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1 **Commentary Plant and Soil 2014**

2

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

5

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8

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17

18 **Abstract**

19 Understanding the regulation of calcium uptake, xylem transport and its impacts on growth and leaf  
20 gas exchange is a subject that has received insufficient recent attention. Calcium (Ca) is unique  
21 within the group of key elements required for plant growth in that it also has a role in cellular  
22 signalling via regulation of changes in its cytoplasmic concentration. Its mobility, within the plant, is  
23 however somewhat constricted by its chemistry and cellular signalling role, and its adsorptive  
24 capacity within the aopoplast and the xylem. Supply and demand for Ca is achieved by a homeostatic  
25 balance which if perturbed can cause a number of distinctive physiological conditions, often related  
26 to Ca deficiency. In this issue Rothwell and Dodd present experiments with bean (*Phaseolus vulgaris*)  
27 and pea (*Pisum sativum*) plants grown in a field soil exposed to the processes of soil liming  
28 (application of Ca carbonate (CaCO<sub>3</sub>)). Given that there is evidence of free Ca in the xylem sap  
29 altering stomatal conductance it is reasonable to ask the question does liming elevate Ca in the  
30 transpiration stream which may explain the observed reduced growth which they hypothesise is due  
31 to Ca-induced stomatal closure. They show that liming doubled soil exchangeable Ca, reduced  
32 stomatal conductance and shoot biomass in both species compared with unlimed controls.  
33 However, xylem sap Ca concentration increased only in bean. Interestingly, the same was not true  
34 for the pea where the root xylem sap concentration remained unchanged despite an increase in soil

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

39

## 40 **Commentary**

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### 42 *Calcium a multitasking element*

43

44 Trying to understand how Ca ions ( $\text{Ca}^{2+}$ ) move within the transpiration stream is a problem that has  
45 received various levels of attention over many years. The processes which determine the flux and  
46 distribution of ions from roots can have a particular importance in determining development,  
47 growth and the physiological performance of the shoot (Gilroy et al 1993, White and Broadley 2003,  
48 Karley and White 2009, Gilliham et al 2011, Hawkesford et al 2012). The flux of Ca ions, for example,  
49 within the xylem and its delivery to aboveground organs, in tomato (*Lycopersicon esculentum*) fruits,  
50 is critical in determining pericarp development and the production of commercially acceptable fruits  
51 (Guichard et al 2001, Suzuki et al 2003). The supply of  $\text{Ca}^{2+}$  to shoot apices can alter cell division and  
52 expansion by influencing cell and vacuole osmotic content and cell wall formation (Hawkesford et al  
53 2012). Calcium ions also aid in maintaining cellular stability and membrane integrity and are involved  
54 in stress perception signalling response cascades (Suzuki et al 2003, White and Broadley 2003,  
55 McAinsh and Pittman 2008, Kudla et al 2010), and more recently have been shown to have a  
56 regulatory function within the nucleus (Mazars et al 2009). The role of Ca in generating changes in  
57 stomatal aperture is also well recognised (Mansfield et al 1990) and Ca flux in the xylem has been  
58 implicated as a regulator of transpiration (Atkinson et al 1989, 1992, Atkinson 1991). The dual role of  
59  $\text{Ca}^{2+}$  in providing a nutritional substrate required for growth, as well as, acting in a quantitative  
60 signalling response element appears paradoxical, but this duality is achieved through tight  
61 cytoplasmic regulation of Ca concentration and sub-cellular partitioning of Ca to vacuoles and in  
62 some cases specific cell types (idioblasts) which store insoluble Ca salts [e.g. Ca oxalate] as well as  
63 the apoplast (Hirschi 2004, Volk et al 2008, Franceschi and Nakata 2005, Helper 2005, He et al 2011,  
64 Gilliham et al 2011). While clearly cytoplasmic Ca status is at the core of a number of specific stress  
65 induced Ca signalling systems cascades further elucidation of these biochemical and molecular  
66 events should facilitate knowledge on how to manipulate these processes (Nakata and McConn  
67 2007, McAinsh and Pittman 2008, Kudla et al 2010, Dodd et al 2010). This may be particularly  
68 relevant for practical crop strategies designed to reduce food waste by increasing shelf-life (see  
69 suggestions of Park et al 2005).

