

RESEARCH PAPER

Accumulation of calcium in the centre of leaves of coriander (*Coriandrum sativum* L.) is due to an uncoupling of water and ion transport

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Abstract

The aim of this study is to understand the parameters regulating calcium ion distribution in leaves. Accumulation of ions in leaf tissue is in part dependent on import from the xylem. This import via the transpiration stream is more important for ions such as calcium that are xylem but not phloem mobile and cannot therefore be retranslocated. Accumulation of calcium was measured on bulk coriander leaf tissue (*Coriandrum sativum* L. cv. Lemon) using ion chromatography and calcium uptake was visualized using phosphor-images of ⁴⁵Ca²⁺. Leaves of plants grown in hydroponics had elevated calcium in the centre of the leaf compared with the leaf margin, while K⁺ was distributed homogeneously over the leaf. This calcium was shown to be localised to the mesophyll vacuoles using EDAX. Stomatal density and evapotranspiration (water loss per unit area of leaf) were equal at inner and outer sections of the leaf. Unequal ion distribution but uniformity of water loss suggested that there was a difference in the extent of uncoupling of calcium and water transport between the inner and outer leaf. Since isolated tissue from the inner and outer leaf were able to accumulate similar amounts of calcium, it is proposed that the spatial variation of leaf calcium concentration is due to differential ion delivery to the two regions rather than tissue/cell-specific differences in ion uptake capacity. There was a positive correlation between whole leaf calcium concentration and the difference in calcium concentration between inner and outer leaf tissue. Exposing the plants to increased humidity reduced transpiration and calcium delivery to the leaf and abolished this spatial variation of calcium concentration. Mechanisms of calcium delivery to leaves are discussed. An understanding of calcium delivery and distribution within coriander will inform strategies to reduce the incidence of calcium-related syndromes such as tip-burn and provides a robust model for the transport of ions and other substances in the leaf xylem.

Key words: Calcium, *Coriandrum sativum*, distribution, ion chromatography, leaves, radioisotope, spatial variation, transpiration, uptake.

Introduction

Calcium is required in large amounts by the plant and accumulation can exceed 0.1–1% dry matter (White, 2001; White and Broadley, 2003). It is a major component of the cell wall, binding pectin molecules together. In the cytosol, calcium is central to intracellular signalling, whereas, in the vacuole, the accumulation of calcium salts can represent a significant contribution to osmotic homeostasis (Fricke *et al.*, 1995).

Calcium is almost completely immobile in the phloem and, since it cannot be re-translocated from leaves following deposition (Biddulph, 1953; Swanson and Whitney, 1953), will accumulate over time at the end of the transpiration stream. In grasses, a dominant transpirational flow along the leaf can lead to high calcium accumulation at the leaf tip and solutes within the transpiration stream accumulate at the epidermis, specifically around the stomata (Fricke,

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2004). The different geometries of dicotyledonous leaves result in different patterns of xylem water flow across the leaf and potentially different patterns of ion accumulation.

A number of plant syndromes have been associated with a heterogeneous distribution of inorganic ions in plant tissues, in particular, calcium. For example, low transpirational flow to tomato fruits can lead to low calcium levels and the development of blossom end rot (Ho *et al.*, 1993). Tip burn is a common problem in leaves of many plants of horticultural importance such as coriander, for which substantial financial losses are associated (Humber VHB, personal communication). Symptoms include leaf browning followed by necrosis at the leaf margin of mature leaves, while younger leaves can appear water-soaked (Chang and Miller, 2005). Calcium has long been recognized as a possible cause of tip burn but there is little formal evidence for its role in this syndrome.

Long-distance movement of calcium through plants is predominantly by bulk flow in the xylem and apoplast. Tissues with a higher rate of transpiration receive larger amounts of calcium than low transpiring tissues (Epstein, 1972). When transpiration is reduced, root pressure is able to drive water and solutes through the plant in the xylem (Henzler *et al.*, 1999). Root pressure has been shown to be significant in transporting calcium to tissues that do not receive sufficient calcium through transpiration. Indeed, it has been suggested that root pressure provides a mechanism for preventing calcium disorders in enclosed organs (e.g. head leaves of cabbage or fruit) where growth-related calcium demand is high and transpiration is low (Palzkill and Tibbitts, 1977).

Preliminary data showed an association between tip burn and calcium deficiency in *Coriandrum sativum* (Kerton, unpublished results). In reaching this conclusion it was discovered that coriander shows a spatial variation in $[Ca^{2+}]$ across the leaf. An understanding of the mechanism by which calcium is distributed within the leaf will facilitate strategies to minimize calcium-related disorders in foliar crops and fruit. In this paper, the causes of the spatial variation of leaf calcium concentration in coriander are investigated and the mechanisms by which calcium is transported and distributed in leaves are discussed.

Materials and methods

Plant material

Coriander seeds (*Coriandrum sativum* L. cv. Lemon) were sown in a 2:1 vermiculite/perlite mix and watered every two or three days with Long Ashton solution which contains 4 mM Ca^{2+} . After 10 d, once both cotyledons had emerged (epigeal stage), the seedlings were transferred to an aerated hydroponics system of Long Ashton solution, care being taken to avoid damaging the roots. Preliminary experiments showed that nutrients in the solution were not significantly depleted over a 7 d period (data not shown), however, the solution was replenished every 2 d. The plants were grown under controlled environmental conditions with a light/dark

regime of 16/8, light/dark temperatures of 23/21.5 °C, a relative air humidity of 70–80%, and *PAR* between 80–120 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For increased humidity conditions, plastic pots were placed over the plants 10 d after sowing.

