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Abscisic acid driven stomatal closure during drought in anisohydric *Fagus sylvatica*

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Abstract

Stomatal closure limits transpiration during drought, restricting water potential decline and delaying the onset of embolism. While critical for ensuring survival during drought, the mechanisms driving stomatal closure during drought remain equivocal. The hormone abscisic acid (ABA) will close stomata in seed plants and is synthesized as leaf turgor declines. ABA driven stomatal closure during drought is particularly apparent in species that are more isohydric. In contrast, in species that have a more anisohydric response to drought, like *Fagus sylvatica*, the importance of ABA in driving stomatal closure during drought is often overlooked or excluded, in place of a hypothesized passive, water potential driven stomatal closure. Here we track ABA levels, leaf water potential and stomatal conductance during a natural summer drought in an individual of *F. sylvatica*. As leaf water potential declined to within 0.3 MPa of turgor loss point during a drought, foliage abscisic acid (ABA) levels increased considerably and stomata closed. Foliage ABA levels correlated with stomatal conductance throughout a drought and post-drought period. These observations make it hard to exclude increased ABA levels as a driving mechanism for stomatal closure during drought in *F. sylvatica*.

Introduction

Drought is an increasing risk for global forests as climate change increases the frequency and severity of droughts (Adams *et al.*, 2017; Allen *et al.*, 2010; Martinez del Castillo *et al.*, 2022). Climate change is already causing increased tree mortality, including in beech forests (Allen *et al.*, 2010; Anderegg *et al.*, 2013; Geßler *et al.*, 2007; Hartmann *et al.*, 2022; Senf *et al.*, 2020). When plants experience long-term rainfall, deficit soil water potential declines and water in the xylem experiences increasingly negative tension until a critical threshold is breached and the adhesion and cohesion forces can no longer hold the column of water together causing an embolism in the xylem tissue (Brodribb and Hill, 1999; Cardoso *et al.*, 2022; Sperry and Pockman, 1993; Tyree and Sperry, 1989). Embolism is irreversible in most cases and if sufficient embolism accumulates, hydraulic conductivity cannot match residual transpiration at which point water potentials catastrophically decline leading to tissue damage and even whole plant death (Brodribb and Cochard, 2009; Cardoso *et al.*, 2020a; Charrier *et al.*, 2016; Mantova *et al.*, 2023). During periods of water deficit plants are able to limit evaporation quickly by closing stomata, which reduces declines in water potential, limiting the chance of embolism during drought (Brodribb *et al.*, 2021; Brodribb and McAdam, 2013; Lamarque *et al.*, 2018; Martin-StPaul *et al.*, 2017).

One strategy that allows plants to occupy seasonally dry environments is the evolution of highly embolism resistant xylem that can transport liquid water at very negative water potentials (<-5 MPa) without experiencing embolism (Larter *et al.*, 2017; Skelton *et al.*, 2017). The building of highly embolism resistant xylem is not the only strategy that allows plants to live in environments that experience a seasonal drought, some species have evolved the capacity to greatly reduce transpiration, by very effectively closing stomata and having very low cuticular conductance which minimizes residual transpiration to such an extent that water potentials decline very slowly under drought conditions (Duursma *et al.*, 2019; Kane *et al.*, 2020; Klein, 2014). These two very different strategies to survive drought form the basis of the isohydric/anisohydric continuum (Jones, 1998; Leuschner *et al.*, 2022; McDowell *et al.*, 2008; Tardieu and Simonneau, 1998). Species classified as more isohydric tend to have stomata that are more effective at closing in response to drought, consequently they often have more vulnerable xylem, and can disconnect plant water potential from soil water potential very effectively during drought. More anisohydric species tend to have stomata that are less responsive to declines in water potential, more embolism resistant xylem, smaller hydraulic safety margins, and a stronger relationship between soil and plant water potential during drought (Attia *et al*., 2015; Jones, 1998; Leuschner *et al*., 2022; Martínez-Vilalta and Garcia-Forner, 2017; McDowell *et al*., 2008; Sade *et al*., 2012; Tardieu and Simonneau, 1998). The isohydric/anisohydric distinction can be viewed as a spectrum rather than two categories that all land plants will fit neatly into (Klein, 2014; Martínez-Vilalta and Garcia-Forner, 2017).

