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Commentary Plant and Soil 2014

Is xylem sap calcium responsible for reducing stomatal conductance after soil liming?

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Abstract

Understanding the regulation of calcium uptake, xylem transport and its impacts on growth and leaf gas exchange is a subject that has received insufficient recent attention. Calcium (Ca) is unique within the group of key elements required for plant growth in that it also has a role in cellular signalling via regulation of changes in its cytoplasmic concentration. Its mobility, within the plant, is however somewhat constricted by its chemistry and cellular signalling role, and its adsorptive capacity within the aopoplast and the xylem. Supply and demand for Ca is achieved by a homeostatic balance which if perturbed can cause a number of distinctive physiological conditions, often related to Ca deficiency. In this issue Rothwell and Dodd present experiments with bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) plants grown in a field soil exposed to the processes of soil liming (application of Ca carbonate ($CaCO_3$). Given that there is evidence of free Ca in the xylem sap altering stomatal conductance it is reasonable to ask the question does liming elevate Ca in the transpiration stream which may explain the observed reduced growth which they hypothesise is due to Ca-induced stomatal closure. They show that liming doubled soil exchangeable Ca, reduced stomatal conductance and shoot biomass in both species compared with unlimed controls. However, xylem sap Ca concentration increased only in bean. Interestingly, the same was not true for the pea where the root xylem sap concentration remained unchanged despite an increase in soil

available Ca. Given that stomatal conductance decreased in both species, but in response to a lime-induced increase in xylem sap Ca in only one; this questions the role of Ca in inducing stomatal closure. They propose that their data suggest that as yet unidentified antitranspirant causes stomatal closure in both species not the increase in xylem sap Ca *per se*.

Commentary

Calcium a multitasking element

Trying to understand how Ca ions (Ca^{2+}) move within the transpiration stream is a problem that has received various levels of attention over many years. The processes which determine the flux and distribution of ions from roots can have a particular importance in determining development, growth and the physiological performance of the shoot (Gilroy et al 1993, White and Broadley 2003, Karley and White 2009, Gilliham et al 2011, Hawkesford et al 2012). The flux of Ca ions, for example, within the xylem and its delivery to aboveground organs, in tomato (*Lycopersicon esculentum*) fruits, is critical in determining pericarp development and the production of commercially acceptable fruits (Guichard et al 2001, Suzuki et al 2003). The supply of Ca^{2+} to shoot apices can alter cell division and expansion by influencing cell and vacuole osmotic content and cell wall formation (Hawkesford et al 2012). Calcium ions also aid in maintaining cellular stability and membrane integrity and are involved in stress perception signalling response cascades (Suzuki et al 2003, White and Broadley 2003, McAinsh and Pittman 2008, Kudla et al 2010), and more recently have been shown to have a regulatory function within the nucleus (Mazars et al 2009). The role of Ca in generating changes in stomatal aperture is also well recognised (Mansfield et al 1990) and Ca flux in the xylem has been implicated as a regulator of transpiration (Atkinson et al 1989, 1992, Atkinson 1991). The dual role of Ca^{2+} in providing a nutritional substrate required for growth, as well as, acting in a quantitative signalling response element appears paradoxical, but this duality is achieved through tight cytoplasmic regulation of Ca concentration and sub-cellular partitioning of Ca to vacuoles and in some cases specific cell types (idioblasts) which store insoluble Ca salts [e.g. Ca oxalate] as well as the apoplast (Hirschi 2004, Volk et al 2008, Franceschi and Nakata 2005, Helper 2005, He et al 2011, Gilliham et al 2011). While clearly cytoplasmic Ca status is at the core of a number of specific stress induced Ca signalling systems cascades further elucidation of these biochemical and molecular events should facilitate knowledge on how to manipulate these processes (Nakata and McConn 2007, McAinsh and Pittman 2008, Kudla et al 2010, Dodd et al 2010). This may be particularly relevant for practical crop strategies designed to reduce food waste by increasing shelf-life (see suggestions of Park et al 2005).

