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ORIGINAL PAPER

Two-year dynamics of foliage labelling in 8-year-old *Pinus pinaster* trees with ^{15}N, ^{26}Mg and ^{42}Ca—simulation of Ca transport in xylem using an upscaling approach

Laurent Augusto · Bernd Zeller · Andrew J. Midwood · Christopher Swanston · Etienne Dambrine · André Schneider · Alexandre Bosc

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Abstract
• ***Introduction*** Atmospheric deposition is an important input of major nutrients into forest ecosystems. The long-term goal of this work was to apply stable isotope methodology to assess atmospheric nutrient deposition in forest systems.
• ***Materials and methods*** A labelling experiment of foliage with stable isotopes of primary and secondary macro nutrients (^{15}N, ^{26}Mg and ^{42}Ca injected into the stem sapwood) was carried on standing trees to monitor interactions between canopy and precipitations. ^{15}N rapidly reached the foliage; however, Mg and Ca were not detected in foliage until more than a year after injection.
• ***Results and discussion*** The delay in mobilization of Mg and Ca prevented us from accurately modelling deposition contributions of these two elements. Nonetheless, an upscaling approach based on published results on Ca transport in shoots xylem was used to simulate our results. These simulations of Ca transport at the tree scale were consistent with our experimental data.
• ***Conclusion*** This consistency suggested that mechanisms of nutrient transport are the same at the different scales. Nitrogen was rapidly transported in the xylem to foliage, probably mainly by mass flow. Conversely, transport of Mg and particularly Ca was considerably delayed, probably due to successive cation exchanges along the xylem vessels.

Keywords Labelling · Nutrient · Xylem · Transport · Pressurized injection

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L. Augusto (✉) · A. Schneider
INRA, UMR 1220 TCEM,
BP81, 33883 Villenave d'Ornon Cedex, France
e-mail: laugusto@bordeaux.inra.fr

B. Zeller · E. Dambrine
INRA, UR 1138 BEF,
54280 Champenoux Cedex, France

A. J. Midwood
Macaulay Institute,
Craigiebuckler,
Aberdeen AB15 8QH Scotland, UK

C. Swanston
Northern Research Station, US Forest Service,
410 MacInnes Drive,
Houghton, MI 49931, USA

A. Bosc
INRA, UR 1263 EPHYSE,
33140 Villenave d'Ornon, France

1 Introduction

In forest ecosystems, understanding nutrient dynamics is important for overall forest management strategies and assessing a range of environmental factors. One such factor is atmospheric deposition of nutrients which can be a major flux and can make a significant contribution to ecosystem nutrient input–output budgets (Ranger and Turpault 1999). The atmospherically derived flux of nutrients can in some circumstances approach the same order of magnitude as soil derived fluxes for major nutrients, with the exception of phosphorus which is deposed at very low rate (N, K, Ca and Mg; e.g. Moreno-Marcos and Gallado-Lancho 2002). Atmospheric deposition of nutrients into forest ecosystems is therefore important, but characterising this flux is not straightforward, and there has been considerable debate as to the best approach. Among the different methods dedicated to the measure of atmospheric deposition into

forests, the most commonly used (e.g. De Schrijver et al. 2008) is the canopy budget method (Staelens et al. 2008). This quantifying approach is easy to carry out but relies on two important assumptions: (1) a reference element, like sodium, is not exchanged at the foliage–precipitation interface and (2) particles containing the major nutrients are assumed to have the same mass median diameter as for the reference element and consequently have similar deposition dynamics (i.e. a similar 'forest stand deposition'/'open-field deposition' ratio). Here, we focused on this second assumption, which arguably is more controversial and difficult to fully validate (Staelens et al. 2008). Initially, our aim was to homogeneously label trees in the field and then monitor subsequent nutrient deposition dynamics in order to investigate the second assumption. Indeed, homogeneously labelled foliages would enable us to use the isotopic dilution framework and to test whether the deposition ratio is the same for the all studied nutrients. To do this, we designed an in situ labelling experiment on standing trees using stable isotopes of major nutrients (^{15}N, ^{26}Mg and ^{42}Ca). Over a 2-year period, it became clear that this approach would not be viable; nevertheless, our data did provide important insights into nutrient transport in trees. In particular, we were able to use an upscaling approach to simulate calcium transport in xylem, which successfully explained the observed fate of this element. The simulation approach, its results and potential contribution to the knowledge of nutrient transport in plants are presented.

2 Materials and methods

2.1 Species, study area and experimental stand

The *des Landes* forest is the largest man-made forest in Europe, covering more than 900,000 ha in south-western France. It was created during the second half of the nineteenth century and initially was managed for the production of resin. For the last few decades, however, the primary product has been wood, mainly destined for paper mills. It is mainly composed of even-aged stands of maritime pine (*Pinus pinaster* (Ait.)) growing on sandy podzols which are extremely poor in nutrients. In this context, the input flux of nutrients through atmospheric deposition is a major contribution to the maintenance of the soil fertility (Augusto, unpublished data).