European beech *Fagus sylvatica* L. is classically characterized as an anisohydric species (Hesse *et al*., 2022; Leuschner *et al*., 2022; Pretzsch *et al*., 2014), although the degree of isohydricity in this species appears to be flexible across rainfall gradients with populations originating from drier environments exhibiting more isohydric stomatal control while plants from wetter regions having more anisohydric stomatal responses to water potential (Nguyen, 2016; Nguyen *et al*., 2017). Leuschner *et al*. (2022) determined that by most metrics, including the relationship between leaf and soil water potential and the size of the hydroscape that *F. sylvatica* should be classed as an anisohydric species, while their data on the relationship between leaf water potential and leaf stomatal conductance indicate a slightly more isohydric behavior that may shift throughout the season from more isohydric to more anisohydric and that the stomata of *F. sylvatica* are very sensitive to declining leaf water potential and changes in vapor pressure deficit. Leuschner *et al.* (2022) states that a root borne signal is the most likely driver of stomatal closure during seasonal drought in *F. sylvatica* and speculates that this signal may be a hormone but stops short at implicating abscisic acid (ABA). ABA is a dynamic plant hormone well known to close stomata during drought in seed plants (Beardsell and Cohen, 1975; McAdam *et al*., 2016; Munemasa *et al*., 2015; Mustilli *et al*., 2002; Nguyen, 2016; Sussmilch *et al*., 2017a). ABA levels rise in leaf tissues as water potentials decline (Wright, 1977) and in response to VPD (Cardoso *et al*., 2020b; McAdam *et al*., 2016). ABA is primarily synthesized in the leaf mesophyll and not the roots as was once suspected (Holbrook *et al*., 2002; McAdam and Brodribb, 2018, 2016; Zhang *et al*., 2018). As leaves dehydrate, ABA is produced once mesophyll cells lose turgor presumably because of changes to a critical membrane-cell wall interaction (Bacete *et al*., 2022; Cardoso *et al*., 2020b; McAdam and Brodribb, 2016; Sussmilch *et al*., 2017b). ABA production during drought has been shown to differ between anisohydric and isohydric species, with isohydric species relying on ABA to close stomata during drought, while some anisohydric species exhibit a switch from having stomata controlled by ABA to water potential driven stomatal closure on repeated droughts and rewatering (Nolan *et al*., 2017; Tardieu and Simonneau, 1998). Or in some cases supposedly not requiring ABA to close stomata during drought at all (Tardieu and Simonneau, 1998; Thomas *et al*., 2000; Torres-Ruiz *et al*., 2015).

Here we sought to document potential relationships between ABA level and gas exchange during a natural drought in a repeatedly-sampled individual of *F. sylvatica*. We tracked leaf gas exchange, leaf ABA level, leaf water potential in a specimen tree of *F. sylvatica f. purpurea* during a natural drought in West Lafayette, IN, USA in the summer of 2021. In addition, we measured the turgor loss point of leaves from the same tree to determine if there was a relationship between the water potential at which ABA is produced in the field and when leaves lose turgor. *F. sylvatica* was selected due to its importance in European forests, the imminent threat climate change poses to this species (Geßler *et al*., 2007; Knoke *et al*., 2006), and the equivocal role of hormones in stomatal regulation in this species (Leuschner *et al*. 2022).

Materials and methods

Plant material

A mature specimen tree of *F. sylvatica f. purpurea* L. (Fagaceae) growing on the Purdue University campus in West Lafayette, Indiana, USA (40.425224, -86.914636) was selected for these observations. *F. sylvatica f. purpurea* is a cosmopolitan ornamental tree with a naturally occurring mutation that makes the leaves appear red or purple (Nonić *et al*., 2017). All individuals in cultivation are clones of a single, now extinct, tree called Mutterblutbuche that arose naturally in the Heinleite hills south of Sondershausen in the German state of Thuringia (Lutze, 1892; Sargent, 1894). The site of origin of this cultivar receives 600-700mm of precipitation annually (www.dwd.de) which would mean that this individual is most likely anisohydric based on the work of Nguyen *et al.* (2017), who observed more isohydric individuals native only to regions that received less than 550 mm of rainfall per year. The individual in this study was grown under higher annual precipitation with West Lafayette, Tippecanoe County, IN, USA receiving approximately 900mm per year (www.noaa.gov).

Data collection

Three leaves were measured on six campaigns spread over the course of 93 days from summer (July) to autumn (October) 2021. Gas exchange was measured on sunny days in three, attached, sun exposed leaves using an infrared gas analyzer (LI-6800 Portable Photosynthesis System; LI-COR Biosciences, Lincoln, NE, USA). The conditions in the cuvette were set to ambient CO2, ambient VPD and a saturating light intensity of 1500 µmol m-2 s-1 to measure instantaneous CO2 assimilation and stomatal conductance. Immediately following gas exchange measurements leaves were excised, wrapped in damp paper towel, and enclosed in plastic zip top bag to halt transpiration. Leaves were then taken back to the lab where leaf water potentials were measured using a Scholander pressure chamber (PMS Instrument Company, OR, USA) with microscope. After slow depressurization of the Scholander chamber, leaf tissue was harvested into a tube to measure leaf ABA content.

