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The kinetics of calcium and magnesium entry into mycorrhizal spruce roots

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Abstract. The entry of calcium and magnesium from external sources into mycorrhizal roots of 3-year-old Norway spruce trees (*Picea abies* [L.] Karst.) was monitored. Roots of intact plants were exposed for various periods of time, ranging from 2 min to 48 h, to nutrient solutions which contained the stable-isotope tracers ^{25}Mg and ^{44}Ca. After labelling, samples of roots were excised from the plants, shock-frozen, cryosubstituted and embedded. The resulting isotope composition in this material was analysed by a laser-microprobe-mass-analyser (LAMMA) at relevant positions within cross-sections of the roots. For both elements, we determined (i) the fractions of the isotopes originating from the plant prior to labelling, and (ii) the fraction of isotopes originating from the corresponding tracer that penetrated into the root. Both divalent cations rapidly penetrated across the cortical apoplast and reached the endodermis. After 2 min of exposure to the labelling solution, an initial transient gradient of the tracers could be observed within the root cortex. Subsequently, calcium as well as magnesium equilibrated between the apoplast of the entire cortex and the external tracer with a half-time, $t_{1/2}$, of about 3 min. In contrast, the kinetics of radial movement into the vascular stele showed a delay with a $t_{1/2}$ of 100–120 min. We take this as strong evidence that there exists a free apoplastic path for divalent cations in the cortex and that the endodermis is a major barrier to the further passage of Mg and Ca into the xylem. While ^{25}Mg in the labelling solution exchanged rapidly with Mg in the cortical apoplast, the exchange across the plasma membrane with Mg present in the protoplasm of the same cortical cells was almost 2 orders of magnitude slower. The kinetics of Ca and Mg entry at +6 °C were similar to those obtained at a root temperature of +22 °C.

Key words: Calcium – Magnesium – Mycorrhiza – Ion uptake – *Picea* (root) – Stable isotope (tracer)

Introduction

One primary function of fine roots in most terrestrial plants is the uptake of mineral nutrients. For obvious reasons, it is a necessity for the plant to be in control of uptake, release, and exchange of solutes. In principle, there are two simple, not necessarily exclusive, ways for nutrient uptake and transport by roots, namely the apoplastic and symplasmic routes.

The simplest model for the uptake of nutrients by roots is to assume that nutrients traverse the apoplast by diffusion, facilitated by the flow of water in the transpiration stream. This rather simplistic view of transport via the 'bulk flow' requires a more or less free radial access from the rhizosphere across the root into the xylem vessels. In this case, the only driving forces required would be diffusion and the transpiration stream. This model, however, appears to be inconsistent with some observations concerning nutrient uptake. In the roots of many higher plants, relevant amounts of hydrophobic material are incorporated into the otherwise hydrophilic walls of certain cells, particularly in the characteristic casparian band, present in the structurally distinguished cell layer, the endodermis (Zeier et al. 1999). Since the early sixties, researchers have raised the question of whether or not the endodermis acts as a diffusion barrier to the free passage of water and ions (e.g. van Fleet 1961; Bonnett 1968; Clarkson and Robards 1975). If this were the case, water and solutes would have to pass through at least two membranes in order to cross the endodermal cells, which would offer extensive mechanisms to control entry and transport (Clarkson 1996) e.g. through ion channels and aquaporins, as well as protection against leakage of water and solutes from the xylem. The endodermis might be traversed via "general" endodermal cells or, in a more

Abbreviation: LAMMA = Laser microprobe mass analyser

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The authors dedicate this paper to Prof. Dr. Dr. h. c. mult. H. Ziegler on occasion of his 75th birthday

specialized way, via dedicated, i.e. passage cells (Peterson and Enstone 1996).

In this study, we investigate the kinetics of entry and the path of mainly calcium and magnesium ions into the root and the functional significance of the endodermis. In many tree species, such as the spruce analysed here, the endodermis is of particular importance, since the roots do not show any sign of typical exodermal structures. Is the casparian band the postulated diffusion barrier for the passage of water and solutes into and out of the root, as suggested by previous work (e.g. Perumalla and Peterson 1986; Peterson et al. 1993)? For our study, we did not use substitutes as tracers for the ions in question but stable isotopes of Mg, K, and Ca were used, to follow the path of ions of the same elements into the mycorrhizal roots of intact small spruce trees. This is a direct and minimally invasive method, combining a functional and structural approach that minimizes physiological disturbances, such as changes in osmotic or hydrostatic pressure or ionic composition caused, for example, by root amputation. We used intact soil-raised plants under nearly natural conditions throughout the experiments. For the first time the complete kinetics of tracer ions were monitored at the cellular level by microbeam techniques.

Material and methods

Trees. Norway spruce (*Picea abies* [L.] Karst.) plants were germinated from seeds originating from the German provenance Harzvorland/Westerhof. The seedlings were raised in sand for 3 months. Single seedlings were then transplanted and grown for 3 years in a greenhouse in pots containing 1000 ml nursery soil. The roots were mycorrhizal to a high degree (>95%). A total of 69 (3 for each exposure time period) plants were used for these experiments. These trees had reached a height of approximately 25 cm above ground. The experiments were carried out at the end of the natural growth period at the end of September. At the time of depotting, the element composition of the centrifuged (2300 *g*, 30 min, corresponding to a water potential of circa 50 hPa) soil solution, was as follows: 7.13 Ca, 1.67 Mg, 5.42 K, 1.10 Na, 0.06 Al, pH of the soil solution: 5.90, pH of the soil (in KCl): 6.36 (all values in mol · m^{-3}).