Trees selected for this study were within an 8-year-old stand with a density of 2,500 trees ha^{-1}. Mean tree height and mean stem diameter at breast height were 4.8±0.1 m and 8.2±0.6 cm (mean value±1 standard error), respectively. The needle mass of the trees was estimated using allometric relationships (Porté et al. 2000) based on the diameter of all branches measured 10 cm from their base. Biomass ranged from 1.08 to 3.97 kg tree^{-1} for current-year needles and from 0.73 to 2.97 kg tree^{-1} for 1-year-old needles (dry weight biomass). Older needles were ignored as they represent less than 1% of the foliage in young maritime pine trees (Porté et al. 2000). The three trees which were labelled represented a total foliage biomass of 9.12 kg. Using unpublished results derived from maritime pines of similar age and located in the same area (Trichet [INRA, France], personal communication), we assumed the following nutrient concentrations of tree foliage: N= 10.0 g kg^{-1}, K=4.5 g kg^{-1}, Ca=2.5 g kg^{-1} and Mg= 1.3 g kg^{-1}. Total nutrient contents (kilogramme nutrient per tree) were calculated for each tree.

2.2 Composition of the solution for injection

Swanston and Myrold (1998) proposed the injection of ^{15}N at a level equivalent to 1% of the total nitrogen content of the tree (kilogramme nutrient per tree). A similar approach was used in the present study, but with slightly lower percentages of ^{15}N (0.6%) and ^{42}Ca (0.7%). As the natural abundance of ^{26}Mg is high (11.0%), the injected dose was increased to 2.4% of the estimated total Mg content of the foliage. We planned on using ^{41}K isotope enrichment; however, the high natural abundance ^{41}K (6.7%) and the cost of the isotope was prohibitive for this study. The solutions for injection were prepared with KNO_3, $CaCO_3$ and MgO. Calcium carbonate and magnesium oxide were first completely dissolved by adding volumes of HCl (0.5 mol L^{-1}) under agitation (Ca^{2+} and Mg^{2+} in solution). Ultra-pure water was then added to achieve the chosen concentrations: 4.3 g N L^{-1}, 1.4 g Ca L^{-1} and 2.1 g Mg L^{-1}. Malic acid is one of the primary constituents of sap in most trees and was also included in the solution for injection at a concentration of 0.4 mmol L^{-1} as previously proposed (Swanston and Myrold 1998). The pH of the solution was adjusted to 5.4 with HCl or KOH, and 1.0 g L^{-1} of acid fuchsin dye was added (Umebayashi et al. 2007). Dye was added to enable the solution injected into the trees to be traced visually (see below the preliminary tests) and to enable any leaching that occurred during the injection into the stem to be spotted. Labelling solutions were prepared with compounds with the following isotopic enrichment (atom percent): $K^{15}NO_3 \geq 98\%$, $^{42}CaCO_3$=79% and ^{26}MgO=98.75%. Considering the composition of the solution and the amount of isotopes to be injected, the volume to be introduced into the sapwood was 14.8 ml kg needles^{-1}.

2.3 Stem injection procedure

The first attempts we made using the common passive injection technique (Swanston and Myrold 1998) failed as

the injection holes were systematically clogged by the resin of the trees 1 to 4 h after the beginning of the process (even so some dye was found up to 0.9 m above the injection point; data not shown). To prevent the resin from clogging the system, we decided to inject isotopes under pressure, reasoning that by accelerating the injection process, the isotopes could be delivered into the stemwood before resin occluded the injection site. The isotopes were injected at bud break (2 May 2005) into three trees selected for labelling. Three additional trees were used as controls (no stem injection). Six coach screws (length=45 mm, diameter= 8 mm) were completely drilled (diameter=4 mm) through their longitudinal axis. A valve was inserted into the top of each coach screw. After bark and phloem were removed, holes (diameter=6 mm) were drilled perpendicularly into the sapwood. A coach screw was inserted into each hole. Orians et al. (2004) observed that in some tree species, there is a degree of sectoriality to sap flow that could lead to preferential conduction of an injected compound to only one part of the canopy. Consequently, two injection points were located on each tree diametrically opposed and at different heights on the trunk (approximately 15 and 60 cm from the ground). Once all trees were equipped with coach screws, a manual side lever grease gun (Umeta 75/PKU, Germany), previously rinsed with acid (HCl 0.5 mol L^{-1}) and washed with ultra-pure water, was filled with the labelling solution. The grease gun was connected to the valves in the coach screws and used to force the isotope solutions into the stemwood of the trees under pressure (~1.6 ml h^{-1}), which was rated at ~800 bars by the manufacturer. For each tree, the dose solution was split equally between the two injection points. At the end of the injection, the wounds inflicted on the tree during the injection procedure were covered with an inert mastic general purpose garden sealant, and the trees were left undisturbed for 1 month (no sampling of needles, see below).

2.4 Tree sampling

The three labelled trees and three control trees were sampled over a period of 2 years. At each sampling date (for ^{15}N analyses: 23rd March (pre-injection), 31st May, first July, 20th October 2005; eighth February, 28th March 2006; 21st March 2007; for ^{42}Ca and ^{26}Mg: first July, 11th August 2005; 28th March 2006; 21st March 2007), ten current-year needles and ten 1-year-old needles were collected. The needles were harvested from the outer half of branches in the lower half of the canopy at each cardinal direction (N, W, E and S). The harvest was repeated following the same pattern, but on the inner half of the branches. Finally, all needles of the same age were pooled to form a composite sample for each tree. At some dates (23rd March 2005, 31st May 2005, 20th October 2005 and 21st March 2007), the same sampling procedure was applied in the upper half of the canopy giving two more composite samples (current-year needles and 1-year-old needles) per tree.