Leaf ABA content was measured by physicochemical methods with an added internal standard according to McAdam (2015). The mass of the fresh foliage sample was recorded on an analytical balance (±0.0001 g, OHAUS Corporation, Parsippany, NJ, USA) where fresh leaf tissue was placed into a 25 ml tube and completely covered in -20°C acetone containing 250 mg l−1 butylated hydroxytoluene. The leaf tissue was then chopped into fine pieces and stored in a -20°C freezer overnight. The leaf tissue was homogenized and 15 ng of [2H5]ABA was added to each sample as an internal standard before extracting overnight at 4°C. An aliquot of supernatant, approximately 2 ml, was taken from each sample and dried down in a vacuum sample concentrator (Labconco, MO, USA), the ABA and internal standard were then resuspended in 200 µl of 2% acetic acid in water (v v−1), after which it was centrifuged at 14,800 RPM for 4 minutes. 100 µl was taken for quantification of ABA and internal standard levels using an Agilent 6460 series triple quadrupole LC/MS (Agilent, CA, USA). After quantification, the homogenized leaf samples were dried at 70°C, and leaf dry mass was determined by subtracting the mass of the clean empty tube from the mass of the tube containing the dried homogenized leaf material.

Leaf chlorophyll a content was measured from the same sample as ABA after extraction in -20°C acetone containing 250 mg l−1 butylated hydroxytoluene. 5 µl of the liquid from each sample was placed onto a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) pedestal and absorbance at 433nm was measured this was then quantified using a standard curve (ThermoFisher Scientific, 2018). The total volume of acetone in the sample was determined gravimetrically by obtaining the mass of the total homogenized sample in acetone, then drying the sample at 70°C and determining dry mass of the vial and sample. The volume of acetone was determined by multiplying the mass change and the density of acetone at standard atmospheric pressure at 180 m above sea level and adding back the 2 ml taken for ABA analysis and the 5 µl take for spectrophotometry. Dry mass of the sample was then determined by cleaning the tube and redrying the vial to determine the mass of the clean tube which was subtracted from the mass of the dry sample with no acetone (Kane and McAdam, 2023).

Table 1: Mean physiological data (±SE, n=3) collected periodically on given days after winter solstice (DAWS) (corresponding day of the year (DOY) is also shown) through an unusually dry period in West Lafayette, Indiana during the summer and autumn of 2021 in *Fagus sylvatica f. purpurea*. Physiological data include leaf water potential (MPa), foliage ABA level (ng g-1 DW), leaf chlorophyll a content (mg g-1 DW), stomatal conductance (mol m-2s-1), photosynthetic rate (Asat, µmol CO2 m-2s-1), and intrinsic water use efficiency (WUEi, µmol mol-1).

DAWS	DOY	Leaf water potential (MPa)	Foliage ABA level (ng g^{-1} DW)	Leaf chlorophyll *a* content (mg g^{-1} DW)	Stomatal conductance (mol $m^{-2}s^{-1}$)	A_{sat} (µmol CO_2 $m^{-2}s^{-1}$)	WUE_i (µmol mol^{-1})
210	200	-0.96 ± 0.26	47.2 ± 2.6902	7.1 ± 0.61	0.22 ± 0.02	14.0 ± 0.98	62.6 ± 5.5
237	227	-1.93 ± 0.09	94.4 ± 12.1	7.09 ± 0.23	0.056 ± 0.01	6.47 ± 1.3	114 ± 1.0
255	245	-1.77 ± 0.04	136 ± 37.3	7.65 ± 0.88	0.03 ± 0.006	4.94 ± 0.52	132 ± 7.6
271	261	-1.73 ± 0.08	64.1 ± 13.1	6.87 ± 0.53	0.04 ± 0.01	5.57 ± 0.97	123 ± 6.7
283	273	-0.90 ± 0.06	54.6 ± 10.2	5.54 ± 0.42	0.10 ± 0.001	9.88± 0.13	97.4 ± 1.5
303	293	-0.76 ± 0.02	45.2 ± 2.7	4.88 ± 0.05	0.10 ± 0.012	7.70 ± 0.55	78.4 ± 13

Turgor loss point was determined by pressure-volume curves (Tyree and Hammel, 1972). Unstressed leaves were wrapped in damp paper towel, bagged in plastic and rehydrated overnight by recutting the petiole under deionized water in a shallow glass dish, being sure to keep the bag, towel, and lamina out of the water. The leaves were then measured in a Scholander pressure chamber (PMS Instrument Company, OR, USA) and immediately moved onto an analytical balance and the mass recorded (±0.0001 g, OHAUS Corporation, Parsippany, NJ, USA). This was repeated 9 times with 6 leaves over the course of 6 hours as leaves dehydrated slowly on the bench. The leaves where then scanned for leaf area using an Epson Perfection V39 Flatbed

Scanner (EPSON, Japan). The leaves were then dried at 60°C for at least 72 h and dry weight taken. The curves were analyzed using the PVAST spreadsheet according to (Bartlett *et al*., 2012).

