decreased at a slower rate than that of branches in which ABA levels did not accumulate on rapid bench dehydration. Ψ_l decreased to -1.83 MPa in never-before stressed branches and -3.96 MPa in branches from plants under long-term drought at 6 h (Figure 6). The ability to synthesize ABA on rapid bench dehydration recovers in *U. californica* plants after rewatering (Figure 7). In plants that were drought stressed for 10 d after peak ABA levels were measured then rewatered to saturating soil water capacity, branches harvested 3 d after rewatering and then rapidly dehydrated on the bench were able to synthesize ABA over 10 h (Figure 7). Mean foliage ABA levels in these branches increased from 0.26 to 1.97 μg g^{-1} FW in 10 h (Figure 7).

Ψ_λ ατ πεακ φολιαγε ABA λεελ ςορρεσπονδς το $\Psi_{\tau\lambda\pi}$

In the angiosperm species *U. californica* mean turgor loss point (Ψ_{tlp}) in never-before stressed plants was -3.66 ± 0.18 MPa (Figure 8). In leaves collected from plants approximately 10 d after peak foliage ABA level, when Ψ_l had declined to -6 MPa, Ψ_{tlp} was not significantly lower than never-before stressed plants (t5.06, = 1.2042, p = 0.2817), with a mean Ψ_{tlp} of -3.58 ± 0.20 MPa (Figure 8). Similarly, in the conifer species *C. rhomboidea* , mean Ψ_{tlp} in never-before stressed plants was -2.83 ± 0.34 MPa (Figure 8), which was not significantly different than mean Ψ_{tlp} in branches of plants that had experienced drought for at least 10 d after peak foliage ABA levels (-3.04 ± 0.16 MPa, t7.98 = -1.69, p = 0.1296).

Discussion

Here we document the occurrence of p-type ABA dynamics in an angiosperm species outside of Fabaceae, the evergreen Lauraceae species *U. californica* . In *U. californica* foliage levels of ABA during long term drought showed a highly similar pattern as $\Psi_l$declined to that of the classical model species that initially characterized p-type responses of foliage ABA levels during long term drought, the conifer species *C. rhomboidea* (Brodribb & McAdam, 2013). This result, coupled with previous reports of p-type ABA dynamics in species from two genera in Fabaceae (Nolan *et al.* , 2017; Yao*et al.* , 2021a; Yao *et al.* , 2021b), all adapted to seasonally dry or arid environments and with highly embolism resistant xylem (no leaf death was seen in our drought experiment in either species experienced a Ψ_l of -6 MPa), suggests that the evolution of the p-type ABA response to long-term drought is linked to the evolution of highly resistant xylem, and is not just a conifer and Fabaceae specific phenomenon. Highly resistant xylem has evolved frequently across angiosperm species (McAdam & Cardoso, 2018) suggesting that if the two are linked, highly embolism resistant xylem and the p-type ABA dynamic (Brodribb *et al.* , 2014), this ABA response to drought could be quite commonly observed across angiosperms. Taken together our results demonstrate the occurrence of a p-type ABA response now in two highly divergent angiosperm families, the early diverging Lauraceae and the Fabaceae (Nolan *et al.* , 2017; Yao*et al.* , 2021a; Yao *et al.* , 2021b), future studies are needed to investigate whether this response is common across angiosperms with highly resistant xylem. The absence of high levels of ABA under long term drought in angiosperm implies that, like in conifers, the stomata of all p-type species may be closed passively by low Ψ_l under long term drought (Brodribb & McAdam, 2013; McAdam & Brodribb, 2015). This is a controversial hypothesis for angiosperm stomatal biology (Franks, 2013; Merilo *et al.* , 2017), especially given that angiosperm ABA biosynthetic and signalling mutants have stomata that are insensitive to changes in leaf water status (McAdam *et al.* , 2016; Cernusak *et al.* , 2019; Brodribb*et al.* , 2021). It has been suggested that passive regulation of stomatal aperture in response to changes in leaf water status is absent from this group of land plants (McAdam & Sussmilch, 2020). Recently in characterizing p-type ABA dynamics in *Caragana* , Yao *et al.*(2021a) suggested that ethylene might be closing stomata during drought and on recovery from drought when ABA levels are low but stomata are not yet open to maximum apertures. Further work is required to address whether the stomata of p-type angiosperms are closed at low Ψ_l passively via low cell turgor pressure, or via an alternative metabolic signal such as ethylene (Hasan *et al.* , 2021).

