Variability among species in the apoplastic pH signalling response to drying soils

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Abstract

After the imposition of soil drying treatments, an elevation of xylem sap pH is one of the earliest observable responses in many herbaceous model plant species. It is theorized that alkalization of sap results in a concurrent elevation in abscisic acid (ABA) concentration delivered to transpiring tissues by preventing Henderson–Hasselbalch-regulated partitioning between the apoplast and symplast. However, here it is demonstrated that the sap alkalization response to soil drying is far from universal in higher plant species. Tests were conducted to determine how universal the pH response to drying soil was in a range of perennial species from a diverse range of plant families. The response was not found in the majority of the 22 species tested. Four species exhibited significant increases in pH, but the majority showed no significant change in xylem sap pH. There was no evolutionary relationship between the species that showed alkalization under drought stress. However, the species that alkalized sap also exhibited good control over internal water status and were the most isohydric species of those tested. None of the species exhibiting anisohydric responses alkalized xylem sap under drought stress. Regardless of alkalization response, plants still retain the ability to respond to changes in xylem sap pH when manipulated by alkaline buffer foliar sprays. This finding indicates that plants have conserved the ability to respond to changes in xylem pH and redistribute ABA, even if they do not currently utilize the mechanism when exposed to drought stress. It was found in Buddleja davidii, Euonymus fortunei, and Hydrangea serrata that the xylem sap pH response to water deficits mirrored the natural pH changes that occur as sap is transported to the leaves, indicating that plants need to be able to have naturally occurring alkalization processes in place for them to be up-regulated under drought stress.

Key words: ABA, apoplast, alkaline buffers, pH signalling, soil drying, xylem sap.

Introduction

One of the first responses that can be observed after the imposition of soil water deficits is xylem sap alkalization (Bahrun et al., 2002; Sobeih et al., 2004). It is hypothesized that sap alkalization is a component of the long-distance biochemical signalling mechanism that closes stomata and thus limits water loss in drying soils. Xylem sap alkalization is not a signal per se (Wilkinson and Davies, 1997) but facilitates the mobilization and transport of the phytohormone abscisic acid (ABA). Root-sourced ABA transported in the xylem is thought to control transpiration (Israelsson et al., 2006) and growth responses to soil drying (Bacon, 1999). As a weak organic acid, the dissociation of ABA is governed in accordance with Henderson–Hasselbalch kinetics; as xylem sap pH rises, ABA is more in the acid form, is loaded into the xylem lumen, and subsequently is confined to the apoplast (Slovik et al., 1992). Plants experiencing soil water deficit generate a high apoplastic pH, which drives ABA partitioning into the apoplast and away from the symplast. Therefore, ABA is transported in the transpiration stream to the sites of action. However, in unstressed well-watered plants, the apoplastic pH remains low, ABA is sequestered into the symplast, and consequently a far lower ABA concentration reaches the stomata and growing tissues in the leaves via the transpiration stream.
Rapid changes in apoplastic ABA concentration driven by changes in pH allow for increases in ABA concentrations without the time lag required for de novo synthesis. However, pH alkalization is not an obligatory requirement for an ABA response to soil drying, as greater quantities of xylem and bulk leaf ABA are also produced in drying soils (Zhang and Davies, 1989, 1990). Apoplastic pH, along with ABA synthesis, recirculation, and delivery rates, will contribute to determining and controlling the ABA concentration that reaches stomatal guard cells and growing cells. In drying soils, xylem sap ABA and pH can increase prior to, or concurrently with, changes in internal water status and reductions in growth and stomatal conductance (g_s) (Schurr et al., 1992; Dodd et al., 2003). Under prolonged and severe water deficits, hydraulic signals will also play a key role in regulating g_s (Saliendra et al., 1995; Comstock and Mencuccini, 1998) and growth (Nonami and Boyer, 1989). However, root-sourced signals are able to act independently of the hydraulic effects of drought (Gollan et al., 1986).