At the final date (21st March 2007), the trees were cut down. Stemwood was sampled by cutting 2–3-cm thick disks with a manual saw. Eleven disks per tree were cut as follows: at the level of the injection points (15 and 60 cm from the ground); at 10, 20 and 40 cm above the injection points (25, 35, 55, 70, 80 and 100 cm from the ground); and at 200, 350 and 500 cm from the ground. For the control treatment, the sampling design was 0, 50, 100, 200, 350 and 500 cm from the ground. All living needles were harvested and separated according to their age and tree. They were dried at 70°C and weighed.

2.5 Analyses, calculations and statistics

Needles were gently washed with distilled water and frozen at −80°C to facilitate final grinding. The samples were then dried at 70°C for 48 h, coarsely ground and finally finely ground in a ball mill (Retsch PM4 planetary grinder). Bark was separated from the wood samples, and the wood disk was cut into pieces with a small axe. A representative fraction of the bulk sample of wood was then prepared for analysis using the same methodology as above.

Nitrogen concentration and N isotopic composition were determined using an online continuous flow CN analyser (Carlo Erba NA1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Mg and Ca isotope ratios were measured in nitric acid digests of the vegetation using an inductively coupled plasma-mass spectrometer (ICP-MS; 7500i and 7500ce Agilent Technologies (UK) Ltd, Stockport, Cheshire, UK; Weatherall et al. 2006). All isotope ratio measurements were based on ten replicates and three measurements per mass. Correction for mass discrimination during the isotope analysis was made by analyzing a standard solution of known isotopic composition after every ten samples. A mass discrimination term was then calculated across the entire analysis run and a linear correction applied by interpolation to the intervening samples. This procedure was checked by monitoring the isotope ratio of a quality control material (acid digest of vegetation quality control and standards internal to the laboratory) analyzed with the samples.

We calculated two theoretical values for isotopic signatures of needles. The ‘standard values’ are the global natural abundances of ^{15}N, ^{42}Ca and ^{26}Mg. The ‘maximum values for foliage’ correspond to the theoretical isotopes ratios in the needles if all injected isotopes had been transported into foliage (values calculated assuming the following nutrient concentrations of tree foliage: N= 10.0 g kg^{-1}, Ca=2.5 g kg^{-1} and Mg=1.3 g kg^{-1}).

The percentage of isotope recovery in the foliage was calculated at the final date using the tree biomass, isotope

abundances, nutrient concentrations and the injected dose. For the aerial woody compartments, we assumed that the isotope abundances in the branches and in the stembark were similar to the mean value measured in the stemwood. Biomass and nutrient concentrations of branches and stembark were estimated with allometric relationships (Porté et al. 2002) and data published on *P. pinaster* (Augusto et al. 2008). We used the same approach for the coarse root compartment (Ritson and Sochacki 2003; Trichet, personal communication). Isotope losses through litterfall were estimated based on the weight of the oldest cohort of needles, their nutrient concentration and the isotope abundance. Losses through the turnover of the tree's fine roots were also estimated. The fine root biomass of each tree (kilogramme) was estimated as follows:

$$\text{Fine roots}_{\text{tree}} = \left(\frac{\text{Fine roots}_{s\tan d}}{S\tan d_{\text{density}}}\right) \times \left(\frac{\text{SB}_{\text{tree}}}{\text{SB}_{s\tan d}}\right) \quad (1)$$

where SB_{tree} and SB_{stand} were the stem basal area of the tree and the mean stem basal area of the trees of the stand, respectively. Stand density was 2,500 trees ha^{-1}, and Fine $roots_{stand}$ was fixed at 4 Mg ha^{-1} based on local studies (Bakker et al. 2009). Since the fine root biomass of each tree was calculated, the isotope losses were estimated using the same method as for litterfall and assuming a root annual turnover of 1. The global recovery percentage of a tree was estimated as the sum of all recovery percentages and losses.

All results were expressed as a mean value±1 standard error of the replicates (in percents of atom). The coefficient of variation (hereafter referred to as CV) was calculated as the 'standard deviation/mean' ratio. Differences between treatments were tested using the Bonferroni *t* test and ANOVA with the 'repeated measurements' option so as to take into account the dependency of values collected at different sampling dates.

2.6 Simulation of calcium transport

Our initial aim was to use an isotopic dilution technique to test whether the three nutrients studied had similar deposition dynamics. Unfortunately, no homogeneous labelling of the foliage was achieved after 2 years, so that our investigations related to this purpose could not be carried on any further. In response to the rather unexpected findings regarding poor Ca and Mg transport, we went through scientific literature to find plausible explanations instead. This attempt was done using an upscaling approach to simulate calcium transport in xylem.

We only found one reference which enabled us to make some quantitative calculations. Ferguson and Bollard (1976; hereafter referenced to as 'laboratory' experiment) monitored in the laboratory the kinetics of ^{45}Ca elution from small sections of 1-year-old shoots collected from apple trees. We compared these results, which were obtained in well-controlled conditions on small shoots, with our field experiment using trees (hereafter referenced to as 'field' experiment).