Stable isotope labelling. Plants were transferred to a growth chamber (day: 16 h, +23 °C, 60% relative humidity, light flux: 280 µmol photons m^{-2} s^{-1}; night: 8 h, +15 °C, 90% relative humidity, darkness; slow light/dark changes for dusk and dawn within 1 h) for 10 d and then removed from the soil. Any soil particles visibly adhering to the finest roots were removed using a fine brush under stereomicroscopic observation. No wounding of the root system could be detected by stereomicroscopic observation. Rhizomorphs or hyphae penetrating into the soil were rarely detected. Mycorrhization appeared to be uniform, as typically observed for only one species of fungus, but the fungal species was not determined. The entry kinetics were studied at root chamber temperatures of +22 °C and +6 °C under otherwise identical conditions in parallel experiments. The root systems were pre-incubated at the selected temperatures overnight in 200 ml of a solution of 1 mol · m^{-3} each $MgCl_2$, KCl, $CaCl_2$ and NaCl at pH 4.5. Then a 15%–20% fraction of the root system was selected for labelling while the remainder was kept in wet cellulose. The selected roots were labelled by substitution of the pre-incubation solutions with solutions of chemically identical composition, but all of the Mg, K, and Ca was replaced by enriched preparations of the stable isotopes ^{25}Mg, ^{41}K and ^{44}Ca. Three plants each were used as controls prior to exposure to any solutions, prior to labelling, and after labelling for 2 min, 4, 8, 16, 32, 64, 128, 256, 512 min and 48 h. Table 1 shows the isotopic composition of the enriched labels as claimed by the supplier. Control measurements by laser microprobe mass analysis confirmed the suppliers specification of the label compositions. During exposure times longer than 32 min, the labelling solutions were aerated and, in the case of the 48-h incubation time, renewed once after 24 h.

Shock-freezing and cryosubstitution. Root samples, 1–2 cm long, with several of the mycorrhizal finest roots were excised, quickly blotted onto filter paper, and immediately shock-frozen by immersion into liquid propane, near its freezing point at −190 °C. The shock-frozen samples were transferred into 20-ml polypropylene scintillation vials (No. 6008117; Packard) containing 10 ml water-free acetone with 0.5% OsO_4 and kept for 3 d at −80 °C. The samples were slowly warmed up first to −20 °C within 24 h and subsequently to room temperature (+20 °C) within 16 h. The cryosubstituted samples were washed five times for 1 h in water-free acetone.

Embedding and ultramicrotomy. The samples were infiltrated in a graded series of embedding medium plus acetone (1 + 3; 1 + 1; 3 + 1; 1;1; each step 8 h). The low-viscosity medium after Spurr (1969) and Mascorro et al. (1976) was modified: viscosity 20 centipoise, 5 g ERL 4206 (vinylcyclohexene dioxide), 10 g HXSA (hexenyl succinic anhydride), 0.4 g Araldite RD2 (DY026), 0.15 g DMAE (dimethyl aminoethanol), all obtained from TAAB (RG78NA; Reading, Berks., UK). Polymerization took place under exclusion of oxygen in "easy molds" (#2208-156, 5.6 mm diameter from LAB, obtained from TAAB). Cured blocks were trimmed for best areas, and those re-embedded in the following medium: viscosity 200 centipoise; 54.5% (w/w) DDS (dodecenyl succinic acid anhydride); 25% Epon 812; 20% Araldite CY212; 0.5% 2,4,6-tri-(dimethylaminomethyl)-phenol (all obtained from SERVA). Sections, 0.5 µm thick, were cut using an ultramicrotome (Ultracut; Reichert-Jung, now Leica Mikrosysteme Bensheim, Germany), using a glass knife without floating liquid, since floating the sections on liquid would result in material loss or redistribution (Harvey et al. 1976).

Analysis by laser-microprobe-mass-analyser (LAMMA). The structurally best-preserved samples (69 out of 500 roots) were used for analysis. Sections were analysed using the LAMMA 500 (Leybold, Köln, Germany). The instrument is described in detail elsewhere (Hillenkamp et al. 1975; Heinen et al. 1980). In brief, the LAMMA 500 is a laser light microscope with a high-energy pulsed laser fitted to a time-of-flight mass spectrometer. A small area in a 0.5-µm thin section, mounted on a 3-mm copper sandwich grid (typically 75 mesh), was selected for analysis by light microscopic observation

Table 1. Natural isotope compositions of the elements Ca, Mg, K (after Weast 1989), and compositions of the stable-isotope tracers. Composition in % of total number of atoms. Only the relevant isotopes are shown