Xylem sap alkalization in response to drought stress has been found repeatedly in plants; however, the diversity of species it has been observed in, to date, is low. Therefore, there is a scarcity of data on how universal a response it is. Not all species previously studied have shown apoplastic alkalization under soil drying. In addition, experiments where there has been no significant alkalization have probably gone unreported, due to there being nothing special to report from such findings. The species that have been shown to increase xylem sap pH under drought stress are tomato (Solanum lycopersicum) (Wilkinson et al., 1998), maize (Zea mays) (Bahrun et al., 2002), Capsicum annuum (Hamad et al., 2004), runner bean (Phaseolus coccineus) (Hartung and Radin, 1989), barley (Hordeum vulgare), grapevine (Vitis vinifera) (Stoll et al., 2000), soybean (Glycine max) (Liu et al., 2003), and sunflower (Helianthus annuus) (Schurr et al., 1992). No significant effects have been observed in sunflower, asiacic dayflower (Commelina communis) (Jia and Davies, 2007), black walnut (Juglans nigra), black willow (Salix nigra), sugar maple (Acer saccharum) (Loewenstein and Pallardy, 1998b), Cotinus coggyria, and Hydrangea (Wilkinson and Davies, 1997; Cameron et al., 2002). Both castor bean (Ricinus communis) (Schurr and Schulze, 1996) and Forsythia×intermedia (Cameron et al., 2002) have been reported to exhibit xylem sap acidification under drought stress.

Attempts have been made to correlate xylem sap pH and soil water status under wild growing conditions. Thomas and Eamus (2002) found correlations between xylem sap pH in dry seasons compared with wet seasons in six Australian savannah tree species. However, Loewenstein and Pallardy (1998a) found no correlation with xylem sap pH and environmental conditions in three temperate deciduous tree species. In addition, Auge et al. (2000) were unable to find a correlation between xylem sap pH and leaf g_s experienced throughout the growing season in 10 out of 11 temperate forest species.

Other environmental factors that can induce changes in xylem sap pH include flooding (Jackson et al., 2003; Else et al., 2006), CO₂ (Savchenko et al., 2000; Hedrich et al., 2001), light intensity (Mühling and Lauchli, 2000; Stahlberg et al., 2001; Felle and Hanstein, 2002), vapour pressure deficit (Jia and Davies, 2007), soil nutrient status (Kirkby and Armstrong, 1980; Mengel et al., 1994; Mühling and Lauchli, 2001; Dodd et al., 2003; Jia and Davies, 2007), plus the diurnal variation in apoplastic pH (Schurr and Schulze, 1996). However, when these environmental factors are altered in a manner that leads to an increase in apoplastic pH, it is associated with a decrease in gs resulting from the concurrent increase in ABA delivery. It should be noted that if a plant is experiencing a stress other than soil drying, that also alters sap pH, then the alkalization due to soil drying may not be observable. Alkaline buffer solutions, of a pH equivalent to that found in the xylem of plants experiencing soil drying, sprayed onto foliage have been demonstrated to be effective at controlling transpiration (Bacon et al., 1998; Wilkinson et al., 1998). The alkaline buffer artificially manipulates the apoplastic pH and thereby affects ABA delivery to the guard cells, with the responses of Forsythia plants mimicking those of plants experiencing soil water deficits (Wilkinson and Davies, 2008).

This study was specifically aimed at providing a contrast to previous studies that have been performed chiefly on herbaceous, annual species; mainly woody perennial species were chosen for study here. Twenty-two species from disparate families were chosen to better represent the vascular plant kingdom. This also allowed testing of the previously stated hypothesis by Wilkinson and Davies (2008) that pH-driven partitioning of ABA in the xylem plays a key role in preventing sympatric uptake of ABA in tall woody species. By imposing water deficits in a standard regime to all species and then testing xylem sap changes, it was possible to determine if alkalization occurred and if it was associated with isohydricity. Experiments were then conducted to ascertain whether the natural xylem sap pH response to soil drying determines if a species possesses the capacity to respond to changes in xylem sap pH. Jia and Davies (2007) found in Commelina communis, tomato, and sunflower that xylem sap pH was higher when extracted from leaves than when extracted from the stem base, indicating that an apoplastic pH gradient exists. Studies were conducted to determine if xylem sap pH is alkalized as it ascends in the apoplast in all species, or if it is restricted to species that also exhibit xylem sap alkalization under soil water deficits.