Because the xylem has a high cationic exchange capacity (Marschner 1995), we hypothesized that the movement of Ca in the sap could be seen as the transport of a cation in a chromatographic column, as already assumed by Biddulph et al. (1961). In other words, the upward movement of Ca in the xylem was considered as almost completely controlled by exchange reactions (Biddulph et al. 1961; Bell and Biddulph 1963). The rate of ascension was hence assumed not to depend only on the water mass flow but mainly on the flux of cations able to exchange with Ca (Shear and Faust 1970), weighted by their rank in the lyotropic series (Bell and Biddulph 1963), and on sapwood volume (Evans 1964). Because Ca (and Mg) is mainly taken up by roots as cations and by passive process (Marschner 1995), Ca flux was here estimated by multiplying the flux of plant transpiration by the Ca concentration in the soil solution:

$$\frac{\text{Ca flux}}{\text{VOL}_{\text{sap}}} = \frac{\left(\text{TRSP}_{\text{H}_2\text{O}} \times [\text{Ca}]_{\text{sol}}\right)}{\text{VOL}_{\text{sap}}} \quad (2)$$

where $TRSP_{H_2O}$ was the transpiration of water through the plant, $[Ca]_{sol}$ was the concentration of Ca in the solution supplying the sap and VOL_{sap} was the volume of the sapwood cylinder.

To test the comparability of the field experiment (our study) with the laboratory experiment (Ferguson and Bollard 1976), we assumed a 30% Ca recovery in foliage consistent with our findings after 2 years (30% was the percent recovery of ^{42}Ca in the foliage at the end of our survey). If the two experiments are comparable, then the homothetic Eqs. 3 and 4 should be verified for 30% of recovery of ^{42}Ca (or ^{45}Ca):

$$\frac{\text{Ca flux}_{\text{field}}}{\text{VOL}_{\text{sap-field}}} = \frac{\text{Ca flux}_{\text{lab}}}{\text{VOL}_{\text{sap-lab}}} \quad (3)$$

and

$$\text{TRSP}_{\text{H}_2\text{O-field}} = \text{TRSP}_{\text{H}_2\text{O-lab}} \times \frac{[\text{Ca}]_{\text{sol-lab}}}{[\text{Ca}]_{\text{sol-field}}} \times \frac{\text{VOL}_{\text{sap-field}}}{\text{VOL}_{\text{sap-lab}}}. \quad (4)$$

For the laboratory values (Ferguson and Bollard 1976), we used the dataset with shoots collected during the growing season (April) because we considered that most of the sap flux occurs at this period ($[Ca]_{sol\text{-}lab}$ = Ca concentration of the elution solution (2.0×10^{-3} mol L^{-1})).

The sapwood volume was estimated according to the size of the shoot assuming a sapwood proportion of 0.95 ($VOL_{sap-lab}=3.2\times10^{-3}$ L). The volume of solution which passed through the sapwood for 30% of ^{45}Ca recovery was estimated from a figure given in the article cited above ($TRSP_{H_2O-lab}\approx28.9\times10^{-3}$ L with a rate of elution of 4.5 ml h^{-1}).

For the field values, $[Ca]_{sol\text{-}field}$ was estimated based on a single soil sampling on 14 April 2009. We assumed that one sampling would be indicative of the mean chemical composition of the soil solution over a year, as previously reported in six stands growing on acidic soils (Augusto and Ranger 2001; CV=14–31% for Ca concentration). Five soil cores were sampled down to around 45 cm. The cores were split into three volumes (corresponding to 0–15, 15–30 and 30–45 cm soil layers; litter was ignored and was minimal with a thickness≈1 cm). Soil samples were immediately sieved (mesh size=4 mm) and analyzed according to Schneider (2003). The calculated concentration of Ca in the soil solution was estimated by weighting the concentration of each soil layer by the density of fine roots of maritime pine stands located in the same experimental forest (Bakker et al. 2009). These results showed that 85% of the fine roots were in the 0–45-cm layer.

The stem volume of the field experiment was estimated according to the stem base area of the labelled trees and to the length between the injection points and the foliage areas of the lowest branches sampled. The volume of sapwood was calculated assuming a sapwood proportion of 0.88 (data from Augusto et al. 2008).

Once all values required by Eq. 4 were available, $TRSP_{H_2O-field}$ was calculated. Finally, we used the ecophysiological GRAECO model (Loustau et al. 2005) to simulate the cumulated transpiration of the labelled trees from the date of injection (2 May 2005) until the last tree sampling (21 March 2007). The cumulated transpiration flux of trees was compared to $TRSP_{H_2O-field}$, which was the theoretical transpiration flux needed to explain our results on ^{42}Ca at final sampling. Parameters of the GRAECO model were established on characteristics of our stand (e.g. available soil≈0–50 cm; understory height≈25 cm).

3 Results

3.1 Needles

The isotopic signature of N before injection (23 March 2005) was very homogenous among trees (CV<1%; Table 1). We assumed similar homogeneity for ^{42}Ca and ^{26}Mg before injection. During the 2-year period of the experiment, the isotopic signature of control trees was constant and at natural abundance for each of the measured isotopes (0.371±0.001 at.% (atom percent) ^{15}N, 0.661±0.018 at.% ^{42}Ca and 11.12±0.03 at.% ^{26}Mg).