70

71 *Mobility, partitioning and homeostasis*

72

73 The mobility of  $\text{Ca}^{2+}$  in the plant is known to be low. Many species, but not all, have suberized cell  
74 walls within the Casparian band of the root system which restricts radial water and apoplastic solute  
75 movement into, and out, of the root stele (Clarkson 1984, Moore et al 2002). Solutes, like Ca, are in  
76 essence forced into a passage involving the cytoplasm, plasmodesmata and aquaporins, which has  
77 both challenges and consequences with respect to the achievable rate of cellular Ca flux. The  
78 potential limitations in symplastic cell to cell diffusion of Ca requires that the supply of free Ca is  
79 maintained but cytoplasmic concentrations are kept at  $\mu\text{M}$  levels to avoid precipitation of Ca  
80 phosphates and cell death. Therefore the entry of Ca into the cytoplasm has to be as tightly  
81 controlled as does its cytosolic removal (White and Broadley 2003, Gilliham et al 2011). We are now  
82 beginning to acquire a molecular understanding of the regulation of membrane transporters which  
83 determine Ca partitioning at the cellular level (de Freitas et al 2011). Subsequent movement of Ca  
84 within the apoplast and the xylem is slowed due to its divalent ability to bond (cation ion exchange  
85 capacity CEC), for example, with anionic charges on substances such as pectates, phospholipids and  
86 carboxyl groups in cell membranes and walls (Ferguson and Bollard 1976, White 2001, see also the  
87 references within Gilliham et al 2011). This process is described by the isotopic data recorded for  
88 calcium and magnesium exchange with the surrounding tissues as sap moves up the xylem (Metzner  
89 et al 2010). Limited mobility of Ca is a unique characteristic among the key plant nutritional  
90 elements required for growth. It can lead to the irreversible binding of  $\text{Ca}^{2+}$  (and other cations) to  
91 the negatively charged inner surfaces of functional xylem cells, retarding the rate of ion distribution.  
92 While limitations in the rate of cytoplasmic movement support suggestions that  $\text{Ca}^{2+}$  show little  
93 redistribution (phloem-fed tissues) over any significant distance within the plant (Karley and White  
94 2009). Upon xylem delivery, whether initially or subsequently when in the cytoplasm it appears that  
95 Ca sequestration predominates; appearing extra-cellularly in the apoplast (He et al 2012), or vacuole  
96 (as salts of phosphoric, oxalic or phytic acids) within idioblasts, or extra cellular (Webb 1999,  
97 Franceschi and Nakata 2005, Volk et al 2008, Hawkesford et al 2012). Despite clear increases in  
98 insoluble Ca in leaves in response to increased Ca supply, the relationship between Ca supply and  
99 oxalate formation and the different forms of Ca oxalate (soluble and insoluble) does not appear  
100 simple and may show leaf ontogenic change (Zindler-Frank et al 2001). It is equally apparent that not  
101 all species show Ca sequestration which is based on, either Ca oxalate, or even the formation of  
102 insoluble Ca salts (see Hawkesford et al 2012).

103

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

117

#### 118 *Supply and demand*

119

120 Breakdown, or limitations, in the xylem supply of  $\text{Ca}^{2+}$ , can have particularly important and dynamic  
121 consequences on the growth rate of rapidly expanding tissues. The condition known as blossom end  
122 rot (BER) is just one of several Ca deficiency derived physiological conditions seen in fruits such as  
123 tomato (Bangerth 1979, Adams and Ho 1992, White and Broadley 2003, Ho and White 2005, Karley  
124 and White 2009). It is also often the case that the tissues most at risk from suffering an imbalance in  
125 their Ca supply and demand are those where transpiration rates are generally lower than other  
126 competing aboveground organs. Inferences such as this lead to suggestions that it is the  
127 transpiration rate that is a critical determinant in the quantitative delivery of  $\text{Ca}^{2+}$  to the shoot, along  
128 with control over the proportional allocation of  $\text{Ca}^{2+}$  to various organs and tissues, because these  
129 tissues have different transpiration rates (Karley and White 2009), e.g. the low transpiration of inner  
130 leafy rosette regions of many of the Brassica family. Increasing leaf sap flow artificially through the  
131 application of ABA can increase Ca movement which reduces the incidence of BER (de Freitas et al  
132 2014). There is however evidence that transpirational water movement is not always a universal  
133 determinant of Ca movement. In some cases it appears that water transport and Ca movement  
134 become uncoupled and can explain the non-uniform distribution of Ca in some leaves (Atkinson  
135 1991, Kerton et al 2009, Metzner et al 2010). The notion that Ca allocation to plant organs is  
136 influenced by differences in transpiration rate is supported by the appearance, initially, of BER in  
137 specific tissues regions or organs associated with low transpiration (Ho and White 2005, de Freitas et

