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Calcium in the Xylem and Its Influence on the Behaviour of Stomata

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Phil. Trans. R. Soc. Lond. B 1993 **341**, 67-74
doi: 10.1098/rstb.1993.0092

Calcium in the xylem and its influence on the behaviour of stomata

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SUMMARY

Calcium is known to play an important role in regulating guard cell turgor and the movements of stomata. The concentrations of calcium ions in xylem sap are often higher than 1 mol m^{-3}, which would be sufficient to influence, or interfere with, stomatal function if such concentrations were delivered to points of evaporation in the vicinity of the guard cells.

This paper presents some recent experimental evidence concerning the way in which a plant's calcium status affects the amount of free calcium in the xylem, and the effect this may have on the diurnal pattern of stomatal behaviour. Changes in the rhizospheric supply of calcium have a major influence on the concentrations in the xylem. In *Commelina communis* an eightfold increase in rhizospheric calcium led to an increase in the xylem sap in the shoot of approximately sixfold. Very high concentrations of xylem calcium were associated with reduced stomatal opening, and injection of a pulse of calcium ions into the xylem via a catheter caused stomatal closure. Calcium-induced suppression of stomatal aperture does not inflict permanent damage upon the guard cells, because stomatal aperture recovered quickly when the calcium concentration in the xylem was reduced.

The experimental data presented suggest that the amount of calcium delivered by the transpiration stream to points of evaporation needs to be regulated if interference with stomatal behaviour is to be avoided. This regulation is likely to occur in tissues such as the mesophyll. The roots may also play an important part in controlling the delivery of calcium into the xylem and evidence is presented of malfunctioning of the regulatory mechanism in roots when plants are exposed to high calcium in the rhizosphere.

Some of the data presented are for a calcifuge, *Lupinus luteus*, and the possibility is discussed that disturbances in stomatal behaviour contribute to the physiological problems of such plants in the presence of high rhizospheric calcium.

1. INTRODUCTION

It was recognized many years ago that calcium might play a part in the functioning of stomata (Iljin 1957). It is, however, only over the past 8 years that the role of calcium ions in the processes leading to stomatal closure has been clearly demonstrated. It is now known that increases in cytoplasmic calcium within the guard cells precede stomatal closure when it is induced by abscisic acid (McAinsh *et al.* 1990; Schroeder & Hagiwara 1989, 1990), and an apparent requirement for calcium when some other agents such as darkness cause stomatal closure (Schwartz 1985) suggests that it may be of more general significance.

Experiments with pieces of epidermal tissue isolated from the rest of the leaf enable the ionic content of the apoplast around the guard cells to be manipulated. Mature guard cells have no plasmodesmatal connections with their neighbours, and consequently the ionic content of the medium used for incubating the epidermis can be assumed to be that in contact with the guard cell protoplasts. When the calcium concentration in the medium is changed there are large effects on stomatal aperture (figure 1). These are almost certainly the result of changes in turgor of the guard cells, rather than that of the surrounding cells, because the volume of totally isolated guard cell protoplasts is similarly affected (figure 1).

The large inhibition of stomatal opening by 0.5 mol m^{-3} calcium raises important questions about the destination of calcium ions that are delivered to leaves via the transpiration stream. Concentrations of total calcium in xylem sap are highly variable between species but generally exceed 0.5 mol m^{-3}, and they are often much greater than this (Atkinson *et al.* 1992). For example, in xylem exudation from tomato, Armstrong & Kirkby (1979) found calcium concentrations between 5 and 7 mol m^{-3}.

Crowdy & Tanton (1970) and Tanton & Crowdy (1972) applied lead-ethylenediaminetetra-acetic acid chelate to detached shoots to discover how water in

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Phil. Trans. R. Soc. Lond. B (1993) **341,** 67–74
Printed in Great Britain

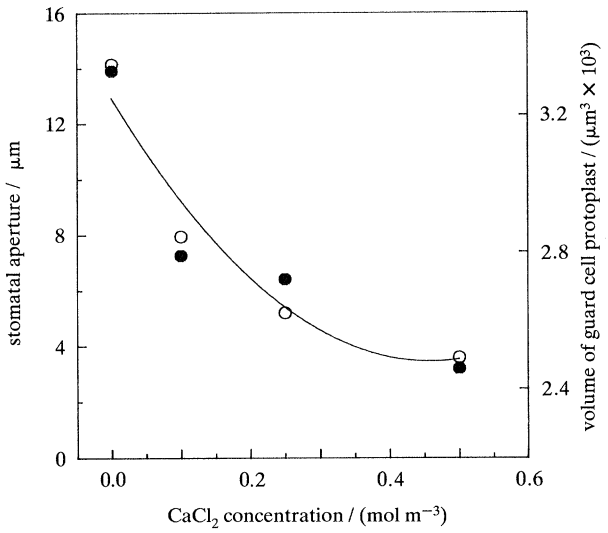

Figure 1. Inhibition of stomatal opening (open circles) and reduction in guard cell protoplast volume (filled circles) of *Commelina communis* L. by calcium ions. The curve was fitted using a second degree polynomial equation. Based on data of De Silva *et al.* (1985) and Smith & Willmer (1988).

the transpiration stream is distributed after it enters leaves. They found that the lead chelate accumulated in and around the guard cells, apparently identifying them as major points of evaporation. This suggested that solutes in the transpiration stream would accumulate in the vicinity of the stomata unless there were some regulatory process (e.g. uptake into cells adjacent to the pathway of water movement) to prevent their reaching those points of evaporation. The dangers of false deductions from the distribution of tracers are discussed elsewhere in this volume by Canny. It is nevertheless important to explore what controls exist over the transport of calcium ions towards the stomata.