Similarities in the dynamics of ABA, catabolite and conjugate levels, as well as the inhibition of dehydration-induced ABA biosynthesis during drought between the p-type angiosperm and conifer species suggests that there is a shared mechanism driving the decline in ABA levels under long term drought stress in seed plants. By rapidly dehydrating branches on the bench we could assess the ability of leaf tissue to rapidly synthesize ABA in response to dehydration (Wright & Hiron, 1969). This technique allowed us to study ABA biosynthetic capacity without the need to quantify the expression of key ABA biosynthetic genes, which can be costly and time consuming and requires a detailed understanding of the homologues of key genes (Sussmilch *et al.* , 2019). Despite considerable investment in genome sequencing in the past decade (Kress*et al.* , 2022), we still do not have for any species with highly resistant xylem to embolism. We show that ABA biosynthesis is highly active and rapid in unstressed branches that are rapidly dehydrated on the bench, like numerous early studies into ABA biosynthesis in plants (Wright & Hiron, 1969; Pierce & Raschke, 1980; Davies *et al.* , 1981). This ability is eliminated in branches that are taken from plants when ABA levels are low under long-term drought and rehydrated overnight on the bench before dehydration (Figure 4). A key limitation to this method is that it relies on quantifying a change in ABA level which is only possible if there are low levels of the hormone at the start of the experiment, hence we are unable to use this method to determine precisely when dehydration induced ABA biosynthesis was deactivated during drought, because for much of a drought ABA levels were high (Figure 1). We could speculate that the point at which ABA biosynthesis was deactivated corresponded to the Ψ_l close to when ABA levels peaked during drought. Coincidently, we found that the Ψ_l at which peak ABA levels occurred was very similar to the Ψ_{tlp} (Figure 8). Work is required to confirm that the expression of key ABA biosynthetic genes such as Nine-cis-epoxycarotenoid deoxygenase (*NCED*) genes are no longer upregulated on rapid dehydration in branches that do not rapidly synthesize ABA when dehydrated on the bench (Hasan *et al.* , 2021). As cells loose turgor or lose volume ABA biosynthesis is triggered (Pierce & Raschke, 1980; Davies *et al.* , 1981;

Creelman & Mullet, 1991; McAdam & Brodribb, 2016), yet there has been very little work conducted on plant tissue that has been dehydrated to a Ψ_l more negative than Ψ_{tlp}.. Consequently, the potential causes of ABA biosynthesis cessation at a Ψ_l more negative than Ψ_{tlp} are highly speculative. Explanations range from an absent trigger for *NCED* expression once membrane pressure on the cell wall ceases (Bacete *et al.* , 2022); cellular processes such as transcription and translation of RNA ceasing at a Ψ_l more negative than Ψ_{tlp} (Dhindsa & Cleland, 1975); or carotenoid precursors for ABA biosynthesis, often sorted in chloroplasts, may be depleted because of increased in the de-epoxidation state of the xanthophyll cycle (Munné-Bosch & Alegre, 2000), reducing availability for conversion to ABA. Munné-Bosch and Alegre (2000) found in the extremely drought resistant *Rosmarinus officinalis* , in which 50% of the xylem experiences embolism at a Ψ_l at -8 MPa (Brodribb *et al.* , 2017) during a severe summer drought the levels of ABA carotenoid precursors violaxanthin and neoxanthin declined by more than 85% maximum levels. We show that the ability to recover ABA biosynthesis can occur, with ABA levels accumulating, but not to levels in never-before stressed branches 4 days after rewatering. This recovery might reflect the rapid recovery of carotenoid levels upon rehydration (Munné-Bosch & Alegre, 2000). Future studies in herbaceous species at a Ψ_l more negative than Ψ_{tlp} could help elucidate whether the mechanism driving ABA biosynthesis cessation after $\Psi_{tlp}$is universal to all plants. These experiments can only be done in strictly controlled environments since a slight decrease in Ψ_l once turgor is lost could trigger lethal embolism as the two occur at very similar Ψ_l in herbaceous plants (Skelton *et al.* , 2017). There is a hypothesis that ABA biosynthesis is triggered by a loss of cell volume and not a change in cell turgor (Sack *et al.* , 2018), our results refute the hypothesis ABA biosynthesis ceases in p-type species yet cell volume presumably continues to decline at a fairly linear rate during drought. This corroborates recent data that indicates an intact cell wall is essential for the biosynthesis of ABA when cells are exposed to solutions of high osmotic potential (Bacete *et al.* , 2022).

Once ABA biosynthesis ceases at a Ψ_l more negative than Ψ_{tlp} our results suggest that continual conjugation of ABA, presumably into ABA-GE, which is the primary, if not only, conjugate for ABA (Milborrow, 1970), and not catabolism of the remaining is the main driver for a decline in foliage ABA levels once ABA biosynthesis ceases. It is believed that phloem flux is greatly reduced during drought (Hartmann *et al.* , 2013; Sevanto, 2014), and our results from a girdling experiment in *C. rhomboidea* demonstrate that girdling the phloem does not change the p-type ABA dynamic during drought or the accumulation of ABA conjugates. By quantifying the levels of PA during long term drought, we are able to categorically rule out catabolism was the primary driver of a decrease in ABA levels after Ψ_{tlp}. Our results demonstrate that catabolism of ABA into PA did not significantly increase after peak ABA levels. Interestingly, the dynamic of PA levels and ABA levels during drought suggests that PA levels and the rate of ABA catabolism could be a simple function of current ABA level.

Conclusion

Here we characterize the mechanism behind the p-type response of ABA dynamics exhibited by highly drought tolerant species under long term drought. The use of rapid bench dehydration to assess ABA biosynthesis revealed that ABA biosynthesis on dehydration is inhibited once plants are drought stressed to Ψ_{tlp}. Continual conjugation of ABA appears to drive a decline in ABA levels once biosynthesis is inactivated. Future work is needed to assess the occurrence of the p-type behaviour in a wider range of species of seed plants, including in herbaceous species which usually die after a slight decrease in Ψ_l below Ψ_{tlp}.

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We thank Amanda Cardoso who conducted a preliminary experiment that revealed a p-type ABA response in *Umbellularia* ; Mike Mickelbart and Gyeong-Mee Yoon for helpful comments on experimental design and text; and John Ross, without whose mentorship and advice on hormone analysis, this study would not have been possible. We acknowledge the use of the Metabolite Profiling Facility of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute for quantifying hormone levels. Support for this project came from the USDA National Institute of Food and Agriculture Hatch project 10104908 and the National Science Foundation grant IOS-2140119.