The pressure injection approach enabled the labelling solution to be successfully introduced into the trees. However, in the case of one tree, a few millilitres of solution (visually estimated as ≈2–5 ml) were lost when the pressure imposed by the grease gun inside the tree was too high. The loss of the injected solution was due to leaching through wood and bark (at a distance of ~10 cm from the injection point).

Except during and just after the bud break period, no temporal trend was observed in the concentration of nitrogen in the needles (Table 1). Current-year needles had slightly higher N concentrations than 1-year-old needles. No significant difference was observed between labelled trees and control trees even if there appeared to be an insignificant trend, at least in the 2005–2006 winter in current-year needles. The nitrogen concentration was lower (6 to 10 $g\,kg^{-1}$) than expected (10 $g\,kg^{-1}$).

After injection, labelled trees showed higher ^{15}N abundance than control trees as expected (Fig. 1). These differences were significant at all dates ($P<0.05$). Nitrogen isotope composition was quite heterogeneous 1 month after injection (CV: current-year needles=89%; 1-year-old needles=14%) partly due to the partial loss for one tree of the labelling solution. Heterogeneity decreased over time, mainly in current-year needles (CV: first July 2005=37%; 20th October 2005=20%; eighth February 2006=5%; 21st March 2007=3%). After 1 month, the isotopic signal decreased. One-year-old needles showed a similar trend but of lower magnitude. Although the ^{15}N signal decreased strongly, labelling was still clearly detectable 1 year after the injection (current-year needles, 0.47±0.02 at.% ^{15}N; 1-year-old needles, 0.43±0.00 at.% ^{15}N). Two years after the injection, the labelling was relatively constant and homogenous among cohorts and trees (0.43±0.00 at.% ^{15}N; $\delta^{15}N=191\pm21‰$). At this date, 7–11% of the injected ^{15}N was recovered in the labelled foliage. As needle biomass was not monitored during the experiment, it was not possible to calculate the recovery percentage in a reliable way for other dates. The final total foliage biomass was 1.91–5.86 kg for the labelled trees, which confirmed the validity of the allometric relationships used (data not shown). The estimated ^{15}N recovery was 42–62% for the total aboveground biomass (stem + canopy). When coarse root biomass, litterfall and fine root turnover were also taken into account, the estimated recovery (based on several assumptions) was 73% for the tree from which some solution leaked and ≥94% for the other trees.

Initially, there was no statistically significant difference in the ^{15}N abundance between needle cohorts ($P\geq0.16$), but heterogeneity of the ^{15}N abundance in the few months after injection prevented any difference from being detected. In the latter half of the experiment, the signals of the cohorts

Table 1 Mean ^{15}N abundance values of needles in relation to the time after labelling and position in the canopy

Sampling date	Canopy position	Needle age (years)	[N] (mg g^{-1})	^{15}N abundance (at.%)
23rd March 2005 (before injection)	Lower half	1	8.9±0.7	0.37±0.00
	Lower half	2	6.3±0.5	0.37±0.00
31st May 2005 (after injection)	Lower half	1	12.2±1.9	1.45±0.91
	Upper half	1	10.0±0.8	0.49±0.06
	Lower half	2	7.3±0.3	0.47±0.05
	Upper half	2	6.8±0.3	0.39±0.01
20th October 2005	Lower half	1	7.3±1.1	0.58±0.08
	Upper half	1	7.6±1.0	0.46±0.02
	Lower half	2	6.1±0.8	0.48±0.05
	Upper half	2	5.6±0.7	0.41±0.01
21st March 2007	Lower half	1	10.5±1.0	0.43±0.01
	Upper half	1	10.1±0.1	0.43±0.01
	Lower half	2	8.6±0.2	0.44±0.02
	Upper half	2	8.9±0.4	0.43±0.01

Mean±1 standard error

had clearly converged (Fig. 1). No significant difference was detected between needles from the lower half of the canopy and those from the upper half (Table 1) on the sampling dates when homogeneity within each tree was explored (31st May 2005, 20th October 2005 and 21st March 2007; $P \geq 0.17$). However, there appeared to be a trend towards greater enrichment in the lower half of the canopy during the first year.

No significant change, or even apparent trend, of isotope composition was detected for Ca (Fig. 2; $P=0.80$) or Mg (Fig. 3; $P=0.90$) during the first year of the survey. ^{42}Ca and ^{26}Mg abundances in both control trees and injected trees remained close to the natural abundance level during the first half of the experiment. Concentrations of ^{42}Ca and ^{26}Mg in foliage rose in the second year of the survey (Figs. 2 and 3). However, the signal was heterogeneous within the canopy of each tree, mostly for Ca (CV=34%). The final recovery of ^{42}Ca and ^{26}Mg in the foliage was 30% and 17%, respectively. Considering the heterogeneity of the isotopic signals, these figures should be treated with a degree of caution.