We have conducted some experiments to address two important questions:

(1) Is calcium in the transpiration stream transported in sufficient quantities to the environs of the stomata to play a part in regulating guard cell turgor (or, if it arrives in excess, to interfere with stomatal operation)?

(2) Are there effective mechanisms within leaves for regulating the amounts of calcium being delivered to the apoplasts of the guard cells?

2. CALCIUM AND STOMATAL BEHAVIOUR OF *TRITICUM AESTIVUM* AND *COMMELINA COMMUNIS*

A preliminary experiment was performed with whole plants of *Commelina communis* grown in Long Ashton nutrient solution culture with two different rhizospheric concentrations of calcium, namely 2 and 8 mol m^{-3}. This led to plants with widely different calcium concentrations in the xylem sap and the abaxial and adaxial epidermal tissues (table 1). The concentration of 1.75 mol m^{-3} in the xylem sap of the calcium-rich plants would be sufficient to inhibit stomatal opening strongly if it were delivered directly into the apoplast adjacent to the guard cells, judging from the dose–response relationship in figure 1. On the contrary, however, the total leaf conductance was found to be markedly higher in the plants grown with the greater concentration of calcium (table 1).

These results could be interpreted as indicating that stomatal aperture is not suppressed by a higher concentration of calcium in the xylem, but we have performed other experiments suggesting that calcium in the transpiration stream can inhibit stomatal opening if the concentration is high enough. Figure 2 shows the rates of transpiration from detached leaves of wheat (*Triticum aestivum*) that had been placed with their cut ends in water or three different concentrations of $Ca(NO_3)_2$. The excision of the leaf induced some stomatal closure that was expressed as a declining transpiration rate over a 7 h period in the controls, but the inhibitory effect of calcium can be clearly seen over and above this. It was also found that if 8 mol m^{-3} $Ca(NO_3)_2$ was introduced into the xylem of intact leaves using a very fine hypodermic needle carefully placed into the mid-vein, stomatal closure was quickly induced (figure 3). Gas exchange was always measured for some time prior to the start of a feed and the solution flow was started prior to insertion. When only distilled water was introduced, there was no significant effect on any of the gas exchange parameters measured (data not shown).

In view of the data in figure 2, and based on the

Table 1. *Calcium concentrations found in the xylem sap and in the abaxial or adaxial epidermal tissues, and leaf conductance of* Commelina communis *L. plants grown with 2 and 8 mol* m^{-3} *calcium*

(Values are means of six replicates for xylem sap and four replicates for epidermal tissue ± s.e. Asterisk shows significant differences at $p < 0.05$ between treatments based on Student's '*t*' test. Data obtained from Atkinson (1991*a*).)

calcium concentration					
applied to rhizosphere	xylem sap	epidermal tissue		epidermal tissue calcium: abaxial to adaxial ratio	leaf conductance
		adaxial	abaxial		
(mol m^{-3})	(mol m^{-3})	(μmol g^{-1} dry mass)			(mmol m^{-2} s^{-1})
2.0	0.63 ± 0.04	69.2 ± 2.9	65.8 ± 4.3 (ns)	0.96 ± 0.09	265 ± 26
8.0	1.75 ± 0.22	104.5 ± 13.1	127.6 ± 16.4 (ns)	1.22 ± 0.08	429 ± 22
	*	*	*	ns	

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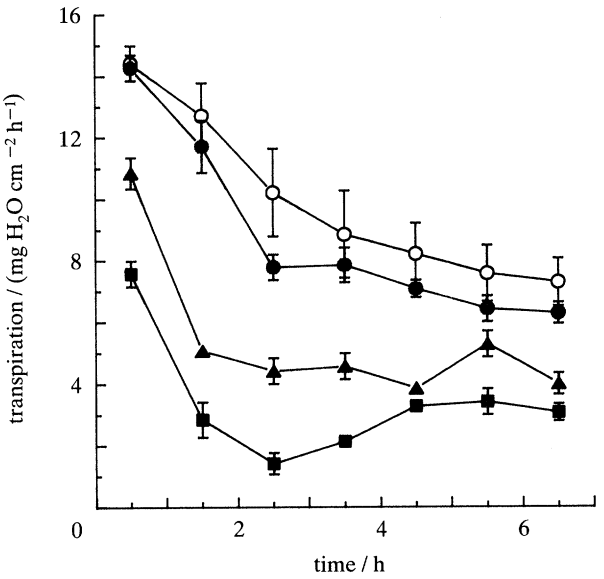