Figure Legends

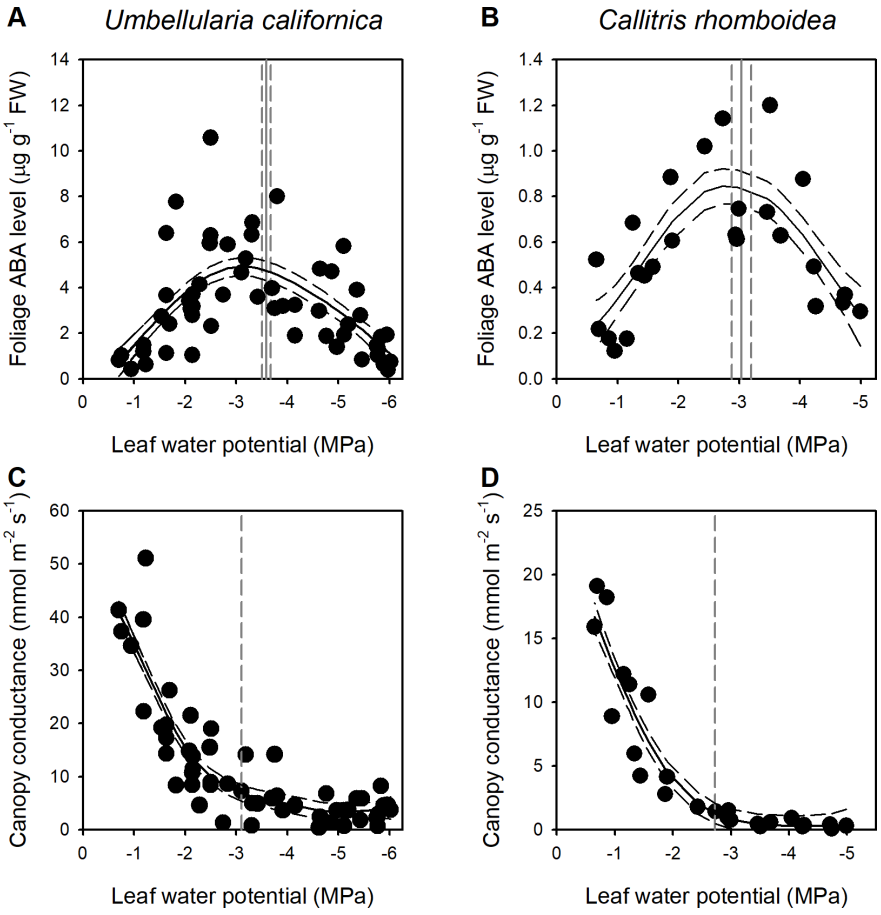


Figure 1. Midday foliage abscisic acid (ABA) level as leaf water potential (Ψ_l) declines during soil drought in angiosperm species *Umbellularia californica* (A) and conifer species *Callitris rhomboidea* (B). Mean leaf water potential (Ψ_l) at turgor loss point and standard errors are shown as grey solid and dashed lines vertical dashed grey line in A and B. Midday canopy conductance during soil drought in *U. californica* (C) and *C. rhomboidea* (D). Vertical dashed lines in C and D represent the Ψ_l at which peak foliage ABA level occurred. Generalized additive model (GAM) curves and standard errors are represented in all relationships by solid and dashed black lines, respectively.