Isotope	40	42	44
Ca-[natural]	96.94	0.65	2.09
44-Ca-label	2.90	0.06	97.00
	24	25	26
Mg-[natural]	78.99	10.00	11.01
25-Mg-label	2.29	96.90	0.81
	39	41	
K-[natural]	93.26	6.73	
41-K-label	4.20	95.80	

and then evaporated by a flash of a focussed UV light beam. Under our conditions, the laser perforations were adjusted to approximately 1–1.5 μm in diameter. In the case of xylem cell walls, as well as in the case of cell protoplasts, the diameter of the laser beam was smaller than the analysed structure. However, in the case of the apoplast of cortical cell walls the analysed area may also occasionally have included material from hyphal cells intruding into the cortex. The size relations can be seen in Fig. 1b. The evaporated material was analysed in the attached time-of-flight mass spectrometer. The resulting voltage signal at the detector was processed, and the integral of the peaks expressed in relative units. Quantitative analysis giving absolute amounts of material is nearly impossible without well-matched standards. However, relative element concentration measurements, comparing LAMMA signals at different locations within one section, comparing different samples, and isotope ratios were performed with a high accuracy of typically 1%–2%.

Correction of the percentage of isotope label. The isotope ratios measured from a single-spot analysis taken from a labelled specimen (see Fig. 1) do not necessarily represent the true ratio of the label taken up from the labelling solution and the fraction of element present before the onset of the labelling. This is due to the fact, that neither the "old" material, originally present in the plant nor the label are isotopically pure. As shown in Table 1, the originally present elements Ca and Mg both consist of a mixture of isotopes (i.e. Mg consists of the natural mixture of the isotopes ^{24}Mg, ^{25}Mg, ^{26}Mg). Similarly, labels are mixtures of isotopes (i.e. the ^{25}Mg-enriched tracer containing small fractions of ^{24}Mg and ^{26}Mg). In some samples – especially during longer incubation times – this mixture of isotopes in the label significantly changed during the labelling periods due to material released from the roots into the labelling solution. The isotopic compositions of the labelling solutions were monitored and corrected for by a matrix correction algorithm. The correction procedure used was described in detail in Fain and Schröder (1987).

For each data point shown in the results we analysed thin sections from three roots, each from a different plant (for more detail, see Kuhn et al. 1995). We obtained 30–50 LAMMA spectra for each sample type (e.g. xylem cell wall) as defined below. A number of at least 3 sections from each root sample were used to obtain the preset limit of 90–150 spectra per sample type. Only favourable points of analysis were used to ensure that the lateral resolution of analysis was adequate for the sample type. Spectra not meeting this requirement with confidence upon light-microscopic examination of the laser perforation were discarded. The mean of the averages computed from the spectra of each plant and the corresponding standard deviation of the mean were computed according to Peter's formula: $SD(m) = 1.25 \cdot ({}^{n}\Sigma_i|X_i - X_m|)/[n \cdot (n-1)^{1/2}]$ (Squires 1971). X_m is the mean value of a number n of single spectra X_i. To determine the isotope ratios of the labelling solution, 50-μl aliquots from the solutions were sprayed onto nitrocellulose support films on copper grids, and the resulting micro-droplets were analysed by LAMMA. The average isotope ratios of these samples were determined from 50 spectra.