Harvesting and Ca^{2+} analysis

Leaf tissue was removed and separated into inner and outer sections prior to sap extraction. Approximately 5 mm of leaf margin tissue was removed to comprise the ‘outer section’. The remaining tissue from the petiole/leaf junction was termed the ‘inner section’. Separated tissue was placed in 0.5 ml microfuge tubes and frozen at -20 °C. To extract leaf sap, frozen tissue was macerated in the tube using a plastic rod. The tube was pierced at the top and base with a small hole, placed inside a clean microfuge tube, and centrifuged at 13 000 rpm for 10 min. Bulk leaf sap accumulated in the lower tube and was diluted (1:20) with deionized water as soon as centrifugation was completed. The resulting diluted sample was then frozen at -20 °C, until analysis.

The concentration of cations within the diluted sap was determined using ion chromatography using the Dionex® DX-120 (Dionex Corporation, California, USA). Samples were diluted with cation eluent (22 mM H_2SO_4) prior to injection. The concentration of Ca^{2+} was determined with reference to a 1 mM standard using accompanying software (Peaknet® version 5.11).

Following calcium uptake using $^{45}Ca^{2+}$

A Long Ashton nutrient solution was modified to contain the same calcium concentration as found in the xylem of a coriander plant grown in a solution containing 4 mM Ca^{2+} (0.4 mM Ca^{2+} , Kerton, unpublished data) and radioactive calcium-45 was added to a final activity of around 50 kBq ml^{-1} . Pairs of 20-d-old plants that had been grown in 4 mM Long Ashton were excised at the petiole under a cold nutrient solution to prevent xylem embolism and held in a 2 ml microfuge tube containing cold nutrient solution whilst the other leaves were prepared. This experiment was repeated with excised leaves placed in a 40 mM Ca^{2+} solution.

Each leaf was placed in a microfuge tube, via a small hole in the lid, containing 1 ml of the 50 kBq radioactive solution described above and weighed. The leaves were left to transpire and were periodically reweighed. Once 0.02 g or 0.2 g water had been transpired from each leaf, they were removed and placed, adaxial surface down, on to cling film. Transpired water was used rather than a time-course due to the natural variation in transpiration rate between plants. Typically, the leaves transpired 0.01 g of water in 1–2 h which corresponded with a mean transpiration rate of $17.81 \pm 2.74 \text{ g h}^{-1} \text{m}^{-2}$. In order to allow subsequent alignment of images, four paper discs were soaked in a radioactive solution to which pen ink had been added and positioned around the leaf, held in place with two strips of adhesive tape. The ‘leaf packet’ was labelled and a photograph was taken with a digital camera. The leaf packet was frozen in liquid nitrogen.

Frozen leaf packets were removed from the liquid nitrogen, placed adaxial surface down on a phosphorscreen and stored at $-80\text{ }^{\circ}\text{C}$. Leaves were exposed for 10 h after which time the leaf packets were removed and the phosphorscreen imaged. The screen was scanned using a Molecular Imager FX, (Bio-Rad Laboratories Ltd, Hertfordshire) and the computer program, Quantity One.

Digital images were converted to black and white, bitmap outlines. The phosphor-images were made transparent and placed over the outlines using the images of the four radioactive paper discs to align the two images. Image preparation was carried out using CorelDRAW® Version 13.

Stomatal density and rate of leaf evapotranspiration

First leaves of coriander at day 20 were placed under a light microscope (Olympus BH-2) with a light source positioned above the leaves allowing clear observation of stomata. Stomata were counted on the abaxial surface at 10 positions on each of 10 leaves for both inner and outer regions, making a total of 100 counts for each.

Evapotranspiration was measured using a method modified from Hoad *et al.* (1996). A hole of known size was made in a small piece of laboratory film and folded over the leaf. The leaf was sealed within the laboratory film and orientated so that the hole was secured over either the inner or outer part of the leaf, limiting evapotranspiration to the selected area. The leaf was detached from the plant by excising the petiole under water. The petiole was then pushed through a hole in the lid of a 1.5 ml microfuge tube containing distilled water and sealed with Blu-tack® (Bostik, Australia). The leaf and tube were placed on a microbalance, positioned under a lamp, and an air pump was used to circulate air gently around the balance chamber.

The loss of water was recorded over time. Inner and outer sections of the same leaf were used to record water loss, providing paired data for statistical analysis. A baseline loss of water was calculated using intact laboratory film and subtracted from all readings. A tangent to the water loss curve during the first 45 min was taken as representative of the initial leaf conductance, g_{leaf} .

In a separate approach, relative transpiration rates were estimated using the cooling effects of evaporation. Thermal images of intact leaves of coriander were taken using a ThermaCAM SC640 thermal imaging camera (FLIR Systems, Kent). Using accompanying software (ThermaCAM Quick Report, version 1.1) it was possible to separate each leaf into inner and outer sections, facilitating the detection of any temperature difference between the two regions.

Measuring isolated tissue uptake properties

Plants were transferred to hydroponics at the epigeal stage and were provided with a modified Long Ashton nutrient solution containing zero calcium. A 'no calcium' solution was used to minimize the initial difference between inner and outer $[\text{Ca}^{2+}]_{\text{leaf}}$. At day 20 from sowing, 10 d later, leaf squares of equal dimensions were cut from the inner and outer sections of first leaves. Replicate samples were

harvested and analysed for $[\text{Ca}^{2+}]_{\text{leaf}}$. The leaf squares were then floated on a sterile, filtered, Long Ashton solution supplemented with $\text{Ca}(\text{NO}_2)_3$ so that $[\text{Ca}^{2+}]$ was 40 mM. Light and temperature conditions were as described in the section headed 'Plant material' and after 48 h the leaf squares had not deteriorated in appearance. The leaf squares were removed from the solution, briefly blotted on tissue paper, and the sap was extracted for $[\text{Ca}^{2+}]_{\text{leaf}}$ analysis.