138 al 2014). What is less clear is a functional link between below average tissue concentrations of free  
139  $\text{Ca}^{2+}$  in BER expressing tissues relative to the total organs Ca content (Petersen and Willumsen 1992),  
140 combined with a lack of sequential evidence demonstrating BER cause and effect (Nonami et al.  
141 1995). Low transpiration rates, induced by decreases in the vapour pressure gradient (leaf to air),  
142 can be linked to Ca deficiency symptoms (Holder and Cockshull 1990, Kerton et al 2009). The  
143 consequences of this, at least with tomato, are that plasma membranes show distinctive signs of  
144 cellular Ca precipitation when grown under conditions known to induce BER (Suzuki et al 2003).  
145 These precipitates are located in parenchyma cells close to tracheids and the vascular bundles.  
146 While the transpiration rate *per se* may not directly influence the loading of Ca in the transpiration  
147 stream, the transpirational flux will determine the xylem sap concentration and in turn its shoot  
148 delivery rate as factor of loading rate multiplied by transpiration flux.

149  
150 It is apparent, commercially, that cellular Ca concentration has an important influence on fruit  
151 texture and the avoidance of disorders such as bitter pit in apple (Nielsen et al 2005). Despite the  
152 application of post-harvest 'remedial' treatment by dipping fruit (Ca cuticular entry and movement  
153 by apoplastic diffusion) in Ca based products to reduce the occurrence of bitter pit in-store,  
154 considerable attention is given to Ca supplementation during fruit growth. It is clear that this  
155 exogenous source of Ca is present within fruit tissues post-harvest, but there is also evidence that  
156 endogenous Ca uptake declines with fruit development. Many studies, but not all, imply that fruit Ca  
157 content is determined early in the growth cycle and once beyond the cell division phase Ca uptake  
158 can decline and the rapidly expanding fruit induces the cellular Ca concentration to decline (Quinlan  
159 1969, see review by Saure 2005). The explanation often proposed for this change in response to  
160 endogenous root-derived Ca is that the fruit xylem transport system becomes non-functional  
161 (Drazeta et al 2004). The consensus is that supplementary Ca sprays, to avoid deficiency during fruit  
162 growth, require application to the fruit (direct uptake via trichomes and stomata on the fruit  
163 epidermis) because of the absence of Ca transport from leaves. Saure (2005) suggests that the  
164 problem with xylem Ca delivery in fruit is not having to cope with deficiency in transport channels, or  
165 a weak the transpirational driving force, but overcoming the plant's need to limit Ca transport during  
166 rapid growth. Again, evidence from both post-harvest application of Ca and uptake during growth  
167 suggests that Ca movement within the fruit occurs, but differences in its measured Ca distribution  
168 occur primarily due to variation in cell growth patterns and utilisation within the fruit (see Saure  
169 2005). This type of variation in Ca partitioning can also be explained by cellular changes in the  
170 expression of a  $\text{Ca}^{2+}/\text{H}^{+}$  tonoplast transporter protein (CAX) (Conn et al 2011, de Freitas et al 2011).  
171 In tomato the sCAX1 transporter expressing phenotypes showed increased total fruit Ca and shelf-