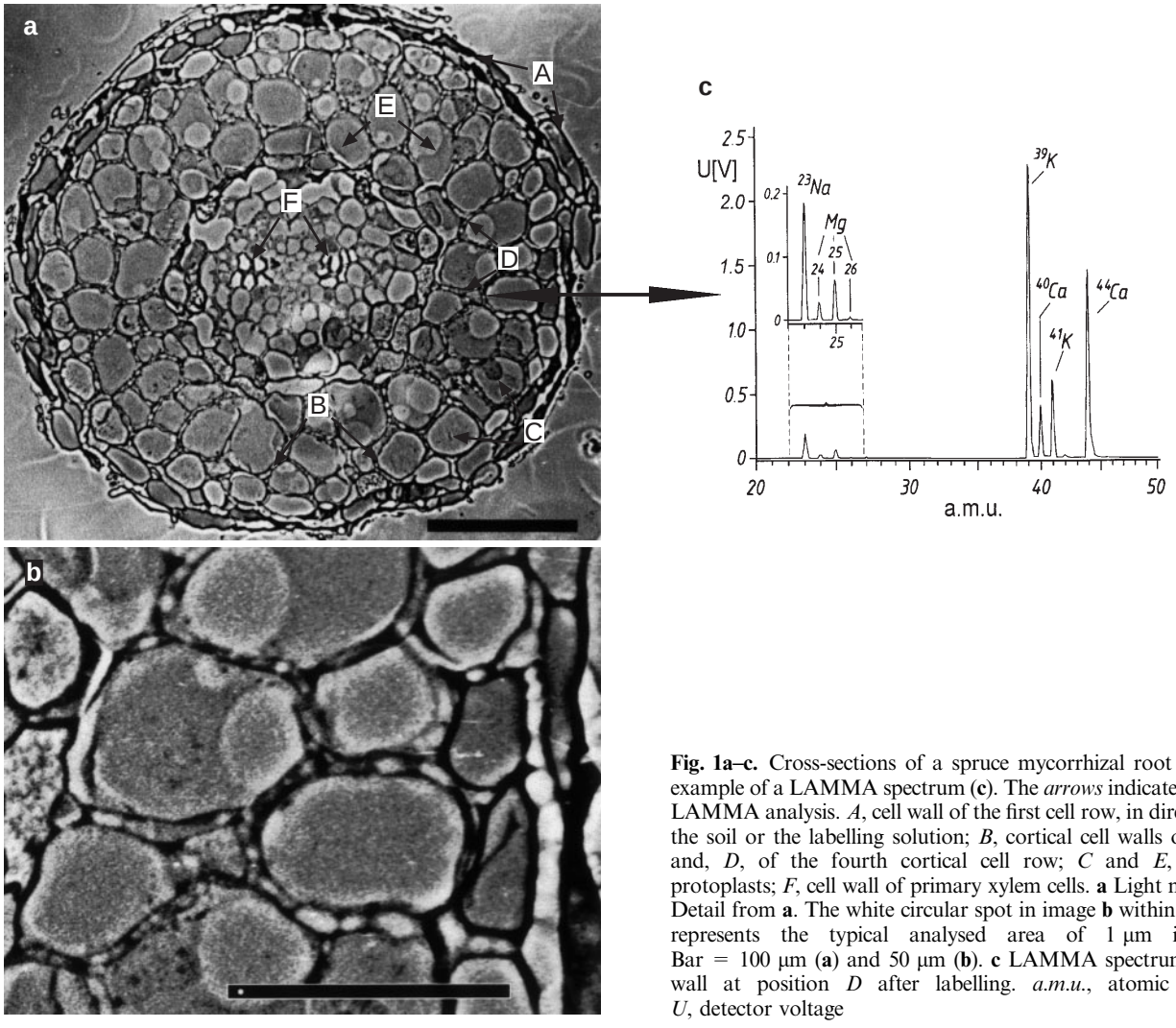

Fig. 1a–c. Cross-sections of a spruce mycorrhizal root (**a**,**b**) and an example of a LAMMA spectrum (**c**). The *arrows* indicate the points of LAMMA analysis. *A*, cell wall of the first cell row, in direct contact to the soil or the labelling solution; *B*, cortical cell walls of the second and, *D*, of the fourth cortical cell row; *C* and *E*, cortical cell protoplasts; *F*, cell wall of primary xylem cells. **a** Light micrograph. **b** Detail from **a**. The white circular spot in image **b** within the scale bar represents the typical analysed area of 1 μm in diameter. Bar = 100 μm (**a**) and 50 μm (**b**). **c** LAMMA spectrum from a cell wall at position *D* after labelling. *a.m.u.*, atomic mass units; *U*, detector voltage

Results

In order to analyse diffusion processes and diffusible elements in tissues, two requirements must be fulfilled. Diffusion has to be stopped rapidly, and the structural preservation of the sample has to be sufficiently good to allow localizing analysis. Shock-freezing is the only available method to fulfill both goals. However, because of the high water content, the relatively large size (ca. 300–500 μm diameter) of the finest roots, and the thermal insulation properties of plant cell walls, the conditions are extremely unfavourable for the object of our study.

Under our experimental conditions the light-microscopical appearance (Fig. 1) of the cryosubstituted samples was typical for ectomycorrhizae of spruce trees and similar to conventional preparations (Kottke and Oberwinkler 1986, 1990). All analysed finest roots appeared monotypically mycorrhizal. A single-layered Hartig net extended to the endodermis, while the surrounding hyphal mantle was small. We could not detect any evidence for the presence of an exodermis in the mycorrhizal spruce roots. Light-microscopic imaging of the autofluorescence, as well as the berberine-aniline blue staining procedure, usually taken as the most valid procedure (Brundrett et al. 1988) for the detection of cell wall modifications, positively detected Casparian bands and suberin lamellae in the endodermis but showed no indication of the presence of an exodermis. This is in agreement with observations of C.A. Peterson (Department of Biology, University of Waterloo, Ontario, Canada, personal communication) that an exodermis is typically absent in gymnosperms.

Stable-isotope labelling and LAMMA analysis. Roots that were incubated in labelling solutions were still attached to the plants. Spectra obtained from roots after a 48-h incubation time served as a positive control showing that all tracer isotopes ^{25}Mg, ^{41}K and ^{44}Ca, could be detected and isotope ratios determined by LAMMA (Fig. 1).

In contrast, no Mg or Ca label was detectable in the xylem and the cortex of control samples that were briefly dipped into the labelling solutions prior to processing. Label was only found on the external surface of the roots, associated with the outward facing cell walls of the outermost cell layer in direct contact with the labelling solution. We take this as an indication that relocation of the divalent cation label does not occur during sample preparation. Under these conditions, we determined the lower detection limit of the ^{44}Ca and ^{25}Mg isotope tracers as 1% of total element.

Entry kinetics of calcium. The shortest time period selected for the kinetical experiments was 2 min. Shorter incubation times did not seem feasible in view of the fact that the time necessary for complete mixing of solution in the boundary layer would not be much shorter. Vigorous mixing of the solutions was not applied, since this could result in inadvertent damage to the fine root. Nevertheless, in the first set of experiments we found detectable label in the cell walls of the cortex in the sample taken 2 min after the onset of labelling. However, no label was detectable within the cortical protoplasts. A detailed analysis of samples treated for 2 min or 4 min, respectively, showed a statistically significant gradient of the ^{44}Ca-label with the cell walls between the outer two and inner two of the, typically, four rows of cortical cells (Fig. 2). The existence of this gradient gives further evidence that no relevant relocation of Ca and Mg occurred during the preparation procedure. The gradient appeared less pronounced at the 4-min time point and disappeared completely after longer incubation times.