Localisation of accumulated calcium using EDAX

Energy dispersive analysis of X-rays (EDAX) was carried out using the FEI XL-30 FEG ESEM at the Centre for Electron Microscopy, University of Birmingham. Plants grown under control conditions in hydroponics containing 4 mM Ca^{2+} were selected for EDAX. A small piece of leaf tissue was held on the SEM sample stage (Quorum Polar-Prep 2000) in a mixture of colloidal graphite and Tissue-Tek®. This was rapidly frozen in N_2 slush under a vacuum at $-180\text{ }^{\circ}\text{C}$ and placed in the preparation chamber of the SEM. The tissue was not coated. The tissue was fractured and moved to the SEM stage at around $-100\text{ }^{\circ}\text{C}$. A beam voltage of 15 keV was chosen along with an acquisition time of 300 s and a beam spot size of 4 (approximately 3 nm in diameter). By fracturing the tissue, data could be acquired for vacuoles by locating ruptured cells. The beam was positioned on a site of interest and spectra were collected for six mesophyll cells and six epidermal cells for both the outer and inner regions of four leaves. Since previous results had revealed a homogeneous distribution of potassium concentration across the leaf, as did preliminary EDAX data, the data were presented as Ca:K ratios and analysed statistically using the non-parametric Sheirer-Ray-Hare test (an extension of Kruskal-Wallis using ranked data).

Results

Calcium distribution

Coriander plants grown in hydroponics containing a Long Ashton nutrient solution (containing 4 mM Ca^{2+}) had a greater concentration of calcium in the centre of the leaf than at the leaf edge. In addition, calcium concentration within the oldest leaves of coriander increased over 28 d (Fig. 1a). Despite variation in individual data points ANOVA indicated a significant effect of both time ($P < 0.000$) and position ($P < 0.000$). By comparison, there was no difference in concentration between days 5 and 28 of the phloem mobile cation, potassium (Fig. 1b; t test: $P = 0.26$). Moreover, there was no difference in the concentration of K^+ between the inner and outer sections over time (Fig. 1b; ANOVA: $P = 0.104$). These data are consistent with the accepted view that potassium, but not calcium, is translocated in the phloem.

Radioactive calcium-45 was used to visualize the fate of recently taken up Ca^{2+} in leaves that were either saturated or unsaturated with calcium. The unsaturated leaves were pretreated in a nutrient solution containing 0.4 mM calcium

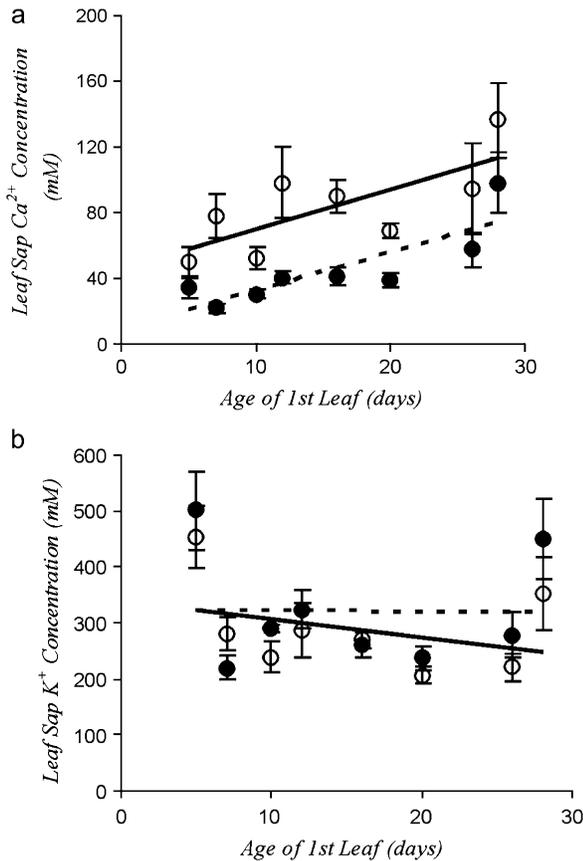


Fig. 1. Calcium (a) and potassium (b) concentration of inner (open symbols, solid line) and outer (closed symbols, broken line) regions of first leaves taken from days 5 to 28 from first leaf emergence. ANOVA showed a significant effect of time and region ($P < 0.000$) for calcium but not for potassium ($P = 0.104$). Data presented as means \pm SE with linear trend lines for each section. ($n > 5$ for each time point, $n = 54$ for each section).

which had previously been shown to be the xylem concentration of coriander plants (Kerton, unpublished results). In leaves that had been pretreated with 0.4 mM calcium, there was no large migration of calcium-45 from its entry point through the petiole to the outer edge of the leaf. This was the case for leaves that had been fed radiotracer for approximately 1 h or 10 h (Fig. 2a). In marked contrast, when the cut petioles were placed in 40 mM Ca²⁺ for over 10 h the Ca-45 did not accumulate at the leaf centre but moved rapidly to the leaf edge (Fig. 2b).