Rain fall data were collected from the Purdue University Weather station at the Purdue Airport (LAF) located around 2 km from the field site (wunderground.com). Drought status was determined from the U.S. Drought Monitor (droughtmonitor.unl.edu) which uses data from NOAA and NIDIS.

Data analysis

Exponential decay single 2 parameter regressions were fit on to the raw data for foliage ABA levels, stomatal conductance, and leaf water potential using SigmaPlot 10.0 (SYSTAT, CA, USA). A linear regression was fit to foliage ABA vs leaf water potential using SigmaPlot 10.0 (SYSTAT, CA, USA). All time course data was analyzed using a one-way ANOVA and post hoc Tukey's test. All time-course data are plotted against days since winter solstice which allows for a standardized comparison of phenological data for readers in both northern and southern hemispheres. The intrinsic water use efficiency was calculated as the quotient of CO2 assimilation rate by stomatal conductance for each leaf measured.

Results

During the summer of 2021 Tippecanoe county, IN, experienced an abnormally dry period (D0 –which is the mildest drought category used by the U.S. Drought Monitor, it is based on low percentile values for a combination of key indices including soil moisture levels and local stream flow dropping into the lowest 20th percentile of historic records, and a standardized precipitation index below -0.5), this period lasted from summer into the early autumn 232-281 days after winter solstice (DAWS) (Figure 1A insert). Prior to the onset of this dry period levels of leaf ABA were low at 47.28 ± 2.69 ng g-1 at 210 DAWS (Figure 1A insert) this corresponded to the time point when we measured the highest rates of stomatal conductance during this study at 0.227 ± 0.027 mol m-2 s-1 (Figure 1B) and an average leaf water potential of -0.96 ± 0.27 MPa (Figure 1C) these are both significantly higher than the subsequent 3 measurements which took place during the dry period. Leaves measured at 237 DAWS, 5 days into the abnormally dry period and 30 days since at least 20 mm of rain had fallen, had higher levels of leaf ABA content, with mean levels doubling (Figure 1A) and a mean leaf water potential declining to -1.93 ± 0.09 MPa (Figure 1C) and stomatal conductance of 0.057 ± 0.012 mol m-2s-1 (Figure 1B). The highest levels of leaf ABA were measured 255 DAWS, 22 days into the abnormally dry period, when levels had increased to 136.53 ± 37.39 ng g-1 (Figure 1A) this corresponded with the lowest rates of stomatal conductance measured during the study period at 0.038 ± 0.006 mol m-2s-1 (Figure 1B) both measurements being significantly different from the prestress measurements. 271 DAWS 10 days before the end of the abnormally dry period, and 3 days after a brief rainfall event of 3.05 mm, the three measured leaves were variable with one leaf recording a leaf ABA content of 39.96 ng g-1 (Figure 1A red circle) this leaf had the highest rate of stomatal conductance and leaf water potential measured at this time point, at 0.066 mol m-2s-1 (Figure 1B red circle) and -1.53 MPa (Figure 1C red circle), respectively. The other two leaves measured on this day had higher levels of leaf ABA content and more negative water potentials which corresponded with lower rates of stomatal conductance (Figure 1A, B, C white circles). After the dry period ended at 281 DAWS the next leaves collected on 283 DAWS and 303 DAWS showed reduced leaf ABA content, less negative water potentials, and higher stomatal conductance relative to the dry period. By 303 DAWS leaf ABA levels had fallen to the lowest measured during the study period at an average of 45.26 ± 2.78 ng g-1 (Figure 1A), this corresponded with the lowest average water potential measured during the study period -0.77 ± 0.03 MPa, yet stomatal conductance did not recover to the maximum rates measured in early summer. CO2 assimilation (Asat) rate through the dry period was like that of stomatal conductance declining from a maximum of 14.03 ± 0.98 µmol CO2 m-2s-1 210 DAWS to a low of 4.95 ± 0.53 µmol CO2 m-2s-1 255 DAWS (Table 1). Asat did not return to predrought levels after the end of the dry period only returning to 9.88 ± 0.14 µmol CO2 m-2s-1 283 DAWS (Table 1). An increase in water use efficiency was observed during the dry period increasing significantly from 62 ± 5.5 µmol mol-1 at 210 DAWS to 114 ± 1 µmol mol-1 at 237 DAWS only 5 days after the onset of the abnormally dry period. After rainfall, WUE returned to a similar pre-drought level despite stomata not reopening to maximum apertures (Figure 1B insert). Mean leaf chlorophyll a content was stable for the duration of the dry period with levels around 7.11 ± 0.61 mg g-1 at 210 DAWS but once the dry period ended chlorophyll a content began declining to 5.54 ± 0.43 mg g-1 DW at 283 DAWS two days after the end of the dry period then to 4.89 ± 0.05 mg g-1 DW 28 days after the autumn equinox at 303 DAWS (Figure 1D).