Figure 2. Transpiration rates of detached leaves of *Triticum aestivum* cv Wembley determined gravimetrically over 7 h. Individual leaves were placed in distilled water (open circles), 4 (filled circles), 8 (filled triangles) or 16 mol m^{-3} Ca (filled squares). Values are means of five replicates ± s.e. Data obtained from Atkinson *et al.* (1990).

concentrations of calcium that have been found in the soil solution (Epstein 1972), we performed an experiment on *Commelina communis* using a wider range of rhizospheric calcium concentrations than those in the study that produced the data in table 1. Seedlings were raised in vermiculite in a controlled environment room at a temperature of 27° ± 0.5°C with an average photon flux density of 275 μmol m^{-2} s^{-1}. They were supplied for 1 week with a full strength modified Hoagland's nutrient solution (Epstein 1972) and then divided into three groups receiving the same nutrient

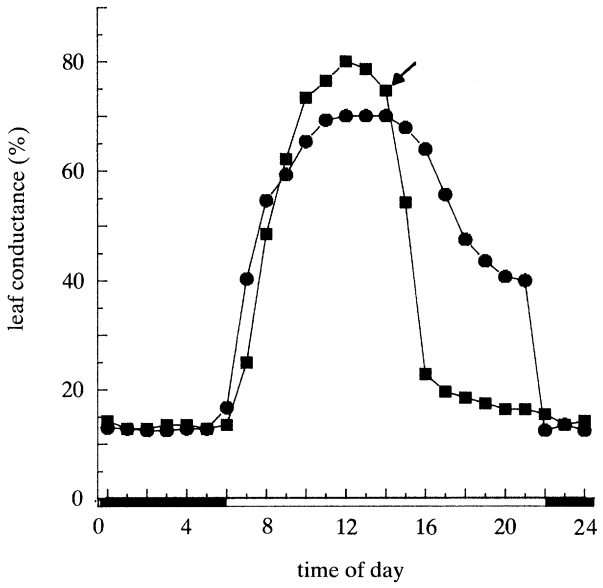

Figure 3. Diurnal leaf conductance measured with a viscous flow porometer in *Triticum aestivum* plants, on day 1 (circles) and on day 2 (squares). The arrow shows the time on day two when the leaf was fed with 8 mol m^{-3} $Ca(NO_3)_2$ via a xylem catheter. Data obtained from Atkinson *et al.* (1990).

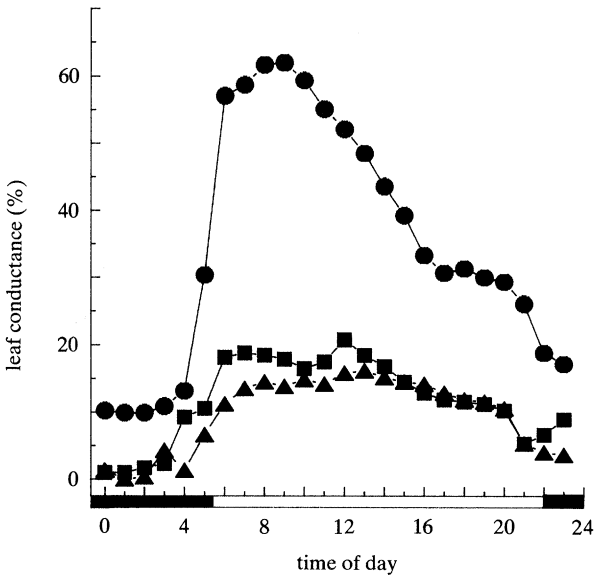

Figure 4. Diurnal leaf conductance measured by viscous flow porometry in *Commelina communis* L. plants grown with 2 (triangles), 4 (circles) and 15 (squares) mol m^{-3} Ca. Values are means of six replicates ± s.e.

solution but within which the calcium concentration was altered to be 2, 4 or 15 mol m^{-3}. The diurnal pattern of stomatal behaviour was measured using a six-channel viscous flow porometer (Atkinson *et al.* 1990). This instrument applies air under slight pressure to one leaf surface (in this case the abaxial) and enables the resistance or conductance to viscous flow (through the leaf and out via the other surface) to be determined. The data obtained depend on stomatal apertures on both adaxial and abaxial epidermes. The diurnal patterns of stomatal behaviour are shown in figure 4, and it is clear that a high rhizospheric calcium concentration can be supraoptimal for this species. The increase in stomatal aperture from 2 to 4 mol m^{-3} calcium is consistent with earlier observations (Atkinson 1991*a*), and it can be suggested that a change of this magnitude is beneficial to the plant in terms of calcium nutrition. The much higher rhizospheric concentration, on the other hand, is clearly capable of inhibiting stomatal opening.