Variation in leaf evapotranspiration

It was hypothesised that the spatial variation in calcium accumulation was caused by a differential evapotranspiration across the leaf surface and this was tested experimentally. Abaxial stomatal density was equivalent for the inner and outer sections (inner: $12.29 \pm 0.43 \text{ mm}^{-2}$, outer: $12.27 \pm 0.54 \text{ mm}^{-2}$. Mann Whitney: $P = 0.436$; $n = 100$). It was observed that stomata were present on both the abaxial and adaxial surfaces. Water loss from the leaf surface, either through stomata or across the cuticle, provided a direct

measurement of leaf conductance, incorporating a transpirational component. There was no significant difference between total leaf conductance (g_{leaf}) of the inner and outer sections (Fig. 3; t test: $P = 0.083$). This was supported by the analysis of a thermal image of a 20-d-old leaf (Fig. 4) which revealed an equal leaf temperature for the inner and outer sections of $25.2 \pm 0.3 \text{ }^\circ\text{C}$ (paired t test: $P = 0.600$). Thus, the rate of water loss was similar for the inner and outer leaf regions and cannot account for the higher calcium accumulation in the centre of the leaf which would have required a higher water loss in the centre of the leaf. Taken together these data are consistent with calcium movement within the inner and outer regions of the leaf being uncoupled from water movement across the apoplast in these two regions.

Uptake of Ca²⁺ by isolated leaf sections

When plants were grown in a solution lacking calcium from day 10, the inner and outer regions of leaves contained a similar calcium concentration; replicate samples of these excised inner and outer regions were subsequently used in calcium uptake experiments. After 48 h incubation on a solution containing 40 mM calcium, there was found to be no significant difference between calcium concentrations in the inner and outer regions of the leaf (Fig. 5; t test: $P = 0.950$). Both leaf regions increased their calcium concentration from $18.23 \pm 1.28 \text{ mM}$ to $166.68 \pm 8.72 \text{ mM}$ over this period suggesting that both are able to take up calcium to high levels when this is available to them, regardless of their position within the leaf.

Manipulation of the difference in $[\text{Ca}^{2+}]_{\text{leaf}}$

Using data from previous experiments, the calcium concentrations of both the inner and outer regions of the leaf were combined to estimate a whole leaf concentration and plotted against the difference between the absolute concentrations of each region. In some cases, the leaves had been fed high levels of calcium via their petioles in order to increase the leaf calcium concentration. The difference in calcium concentration between the inner and outer leaf tissue increased as the leaf calcium accumulated (Fig. 6; Pearson's correlation coefficient = 0.756; $P = 0.000$). The greatest calcium concentrations measured were 542 mM and 442 mM for inner and outer sections, respectively.

Growing the plants under humid conditions reduced transpiration from $127.9 \text{ g h}^{-1} \text{ m}^{-2}$ to $4.6 \text{ g h}^{-1} \text{ m}^{-2}$ and increased the fresh weight:dry weight ratio of the leaf by 2.0% over a 30 d period. Under these conditions calcium delivery was reduced, bringing the calcium concentration below a certain threshold resulting in both the inner and outer sections having similar concentrations (Fig. 7; ANOVA: $P = 0.666$).

Calcium localisation

Energy dispersive analysis of X-rays (EDAX) was carried out to identify the cellular site of accumulation in the leaf. Analysis of the spectra retrieved from EDAX was carried

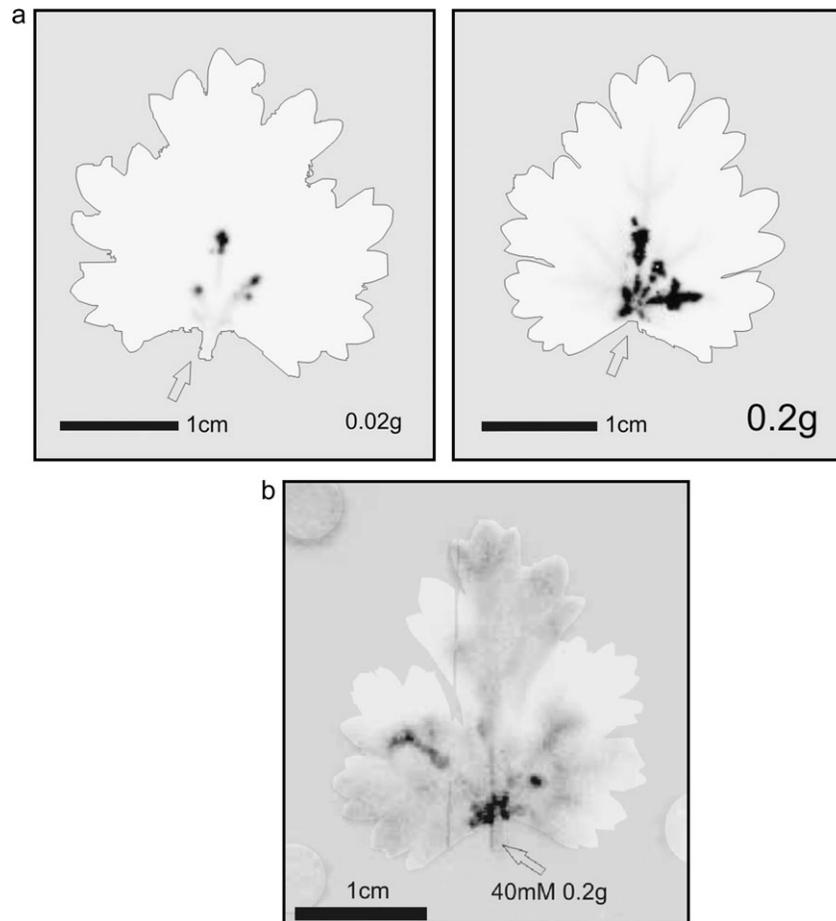


Fig. 2. Phosphor-images of $^{45}\text{Ca}^{2+}$ within *C. sativum*. (a) Leaves were fed 0.4 mM Ca^{2+} and exposed once 0.02 g and 0.2 g water had been lost through transpiration. Images show retention of calcium in the leaf centre. (b) Leaf fed with 40 mM Ca^{2+} and exposed once 0.2 g water had been lost through transpiration. The calcium migrates to the leaf edge at this calcium concentration. Scale bar is equal to 1 cm. Arrow signifies petiole.