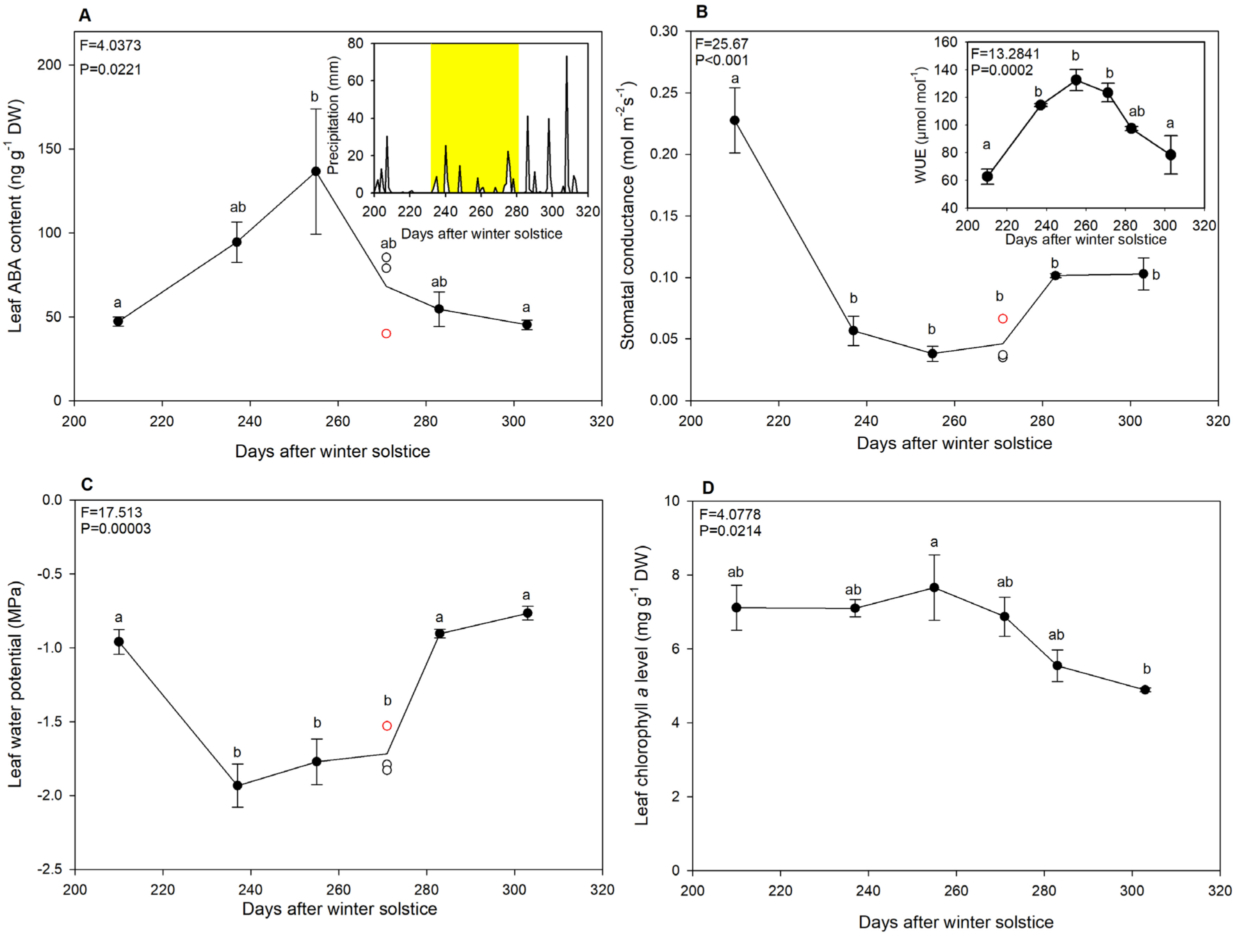

Figure 1: (A) Mean leaf ABA content, (B) stomatal conductance, (C) leaf water potential, (D) leaf chlorophyll a content through a natural drought in an individual of *Fagus sylvatica f. purpurea* in West Lafayette, Indiana, USA. Each black point represents an average of 3 sun exposed leaves measured at midday and are accompanied with standard error bars. The two white and one red point on 271 days after winter solstice show the individual values of leaf ABA content, stomatal conductance, leaf water potential for each leaf collected on that day with the trend line passing through the average of those points the red point represents data collected from the same individual leaf. The insert in A depicts the rain fall during the study period with the yellow area indicating the abnormally dry period (D0). The insert in (B) depicts water use efficiency through the drought. One-way ANOVAs were performed on all time course data and the F-statistic and P value are displayed, Tukey's post hoc tests were performed, and significant differences are denoted by different letters.

Stomatal conductance and leaf ABA content in *F. sylvatica* formed a significant ($P=0.0084$: $R^2=0.3611$) exponential decay relationship ($\text{Stomata conductance}=0.3122*e^{(-0.0187*\text{Leaf ABA content})}$), with stomatal conductance exponentially declining as leaf ABA content increased (Figure 2A). We also observed a significant linear relationship ($P=0.0033$: $R^2=0.4621$) between stomatal