Mobility, partitioning and homeostasis

The mobility of Ca^{2+} in the plant is known to be low. Many species, but not all, have suberized cell walls within the Casparian band of the root system which restricts radial water and apoplastic solute movement into, and out, of the root stele (Clarkson 1984, Moore et al 2002). Solutes, like Ca, are in essence forced into a passage involving the cytoplasm, plasmodesmata and aquaporins, which has both challenges and consequences with respect to the achievable rate of cellular Ca flux. The potential limitations in symplastic cell to cell diffusion of Ca requires that the supply of free Ca is maintained but cytoplasmic concentrations are kept at µM levels to avoid precipitation of Ca phosphates and cell death. Therefore the entry of Ca into the cytoplasm has to be as tightly controlled as does its cytosolic removal (White and Broadley 2003, Gilliham et al 2011). We are now beginning to acquire a molecular understanding of the regulation of membrane transporters which determine Ca partitioning at the cellular level (de Freitas et al 2011). Subsequent movement of Ca within the apoplast and the xylem is slowed due to its divalent ability to bond (cation ion exchange capacity CEC), for example, with anionic charges on substances such as pectates, phospholipids and carboxyl groups in cell membranes and walls (Ferguson and Bollard 1976, White 2001, see also the references within Gilliham et al 2011). This process is described by the isotopic data recorded for calcium and magnesium exchange with the surrounding tissues as sap moves up the xylem (Metzner et al 2010). Limited mobility of Ca is a unique characteristic among the key plant nutritional elements required for growth. It can lead to the irreversible binding of Ca^{2+} (and other cations) to the negatively charged inner surfaces of functional xylem cells, retarding the rate of ion distribution. While limitations in the rate of cytoplasmic movement support suggestions that Ca^{2+} show little redistribution (phloem-fed tissues) over any significant distance within the plant (Karley and White 2009). Upon xylem delivery, whether initially or subsequently when in the cytoplasm it appears that Ca sequestration predominates; appearing extra-cellularly in the apoplast (He et al 2012), or vacuole (as salts of phosphoric, oxalic or phytic acids) within idioblasts, or extra cellular (Webb 1999, Franceschi and Nakata 2005, Volk et al 2008, Hawkesford et al 2012). Despite clear increases in insoluble Ca in leaves in response to increased Ca supply, the relationship between Ca supply and oxalate formation and the different forms of Ca oxalate (soluble and insoluble) does not appear simple and may show leaf ontogenic change (Zindler-Frank et al 2001). It is equally apparent that not all species show Ca sequestration which is based on, either Ca oxalate, or even the formation of insoluble Ca salts (see Hawkesford et al 2012).

The success of Ca homeostasis is clearly apparent from tissue cytoplasmic concentrations of Ca (100-200 nM) that are maintained against a three to four-fold higher (1 to 10 mM) external concentration within the rhizosphere (Gilroy et al 1993, Karley and White 2009). The complexity of cellular Ca homeostasis is achieved by an integrated array of membrane bound transport proteins, calmodulin-binding, P-ATPases and Ca specific ion channels all of which can modulate Ca uptake to meet demand (Gilroy et al 1993, Miedema et al. 2001, White and Broadley 2003, Franceschi and Nakata 2005, McAinsh and Pittman 2008, Volk et al 2008, Karely and White 2009, Kudla et al 2010, Dodd et al 2010, Gilliham et al 2011). Homeostasis can also be shown to be closely linked with apoplastic, extracellular water flow, and transpiration, where cytoplasmic Ca^{2+} are implicated in the regulation of water flow via aquaporins (see Gilliham et al 2011). These authors review the importance of how water flow varies with species, organs, ontogeny and their growing environment and its influence on Ca flow. For example, ABA whole plant spray treatments reduced Ca deficiency in tomato by increasing sap flow and Ca^{2+} movement into the fruit (de Frietas et al 2014).

Supply and demand

Breakdown, or limitations, in the xylem supply of Ca^{2+}, can have particularly important and dynamic consequences on the growth rate of rapidly expanding tissues. The condition known as blossom end rot (BER) is just one of several Ca deficiency derived physiological conditions seen in fruits such as tomato (Bangerth 1979, Adams and Ho 1992, White and Broadley 2003, Ho and White 2005, Karley and White 2009). It is also often the case that the tissues most at risk from suffering an imbalance in their Ca supply and demand are those where transpiration rates are generally lower than other competing aboveground organs. Inferences such as this lead to suggestions that it is the transpiration rate that is a critical determinant in the quantitative delivery of Ca^{2+} to the shoot, along with control over the proportional allocation of Ca^{2+} to various organs and tissues, because these tissues have different transpiration rates (Karley and White 2009), e.g. the low transpiration of inner leafy rosette regions of many of the Brassica family. Increasing leaf sap flow artificially through the application of ABA can increase Ca movement which reduces the incidence of BER (de Freitas et al 2014). There is however evidence that transpirational water movement is not always a universal determinant of Ca movement. In some cases it appears that water transport and Ca movement become uncoupled and can explain the non-uniform distribution of Ca in some leaves (Atkinson 1991, Kerton et al 2009, Metzner et al 2010). The notion that Ca allocation to plant organs is influenced by differences in transpiration rate is supported by the appearance, initially, of BER in specific tissues regions or organs associated with low transpiration (Ho and White 2005, de Freitas et