Xylem sap was collected from the shoots of plants of *Commelina communis* that had been raised with different rhizospheric calcium concentrations as described above. Excised shoots were inserted into a Scholander-type pressure bomb, and 0.3 to 0.4 MPa was applied in excess of the balance pressure in order to produce a flow of xylem sap from the cut end. Sap was collected for 3 min, and was then placed in 1 cm^3 plastic vials and frozen, and determinations of free calcium concentrations were made subsequently (Atkinson *et al.* 1992). The data in figure 5 show a clear relationship between the amount of calcium available in the rhizosphere and in the xylem sap.

To determine whether calcium at the concentrations found in xylem sap might be sufficient to interfere with stomatal behaviour, the sap concentrations found in plants grown at 4 and 15 mol m^{-3} calcium (figure 5) were applied to isolated epidermis

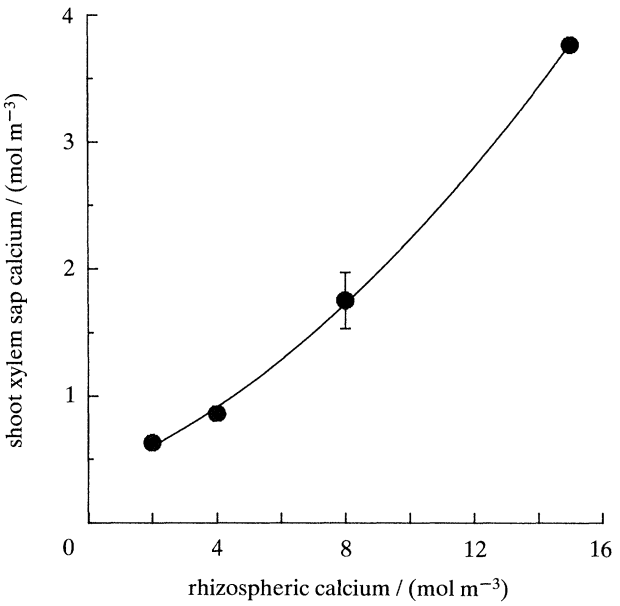

Figure 5. Calcium concentration applied in the rhizosphere of *Commelina communis* L. plants and its relationship with the calcium concentration found in the shoot xylem sap. Values are means of six replicates ± s.e. The curve was fitted using a second degree polynomial equation.

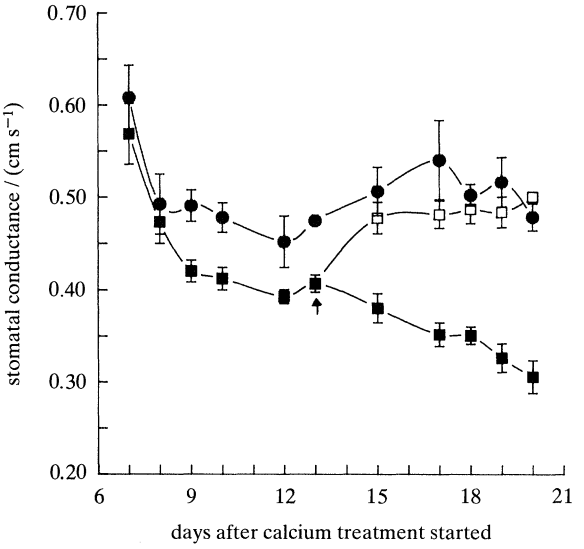

Figure 7. Abaxial stomatal conductance measured with a diffusion porometer for *Commelina communis* L. watered with 4 (filled circles or open squares) and 15 (filled squares) mol m^{-3} Ca. The arrow and the hollow squares show when low calcium was supplied to half of the plants previously grown with high calcium. Values are means of 20 replicates ± s.e.

taken from the same plants. Twenty seven days after the two rhizospheric calcium regimes were applied, strips of abaxial epidermis were removed from the youngest, fully expanded leaves. They were cut into 5 × 10 mm pieces and placed in 10 mol m^{-3} MES (2-[N-morpholino] ethane sulphonic acid) buffer with 50 mol m^{-3} KCl and adjusted to pH 6.15 with KOH. They were incubated for 3 h at 25°C under a photon flux density of 280 μmol m^{-2} s^{-1} under CO_2-free conditions, and then stomatal apertures were measured under the microscope. The results are shown in figure 6 and it will be seen that in both groups of plants stomatal opening was reduced by the concentration of calcium that had been found in the xylem of those plants.