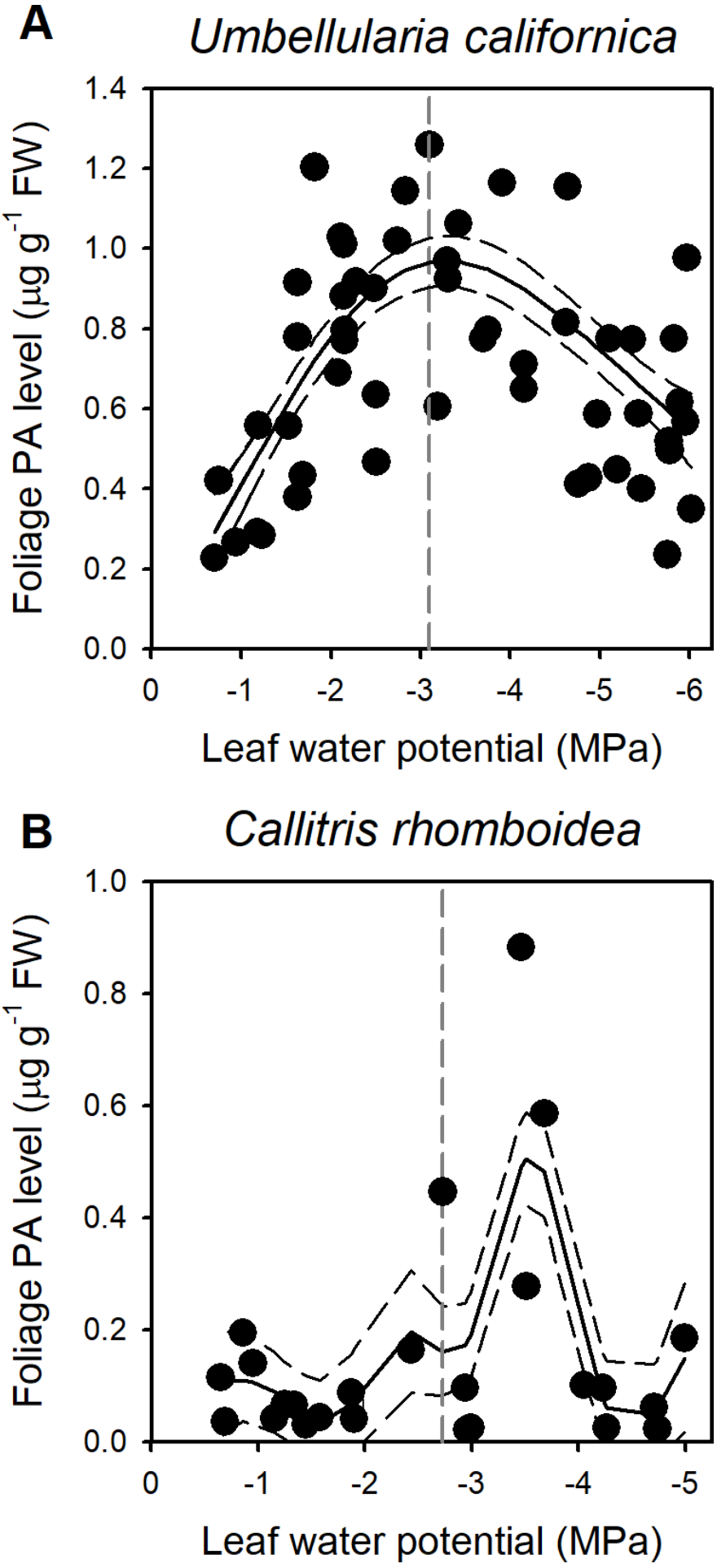


Figure 2. Foliage phaseic acid (PA) levels as leaf water potential (Ψ_l) declines during soil drought in the angiosperm species *Umbellularia californica* (A) and conifer species*Callitris rhomboidea* (B). Generalized additive model (GAM) curves and standard errors are represented by solid and dashed lines, respectively. The grey vertical line depicts the Ψ_l at peak foliage ABA level.

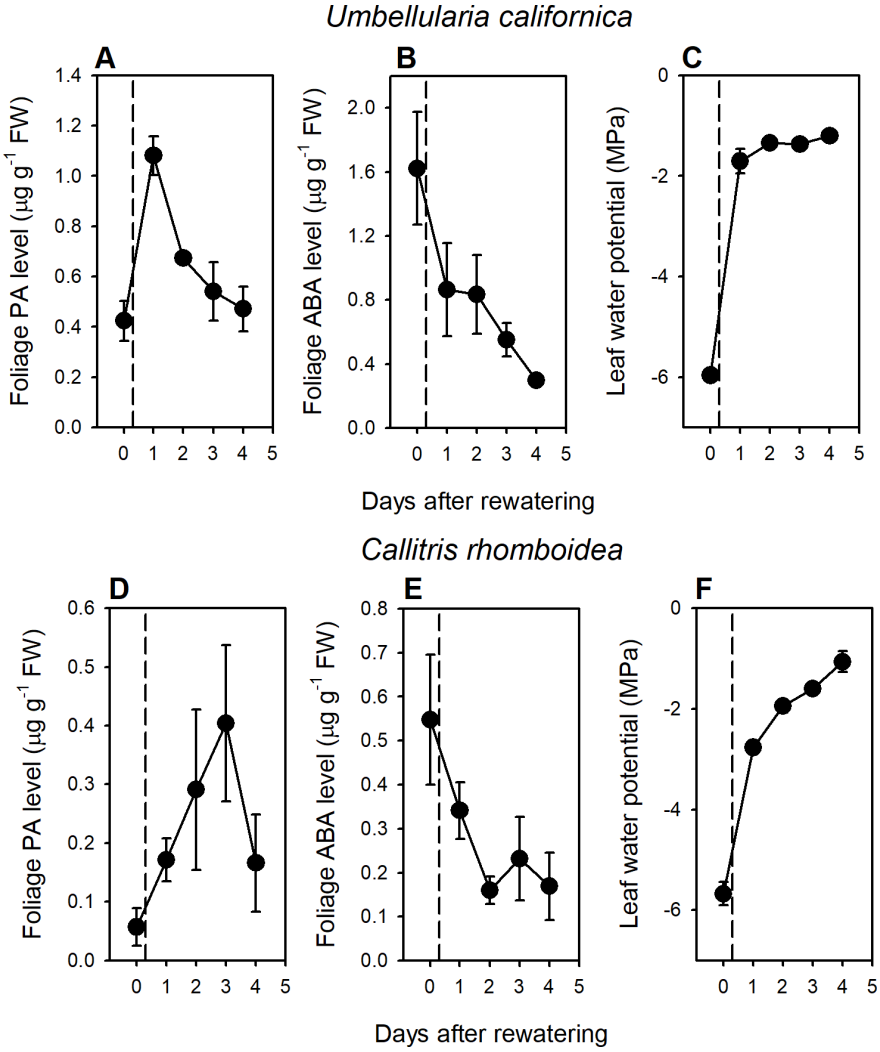


Figure 3. Mean midday foliage phaseic acid (PA) levels (A and D), foliage abscisic acid (ABA) levels (B and E) and leaf water potential (C and F) in plants of the angiosperm species *Umbellularia californica* (A-C) and conifer species *Callitris rhomboidea* (D-F) that were rewatered from a drought-stressed state (denoted by dashed vertical lines) (n= 5, ±SE).