conductance and leaf water potential ($\text{Stomatal conductance} = 0.212+0.0869*\text{Leaf water potential}$). Similarly, we found a significant ($P=0.0066$: $R^2=0.3785$) exponential decay relationship ($\text{Leaf ABA content} =28.1118*e^{(-0.6836*\text{Leaf water potential})}$) between leaf ABA content and leaf water potential over the year in *F. sylvatica*. The highest levels of leaf ABA measured during the study period (>75ng g^{-1}) were measured only when leaves were within ± 0.34 MPa of the average leaf turgor loss point of -2.02 ± 0.09 MPa (Figure 2B).

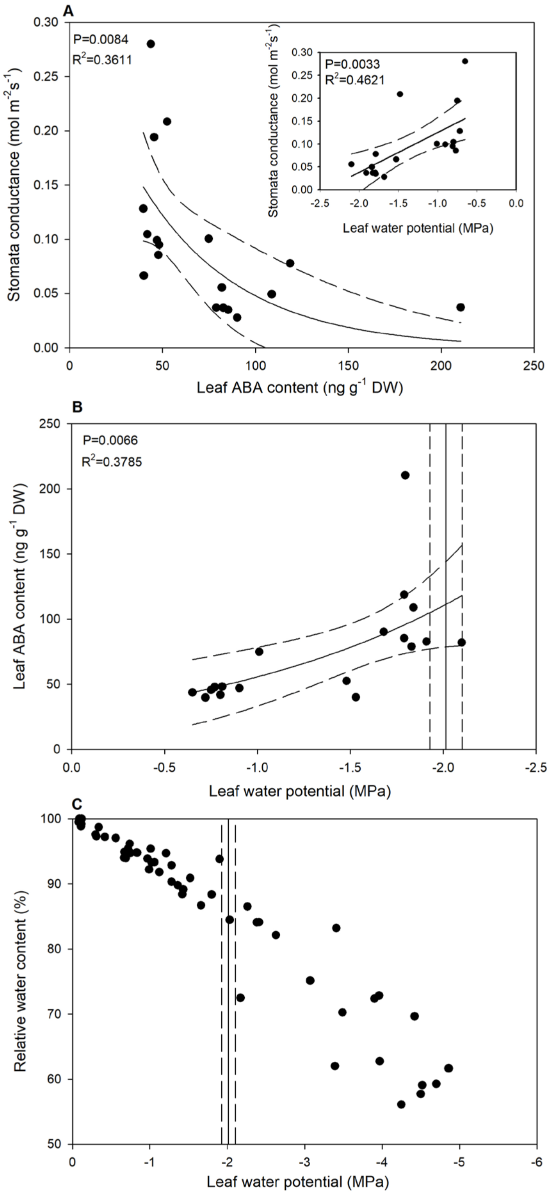

Figure 2: (A) The relationship between leaf ABA content and stomatal conductance and (B) leaf water potential and leaf ABA content for leaves sampled through a natural drought in West Lafayette, Indiana, USA in 2021. Each point represents one sun leaf collected during midday from an individual *Fagus sylvatica f. purpura.* Exponential decay regressions are fitted to the data with dashed lines representing 95% confidence intervals. The vertical solid line in B depicts the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point. Insert in (A) shows the linear relationship between stomatal conductance and leaf water potential. (C) Pressure-volume curve for leaves of *Fagus sylvatica f. purpura.* Each point represents one measurement of one of the 6 leaves with each leaf being measured 9 times during the dry down. The vertical solid line shows the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point.