al 2014). What is less clear is a functional link between below average tissue concentrations of free Ca^{2+} in BER expressing tissues relative to the total organs Ca content (Petersen and Willumsen 1992), combined with a lack of sequential evidence demonstrating BER cause and effect (Nonami et al. 1995). Low transpiration rates, induced by decreases in the vapour pressure gradient (leaf to air), can be linked to Ca deficiency symptoms (Holder and Cockshull 1990, Kerton et al 2009). The consequences of this, at least with tomato, are that plasma membranes show distinctive signs of cellular Ca precipitation when grown under conditions known to induce BER (Suzuki et al 2003). These precipitates are located in parenchyma cells close to tracheids and the vascular bundles. While the transpiration rate *per se* may not directly influence the loading of Ca in the transpiration stream, the transpirational flux will determine the xylem sap concentration and in turn its shoot delivery rate as factor of loading rate multiplied by transpiration flux.

It is apparent, commercially, that cellular Ca concentration has an important influence on fruit texture and the avoidance of disorders such as bitter pit in apple (Nielsen et al 2005). Despite the application of post-harvest 'remedial' treatment by dipping fruit (Ca cuticular entry and movement by apoplastic diffusion) in Ca based products to reduce the occurrence of bitter pit in-store, considerable attention is given to Ca supplementation during fruit growth. It is clear that this exogenous source of Ca is present within fruit tissues post-harvest, but there is also evidence that endogenous Ca uptake declines with fruit development. Many studies, but not all, imply that fruit Ca content is determined early in the growth cycle and once beyond the cell division phase Ca uptake can decline and the rapidly expanding fruit induces the cellular Ca concentration to decline (Quinlan 1969, see review by Saure 2005). The explanation often proposed for this change in response to endogenous root-derived Ca is that the fruit xylem transport system becomes non-functional (Drazeta et al 2004). The consensus is that supplementary Ca sprays, to avoid deficiency during fruit growth, require application to the fruit (direct uptake via trichomes and stomata on the fruit epidermis) because of the absence of Ca transport from leaves. Saure (2005) suggests that the problem with xylem Ca delivery in fruit is not having to cope with deficiency in transport channels, or a weak the transpirational driving force, but overcoming the plant's need to limit Ca transport during rapid growth. Again, evidence from both post-harvest application of Ca and uptake during growth suggests that Ca movement within the fruit occurs, but differences in its measured Ca distribution occur primarily due to variation in cell growth patterns and utilisation within the fruit (see Saure 2005). This type of variation in Ca partitioning can also be explained by cellular changes in the expression of a Ca^{2+}/H^{+} tonoplast transporter protein (CAX) (Conn et al 2011, de Freitas et al 2011). In tomato the sCAX1 transporter expressing phenotypes showed increased total fruit Ca and shelf-

life, while the occurrence of BER increased (Park et al 2005, de Freitas et al 2011). Increased BER was explained by elevated vacuolar Ca combined with reduced cytosolic and apoplastic Ca, leading to membrane dysfunction and leakage (Conn et al 2011). Controlling CAX expression may facilitate an alternative strategy removing the need for post-harvest chemical treatments to increase shelf-life (Park et al 2005).