3.2 Stemwood

The dye was still present at the wound sites which could explain the high enrichment in stemwood near the injection

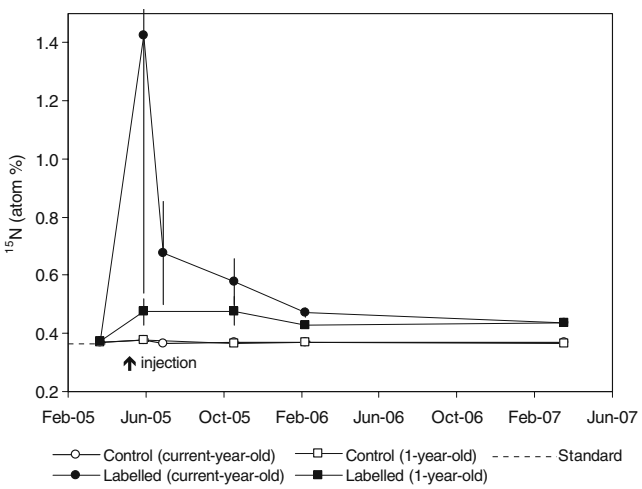

Fig. 1 ^{15}N content of needles, means±1 standard error ($n=3$). The measured natural abundance level of the ^{15}N in the needles is indicated on the graph. *Error bars* could not been drawn for some points because of very low standard error values

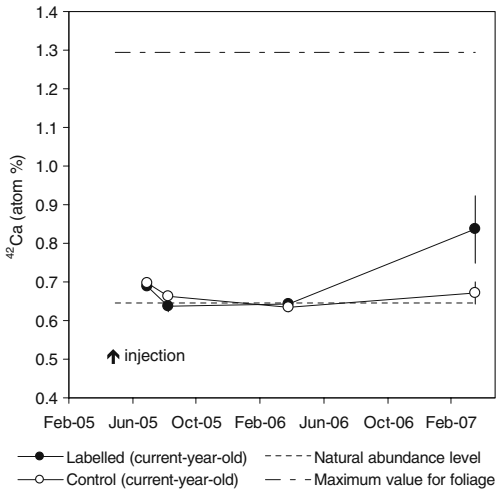

Fig. 2 ^{42}Ca content of needles, means±1 standard error ($n=3$). The accepted natural abundance level of ^{42}Ca is illustrated together with the theoretical maximum isotope level assuming all the injected isotope was in the foliage. *Error bars* could not been drawn for some points because of very low standard error values

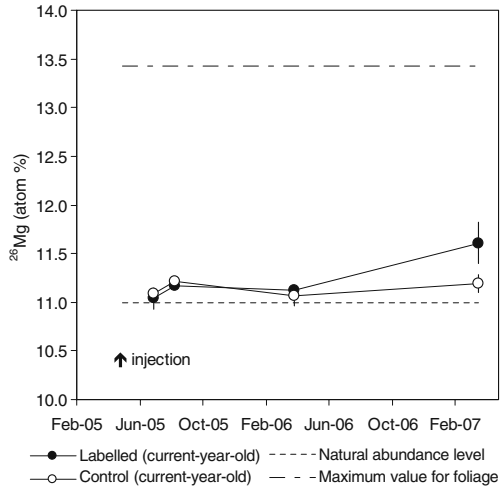

Fig. 3 ^{26}Mg content of needles, means±1 standard error (n=3). The accepted natural abundance level of ^{26}Mg is illustrated together with the theoretical maximum isotope level assuming all the injected isotope was in the foliage. *Error bars* could not been drawn for some points because of very low standard error values

points (data not shown). With the exception of the injection points and the stem volume within 10 cm of the injection points which exhibited high ^{15}N enrichment, the ^{15}N signal was fairly homogenous (CV<5%) and significantly different (P≤0.04) from control trees (0.37±0.00 at.% ^{15}N; δ^{15}N=2±1‰) in each labelled tree (0.50±0.02 at.% ^{15}N; δ^{15}N=356±25‰). The ^{42}Ca and ^{26}Mg abundances of stemwood from labelled trees were not different (P≥0.30) from baseline values in the lower part of the trunk (^{42}Ca=0.65 at.%; ^{26}Mg=11.01 at.%). In the upper part (≥200 cm), some samples showed very high values (^{42}Ca: up to 1.03 at.%; ^{26}Mg: up to 12.09 at.%). However, heterogeneity was high, the values were scattered and no clear systematic distribution was observed (data not shown).

3.3 Simulation of calcium transport

The heterogeneity of the Ca and Mg labelling of the foliage disabled us from using the isotopic dilution technique. Consequently, we were not able to reliably test whether the 'forest stand deposition'/'open-field deposition' ratio was the same for the three studied nutrients. The simulation results for Ca transport were as follows:

The Ca concentration in the different soil layers was 2.4–4.5×10^{-5} mol L^{-1}. Taking into account the fine roots distribution in the soil, the final mean Ca concentration in the solution (i.e. $[Ca]_{sol\text{-}field}$) was 4.0×10^{-5} mol L^{-1}. This value was one to two orders of magnitude lower than $[Ca]_{sol\text{-}lab}$. The stem volume of the field experiment ($VOL_{sap\text{-}field}$) ranged from 3.3 to 8.0 L. This range of values was three orders of magnitude higher than $VOL_{sap\text{-}lab}$.