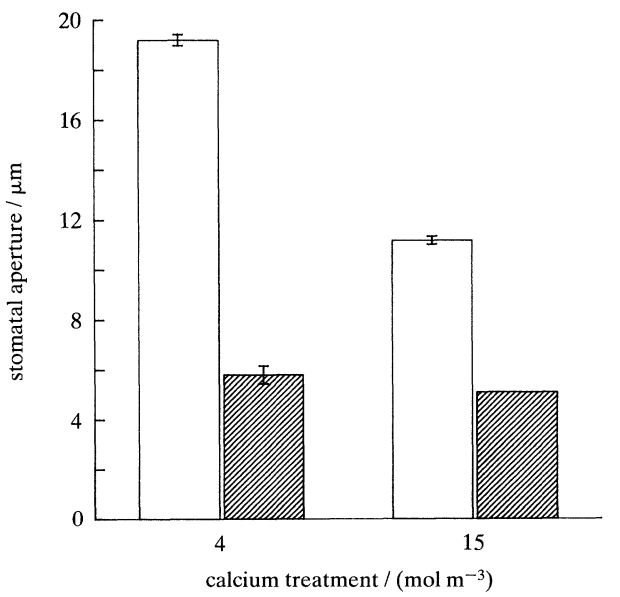

Figure 6. Stomatal aperture of epidermal strips incubated in MES buffer (open bars) or in MES buffer with two concentrations of calcium (shaded bars). The epidermal strips were from *Commelina communis* L. plants grown in 4 or 15 mol m^{-3} Ca. The concentrations of calcium used in the incubation medium were: 0.87 or 3.76 mol m^{-3} Ca for the plants grown with 4 or 15 mol m^{-3} Ca respectively. Values are means of 60 replicates ± s.e.

It was important to determine whether the higher concentrations of calcium in the xylem of plants with calcium-rich rhizospheres exert long-term effects on stomatal apertures. Plants of *Commelina communis* were grown for 13 days with rhizospheric calcium concentrations of 4 or 15 mol m^{-3}, by which time the stomatal conductance on the abaxial surfaces of the leaves of the high calcium plants was reduced by about 15% (figure 7). At this stage, the calcium supply to some of the high calcium plants was reduced from 15 to 4 mol m^{-3} and stomatal conductance was almost fully restored within two days. There was no evidence of any after-effect of the high calcium treatment on stomatal conductance (figure 7).

As a result of these experiments we have concluded:

1. If plants of *Commelina communis* are grown in a calcium rich medium, the concentration of the free calcium in the transpiration stream would be sufficient to suppress stomatal opening if it arrived undiluted in the vicinity of the stomatal guard cells.
2. The concentration of calcium in the transpiration stream is not the same as the concentration of rhizospheric calcium (figure 5).
3. High concentrations of calcium in the transpiration stream can suppress stomatal aperture but there is a rapid recovery upon a return to lower concentrations.

We can also speculate that regulation of the amount

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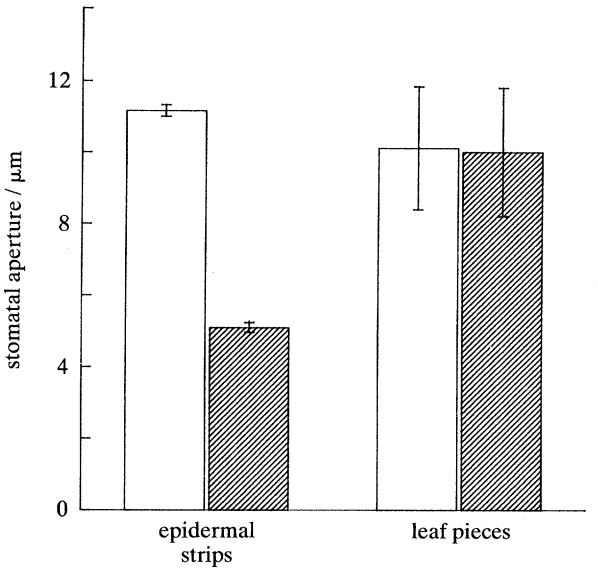

Figure 8. Stomatal aperture in epidermal strips and leaf pieces of *Commelina communis* L. plants grown with 15 mol m^{-3} Ca and incubated either with MES buffer (open bars) or with MES buffer + 3.76 mol m^{-3} Ca (shaded bars), which is the calcium concentration found in the xylem sap of these plants. Values are means of 30 replicates ± s.e.

of calcium delivered by the transpiration stream to the vicinity of the guard cells is of importance in relation to normal stomatal functioning. In plants of *Commelina communis* the capacity of any regulatory mechanism can clearly be exceeded when the rhizospheric calcium concentration is as high as 15 mol m^{-3}.

In an attempt to find evidence of the existence of a mechanism for regulating the access of calcium to the guard cells of *Commelina communis* plants grown with 15 mol m^{-3} calcium, we compared the effect of 3.76 mol m^{-3} calcium (the concentration measured in the xylem sap of these plants) on isolated epidermis and on pieces of leaf with all the tissues intact. Pieces 5 × 20 mm were cut, avoiding the main central vein, from the youngest fully expanded leaves, and were incubated abaxial surface uppermost in an assay solution of the same composition as that used for isolated epidermis. After the 3 h period of incubation the abaxial epidermis was detached and stomatal opening was measured under the microscope. The results in figure 8 show that there was no detectable effect of the calcium on stomata of the leaf pieces, whereas there was substantial inhibition of opening in the isolated epidermis. This suggests that the other leaf tissues, principally the mesophyll, are capable of removing calcium ions and thus regulating the concentration around the stomatal complexes. In this experiment the flux of calcium into the tissues was unlikely to be as high as that in a transpiring plant (figure 4), i.e. the regulatory capacity was not exceeded as it can be in the intact plant.