In further kinetical experiments, involving incubation times ranging from 2 min to 2 d, similar results were obtained regarding the rapid penetration of the cortical cell walls. However, in all structures interior to the endodermis, e.g. the primary xylem, Ca-label could only be detected after longer incubation times. Two independent series of experiments were performed at two different root temperatures in order to detect a possible temperature dependence of ion access and transport into the xylem. The two kinetics presented in Fig. 3 show the percentage of the ^{44}Ca-label at root temperatures of +22 °C and +6 °C. As expected, Ca peaks measured in spectra from the protoplasm of cortical cells and of primary xylem cells were not sufficiently large to obtain precise isotope ratio measurements, and were therefore not included in the figures.

Statistically significant differences in the labeling of cell walls of the different cortical cell layers were only detectable within the first 2–4 min, as shown in Fig. 2.

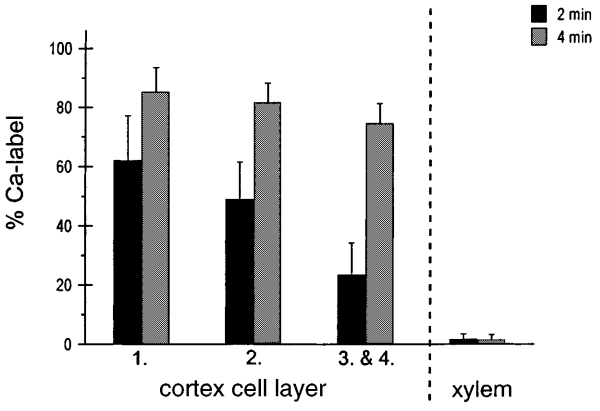

Fig. 2. Radial distribution of ^{44}Ca-label within the cortical cell walls after short exposure times to the label at a root temperature of +22 °C. The percentage of the ^{44}Ca-label within total Ca (total Ca defined as 100%) was analysed at three locations in the cortex and in the primary xylem vessels: (i) in the cell walls of the first, peripheral layer of cortical cells, in direct contact with the labelling solution [*1.*], (ii) in the second cortical cell layer [*2.*], (iii) in the cortical cell layer adjacent to the endodermis, typically the third or fourth cell layer [*3. & 4.*] and (iv) in the xylem. Incubation in the labelling solutions was for 2 and 4 min. Three different mycorrhizal roots from three different plants were analysed for each incubation time. Samples were shock-frozen, cryosubstituted, embedded, and sections analysed by LAMMA. The corrected fraction of the ^{44}Ca-labelling isotope was plotted as a percentage of the total Ca. The error bars represent the SD

Therefore the data obtained from all locations within the entire cortex for longer incubation times were pooled in order to maximize the number of sampling sites. The half-time ($t_{1/2}$) of the Ca-entry kinetics ranged from 2 to 4 min. The observation that the long-term plateau reached almost 100% indicates that there is no residual fraction of Ca in the cortex cell walls that escapes the exchange with external Ca.

The kinetics obtained from cell walls located within the vascular stele were fundamentally different from the data for the cortex (Fig. 3). There was a 100–120 min delay before a larger fraction of ^{44}Ca-label appeared in the cell walls in the stele. In most cases, the strongest Ca signals could be measured in the cell walls of the primary xylem cells. Since we could not find any obvious difference in the isotope composition between the cell walls of the parenchymatic cells and the composition in the primary xylem cells, we focussed our attention on the latter cell type, especially because these cells represent the tissue carrying the axial flow and the long-range transport of Ca ions. The data shown are the pooled isotope ratio data from the xylem cell walls. As observed in the cortex, the final value approached 100% of the label.