Discussion

We find that though *F. sylvatica* is traditionally classed as an anisohydric species (Hesse *et al*., 2022; Leuschner *et al*., 2022; Pretzsch *et al*., 2014), it does not mean that ABA plays no role in driving stomatal closure during drought in this species. Our results suggest that water potential and turgor driven production of the drought stress hormone ABA might drive stomatal closure in *F. sylvatica* during drought. Our data support the conclusion of Leuschner *et al.* (2022) that stomatal control in *F. sylvatica* is sensitive to declining leaf water potential, but we show that this relationship may be mediated by ABA because as leaf water potentials become more negative and stomatal conductance declines, leaf ABA levels rises. We observed significant relationships between stomatal conductance and both forces that could potentially close stomata including increasing ABA levels and declining leaf water potential. While our observations make it impossible to separate the effect of ABA and leaf water potential on stomatal conductance during drought it is tempting to speculate that an increase in ABA level closes stomata initially during drought and that this stomatal closure is facilitated by further declines in leaf turgor, especially if the epidermal pavement cells lose turgor (Buckley, 2019; Rodriguez-Dominguez *et al*., 2016).

Our observations, while limited to a single individual, suggest that ABA driven stomatal closure during drought occurs before embolism induced declines in hydraulic conductivity, or even incipient embolism formation. Published embolism resistance data for *F. sylvatica* suggest that P50 ranges from -1.5 to -3.94 MPa depending on the region of origin as well as position in the canopy (Guan *et al.* 2022; Lemonie *et al.,* 2002). Hacke and Sauter (1995) found in individuals of *F. sylvatica f. purpurea* in Northern Germany that embolism begins to occur in this variety at a water potential more negative than -1.9 MPa with stem P50 occurring around -2.9 MPa. Hacke and Sauter (1995) suggest that in the summer plants of *F. sylvatica f. purpurea* rarely experience water potentials more negative than -1.9 MPa which matches our observations here. During our study period we only observed two leaves with water potentials less than -1.9 MPa both observed on the same day (237 DAWS) during the abnormally dry period. This same day we saw stomatal conductance having declined by more than 75% of that measured prior to the drought (210 DAWS) and these leaves had double the average leaf ABA content. In other work of *F. sylvatica* at Southern European sites experiencing the lowest annual rainfall across the species range, daily leaf water potentials rarely fall below -2 MPa and only in upper canopy leaves (Aranda *et al*., 2000). These and our observations support the conclusions of Guan *et al.* (2022) and Leuschner (2020) that there is a considerable safety margin between the water potential of stomatal closure and P50 in *F. sylvatica*.

ABA has long been known to close stomata (Beardsell and Cohen, 1975; Mittelheuser and Van Steveninck, 1969) and this link is understood to be associated with driving stomatal closure during drought in seed plants (Wright, 1977). While limited, our observations during a drought suggest that this might also be the case for *F. sylvatica f. purpurea*. Nguyen (2016) observed that *F. sylvatica* plants grown in drier conditions showed higher expression of ABA related stress genes and lower water loss rates but did not measure ABA levels. Leuschner *et al.* (2022) speculates that there could be a hormonal control of stomatal closure in *F. sylvatica* but preferred the explanation that a lasting loss of bulk leaf turgor passively closed stomata as the most plausible explanation for the long term (May to August) partial stomatal closure observed in the field when leaf water potential declined between -1 to -1.5 MPa. Our observations cannot rule out the possibility that like many other seed plant species ABA, synthesized by leaves near turgor loss point, is closing stomata under these conditions in *F. sylvatica*. Leuschner *et al.* (2022) also speculates that the signal for stomatal closure originates in the roots because isohydric species can maintain a stable leaf water potential despite soil drying. Modern work has shown that ABA is primarily produced in leaf tissue during periods of water deficit (Holbrook *et al*., 2002; McAdam and Brodribb, 2018, 2016; Zhang *et al*., 2018), and that the signal is driven by a loss of cell turgor. Our results along with the observations of Hacke and Sauter (1995) suggest that this might also be the case in *F. sylvatica*. The embolism onset threshold of -1.9 MPa observed by Hacke and Sauter (1995) in this clone also corresponds closely to the turgor loss point we measured in leaves of *F. sylvatica f. purpurea* which was -2.02 ± 0.09 MPa the lower bound being -1.93 MPa indicating that turgor loss point may be an important drought threshold for *F. sylvatica f. purpurea*. Leuschner *et al.* (2022) questioned whether a hormonal signal like ABA could increase and keep stomata closed for a sustained period ‘weeks or months’, we show here in this single-season study that elevated levels of leaf ABA can be observed during drought in an individual of this species for least 34 days. There are many other reports of sustained high levels of ABA in leaves during long-term drought (Brodribb *et al*., 2014; Thomas et al., 2000; Tombesi *et al*., 2015; Wang *et al*., 2001). Based on the relationships we observed between ABA level, leaf water potential and stomatal conductance, we cannot exclude an influence of leaf water potential acting directly on stomatal conductance in *F. sylvatica* to drive stomatal closure during drought. It could be that ABA and leaf water potential act in concert to close and maintain the closure of stomata during drought, although this speculation will need further study to resolve and in *F. sylvatica* might also be dependent on the geographic origin of the individual (Nguyen *et al*., 2017).