3. CALCIUM AND STOMATAL BEHAVIOUR IN A CALCIFUGE, YELLOW LUPIN

The poor performance of calcifuges in the presence of high concentrations of rhizospheric calcium is well known, but the nature of the physiological malfunctioning caused by excess of calcium has not been satisfactorily explained. The data described above for *Commelina communis* suggest that a species with a limited ability to regulate the rate of delivery of calcium from the xylem into the epidermis might display a decline in stomatal conductance during exposure to high rhizospheric calcium. If this were sufficient to reduce photosynthetic carbon gain, then growth and survival would be affected.

We have begun a detailed study of yellow lupin (*Lupinus luteus* L., wild-type), a calcifuge that has been used in previous studies in stomatal behaviour. Lancaster *et al.* (1977) reported that the stomata of a cultivar of this species (Weiko III) are insensitive to abscisic acid (ABA). The mode of action of ABA at the cellular level involves changes in cytosolic calcium in guard cells (Mansfield *et al.* 1990; McAinsh *et al.* 1992), and an excess of calcium in and around the guard cells might well disturb their ability to show a normal response to ABA. We placed detached leaves of *Lupinus luteus* in either water or ABA solutions of different concentrations, and found that there was a substantial response to ABA comparable to that which is familiar in many other species (figure 9). The plants that provided the leaves used in this experiment had been grown in peat moss with a low calcium content, but there was no evidence of a reduced response to ABA even when plants were grown with abundant calcium (Atkinson 1991*b*). We have thus not been able to confirm the findings of Lancaster *et al.* (1977) and have concluded that the lack of response to ABA may have been a peculiarity of the cultivar they were using.

Having ruled out a disturbance in ABA responses as a cause of the calcifuge habit of this species, we have explored other aspects of its stomatal physiology in detail. Our first studies with yellow lupin used rhizospheric calcium concentrations up to 8 mol m^{-3}

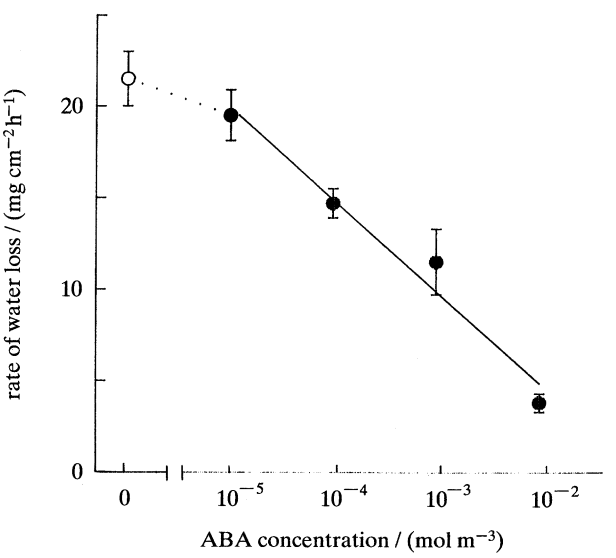

Figure 9. Effect of distilled water (open circles) or different concentrations of ABA (filled circles) on the rate of water loss of detached leaves of *Lupinus luteus* L. Data obtained from Atkinson (1991*b*).

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Table 2. *Final length and leaf extension rates ± s.e. of* Lupinus luteus *L. grown with different rhizospheric concentrations of calcium*

(Values are means of ten replicates ± s.e.)

	leaf no. 3		youngest leaf	
calcium treatment	final length of leaf	rate of leaf extension	final length of leaf	rate of leaf extension
(mol m^{-3})	cm	(mm d^{-1})	cm	(mm d^{-1})
1	3.7 ± 0.1	2.7 ± 0.1	3.7 ± 0.1	3.2 ± 0.2
10	3.4 ± 0.2	2.5 ± 0.1	2.9 ± 0.2	2.5 ± 0.2
15	3.3 ± 0.1	2.4 ± 0.1	2.9 ± 0.1	2.2 ± 0.2

(Atkinson 1991*b*). This was considered to be a high concentration for a calcifuge but it did not induce stomatal closure compared with plants grown at 1 mol m^{-3} calcium (Atkinson 1991*b*). However, when measurements of gas exchange were made over a range of atmospheric water vapour deficits, the coupling between assimilation and stomatal conductance was less clear with plants grown with 8 mol m^{-3} compared with 1 mol m^{-3} calcium. The consequence in terms of the regulation of leaf conductance was to perturb water use efficiency (the amount of carbon gained per unit of water transpired).