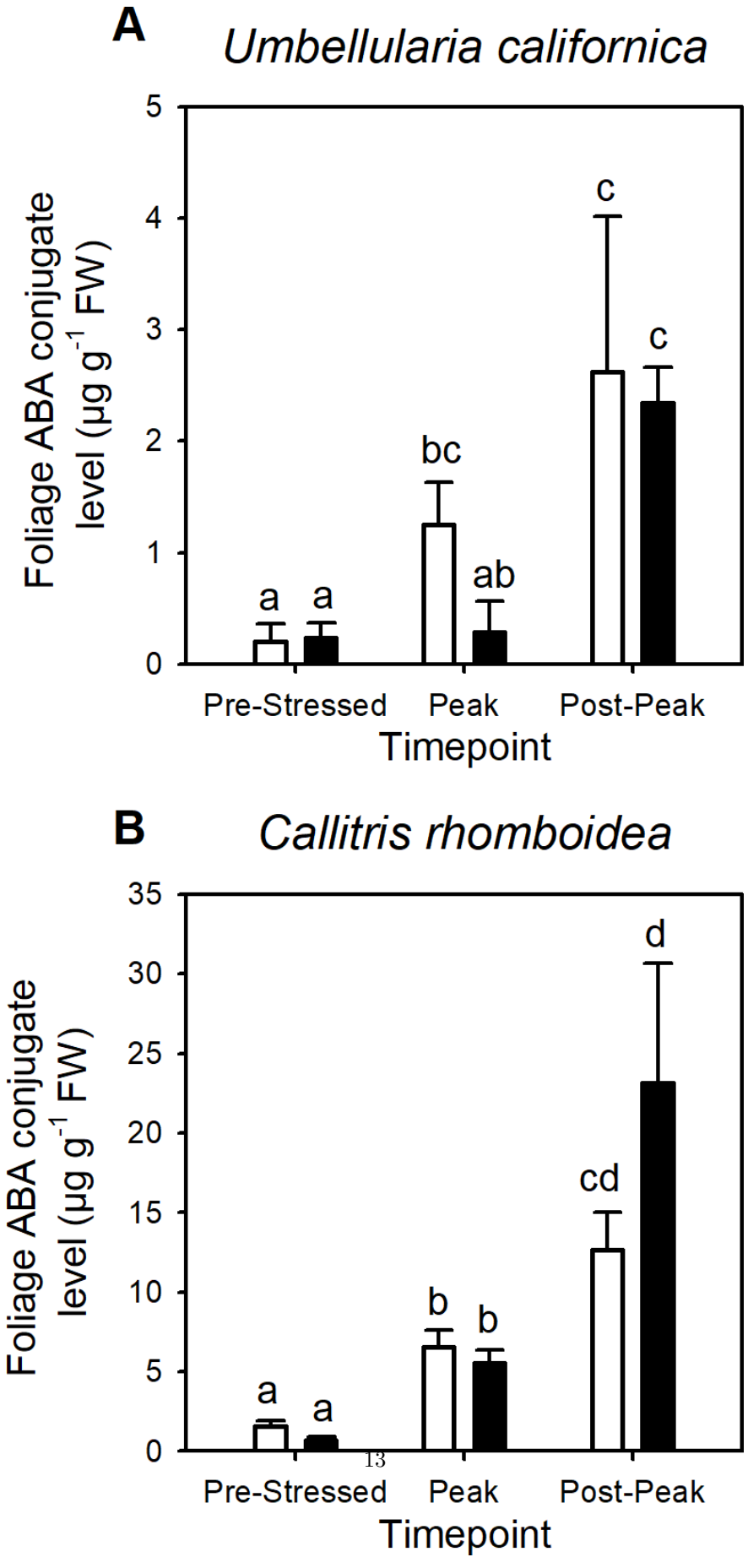
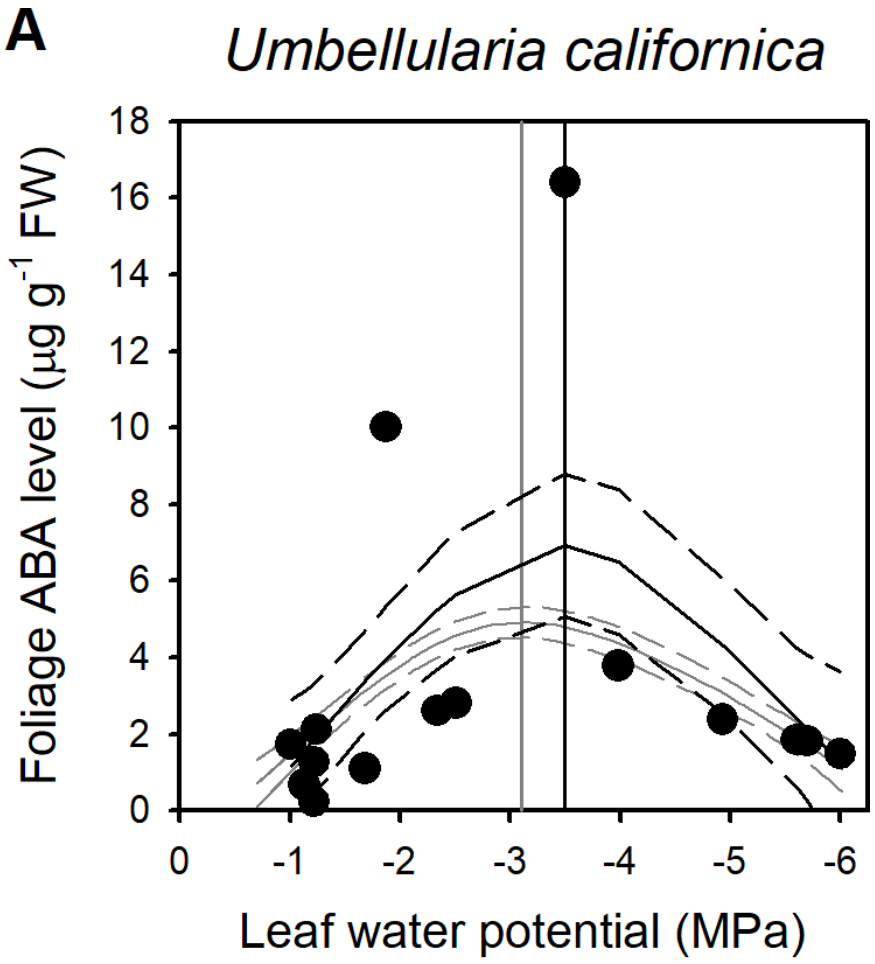