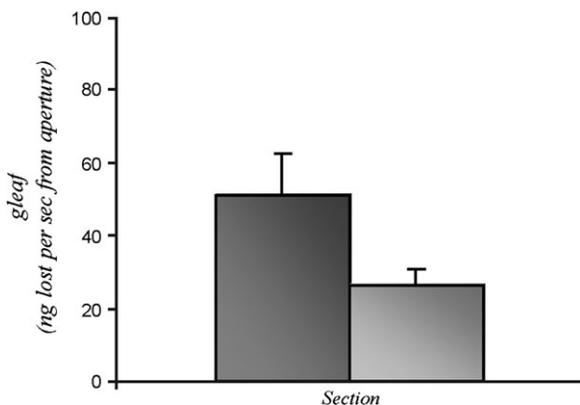


Fig. 3. Comparison of outer (dark) and inner (light) leaf conductance. No significant difference was found between the two regions (Paired *t* test; $P=0.083$). Units are in ng lost per second through the aperture. Data presented as mean \pm SE ($n=8$).

out by plotting the calcium to potassium ratio (Fig. 8). The Ca/K ratio was consistent with calcium accumulation being primarily within the mesophyll vacuoles of the inner section of the leaf. The lowest Ca/K ratio was detected at the outer

epidermal vacuoles (Sheirer–Ray–Hare: region; $P=0.003$. cell type; $P=0.002$).

Discussion

It was observed that *C. sativum* accumulated more calcium in the centre of the leaf than at the leaf margin and the overall leaf calcium concentration increased with time. Use of $^{45}\text{Ca}^{2+}$ indicated that recently imported Ca^{2+} accumulated in the centre of the leaf. The dependence of leaf calcium concentration on transpirational input has been observed in other studies. Konsaeng *et al.* (2005) found that the calcium concentration was higher in older leaves of 17 tropical plant species than younger leaves, while Fricke (2004) reported that calcium accumulated in epidermal cells close to stomata at the end of the transpiration stream in barley (*Hordeum vulgare* L.).

Delivery of calcium by the transpiration stream

An assumption of the present study was that calcium was carried passively, dissolved in the transpiration stream and

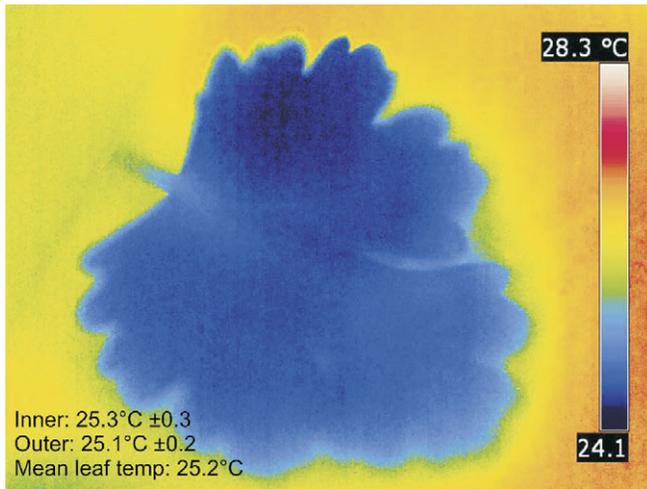


Fig. 4. Analysed thermal image of a transpiring coriander leaf at day 20 from sowing showing an even temperature distribution across the leaf surface (Paired t test; $P=0.600$, $n=5$).

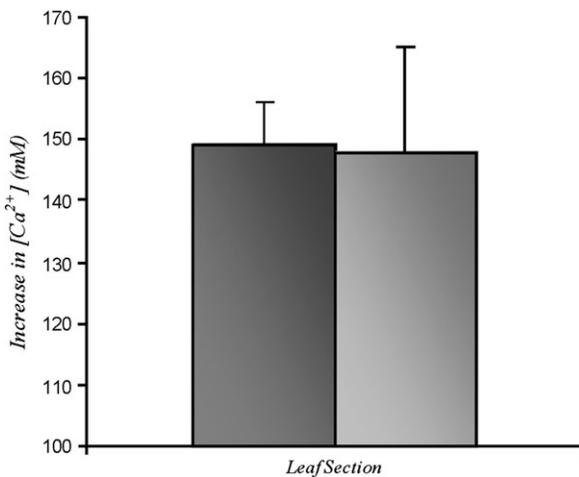


Fig. 5. The change in calcium concentration of isolated leaf tissue from outer (dark) and inner (light) leaf regions. No significant difference was found (t test; $P=0.950$). Data presented as mean \pm SE ($n=5$).

it was initially hypothesised that the higher calcium concentration in the centre of the leaf was due to a higher delivery rate in this region. Poole *et al.* (1996) observed a heterogeneous stomatal density in leaves of *Alnus glutinosa* (L.) which was accompanied by a heterogeneous transpiration across the leaf. However, in *C. sativum*, both stomatal density and the resulting evapotranspiration were equal across the leaf surface.