Calcium soil supply

The role that soil Ca status plays in defining the presences of 'indicator' species (calcifuges and calcicoles) within the landscape is a foundation stone in the development of ecophysiological approaches to mechanistically explain species distribution (Bradshaw et al 1958, 1960, Jefferies and Willis 1964, Rorison and Robinson 2006). The effectiveness of this approach has inspired an array of work based on expanding and illuminating how plants cope with varying levels of exposure to Ca, and the impacts of its salts, on many aspects of soil and plant performance (Kinzel 1983). With the finding that free Ca^{2+} are involved in the process of stomatal closure, it has become apparent that Ca may also have a role in influencing whole leaf gas exchange and that this might also be another chapter in the story of explaining species distribution with respect to variation in soil Ca concentrations (De Silva et al 1986, Mansfield et al 1990, Atkinson 1991). However, there has been little attempt to address the question, particularly in agricultural systems where the direct implications for crop management practices change soil available Ca rapidly, as occurs during remedial liming. It is therefore interesting to see in this issue that Rothwell and Dodd (2014) address the question of Ca inputs, via liming, having a direct impact on crop gas exchange. The positive growth and yield responses of field crops to the liming of acidic soil are very well documented (Tang et al 2003, Karaivazoglou et al 2007). It is more challenging to find studies which have recorded direct negative impacts of lime application in agricultural systems and crops which have been linked to the decrease or increase in the availability of other elements such Al, Zn, Mn, B and P (Vickers and Zak 1978, Sumner 1979, Kochian et al 2004), but more recently the focus has been on soil attributes, such as SOM, nitrogen mineralisation and changes in the microflora, and their impact on aspects of the crop, not the direct influence that Ca^{2+} uptake has on the plant (Haynes and Naidu 1998, Kemmit et al 2006, Fageria and Baligar 2008). Rothwell and Dodd (2014) address the question; does liming elevate xylem sap Ca which limits gas exchange by inducing partial stomatal closure and potentially reduces yields. A positive answer to this question has important implications for liming impacts on crop productivity as managing soil pH is a vital component, in acid soil, which occurs globally over a large proportion of agricultural land. By understanding the possible negative impacts that a flush of

206 soil Ca might have on the regulation of crop gas exchange we might be able to utilise crops and/or
207 growing systems which are more capable of managing soils with higher Ca concentrations, or crops
208 that have the capacity to restrict/regulate more effectively Ca uptake and translocation in xylem sap.
209
210 *Species differences in response to soil calcium*
211
212 To address this question Rothwell and Dodd experiment with bean (*Phaseolus vulgaris*) and pea
213 (*Pisum sativum*) grown in a field collected sandy loam soil to which they applied commercially
214 available agricultural lime at 3 g l^{-1} as Ca carbonate ($CaCO_3$). This rate of application matches that
215 recommended to achieve a soil pH of around 6.5. These plants were grown with the intention of
216 being suitable for enclosing within pressure chambers to extract xylem sap to measure its Ca
217 concentration. The two sets of plants were cultured in slightly different ways (de-topped or a leaflet
218 *mid-rib incision*) and to facilitate the most appropriate method for sap extraction given the structural
219 differences between bean and pea. Importantly, great care was taken over the sampling of the
220 xylem sap, with sap collection occurring over a range of transpiration rates (sap flows) by application
221 of positive pneumatic pressures (see Rothwell and Dodd in this issues for a full explanation). The sap
222 flows achieved included those which had been determined previously (gravimetrically) to match the
223 *in vivo* transpiration rates of the experimental plants. The reasoning and importance of doing this is
224 vital in determining actual xylem sap concentrations, because we know that if we change the
225 transpirational flow, as we do when invasively cutting the xylem column (detoping sap collection),
226 this at best, temporarily, upsets the existing coupling between xylem cell ion loading and the now
227 non-existent transpirational pull. At worst, it may completely uncouple the delivery of solutes to the
228 shoot. This uncoupling, for example, can lead to an overestimation of the concentrations of a xylem
229 solute because we have removed the transpiration pull (flow), permanently relying on root pressure
230 exudation only (which generally induces a lower flux than daytime transpiration), in the absence of
231 changing the rate at which solutes are loaded into the xylem. This concept is well described and
232 utilised by Jackson and his associated co-workers (Jackson et al 1995). These authors also show how
233 changing the volume flux of the transpiration stream can not only influence solute concentration,
234 but also the mass of solutes which are exported from the root to shoot, which is described by the
235 delivery rate (Else et al 1994). It is apparent in studying the movement of Ca^{2+} within the xylem that
236 we have accurate measures of *in planta* xylem sap concentrations which can be used knowing the
237 transpiration flow to derive shoot Ca delivery rates.
238