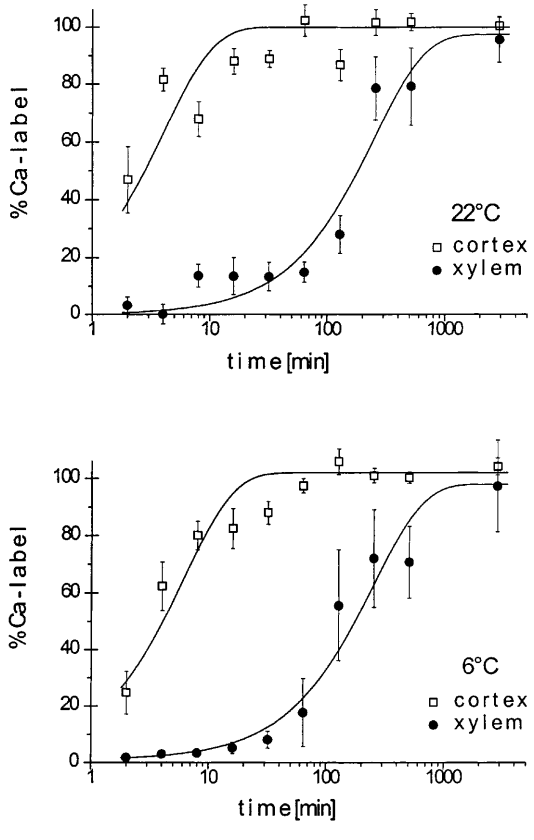

Fig. 3. Kinetics of Ca entry. The finest spruce mycorrhizal roots were incubated for the indicated time periods in labelling solutions. The ^{44}Ca-isotope percentages in cell walls of the cortex and primary xylem cells were determined by LAMMA measurements. Three different mycorrhizae from three different plants were analysed for each pair of data points. Sample preparation and presentation as in Fig. 2. *Top*, labelling at +22 °C root temperature; *bottom*, labelling at +6 °C

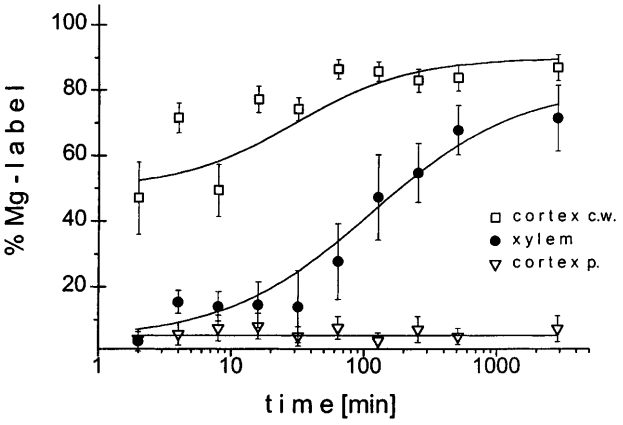

Fig. 4. Kinetics of Mg entry into cell walls of cortical and primary xylem and in the protoplast of cortical cells. Sample preparation and presentation as in Figs. 2 and 3. Root temperature was +6 °C

Even though the first time point at 6 °C seems a bit lower than at 22 °C the overall kinetics are very similar for both temperatures. This is true for the kinetics in both the cortex and the stele.

Entry kinetics of magnesium. The kinetics of ^{25}Mg entry into the cortical cell walls and into the stele (Fig. 4) appear almost identical to the data obtained for Ca (Fig. 3), with the exception that the long-term plateaus do not reach a value of 100% (for possible reasons, see *Discussion*). The data of the low- and high-temperature experiments match well and, therefore, only the kinetics of one case (that of 6 °C) are shown here.

In contrast to the Ca-distribution pattern, we found prominent peaks of Mg in the cortical cell protoplasm. We were therefore able to determine the Mg isotope ratios in these structures with sufficient precision. Surprisingly little of the Mg present in the protoplasts exchanged with the Mg-label during the entire experiment.

Discussion

Many attempts have been made to monitor the path of nutrients into the roots of higher plants. Unfortunately, there is no localizing method available to measure or visualize mineral elements within plant roots in vivo. The various root compartments and their exchange kinetics for some ions have been studied by washout kinetics of roots that were preloaded with tracer (e.g. Pitman 1971; Rygiewicz et al. 1984; Hajibagheri et al. 1988). But due to the applied technique such studies can only provide circumstantial and indirect evidence, and the validity of their interpretation has been subject to debate (e.g. Lüttge 1973). Therefore, localizing techniques are necessary that directly show the fate of ions entering the root. Substitute tracers have been applied in the past in vivo and were localized after (chemically fixed) specimen preparation (e.g. Robards and Robb 1974; Nagahashi et al. 1974). Obviously, two questions are important for the interpretation of such tracer experiments: (i) How suitable is the tracer? (ii) How

suitable are the methods of analysis and the corresponding specimen preparation technique?

The problem of the right tracer. In the past, the elements of interest (including mineral nutrients) have been substituted by presumed representative tracers e.g., fluorescent dyes as substitutes for water or ions, Sr^{2+} or Zn^{2+} for Ca^{2+}, or Rb^{+} for K^{+}. Martin J. Canny (1990, 1995) expressed, in detail, the growing concern about the use of substitute tracers. In view of the detailed knowledge about plant ion channels and their high ion-selectivity (cf. Tester 1990), the use of fluorescent dyes should be excluded because of the different charge and size of these compounds, and ion substitution because of the pronounced ion selectivity of cation channels. For the same reasons even the last "stronghold" of tracer studies involving ion substitution, the replacement of K^{+} by Rb^{+} ions should also be excluded. Reports demonstrating the discrimination of K over Rb at the total-plant level (Marschner and Schimansky 1968; Jeschke 1970) have apparently not received the appropriate attention. The discrimination is explained at the molecular level by the demonstrated selectivity for K over Rb of certain K-channels (Hedrich et al. 1995).