Materials and methods

Universality of the pH response to soil drying

Eighteen rooted cuttings of 22 species (Table 1) were potted into 0.9 dm³ pots using a medium consisting of 100% sphagnum peat with 6.0 kg m⁻³ Osmocote Plus 12-14 month controlled-release fertilizer (The Scotts Co., Surrey, UK) and 1.5 g m⁻³ MgCO₃ (excluded for Rhododendron obtusum and Hydrangea serrata). Plants were then left to...
Table 1. The response of leaf temperature, stem water potential, and xylem sap pH in 22 perennial plant species to being well watered (1.0 ETP), a mild soil water deficit (0.8 ETP RDI), and a severe soil water deficit (0.5 ETP RDI).

Measurements were taken once plants exhibited a significant increase in leaf temperature in a deficit treatment. All plants were pot grown and contained in a climate-controlled greenhouse with supplementary lighting. Treatments consisted of 15 plants with values equalling means from 30 stems, LSD values calculated at $P < 0.05$, df = 29. Treatments that produced significant differences in a parameter compared with the control (1.0 ETP) treatment are highlighted in bold. Arrows illustrate if a species exhibited significant raising or lowering of xylem sap pH in response to soil drying to RDI.

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaf temperature (°C)</th>
<th>Water potential (MPa)</th>
<th>Xylem pH</th>
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<td></td>
<td>1.0 ETP</td>
<td>0.8 ETP</td>
<td>0.5 ETP</td>
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acclimate in the growing environment for 2 weeks. Plants were grouped by species, with treatments randomized in an indoor growing environment [photoperiod 16 h, daytime temperature 20°C, night-time temperature 18°C, relative humidity 35–45%], mean photosynthetically active radiation (PAR) 500 µmol m⁻² s⁻¹]. Regulated deficit irrigation (RDI) was used to apply soil water deficits, as it allows water deficits to be generated in a controlled and gradual manner (Sharp et al., 2009). Daily irrigation was applied in proportions of the estimated potential evapo-transpiration (ETp) of the plants over a 24 h period. Fifteen plants received a well-watered treatment, where containers were filled to capacity by replacing 100% of the daily evapotranspiration (1.0 ETp). Two sets of 15 plants were supplied with 0.8 ETp and 0.5 ETp of the control plants and deemed to be receiving mild and severe soil water deficits, respectively.

Thermal imaging of leaf temperature was used as a surrogate for stomatal conductance. Leaf temperature is known to be a sensitive indicator parameter of leaf conductance to water vapour and thus an acceptable method for approximating leaf gs (Jones, 1999; Jones et al., 2002). Leaf temperatures taken from thermal images were the most practical method of assessing the high numbers of leaves within this study in a representative time frame, and exhibited a very high resolution. Porometers were used to confirm that leaf temperatures were indicative of stomatal closure, but the requirement for porometer calibration for each species, the small needle leaves of certain species, and the high sampling number precluded the use of porometry. The maximum adaxial temperatures of two leaves per plant were recorded using a FLIR SC200 thermal imaging camera (FLIR Systems, Wilsonville, USA). The atmospheric environment and incident radiation were kept unchanged, and relative humidity was kept low (35–45%) in order to reduce the errors in estimating gs from leaf temperatures. Once the soil drying regime generated a significant increase in leaf temperature, further analysis began.

In order to measure stem water potential ($\Psi_{stem}$) and extract xylem sap, the two largest stems per plant were then cut 50 mm from the apex and placed in a Scholander pressure bomb (Plant Moisture Systems, Santa Barbara, CA, USA) with the chamber lined with damp blotting paper. Compressed air was progressively added to the chamber until sap began to appear at the site of excision (viewed with a ×10 hand lens), and the pressure when sap emerged was noted and this was taken be equal to the negative value of $\Psi_{stem}$. The cut stem was then blotted dry and the pressure increased by 0.2 MPa over the balance pressure in order to extract xylem sap. The subsequent sap exuded was collected in 100 mm³ tubes (Eppendorf, Hamburg, Germany) and kept on ice before the pH of the...
sap was measured using a micro PHR-146 pH probe (Lazer Research Labs, Los Angeles, CA, USA). Three species were found to be uncooperative to the measurement of $\Psi_{\text{stem}}$ in pressure chambers. *Forsythia x intermedia* possesses hollow stems, which causes air to leak from the chamber. *Elaeagnus augustifolia* only exudes very low xylem sap contents, and *Trachelospermum jasminoides* produces latex at the sight of excision that obscures observations and sap analysis.