Figure 4. Mean foliage ABA conjugate levels (n=3, ±SE) in leaves taken from branches that were intact (white) or girdled (black) from plants of the angiosperm species *Umbellularia californica* (A) and conifer species *Callitris rhomboidea* (B) prior to drought stress (pre-stressed), at peak ABA levels (peak) and 10 d after peak ABA levels (post-peak). Different letters denote significant differences ($P<0.05$) in mean ABA-GE levels.



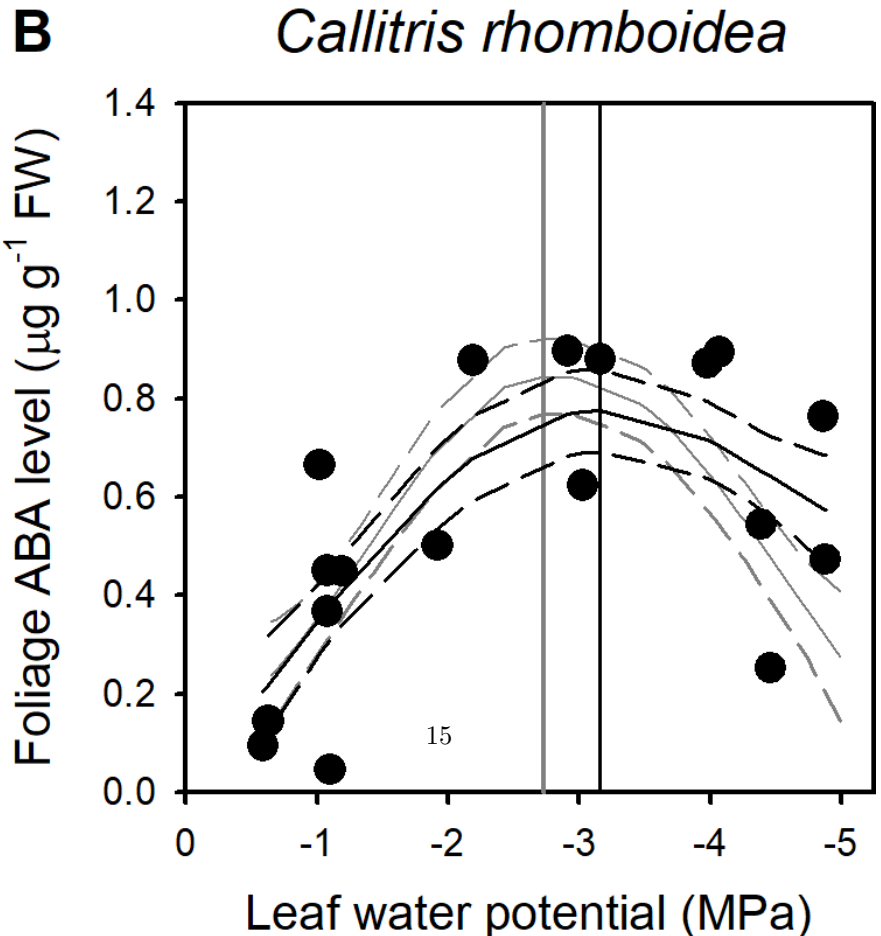


Figure 5. Foliage abscisic acid (ABA) levels as leaf water potential (Ψ_l) declines during soil drought in leaves taken from girdled branches in the angiosperm species *Umbellularia californica* (A) and conifer species *Callitris rhomboidea* (B). Generalized additive model (GAM) curves and standard errors are represented by solid and dashed black lines, respectively, for the ABA data from girdled branches, and in grey from ABA data taken from intact branches. The black vertical line depicts the Ψ_l at peak foliage ABA level in girdled branches, and the grey vertical line the Ψ_l at peak foliage ABA level in intact branches.