We have recently completed some further studies with *Lupinus luteus* and have found that somewhat higher concentrations of calcium in the rhizosphere are necessary before marked inhibitory effects on growth are seen. Plants were grown with 1, 10 and 15 mol m^{-3} calcium, and measurements were made of rates of leaf extension and of the final dimensions of fully expanded leaves. The data in table 2 show that 10 and 15 mol m^{-3} calcium reduced the length of the youngest fully expanded leaves by about 20%. The plants grown with the highest concentration of calcium were noticeably smaller in overall dimensions than those grown with 1 mol m^{-3} calcium, but the plants did not display other signs of injury, e.g. necrosis or yellowing. Stomatal opening was, however, found to be greatly reduced. Figure 10 shows the diurnal course of stomatal behaviour for plants grown with 1 and 15 mol m^{-3} calcium. There was a considerable depression in the maximum conductance attained amounting to nearly 71% in the arbitrary units of the viscous flow porometer. An additional series of measurements on the adaxial leaf surfaces using a diffusion porometer showed a 44% reduction in diffusive conductance in the high-calcium plants, and the transpiration rates of detached leaves were reduced by about 40% (figure 11). The most striking contrast between the high and low calcium treatments was, however, found when leaves were detached from plants that had been subjected to a mild water deficit. Water was withheld for 3 days and by this stage water potential had not declined perceptibly, but when leaves were detached and transpiration rate was determined gravimetrically, there were large differences in the response to water deficit between the two calcium treatments. The leaves from plants grown with 1 mol m^{-3} calcium showed no significant effects of the previously experienced water deficit, but those from plants grown with 15 mol m^{-3} calcium showed a very substantial change, suggesting that the stomata had become hypersensitive to mild water deficit (figure 11).

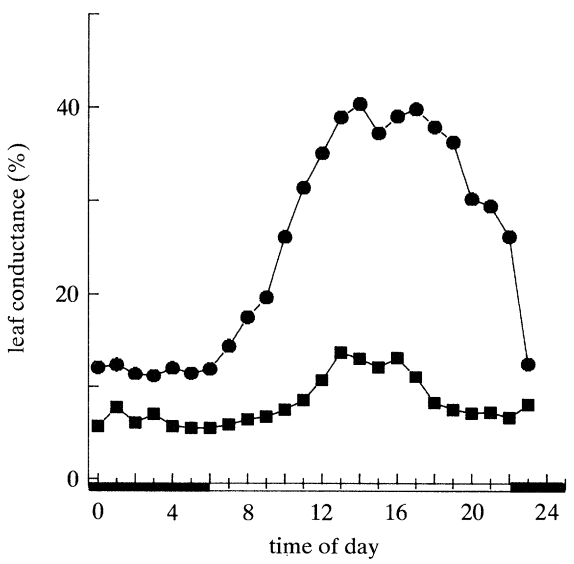

Figure 10. Diurnal leaf conductance measured with a viscous flow porometer in *Lupinus luteus* L. plants grown with 1 (circles) and 15 (squares) mol m^{-3} Ca. Values are means of six replicates ± s.e.

A possible reason for the development of this hypersensitivity was revealed by measurements of the concentrations of free calcium in the xylem sap of roots and shoots. Sap was collected separately from roots and shoots by pressurization within a Scholander bomb, and the free calcium concentrations were determined using an ion specific electrode (Atkinson *et al.* 1992). It was found (figure 12*a*,*b*) that the calcium concentrations in the sap from both roots and shoots were considerably (2.5 to 3 times) higher in the high-calcium plants that were not under water stress. As mild water stress developed, however, the differences became much more marked. In 3 days the calcium concentration in the sap from the roots doubled in the high-calcium plants, while it did not change significantly in the low-calcium plants. Three days later the calcium concentration in the sap from the shoots of the high-calcium plants had shown a $2\frac{1}{2}$-fold increase, but still there was no comparable change in either roots or shoots of the low-calcium plants. Thus the hypersensitivity of the stomata of this species to a

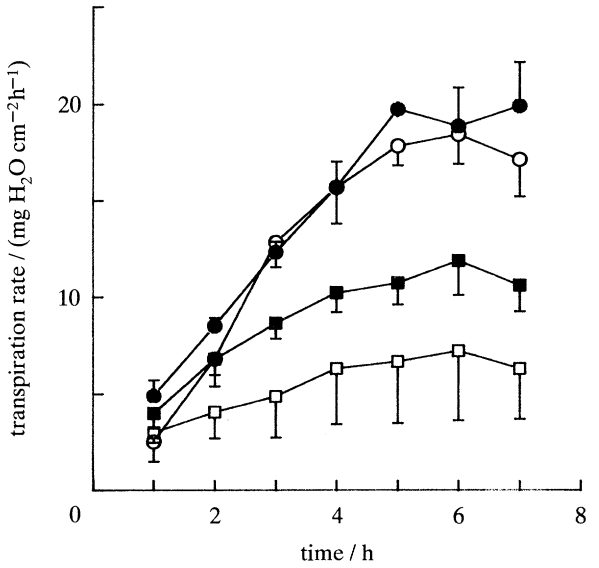

Figure 11. Transpiration rates of *Lupinus luteus* L. grown with 1 (circles) and 15 (squares) mol m^{-3} Ca three days after withholding water. Filled and open symbols represent the well watered and drought treatments respectively. Values are means of six replicates with s.e.