Plants that closed stomata (indicated by significant increases in leaf temperature) under water deficits while exhibiting no change in water potential were considered to be regulating water relations in an anisohydric manner. Those species that had increased leaf temperatures under water deficits at the same time as significant reductions in stem water potentials were considered to be behaving anisohydrically.

Response to apoplastic pH manipulation

Potassium phosphate (KH$_2$PO$_4$:K$_2$HPO$_4$) buffer was sprayed on to the foliage of species that exhibited divergent responses in xylem sap pH to soil drying; *Buddleja davidii* significantly increased xylem sap pH, *Physocarpus opulifolius* decreased xylem sap pH, and *Lonicera periclymenum* exhibited no significant change in xylem sap pH. These species were also chosen because they possessed high stomatal conductance under well-watered conditions. Eight plants of each species were treated with 20 mol m$^{-3}$ potassium phosphate buffer spray iso-osmotically adjusted to either pH 6.0, 7.0, or 8.0. After 2 h, adaxial leaf temperatures were measured on two leaves per plant.

Apoplastic ABA and pH profiles

The changes in pH and ABA concentrations were determined in three species that exuded large volumes of sap and possessed divergent responses in xylem sap pH to soil drying; *B. davidii*, *Euonymus fortunei*, and *H. serrata*. Sap was extracted sequentially from further along the transpiration stream by applying increments of increased pressure in order to generate a profile from roots to leaves. Plants were severed at the root–shoot junction and sap extracted from further along the transpiration stream by the application of increasing pressures from a pressure chamber to both the root zone and the aerial sections. Once a balancing pressure was obtained, then stepwise increases in chamber pressure of 0.1 MPa up to 1.0 MPa over the balance pressure were applied. The volume of sap extracted at each pressure point was measured with a micropipette, the pH recorded, and the ABA content determined by radioimmunoassay (RIA).

The mean total xylem sap volumes collected from roots and from stems are given in Fig. 1. To discount the possibility that changes in sap pH and ABA content resulted from artefacts generated from lysing cell sap at high pressures, sap was also extracted from these three species by immediately pressurizing the chamber to 1.0 MPa above the balance pressure. As there was no sudden significant increase in pH or change in ABA concentration when 1.0 MPa over-pressure was immediately supplied (data not shown), and also because the ABA content is not reduced at higher pressures (as a result of cell contents diluting apoplastic ABA concentrations), the possibility of artefact generation is discounted.

**RIA of xylem sap ABA concentration**

Xylem sap samples were thawed and centrifuged for 5 min at 11 500 rpm, and 20 mm$^3$ of the supernatant was used as a single sample. The ABA concentration was determined by RIA following the protocol of Quarrie et al. (1988) using $[\gamma^3\text{H}]$(±)-ABA at a specific gravity of 2.0 TBq mmol$^{-1}$ (Amersham International, Bucks, UK) and the monoclonal antibody AFRC MAC 252, which is specific for (+)-ABA. The endogenous ABA concentration in the sample was determined by comparing the ratio of tritiated ABA bound to the antibody to endogenous ABA. As a known quantity of tritiated ABA is added, it is possible to calculate the
endogenous ABA concentration in the sample by fitting the data to standard curves generated from samples of known ABA masses.

**Results**

**Universality of the apoplastic pH response to soil drying in perennial plants**

Of the 22 species tested, four exhibited significant increases in xylem sap pH in response to water deficits (B. davidii, Dicksonia Antarctica, Penstemon heterophyllus, and R. obtusum) (Table 1). For 12 species there was no significant difference between the xylem sap pH from well-watered plants and drought-stressed plants. Physocarpus opulifolius, Spiraea japonica, and H. serrata significantly reduced xylem sap pH under soil water deficits. With the exception of R. obtusum, significant reductions in $\Psi_{stem}$ under RDI were not observed in species that exhibited xylem sap alkalization.