Stable isotopes are chemically identical tracers. Isotopes can be used to solve the problems with tracers. Because the chemical properties of isotopes are identical, isotopic labelling is the method of choice – at least in principle. Radioactive tracers have been successfully used in the past in conjunction with autoradiography. However, the lateral resolution obtained with the available tracers of mineral ions appears insufficient in this case. The lateral resolution for ^{45}Ca is limited to about 5 μm because of the high radiation energy of the isotope and the resulting penetration. Moreover, the quantitative determination of both the radio-tracer and the unlabelled element fraction within the tissue is practically impossible. The second limiting factor is the absence of suitable radioactive tracers for certain elements such as magnesium and potassium. However, stable isotopes are available as tracers for all physiologically relevant mineral ions, with the only exception of sodium. Isotope discrimination effects are negligibly small, because isotopes have the same ionic radius, charge and hydration energy, and because no covalent bonds are formed, as in the case of the elements S, O, C and N.

Detection of the isotope tracers by mass spectrometry. In contrast to the "classic" EDXA microbeam analysis, it is possible to detect isotope tracers by LAMMA (Heinen et al. 1980; Schröder et al. 1988; Kuhn et al. 1995, 1997). Since all major isotopes can be detected simultaneously in the same area of analysis, both the labelled fraction and the fraction of material originating from the plant can be determined with a precision of about 1–3%, depending on the isotope.

Specimen preparation. Within the limits of the light-microscopic resolution – and within the lateral resolution of our microprobe – the structural preservation that could be obtained with the shock-freezing, cryosubstitution protocol seemed adequate for localizing analysis at the tissue and cellular levels. Even though direct cryosectioning would be preferable to sectioning after cryosubstitution, the number of samples required to be analysed for physiological kinetics could not be obtained using the former technique. For the present study, 500 root samples were screened for optimal structural preservation and the structurally best preserved 69 samples selected for analysis. Even though the process of cryosubstitution bears the danger of ion loss and redistribution, we have good evidence that such effects do not significantly affect the Ca and Mg distribution during specimen preparation. Previously, Ca and Mg labelling in conjunction with shock-freezing and cryosubstitution were used to study exchange and transport at the cellular level, demonstrating good agreement between cryosubstituted and cryosectioned material (Sahly et al. 1994). The measured gradients (Fig. 2) and the kinetics (Figs. 3, 4) also argue against a redistribution of Ca and Mg. In addition, in control experiments with radioactive ^{45}Ca, the washout of Ca could be shown to be below the confidence limit of 10% (Kuhn 1993). Moreover, any washout of tracer would affect both isotopes identically and, therefore, would not influence the measured isotope ratios. Indeed, from the 2% value of tracer in the xylem at the 2-min time points (Fig. 4) we can conclude that any redistribution of Mg and Ca isotopes must be below this value. The high plateau of tracer at the later points (e.g. at 48 h) must originate from tracer uptake during the physiological experiment, because all samples were processed identically. Even though the solubility of monovalent cations is also low in the (water free) processing solutions, it is higher than in the case of divalent cations and a small fraction of the K present in the tissue may dissolve and be lost and/or redistributed. Therefore, we did not present the kinetics of K entry, even though ^{41}K was applied as a tracer. Further experiments using cryosections are currently in progress to monitor K access and exchange under conditions where loss and redistribution of monovalent ions can be excluded.

What are the factors influencing the entry of cations into the root? Let us consider the example of Ca^{2+} in detail.

(i) Diffusion coefficient. The value for free Ca^{2+} diffusion at 23 °C in water at the low ion concentrations as used in this study is $D = 778\ \mu m^2 \cdot s^{-1}$ (Wang 1953). If we assume an apoplastic path of $x = 200$ μm from the surface of the root up to the endodermis and $t = 3$ min, we can calculate D from the values of the present study according to

$$t = x^2/6D\ (s)$$

$$180 = 200^2/6D\ (s)$$

$$D = 37\ (\mu m^2 \cdot s^{-1}).$$

This value of D is still about 20-fold lower than for free diffusion. Obviously the kinetic of Ca^{2+} entry measured

here is too slow to represent the unrestricted free diffusion in water or the expected faster diffusion in restricted spaces. Consequently the radial movement of Ca^{2+} in spruce roots must be influenced by other factors as well.

(ii) Transpiration stream. If the solvent moves, the solute will move with it. Therefore, a vigorous transpiration stream would be expected to speed up the entry of solutes into roots. This has been shown experimentally in some species (Aloni et al. 1998). In the case of spruce under our experimental conditions, transpiration rates were very low. In view of this, and in view of the fact that Ca^{2+} moved more slowly than expected by free diffusion, one needs to look for other factors. Still, in systems with higher rates of transpiration the radial bulk flow across roots due to the transpiration stream may have a substantial effect on ion transport into roots.

(iii) Binding to mobile and fixed binding sites. This is the major universal effect slowing and limiting the distance of diffusion of Ca in many cases. Exciting new insight about the movement of Ca has come from work on Ca-signalling in muscle (Safford and Bassingthwaighte 1977), neurons (Oheim et al. 1998) and sensory cells, and from the advances in Ca-imaging techniques (Naraghi and Neher 1997). A detailed study of the effects of Ca-binding on Ca-diffusion coefficients yielded values for D ranging from 13 to 65 $\mu m^2 \cdot s^{-1}$ (Allbritton et al. 1992). This value is in agreement with the value of D shown in our study, and may indicate that Ca-binding to mobile and fixed binding sites is probably the major factor modulating the kinetics of Ca^{2+} entry. Likely candidates for fixed Ca-binding sites in the spruce roots would be components of the cell wall that carry large numbers of cation-binding sites. The expected ion chromatographic effect in a presumably similar milieu has been described for xylem transport in excised shoots (van de Geijn and Petit 1979). Since we have little information about the composition of the apoplastic liquid phase in the spruce root cortex, we can only speculate about possible mobile cation-binding sites. In general, one might expect that mobile binding sites may play only a minor role, since they would diffuse out of the root and become unavailable to influence the kinetics of Ca^{2+} entry.