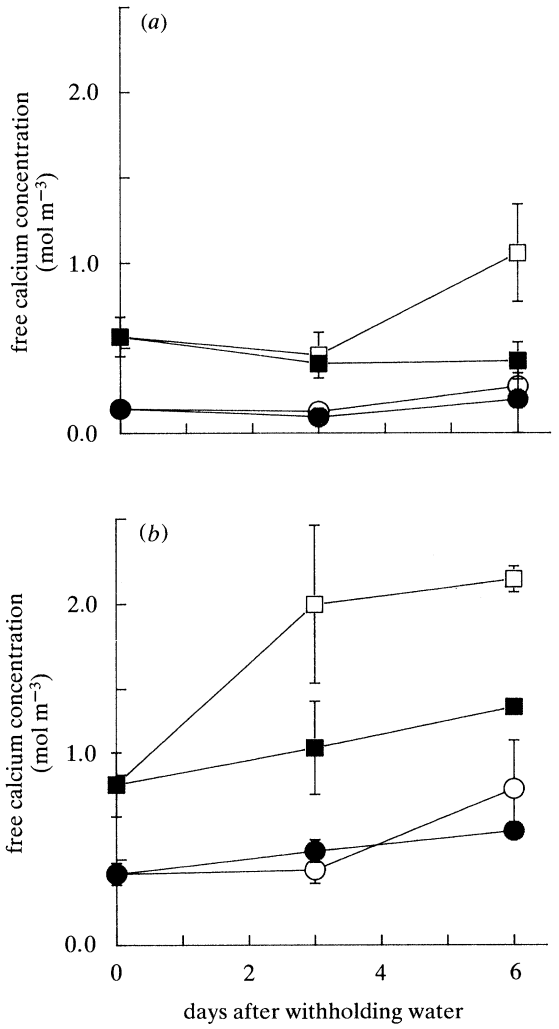

Figure 12. Free calcium concentration in xylem sap from shoot (*a*) and root (*b*) of *Lupinus luteus* L. grown with 1 (circles) and 15 (squares) mol m^{-3} Ca. Filled and open symbols show the well-watered and the drought treatments respectively. Values are means of six replicates ± s.e.

developing soil water deficit in the presence of high rhizospheric calcium may well be the result of a much increased concentration of calcium ions in the leaves, and in consequence a suppression of stomatal opening.

These results strongly contrast with those obtained by Gollan *et al.* (1992), who reported that in plants of sunflower (not a calcifuge) the calcium concentration in xylem sap decreased with soil drying. They argue that changes in the availability of calcium from the soil solution could be the reason for such a decline in calcium concentration. However, judging from the time at which the changes in concentration of calcium in the sap were detected in roots and shoots (figure 12*a*,*b*), our experiments suggest that there may first have been a breakdown in the control of calcium entering the xylem within the roots. In the high-calcium plants there was a marked increase in calcium ions in the root sap after 3 days, but a change in the shoot sap was detected only after 6 days.

Determinations (using a radioimmunoassay) were also made of the changes in the amounts of ABA in roots and shoots occurring concurrently with the changes in calcium concentrations in figure 12*a*,*b*. The concentrations in the bulk tissues were higher in the shoots than in the roots, but there were no differences that could be attributed to the calcium status of the plants. There were no significant increases in ABA content in roots or shoots in either the low or high-calcium plants 3 days after water was withheld (data not shown). This supported the conclusion, from the measurements of tissue water relations, that the degree of water stress was very mild at the time when the concentration of calcium in the xylem of the roots had risen substantially (figure 12*a*,*b*).

4. FINAL REMARKS

Our previous studies have suggested that elevated calcium concentrations in the xylem sap are capable of reducing stomatal conductance (Atkinson *et al.* 1990) but there was little convincing evidence that calcium is an agent used in root-to-shoot signalling (Atkinson 1991*a*). The new data presented here raise the question again, but doubt must still remain whether calcium has an important role in root-to-shoot communication to control plant water relations during periods of soil drying. The absence, during 6 days of soil drying, of an increase in calcium in the xylem sap of the more 'healthy' plants grown with low rhizospheric calcium suggests that stomatal regulation of water relations in these plants is achieved by other means.

We consider that a more realistic interpretation of the new data reported here for the high-calcium plants is that the sharp rise in calcium content of the xylem sap during a mild water deficit is evidence of malfunctioning of cellular regulatory mechanisms within the roots. The calcium concentration in the xylem sap is considerably below that in the rhizosphere in both *Commelina communis* (figure 5) and *Lupinus luteus* (figure 12*a*,*b*), indicating that the calcium in water around the roots does not simply move in solution by mass flow across the cortex. In the case

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of *Lupinus luteus*, the regulation, which is presumably in the endodermal layer, appears quickly to become less effective during water stress. This results in a much higher calcium concentration in xylem sap which may in turn be responsible for a greater degree of stomatal closure than is required to maintain water status. This would lead to an inhibition of photosynthetic carbon acquisition and reduce the plant's competitive affinity.

We are grateful to the Natural Environment Research Council (C. J. Atkinson) and CONACyT, México (L. P. Ruiz), for financial support.

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