$TRSP_{H_2O-field}$ calculated from Eq. 4 ranged from 1,446 to 3,524 L tree^{-1}, which was four to five orders of magnitude higher than $TRSP_{H_2O-lab}$. The cumulated fluxes of transpiration of the labelled trees as simulated by the GRAECO ecophysiological model were 1,587 to 4,870 L tree^{-1} (lines in Fig. 4), which corresponded to 1.1–1.4-fold higher values than $TRSP_{H_2O-field}$ calculated with Eq. 4. The period when this cumulated flux corresponded to the calculated value of $TRSP_{H_2O-field}$ was thus 14th July 2006 to 14th January 2007 (Fig. 4). Then, the delay between the injection of ^{42}Ca and the calculated dates was 1.20–1.71 years. The real delay between the date of injection and the last sampling date of foliage was 1.89 years. So, the calculated dates and the date of analysis for which 30% of the injected ^{42}Ca was recovered in the needles were in the same order of magnitude.

4 Discussion

4.1 Isotopic labelling of trees

4.1.1 Nitrogen

Direct labelling of trees with low-cost ^{15}N and delivered using stem injection produced results comparable with those of Swanston and Myrold (1998). In both cases, foliage isotopic enrichment was quite high and heterogeneous a few weeks after the injection, which suggests a quite high sectoriality of the sap flow (Orians et al. 2004). Indeed, spiral ascent of sap is a common pattern in gymnosperm trees (Kozlowski et al. 1967; Waisel et al. 1972), which could explain the observed short-term heterogeneity.

The transport of nitrogen in the sapwood was rapid (<1 month according to isotopic analyses), in a few days

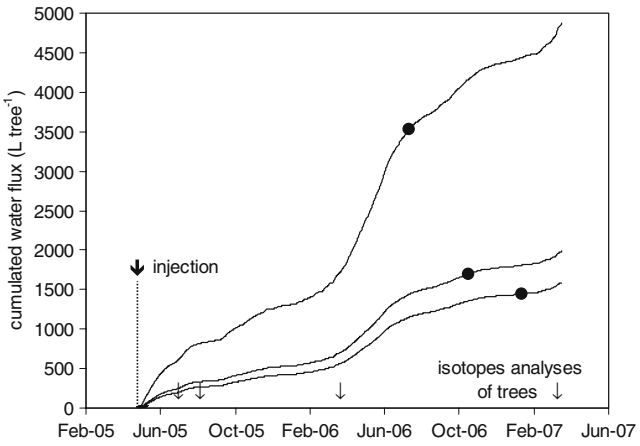

Fig. 4 Simulation of the cumulated water flux by transpiration of the three labelled trees. Calculations were made using the GRAECO model. *Closed circles* indicate the date at which the cumulated water flux corresponded to the value theoretically necessary to reach 30% of ^{42}Ca recovery in the needles (see Eq. 4 and related text)

assuming an undelayed mass flow transport (Meinzer et al. 2001). Thereafter, foliage isotopic enrichment decreased and became homogenous after a few months. Both experiments demonstrated a similar N enrichment after 1 year (around 0.45 at.% ^{15}N), which represents sufficient labelling for biogeochemical study purposes ($\delta^{15}N > 100‰$). Labelling peaked first in young needles: this was a common result in other experiments (e.g. Nadelhoffer et al. 1999), which is generally attributed to the higher physiological activity of this fast-growing foliage.

The recovery percentage of ^{15}N in foliage 1 year after injection was low in our experiment (≈10%) compared to those of Proe et al. (2000a; 40–70%) in 4-year-old *Pinus radiata*. The 'foliage biomass/woody aerial biomass' ratio decreases steeply with tree age (Ritson and Sochacki 2003), and as our trees were older than those used by Proe et al., we suggest that our lower recovery percentage was due to a larger nitrogen sink in the trees' woody components. This hypothesis is consistent with our estimates of the recovery percentage of ^{15}N for the tree total biomass and to published results which showed that stems, stumps, coarse roots and fine roots may contain a very high percentage of the injected nitrogen (Seiter and Horwath 1999; Dail et al. 2009; Garten and Brice 2009).

4.1.2 Calcium and magnesium

Our data indicated that Ca and Mg have very low mobility in this species of pine, similar to results observed for ^{45}Ca on apple seedlings (Shear and Faust 1970) and for ^{26}Mg on pine trees (Payn [Scion Inst., N.Z.], personal communication). These results seem to be in contradiction with those of Brandtberg et al. (2004) which showed a detectable uptake of ^{45}Ca, a radio-isotope, by trees only 1 month after isotopes application onto soils. Since this reference did not show any recovery rate, we tried to estimate them. Taking into account the decrease in radioactivity after 1 month (^{45}Ca half-life= 162.6 days) and assuming a tree foliage biomass of 10 kg tree^{-1}, we estimated that only 0.04–0.31% of the injected ^{45}Ca was present in the foliage in the study by Brandtberg and his colleagues. On the other hand, we consider that at least 10% of the injected ^{42}Ca and ^{26}Mg should have been recovered in the foliage in our experiment to visibly increase the abundance of the injected isotopes (from 0.65 to 0.71 at.% for ^{42}Ca; from 11.01 to 11.36 at.% for ^{26}Mg). This comparison illustrates the difference in sensitivity between the radio-isotopic approach and approaches using stable isotopes. The former is highly sensitive as it measures a radiation flux which was initially absent from the object under study (Göransson et al. 2006), the trees in this case, whereas the latter measures the dilution of a pre-existing isotope pool. Consequently, we conclude that the two studies are not inconsistent with each other and that the methodological difference can explain the discrepancy in the time lag between labelling and a significant response in the tree foliage.