**Response of species with differing apoplastic pH response to soil drying to apoplastic pH manipulation**

Foliar sprays of pH 8.0 phosphate buffer (20 mol m$^{-3}$ KH$_2$PO$_4$) significantly increased adaxial leaf temperatures above those measured on plants sprayed with water controls (Table 2). Increases in leaf temperature were observed in B. davidii, P. opulifolius and L. periclymenum. Foliar sprays of phosphate buffers adjusted to pH 6.0 and pH 7.0 had no significant effect on leaf temperature compared with water control sprays in all three species.

**Apoplastic ABA and pH profiles**

In B. davidii, as sap is extracted from further up the transpiration stream the pH rises (Fig. 1). In H. serrata, as the sap is extracted from further into the stem xylem the pH initially decreases, then returns to starting pH values. Finally, as sap is extracted from the leaf apoplast, the pH starts to rise significantly above stem values. In E. fortunei, the pH does not significantly change as sap ascends through the stems and into the leaves in the transpiration stream. From sap extracted from the root zone it can be demonstrated that the xylem sap pH of H. serrata and B. davidii remains stable as it travels through the roots before reaching the stems. In E. fortunei, there is some alkalization as sap ascends close to the root–shoot junction, but the pH remains stable as it travels through the rest of the roots.

In B. davidii, H. serrata, and E. fortunei, apoplastic ABA concentrations do not change as xylem sap ascends from the root–shoot junction to the leaves (Fig. 2). In the roots, the ABA concentration in the saps extracted from B. davidii and H. serrata remained stable as they ascended to the root–shoot junction. In E. fortunei, the apoplastic ABA concentration was raised as sap ascended to the root–shoot junction. It was found that in E. fortunei, the ABA concentration in xylem sap extracts was significantly lower than in both B. davidii and H. serrata at each extraction point above and below the root–shoot junction.

**Discussion**

This study demonstrates that xylem sap alkalization in response to soil drying is far from universal in the plant
kingdom. Species either alkalize, do not significantly alter their pH, or actually lower xylem sap pH. Only B. davidii, D. Antartica, P. heterophyllus, and R. obtusum exhibited xylem sap alkalization in soil drying treatments. There is no clear evolutionary relationship between apoplastic pH responses. The species tested could also not be divided according to calcifuges/calcicole classification; R. obtusum exhibited xylem sap alkalization, while another calcicole, H. serrata, produced an acidic xylem sap in response to soil drying. The elevations in xylem sap pH values observed are comparatively high for some species, but Wilkinson et al. (1998) observed that tomato xylem sap increased from pH 5.0 to pH 8.0 when soil drying was imposed.

Those species that exhibited sap alkalization in response to soil drying chiefly showed the greatest control of water status under water deficits (i.e. they were behaving isohydrically). It is hypothesized that alkalization allows quick and measured control of stomatal conductance (by its action on ABA concentrations) and thereby leads to increased isohydricity. *Cortaderia selloana* xylem sap pH remained at 5.8 in every single plant in all the treatments, and this was associated with a strongly anisohydric response, with a significant depression in stem water potentials. These findings suggest that anisohydric responses and alkalization of apoplastic pH are in some way linked. However, it is likely that other factors are important, and it has been previously hypothesized that the differences in water-conducting capacity of stems and petioles (Schultz, 2003), aquaporin activity (Levin et al., 2007), as well as perception of ABA at target sites (Tardieu and Simonneau, 1998) are responsible for isohydric behaviour.

Schurr and Schulze (1996) suggested that xylem sap acidification in drought conditions in castor bean was due to changes in phosphate nutrition and delivery. In castor bean and those plants exhibiting xylem sap acidification in this study (*Spiraea japonica, H. serrata,* and *P. opulifolius*), phosphate or nitrate availability could be dominating xylem sap chemistry when drought stress is imposed. As previously stated by Schurr and Schulze (1996) we cannot see any obvious physiological reason for apoplastic alkalization, unless there is transportation of an unidentified acidic drought-responsive compound in these species.