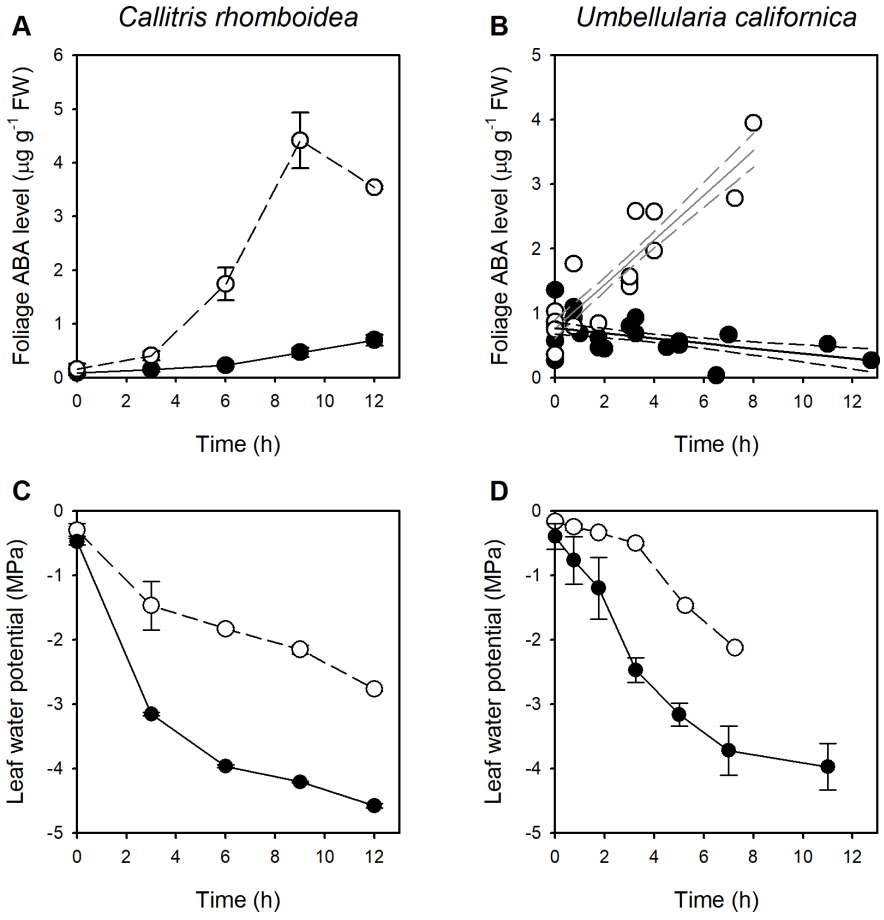


Figure 6. Foliage abscisic acid (ABA) levels (A and B) and leaf water potentials (Ψ_l) (C and D) as branches of the conifer species *Callitris rhomboidea* (A and C) and angiosperm species *Umbellularia californica* (B and D) that were rehydrated overnight were subsequently dehydrated on the laboratory bench for 12 h. Bench dehydration started at time = 0. Branches were taken from either unstressed plants (white) or plants that were exposed to a soil drought for 10 d after peak ABA levels were measured (black). Data represent means and standard errors (n=5) in *C. rhomboidea* and pooled data for 5 branches in *U. californica* . Generalized additive model (GAM) curves and standard errors are represented by black solid and dashed lines for ABA levels in branches harvested from *U. californica* plants exposed to drought for 10 d after peak ABA and grey lines for ABA levels in branches harvested from unstressed *U. californica* plants.