We observed that upon the end of the dry period in 2021 leaf ABA content and leaf water potential recovered to predrought levels, but that even though stomatal conductance increased gas exchange did not return to the levels measured prior to the drought. We believe that there are two possible reasons for this that are not mutually exclusive. Based on the observations of Hacke and Sauter (1995) leaves may have reached water potentials that could cause some low levels of embolism which can reduce maximum stomatal conductance upon recovery (Cardoso *et al*., 2020a; Skelton *et al*., 2017). Given that midday water potentials recovered to predrought levels in our study we believe that it is unlikely that stomatal opening was suppressed by embolism-induced declines in hydraulic conductance. The other, more likely, explanation is that stomatal conductance may be limited by the seasonal phenology of this deciduous species (Abadía *et al*., 1996; Wang *et al*., 2022). The first measurement after the end of the drought (281 DAWS) occurred on 283 DAWS (September 30, 2021), more than 100 days after the summer solstice and a week after the autumn equinox in the Northern Hemisphere. We observed a 31% reduction in average chlorophyll *a* content in leaves of *F. sylvatica f. purpurea* between the initial and final measurement of this study, which likely drove lower assimilation rates and may have been hastened by the high levels of leaf ABA induced by the drought as observed in other deciduous species (Kane and McAdam, 2023). These may have acted independently or in concert with the drought to accelerate leaf senescence (Radin, 1981; Thimann and Satler, 1979a, 1979b). It is not yet settled if drought influences senescence rate in *F. sylvatica* (Frei *et al*., 2022; Mariën *et al*., 2021).

The decrease in leaf water potential and increase in ABA levels, driving stomatal closure during the dry period, caused a two-fold increase in WUE in this individual (Figure 1). This increase suggests a more dramatic influence of ABA or low water potential on stomatal conductance during drought relative to photosynthetic rate, which improves the optimization of water use (Dubbe *et al*., 1978). When ABA levels declined and leaf water potential recovered after the dry period, WUE declined to pre-drought levels, this occurred even though stomata did not open to maximum rates of conductance – which we attribute to autumnal declines in chlorophyll content lowering maximum photosynthetic rates (Figure 1). Our results suggest that declines in stomatal closure during drought have a more profound influence of WUE than declines in chlorophyll content and the associated reduction in maximum photosynthetic rate during senescence. The feedback of lower photosynthesis on stomatal conductance in the autumn as chlorophyll content declines did not improve WUE.

During the abnormally dry period of 2021 in West Lafayette, Indiana, we observed more negative water potentials, reduced stomatal conductance, and higher levels of leaf ABA content in an individual of *F. sylvatica*. Before and after the dry period leaves were at higher water potentials, had higher rates of stomatal conductance, and lower levels of leaf ABA content. The results of this study provides additional evidence that the iso/anisohydric classification may be more correctly viewed as a spectrum made up of a collection of traits rather than two distinct categories (Bryant *et al*., 2022; Chen *et al*., 2021; Jones, 1998; Leuschner *et al*., 2022; McDowell *et al*., 2008; Sade and Moshelion, 2014; Tardieu and Simonneau, 1998). This is especially true in species like *F. sylvatica* which appear to have considerable plasticity in traits that are used for classification as iso/anisohydric depending on the rates of precipitation the provenance receives or the time of year samples are taken (Leuschner, 2020; Leuschner *et al*., 2022; Nguyen, 2016). Our descriptive observations provide several hypotheses that should be tested in future studies, including whether ABA deficiency in *F. sylvatica* results in a wilting phenotype, or if there is variation across the range of *F. sylvatica* in the degree of hormonal control of stomata. Future studies could also address some of the critical limitations of this descriptive work, particularly those associated with a limited number of collections dates, use of a single individual, testing whether osmotic adjustment occurred during drought and lack of predawn water potentials which would all help to further resolve the role of hormonal control of stomata in *F. sylvatica*.

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