The relationship between solute transport and the transpiration stream has been extensively discussed in the literature (reviewed by Canny, 1990a). For example, Canny (1990b) found that the fluorescent xylem tracer, sulphorhodamine, did not accumulate at the end of the leaf veins or at the point of evaporation surrounding the stomata but a few micrometres before the vein termini. It was suggested that the tracer underwent osmotic filtration as the water

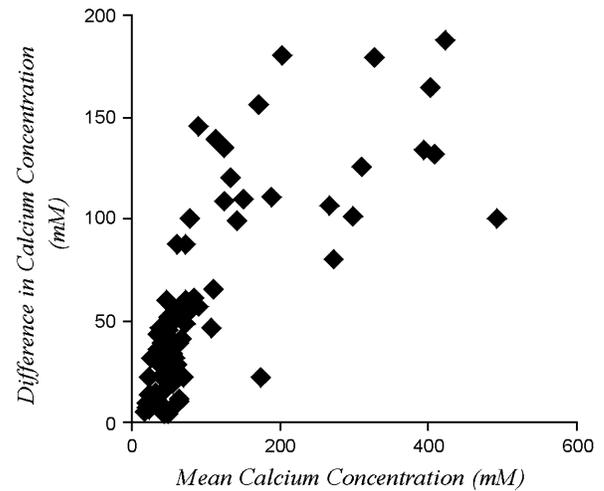


Fig. 6. Correlation between the estimated mean leaf calcium concentration and the difference between inner and outer parts of the leaf (Pearson Correlation Coefficient; $P=0.000$).

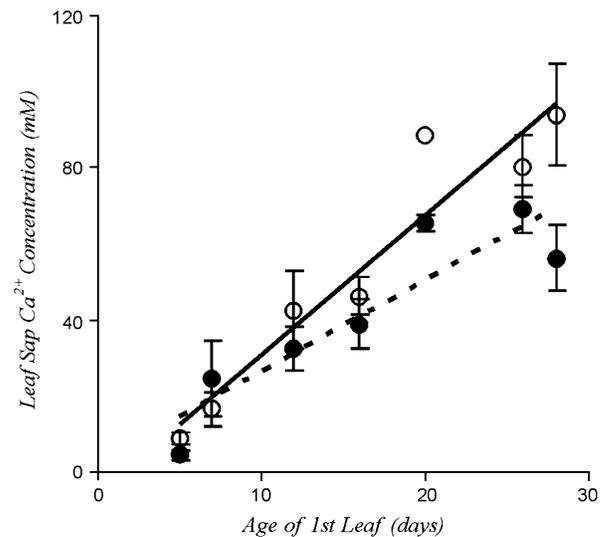


Fig. 7. Calcium concentration of inner (open symbols, solid line) and outer (closed symbols, broken line) regions of first leaves taken from days 5 to 28 from plants grown under high humidity conditions. ANOVA showed no significant effect of region over time ($P=0.666$). Data presented as means \pm SE with linear trend lines for each section ($n > 4$ for each time point, $n > 29$ for each section).

passed from the xylem into the symplast within the leaf (Canny, 1990b). However, in the present study, calcium is unlikely to be concentrated in this way as any soluble calcium would remain in the apoplast, following water movement into the symplast, and would then move to the leaf margin in the transpiration stream. By contrast, the data of the present study suggest that calcium is preferentially taken up by the cells of the inner leaf. Consistent with this, recently imported calcium was retained in the leaf centre even following exposure to the radiotracer for over 10 h (Fig. 2a). This accumulation of Ca^{2+} in the leaf could be saturated by loading the xylem of the leaf petiole with

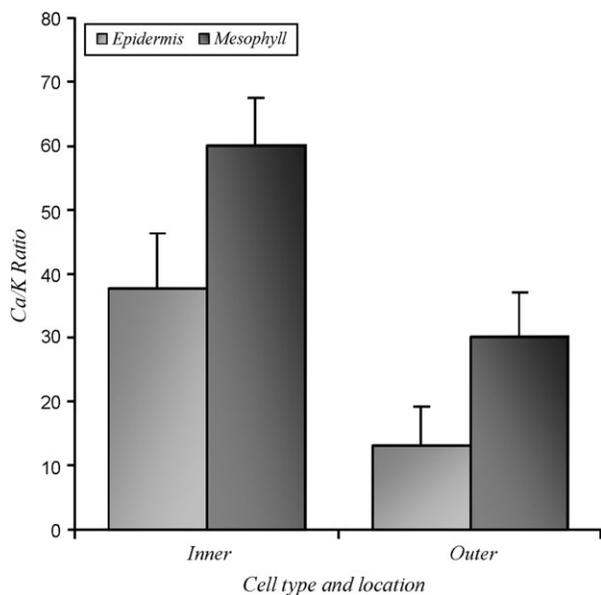


Fig. 8. The calcium:potassium ratios derived from EDAX spectra from coriander leaves at day 20. Accumulated calcium was localised to the inner mesophyll vacuoles of the leaf (Sheirer-Ray-Hare: region; $P=0.003$, cell type; $P=0.002$). Data presented as means \pm SE ($n > 16$).

high non-physiological levels of calcium (40 mM) which resulted in rapid movement of recently imported calcium to the leaf margin. (Fig. 2b).

Taken together, these data suggest that the extent of uncoupling of calcium from the transpiration stream within *C. sativum* as it moves across the leaf is greater in the leaf centre. The uncoupling of calcium from the transpired water has also been reported in the xylem of both monocotyledonous and dicotyledonous species, where xylem loading of calcium was independent of transpiration rate (Atkinson *et al.*, 1992).