Using this approach Rothwell and Dodd (2014) showed that compared with unlimed controls, liming reduced shoot biomass in both bean and pea. There are other studies which show this negative response and it is interesting that in these cases it is also a leguminous species, e.g. crown vetch (*Coronilla varia* L.) and alfalfa (*Medicago sativa* L.) and sorghum (*Sorghum sudanense*) respectively (Vickers and Zak 1978, Sumner 1979). The reduction in biomass corresponded with a significant reduction in stomatal conductance and assimilation for both species. Liming itself doubled soil exchangeable Ca which led to a massive increase in xylem sap Ca concentration from 0.9 to 1.7 mM, but only for bean. Interestingly, for pea, root and leaf Ca concentrations remained unchanged despite an increase in soil available Ca. Having collected xylem sap samples in an appropriate manner Rothwell and Dodd (2014) were able to show how an increase in Ca delivery rate was apparent, on liming, with bean, but not with pea. In fact with pea, Ca delivery declined, most likely due to the observed reduction in stomatal conductance restricting transpiration. The authors conclude that there are species differences in their ability to regulate Ca uptake and delivery to the shoot irrespective of the initial differences within the soil. This very much supports earlier suggestions about these species (Atkinson et al 1992). What is interesting and novel about Rothwell and Dodd (2014) is the suggestion of why stomatal conductance declined in pea in the absence of elevated xylem sap Ca. They propose two possible explanations; the first is that stomatal sensitivity to Ca shows species differences, while the second suggests that perhaps the correlative link implied between bean xylem sap Ca concentration and stomatal conductance was not causative. They rule out the differential species sensitivity by showing similar species responses to artificial Ca supply in a detached leaf transpiration assay. It would be interesting to repeat this sensitivity experiment with plants known to respond to lime induced reductions in stomatal conductance using intact attached leaves. Catheter-type applications of a putative stomatal conductance regulator (Ca and ABA) can be effectively introduce into the xylem stream to induce dynamic changes in sap constituents and corresponding reductions in stomatal conductance (Atkinson et al 1990). This 'topical' application of Ca into the leaf mid-rib allows little more than the xylem Ca stream concentration to change for a well-watered leaf. It might, if the Ca were sourced from the $CaCO_3$ used in the liming treatments to rule out any possible other stomatal closing factors, but such a component naturally occurring seems unlikely. We would expect with agricultural lime in this case "coarse screened limestone" for there to be MgO also present but at >15% (www.aglime.org.uk.). They rule out the possibility of inaccuracy in the measurements of ions within the xylem sap, I think correctly, based on the methodology used. They also consider the possibility of concluding that it may well be an alternative substance rather than Ca^{2+} in the xylem stream that cause stomatal closure in bean. This is an interesting hypothesis which Rothwell and Dodd leave us to think about. It could be considered from another perspective,

it is clearly not the absence of negative physiological responses in pea to the increase in soil available Ca, but the fact that the xylem sap Ca stomatal signal (concentration or delivery) does not change, as it does with bean. This does not however negate, with pea, that an unidentified antitranspirant is produced on soil liming. However, it is perhaps easier to speculate on what that putative pea signal might be rather than the means of establishing proof. It is well known that intracellular Ca is key component in the signalling pathway that leads to symbiosis with nitrogen-fixing bacteria (see reference within McAinsh and Pittman 2008). It is also well documented that Ca via changes in pH can influence the availability of many; particularly trace metals in the soil (see Tyler and Olsson 2001). Here liming could be seen as factor with a stronger case for removing a positive stomatal opening signal, however, given that soil liming is well documented, for example, for reducing the availability and crop uptake of a number of metals such as Cd, Cu, Ni, Al and Zn this notion seems a rather unlikely explanation of the observed response for pea (Bolan et al 2003). The case for indirect phosphate-induced changes in stomata conductance and growth may not be obvious, but could have some relevance here (Murrmann and Peach 1969, Haynes 1982). For example, a high soil Al content, on liming, can initiate a reduction in available phosphate [which can occur with soils high in Ca] (Vickers and Zak 1978, Sumner 1979). Phosphorus deficiency can induce a decline in stomatal conductance, albeit only at low water potentials and the presence of increased ABA (Radin 1984, Jeschke et al 1997). Similarly, with salt stress, increasing root available Ca can overcome the influence salinity on water uptake (Cabanero et al 2004). Again, with the work of Rothwell and Dodd (2014) it is highly unlikely that the field soil used was highly weathered; that it had a high Al content; there was a deficiency in P availability, or that water deficits were responsible for stomatal closure, and this occurred via a root-derived antitranspirant. Hopefully, the work of Rothwell and Dodd (2014) might stimulate opportunities for revisiting crop Ca management and perhaps shifting our focus towards understanding more about what is going on below ground and the mechanism(s) of how pea achieves its regulation of shoot Ca delivery and the possible involvement of a putative novel antitranspirant. More recent novel approaches undertaken by Metzner et al. (2010) suggest that ion exchange capacity of stem and their parenchymal tissues has may not have been fully appreciated as Ca sources/contributors to xylem sap Ca homeostasis. The structural differences between *Phaseolus* and *Pisum* stems may also have functional effective differences in their capacity to maintain Ca homeostasis in the transpiration stream.

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308 References

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