(iv) Uptake into the symplast. While entering the root, a fraction of the tracer ions may exchange with the ion pool of the symplasm. If this exchange were large it would dilute the tracer and retard the apparent tracer front in the apoplast. In the present work, the most precise data on exchange between the cortical symplasm and external ions are for Mg (Fig. 4). Since the measured exchange rate is very small we can conclude that the entry into the root up to the endodermis is faster via the apoplastic path than via the symplasm. Due to the relatively low amount of Ca in the cortical protoplasts, the effect on the kinetics of the Ca^{2+} entry can also be neglected.

(v) Viscosity of the extracellular matrix. The diffusion coefficient, of course, strongly depends on the viscosity of the solute. However, we have too little information on this parameter to calculate how much the entry of ions would be retarded.

In summary, the data indicate that the entry kinetic of calcium into the cortex is primarily retarded by fixed cation-binding sites. The above arguments may also be applied to Mg^{2+} and to the release of ions from the root. The same factors will also influence the movements of anions, although the retardation due to binding sites is presumably much smaller than in the case of cations.

Cell wall modifications. Apparently the Hartig net present in the cortex does not represent a major diffusion barrier. In contrast, the endodermis is a diffusion barrier for Ca and Mg. The measured entry to structures internal to the endodermis is approximately 2 orders of magnitude slower than the entry into the cortex. This indicates a significant and relevant block for the entry of ions into the stele, and for the reverse process, the release of ions from roots. The fact that in both cortex and the stele the Ca ions exchange completely with the tracer after longer exposure times shows that this result is not limited by the ion-exchange properties of the cell walls.

The small increments in the early phase of the uptake kinetics of both Ca and Mg into the stele are rather peculiar. This small fraction of label in the stele was only detectable in the +22 °C kinetics, but was absent in the kinetics taken at +6 °C. A Student *t*-test confirmed that these measurements are statistically significant. One might speculate that the temperature dependence indicates the presence of a radial symplasmic transport system. The small percentage of labelling could be explained by a small radial transport rate and/or mixing with the "old" Ca within the stele, including axial exchange. The sudden larger rise in label in the stele after 100–120 min could result from a "front" of label reaching the cross-section in the root zone, accumulating axially from upstream regions in the transpiration stream.

Temperature dependence. Generally, if transport across a diffusion barrier occurs through cell membranes, it is mediated by transport proteins. Proteins typically show a temperature dependence that does not depend linearly on the temperature. Therefore, a strong temperature dependence would be a good indication of a symplasmic transport of the ion under study. There are several examples where ion uptake and transport into roots were reported to be temperature-dependent (see Johnson and Jackson 1964; Drew and Biddulph 1971; Schimansky 1981). In the kinetics of Mg and Ca, we find no such effect, with the exception of the small initial increment already discussed above. This is a strong indication that uptake of Ca and Mg into the stele can occur via apoplastic pathways, e.g. by small discontinuities in the endodermis such as postulated by Steudle and Jeschke (1983), Kuhn (1993), and Frensch et al. (1996). However, this does not exclude the possibility of symplasmic transport, since the protein machinery in the root system of the spruce may just be well adapted to transport of nutrients at low temperatures. Soil temperatures near 6 °C are more frequent than 22 °C in the natural habitat of this species.

The third stable-isotope tracer in the incubation solutions was ^{41}K. In contrast to divalent cations we could not exclude any loss or redistribution of the monovalent cation K^+. Therefore, no kinetics are shown for potassium. Nevertheless, we found a significant and presumably relevant difference in the labelled K fraction at the late times, when the two temperatures were compared. Both after 512 min and after 48 h, the exchange of K was 10% less at the lower temperature both in the cortex and in the stele. This difference suggests that at least one component in the uptake mechanism of potassium may be mediated by a protein.

The rapid exchange of ions (half-time $t_{1/2}$ = 2–4 min) between the cortex and external sources is highly relevant for plant nutrition since this exchange will not be a limiting factor in the uptake of cationic nutrients into fine roots. This is presumably also the case for neutral solutes and negatively charged ions. Besides, this rapid exchange is also relevant for protocols of sample preparation in the analysis of bulk root samples. In nutrient-uptake studies a rinsing step in order to remove soil debris from the root material prior to analysis should critically be taken into consideration.

Comparison of the half-times of ion exchange that we determined quantitatively within the cortex ($t_{1/2}$ = 2–4 min) and across the endodermis ($t_{1/2}$ = 100–120 min) reveals a difference of almost 2 orders of magnitude. This makes the endodermis the only relevant barrier in the path of nutrients between the rhizosphere and the long-range transport system in mycorrhizal spruce roots.

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