Not all not all plants exhibited alkalization in soil drying, species representing alkalizing, acidifying, or non-responsive types closed stomata in response to *in vivo* modification of xylem sap pH by alkaline foliar sprays. The presence of a response to alkalization of the apoplast in *P. opulifolius* and *L. periclymenum* demonstrates that the ability to generate alkalized xylem sap in drought conditions is not required for the action of pH signals in the apoplast. The basis for conserved responses to alkaline xylem sap could lie in the physicochemical properties of the apoplast-symplast membranes being conserved between species and determining how ABA is partitioned. Therefore, the response to artificial manipulation is passive and governed entirely by the prevailing Henderson–Hasselbalch kinetic state in the tissues.

This study demonstrates that in woody plants, xylem sap alkalization is much less common than in the annual model plant species. However, it is not certain how many other studies have investigated apoplastic pH responses to soil drying but have not reported results, due to no significant differences being found. In addition, there could be synergistic or antagonistic actions of other environmental conditions that affect apoplastic pH (Hedrich et al., 2001; Felle and Hanstein, 2002; Dodd et al., 2003; Jackson et al., 2003; Jia and Davies, 2007). Different species may only modify their apoplastic pH depending on the specific current combination of environmental signals being received. The chemical agent responsible for the natural increase in sap pH under soil water deficits also remains unconfirmed. Proton pumps (ATPases) are hypothesized to play a key role in controlling apoplastic pH by driving H⁺ ions into the sap in well-watered conditions. Apoplastic pH can be altered by inhibiting the action of proton pumps with the use of vanadate (Jia and Davies, 2007). Symplastic proton pumps are thought to generate the significant elevations in apoplastic pH observed when nitrate availability is increased (Kosegarten et al., 1999). The formation of malate anions via the action of nitrate reductase as the sap travels to the leaves has also been implicated in controlling apoplastic pH responses to soil drying (Wilkinson et al., 2007), as has the buffering capacity of nutrients in the transpiration stream (Schurr and Schulze, 1996).

The pH gradients of xylem sap during the ascent through the stem in *B. davidii, H. serrata,* and *E. fortunei* mirror the changes observed in sap after the imposition of soil water deficits. It is hypothesized here that there is a requirement for a species to exhibit alkalization along the transpiration stream under well-watered conditions in order for drought-responsive alkalization to be intensified once soil water deficits are imposed. In species that show sap acidification as it ascends, such as *H. serrata,* this acidification is intensified under drought stress, whereas in species that show stability of xylem sap pH as it ascends through the stem section, such as *E. fortunei,* there is no mechanism present to alter apoplastic pH when soil water deficits are imposed. The lack of a pH response in *E. fortunei* was combined with a low ABA concentration throughout the transpiration stream. The depression of these two major regulatory signals, which allow quick and controlled responses to soil drying, could be contributing to the anisohydric behaviour in this species. The alkalization observed at the very end of the transpiration stream in *H. serrata* is thought to be due to alkalization occurring in the leaf apoplast and may represent leaf-localized control of ABA levels and transpiration around the guard cell. Large pH gradients between the leaf and stem–xylem apoplast are known to exist (Hoffmann and Kosegarten, 1995; Mühling and Lauchli, 2000), and leaf-localized control of ABA concentration by apoplastic pH has previously been demonstrated (Slovak and Hartung, 1992a, b; Slovak et al., 1992).

The findings that plant species with differing apoplastic pH responses to soil drying retain the ability to respond to artificial modifications in sap pH could have potential for use in the horticulture industry. Alkaline buffers could be...
applied to crops to limit excessive transpiration and thereby cut down the need for irrigation when water supplies are limited. In ornamental and fruit crops alkaline buffers could be used to stimulate a spike in xylem ABA concentration to control excessive vegetative growth, and reduce the need for pruning.

In conclusion, the pH response to drying soil is far from universal in perennial species and there is no evolutionary relationship between the species that alkalize sap under drought stress. However, the species that do alkalize sap have good control over internal water status and were the most isohyic species. It was found that plants retain the ability to respond to changes in xylem sap pH regardless of the alkalization response. The xylem sap pH response to water deficits mirrored the natural pH changes that occur as sap is transported to the leaves, indicating that there is a necessity for a plant species to have a naturally occurring alkalization processes in place for it to be up-regulated under drought stress.

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