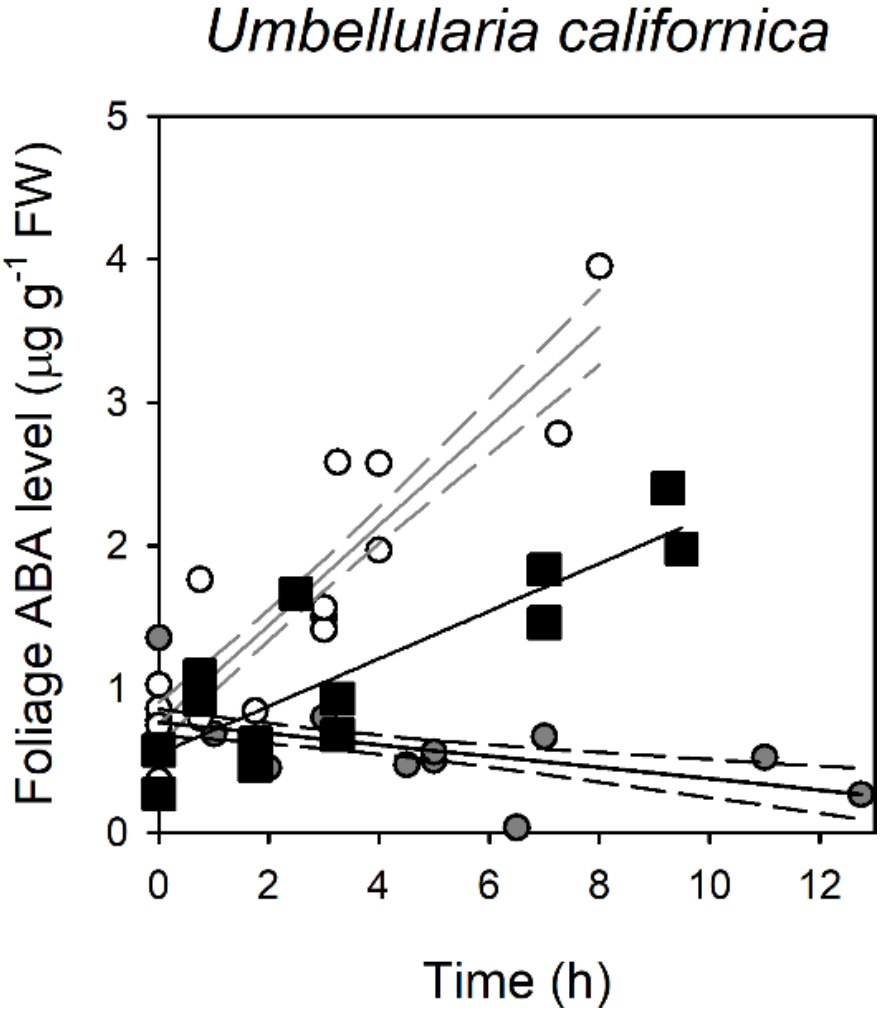


Figure 7. Foliage abscisic acid (ABA) levels as branches of the angiosperm species *Umbellularia californica* that were rehydrated overnight were subsequently dehydrated on the laboratory bench for 12 h. Bench dehydration started at time = 0. Branches were taken from either unstressed plants (white), plants that were exposed to a soil drought for 10 d after peak ABA levels were measured (grey) (data from Figure 4), or plants exposed to soil drought for 10 d after peak ABA levels were measured then rewatered and maintained in saturated soil for 3 d (black squares). Data are taken from leaves collected from 5 branches. Generalized additive model (GAM) curves and standard errors are represented by black solid and dashed lines for ABA levels in branches harvested from *U. californica* plants exposed to drought for 10 d after peak ABA and grey lines for ABA levels in branches harvested from unstressed *U. californica* plants, a significant linear regression is shown for the data collected from a rehydrated plant.

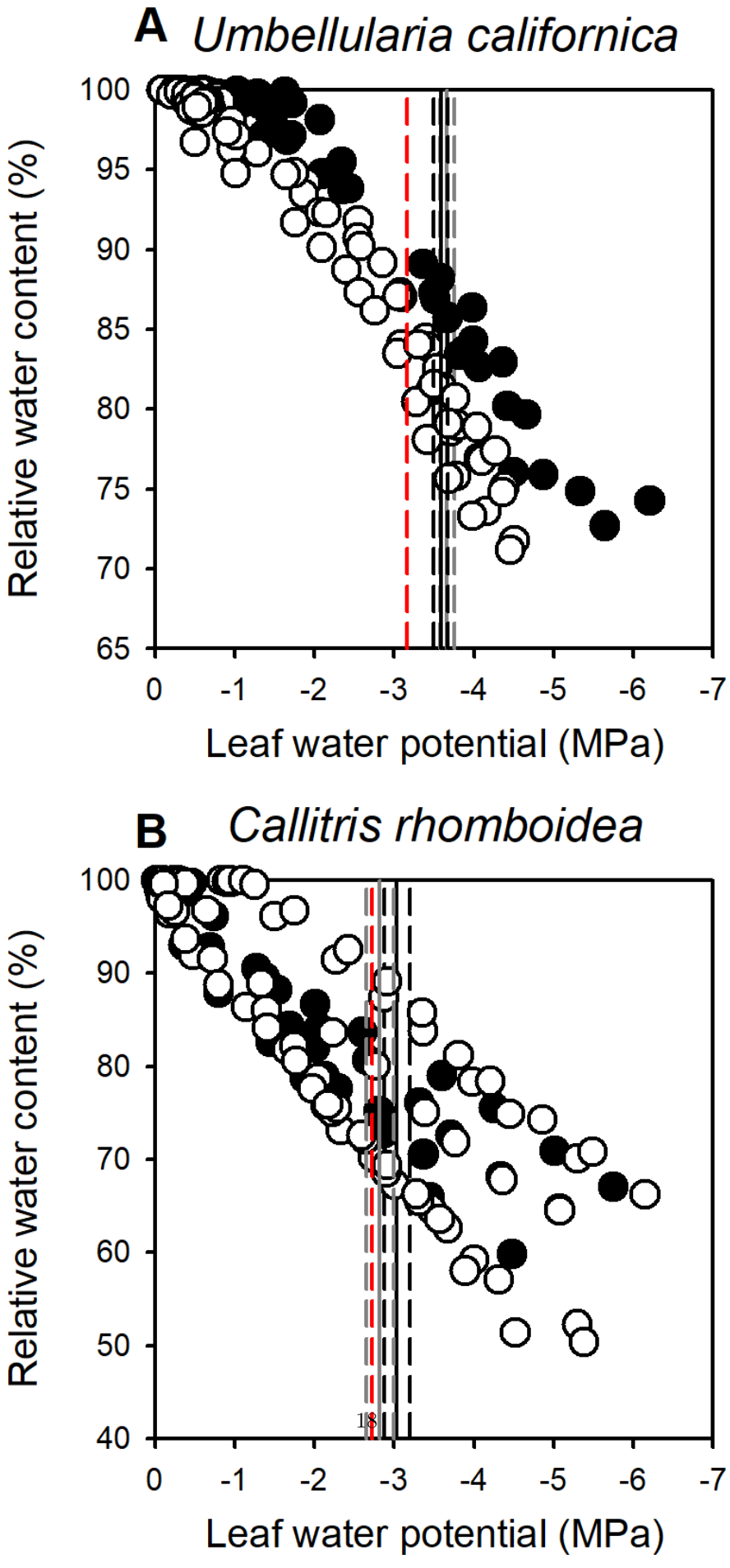


Figure 8. Pressure volume curves for leaves of the angiosperm *Umbellularia californica* (A) and small branches of the conifer *Callitris rhomboidea* (B) (n=5) in never-before stressed plants (black) and plants that had experienced long-term drought and at least 10 d at a leaf water potential (Ψ_l) more negative than when peak foliage abscisic acid (ABA) level occurred (open circles). Red dashed vertical lines represent the Ψ_l at peak foliage ABA level. Black vertical and dashed lines represent mean Ψ_l at turgor loss point (Ψ_{tlp}) and standard error for tissue collected from plants under long-term drought. Grey solid and dashed vertical lines represent mean $\Psi_{tlp}$and standard error for leaves of never-before stressed plants.

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