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

177

#### 178 *Calcium soil supply*

179

180 The role that soil Ca status plays in defining the presences of 'indicator' species (calcifuges and  
181 calcicoles) within the landscape is a foundation stone in the development of ecophysiological  
182 approaches to mechanistically explain species distribution (Bradshaw et al 1958, 1960, Jefferies and  
183 Willis 1964, Rorison and Robinson 2006). The effectiveness of this approach has inspired an array of  
184 work based on expanding and illuminating how plants cope with varying levels of exposure to Ca,  
185 and the impacts of its salts, on many aspects of soil and plant performance (Kinzel 1983). With the  
186 finding that free  $\text{Ca}^{2+}$  are involved in the process of stomatal closure, it has become apparent that Ca  
187 may also have a role in influencing whole leaf gas exchange and that this might also be another  
188 chapter in the story of explaining species distribution with respect to variation in soil Ca  
189 concentrations (De Silva et al 1986, Mansfield et al 1990, Atkinson 1991). However, there has been  
190 little attempt to address the question, particularly in agricultural systems where the direct  
191 implications for crop management practices change soil available Ca rapidly, as occurs during  
192 remedial liming. It is therefore interesting to see in this issue that Rothwell and Dodd (2014) address  
193 the question of Ca inputs, via liming, having a direct impact on crop gas exchange. The positive  
194 growth and yield responses of field crops to the liming of acidic soil are very well documented (Tang  
195 et al 2003, Karaivazoglou et al 2007). It is more challenging to find studies which have recorded  
196 direct negative impacts of lime application in agricultural systems and crops which have been linked  
197 to the decrease or increase in the availability of other elements such Al, Zn, Mn, B and P (Vickers and  
198 Zak 1978, Sumner 1979, Kochian et al 2004), but more recently the focus has been on soil attributes,  
199 such as SOM, nitrogen mineralisation and changes in the microflora, and their impact on aspects of  
200 the crop, not the direct influence that  $\text{Ca}^{2+}$  uptake has on the plant (Haynes and Naidu 1998, Kemmit  
201 et al 2006, Fageria and Baligar 2008). Rothwell and Dodd (2014) address the question; does liming  
202 elevate xylem sap Ca which limits gas exchange by inducing partial stomatal closure and potentially  
203 reduces yields. A positive answer to this question has important implications for liming impacts on  
204 crop productivity as managing soil pH is a vital component, in acid soil, which occurs globally over a  
205 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  $3 \text{ g l}^{-1}$  as Ca carbonate ( $\text{CaCO}_3$ ). 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  $\text{Ca}^{2+}$  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

239 Using this approach Rothwell and Dodd (2014) showed that compared with unlimed controls, liming  
240 reduced shoot biomass in both bean and pea. There are other studies which show this negative  
241 response and it is interesting that in these cases it is also a leguminous species, e.g. crown vetch  
242 (*Coronilla varia* L.) and alfalfa (*Medicago sativa* L.) and sorghum (*Sorghum sudanense*) respectively  
243 (Vickers and Zak 1978, Sumner 1979). The reduction in biomass corresponded with a significant  
244 reduction in stomatal conductance and assimilation for both species. Liming itself doubled soil  
245 exchangeable Ca which led to a massive increase in xylem sap Ca concentration from 0.9 to 1.7 mM,  
246 but only for bean. Interestingly, for pea, root and leaf Ca concentrations remained unchanged  
247 despite an increase in soil available Ca. Having collected xylem sap samples in an appropriate  
248 manner Rothwell and Dodd (2014) were able to show how an increase in Ca delivery rate was  
249 apparent, on liming, with bean, but not with pea. In fact with pea, Ca delivery declined, most likely  
250 due to the observed reduction in stomatal conductance restricting transpiration. The authors  
251 conclude that there are species differences in their ability to regulate Ca uptake and delivery to the  
252 shoot irrespective of the initial differences within the soil. This very much supports earlier  
253 suggestions about these species (Atkinson et al 1992). What is interesting and novel about Rothwell  
254 and Dodd (2014) is the suggestion of why stomatal conductance declined in pea in the absence of  
255 elevated xylem sap Ca. They propose two possible explanations; the first is that stomatal sensitivity  
256 to Ca shows species differences, while the second suggests that perhaps the correlative link implied  
257 between bean xylem sap Ca concentration and stomatal conductance was not causative. They rule  
258 out the differential species sensitivity by showing similar species responses to artificial Ca supply in a  
259 detached leaf transpiration assay. It would be interesting to repeat this sensitivity experiment with  
260 plants known to respond to lime induced reductions in stomatal conductance using intact attached  
261 leaves. Catheter-type applications of a putative stomatal conductance regulator (Ca and ABA) can  
262 be effectively introduced into the xylem stream to induce dynamic changes in sap constituents and  
263 corresponding reductions in stomatal conductance (Atkinson et al 1990). This 'topical' application of  
264 Ca into the leaf mid-rib allows little more than the xylem Ca stream concentration to change for a  
265 well-watered leaf. It might, if the Ca were sourced from the CaCO<sub>3</sub> used in the liming treatments to  
266 rule out any possible other stomatal closing factors, but such a component naturally occurring seems  
267 unlikely. We would expect with agricultural lime in this case "coarse screened limestone" for there  
268 to be MgO also present but at >15% ([www.aglime.org.uk](http://www.aglime.org.uk)). They rule out the possibility of inaccuracy  
269 in the measurements of ions within the xylem sap, I think correctly, based on the methodology used.  
270 They also consider the possibility of concluding that it may well be an alternative substance rather  
271 than Ca<sup>2+</sup> in the xylem stream that cause stomatal closure in bean. This is an interesting hypothesis  
272 which Rothwell and Dodd leave us to think about. It could be considered from another perspective,



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

303

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307

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