There is a clear role for transpiration in determining the spatial variation in Ca²⁺ accumulation as spatial variation in calcium concentration across the leaf was abolished when plants were grown at high humidity under plastic lids (RH >99%). Under low transpiration it can be assumed that xylem sap was entering the leaf at a lower rate with an altered Ca²⁺ concentration, driven by root pressure. Increasing humidity during the night increased the sap flow in tomato (*Lycopersicon esculentum*), but reduced the distribution of calcium from root to shoot (Choi *et al.*, 1999). However, in this study, it is more likely that the delivery rate of calcium to the leaf was so reduced that the amount of calcium in the leaf did not exceed the threshold required to set up the spatial variation measured previously (Fig. 6).

Intrinsic uptake properties of inner and outer leaf cells

In contrast to intact leaves, isolated tissue from the inner and outer regions of the leaf accumulated similar amounts of calcium, consistent with the conclusion that calcium was depleted in the transpirational water reaching leaf margins.

Therefore, the observed spatial variation is assumed to be a function of the cells' position within the leaf. It has previously been established that different cell types have different ion uptake properties. For example, in barley (*Hordeum distichum* L.), phosphorus was found in mesophyll vacuoles and was absent from the epidermis whereas calcium and chlorine accumulated at the epidermis (Leigh and Storey, 1993; Williams *et al.*, 1993).

Since the petiole joins directly to the inner leaf region, cells in this region have the first opportunity to take up calcium from the apoplast. Calcium levels in the cytosol are considered to be in the micromolar range compared to the tens of millimolar considered in the present study. Low cytosolic levels of Ca²⁺ are maintained by molecular chaperones such as calnexin (Hirschi, 2004). To maintain this cytosolic homeostasis, Ca²⁺ must be taken up across the plasma membrane and accumulated in the vacuole (White *et al.*, 1992). Such Ca²⁺ transport across the tonoplast, from cytosol to vacuole, occurs against a large concentration gradient and is therefore likely to be active, using a Ca²⁺-ATPase. One such transporter, ACA11, has been localised to the tonoplast of *Arabidopsis* (Lee *et al.*, 2007). In coriander, the maximum leaf tissue Ca²⁺ concentration measured was as high as 492.73 mM. A calcium concentration of 2.3 mM was measured using ion-selective electrodes in *Riccia fluitans* (Felle, 1988). López-Milan *et al.* (2000) measured a calcium concentration of 10 mM in apoplastic fluid in sugar beet (*Beta vulgaris* Monohil hybrid) under iron stress. Fricke *et al.* (1996) measured a calcium concentration of 40 mM in epidermal cells of barley (*Hordeum vulgare*). In the present study, the degree of Ca²⁺ scavenging by cells of the inner leaf was manifest as the difference between the inner and outer tissue calcium concentration. If inner cells are engaged in such scavenging, uptake saturation levels have not been reached as the difference is maintained up to a concentration of over 492.73 mM.

The vacuole as the origin of calcium from sap samples

The vacuole is a major source of intracellular calcium (Martinoia *et al.*, 2007; White and Broadley, 2003). In addition, there is considerable calcium associated with pectin in the cell walls (Carpita and Gibeau, 1993), although it is unclear how much of this calcium fraction would be removed in the sap extraction procedure. It is not expected that a significant amount would be removed since no acids were used in the extraction procedure. Calcium can also be ionically bound to the cell wall but this would only be displaced by high concentrations of ions further up in the lyotropic series (such as Al³⁺), which are not present in the *C. sativum* leaf tissue. In any case, it is unlikely that sufficient calcium would be bound to account for the large concentration recovered in the leaf sap (up to around 500 mM). Since cytosolic volumes are small and calcium concentrations in this compartment low, it was assumed here that the dominant source of calcium from the extracted leaf sap is soluble calcium from leaf cell vacuoles. However,

as previously mentioned, insoluble calcium may have been carried into the final samples and so cannot be disregarded. EDAX of inner and outer leaf tissue localised the accumulated calcium in the vacuoles of the mesophyll cells of the inner leaf. Similar studies have involved this technique to localise ions on a cellular level within the leaf (Wojcik *et al.*, 2005; Fernando *et al.*, 2006).

In summary, this study has shown that the extent of calcium and water uncoupling within the apoplast of co-riander leaves differs across the leaf and there is no difference between the uptake properties of cells from inner and outer regions of the leaf. The difference in calcium accumulation in inner and outer regions of the leaf is consistent with a depletion of Ca^{2+} in the apoplastic solution as xylem sap moves from the point of import at the petiole in the centre of the leaf to the leaf margins. Cells in the centre of the leaf are therefore exposed to higher apoplastic Ca^{2+} and accumulate more Ca^{2+} in their vacuoles.

The arrangement of xylem networks has a direct effect on water and solute transport within the leaf (Roth-Nebelsick *et al.*, 2001; Loeffe *et al.*, 2007). The association between calcium distribution and the xylem networks of different leaf anatomies may explain the distribution of calcium concentration observed in *C. sativum* and this is the subject of further investigation.

References

- Atkinson CJ, Ruiz LP, Mansfield TA.** 1992. Calcium in xylem sap and the regulation of its delivery to the shoot. *Journal of Experimental Botany* **43**, 1315–1324.
- Biddulph O.** 1953. Translocation of radioactive mineral nutrients in plants. Use of isotopes in plant and animal research. *US Atomic Energy Commission*. TID-5098.
- Canny MJ.** 1990a. What becomes of the transpiration stream? *New Phytologist* **114**, 341–368.
- Canny MJ.** 1990b. Fine veins of dicotyledon leaves as sites for enrichment of solutes of the xylem sap. *New Phytologist* **115**, 511–516.
- Carpita NC, Gibeaut DM.** 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**, 1–30.
- Chang YC, Miller WB.** 2005. The development of upper leaf necrosis in *Lilium* 'Star Gazer'. *Journal of the American Society for Horticultural Science* **130**, 759–766.
- Choi JH, Chung GC, Lee SH.** 1999. Influence of night humidity on the distribution of calcium and sap flow in tomato plants. *Journal of Plant Nutrition* **22**, 281–290.
- Epstein E.** 1972. *Mineral nutrition of plants: principles and perspectives*. New York: John Wiley and Sons.
- Felle H.** 1988. Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: interaction of Ca^{2+} and pH. *Planta* **176**, 248–255.
- Fernando DR, Batianoff GN, Baker AJ, Woodrow IE.** 2006. *In vivo* localization of manganese in the hyperaccumulator *Gossia bidwillii* (Benth.) N. Snow & Guymer (Myrtaceae) by cryo-SEM/EDAX. *Plant, Cell and Environment* **29**, 1012–1020.
- Fricke W.** 2004. Solute sorting in grass leaves: the transpiration stream. *Planta* **219**, 507–514.
- Fricke W, Hinde PS, Leigh RA, Tomos AD.** 1995. Vacuolar solutes in the upper epidermis of barley leaves: intercellular differences follow patterns. *Planta* **196**, 40–49.
- Fricke W, Leigh RA, Tomos AD.** 1996. The intercellular distribution of vacuolar solutes in the epidermis and mesophyll of barley leaves changes in response to NaCl. *Journal of Experimental Botany* **47**, 1413–1426.
- Henzler T, Waterhouse RN, Smyth AJ, Carvajal M, Cooke DT, Schaffner AR, Steudle E, Clarkson DT.** 1999. Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of *Lotus japonicus*. *Planta* **210**, 50–60.
- Hirschi KD.** 2004. The calcium conundrum. Both versatile nutrient and specific signal. *Plant Physiology* **136**, 2438–2442.
- Ho LC, Belda R, Brown M, Andrews J, Adams P.** 1993. Uptake and transport of calcium and the possible causes of blossom-end rot in tomato. *Journal of Experimental Botany* **44**, 509–518.
- Hoad SP, Grace J, Jeffree CE.** 1996. A leaf disc method for measuring cuticular conductance. *Journal of Experimental Botany* **47**, 431–437.
- Konsaeng S, Dell B, Rerkasem B.** 2005. A survey of woody tropical species for boron retranslocation. *Plant Production Science* **8**, 338–341.
- Lee SM, Kim HS, Han HJ, Moon BC, Kim CY, Harper JF, Chung WS.** 2007. Identification of a calmodulin-regulated auto-inhibited Ca^{2+} -ATPase (ACA11) that is localized to vacuole membranes in *Arabidopsis*. *FEBS Letters* **581**, 3943–3949.
- Leigh RA, Storey R.** 1993. Intercellular compartmentation of ions in barley leaves in relation to potassium nutrition and salinity. *Journal of Experimental Botany* **44**, 755–762.
- Loeffe L, Martienez-Vilalta J, Plijola J, Mencuccini M.** 2007. The relevance of xylem network structure for plant hydraulic efficiency and safety. *Journal of Theoretical Biology* **247**, 788–803.
- Lopéz-Millan AF, Morales F, Abadia A, Abadia J.** 2000. Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet: implications for iron and carbon transport. *Plant Physiology* **124**, 873–884.
- Martinoia E, Maeshima M, Neuhaus HE.** 2007. Vacuolar transporters and their essential role in plant metabolism. *Journal of Experimental Botany* **58**, 83–102.
- Palzkill DA, Tibbitts TW.** 1977. Evidence that root pressure-flow is required for calcium-transport to head leaves of cabbage. *Plant Physiology* **60**, 854–856.
- Poole I, Weyers JDB, Lawson T, Raven JA.** 1996. Variations in stomatal density and index: implications for palaeoclimatic reconstructions. *Plant, Cell and Environment* **19**, 705–712.
- Roth-Nebelsick A, Uhl D, Mosbrugger V, Kerp H.** 2001. Evolution and function of leaf venation architecture: a review. *Annals of Botany* **87**, 553–566.

Swanson CA, Whitney JB. 1953. Studies on the translocation of foliar-applied P-32 and other radioisotopes in bean plants. *American Journal of Botany* **40**, 816–823.

White PJ. 2001. The pathways of calcium movement to the xylem. *Journal of Experimental Botany* **52**, 891–899.

White PJ, Banfield J, Diaz M. 1992. Unidirectional Ca²⁺ fluxes in roots of rye (*Secale cereale* L.): a comparison of excised roots with roots of intact plants. *Journal of Experimental Botany* **43**, 1061–1074.

White PJ, Broadley MR. 2003. Calcium in plants. *Annals of Botany* **92**, 487–511.

Williams ML, Thomas BJ, Farrar JF, Pollock CJ. 1993. Visualising the distribution of elements within barley leaves by energy dispersive X-ray image maps (EDX maps). *New Phytologist* **125**, 367–372.

Wojcik M, Vangronsveld J, D'Haen J, Tukiendorf A. 2005. Cadmium tolerance in *Thlaspi caerulescens*. II. Localization of cadmium in *Thlaspi caerulescens*. *Environmental and Experimental Botany* **53**, 163–171.