4.2 Simulation of calcium transport

The parameter values for Ca concentration in solution, volume of sapwood and water transpiration were highly different (two to five orders of magnitude) between the two datasets (laboratory and field experiments). Even though, the simulated delay between the injection of Ca into sapwood and its retrieval in foliage was consistent with our survey results. The differences observed were not surprising since several assumptions and approximations were necessary to complete the simulations. Consequently, we considered that the moderate discrepancy between the two approaches was logical and that the field results were consistent with those obtained in laboratory conditions.

4.3 Interpretation of the results

4.3.1 Nitrogen

Nitrogen is transported as monovalent ions (NO_3^- and NH_4^+) in the xylem, and its conduction seems to be rapid (Marschner 1995). Similarly, nitrogen is intensively translocated among tree compartments (Colin-Belgrand et al. 1996; Proe et al. 2000b; Fife et al. 2008) owing to its high mobility through the phloem (Marschner 1995). Our results were in agreement with this functioning scheme (rapid increase followed by an ~80–90% decrease of the ^{15}N signal in the foliage due to dilution by subsequent uptake of ^{14}N and internal recycling of ^{15}N).

4.3.2 Calcium

Calcium mobility is known to be low because of its very strong adsorption on the cell walls of the xylem (e.g. Kuhn et al. 1995). Indeed, xylem vessels contain fixed negative charges, which are associated with cations and divalent cations, such as Ca, which are more strongly retained in the vessels than monovalent cations (e.g. Zwieniecki et al. 2001). In addition, the mobility of Ca in the phloem is extremely low due to physiological constraints (Marschner 1995), which explain that this nutrient resists retranslocation within the tree (e.g. Colin-Belgrand et al. 1996; Fife et al. 2008). Therefore, the very slow response of the foliage to ^{42}Ca injection into the stem was probably more related to the ions than to the method of injection or to the method of sampling. Moreover, the upscaling of results obtained over a short period in shoots (Ferguson and Bollard 1976) to our experimental conditions (monitoring of standing trees over a period of 2 years) was in reasonable agreement with the

curves of the isotope composition. It suggested that the general theory for ionic exchanges explains most of the Ca rate of transport in the xylem.

We speculate that the conduction of most of ^{42}Ca in the sapwood occurred as a 'slowly advancing front', like in a chromatographic column. In this hypothesis, when ^{42}Ca cations were injected into the sapwood, almost all of them immediately exchanged with Ca already present on the surface of the sapwood vessels (Biddulph et al. 1961). An almost insignificant proportion probably remained in the sap and quickly reached the upper part of the plant (Brandtberg et al. 2004). Once all ^{42}Ca cations were adsorbed near the injection points, they remained immobile unless a flux of further Ca absorbed by the trees from the soil enables exchange allowing 'upward movement' from the injection point (Bell and Biddulph 1963; Jacoby 1967). The movement of the injected isotope was then accomplished by successive exchanges along the tree sapwood and was controlled by the flux of Ca which passed through the stem section (Bell and Biddulph 1963).

4.3.3 Magnesium

Some analogies could be made between Mg and Ca. Indeed, because there are both divalent cations, their transport in the xylem is delayed by the negative charges of the vessels. In the same way, even if the mobility of Mg in the phloem is high, the effective flux of this nutrient through phloem transport is low to moderate (Marschner 1995; Proe et al. 2000b). Our results were supported by this pattern as the introduced ^{26}Mg reached foliage more than 1 year after injection. The lower percentage of recovery of ^{26}Mg in the foliage compared to those of ^{42}Ca may be due to competition among cations because Mg flux is known to be depressed by other cations (Kurvits and Kirby 1980; Heenan and Campbell 1981). Another explanation could be that ^{26}Mg, which reached foliage during the second growing season, was partly remobilized though phloem transport during the following fall (Proe et al. 2000b; Weatherall et al. 2006). The ratio of total concentration in *P. pinaster* needles between current-year-old needles and 1-year-old needles supported this hypothesis (N>1, Mg=1 and Ca<1; Augusto et al. 2008). Indeed, it indicated that nitrogen is intensively remobilized from current-year-needles during the fall season, that Ca is accumulated during all needle life (Fife et al. 2008), whereas Mg has an intermediate fate.

5 Conclusion

The initial objective was to obtain a homogenous labelling of tree foliage to test an assumption on atmospheric deposition. This objective could not be attained because of the labelling delay. Direct labelling of tall trees within a single growing season using stable isotopes is suitable for N but not easy to carry out for Ca or Mg. For stable isotopes, the long time lag between injection and visible labelling and the high variability will likely be problematic in many experimental designs. To improve the ^{42}Ca and ^{26}Mg labelling of trees, we suggest introducing a highly concentrated solution of KCl (van Ieperen and van Gelder 2006) or divalent cations (Bell and Biddulph 1963), which should cause an efflux of the ^{42}Ca and ^{26}Mg adsorbed on the vessels through exchange reactions. Introducing the tracer in a complex form, with EDTA for instance (Ferguson and Bollard 1976), or injecting the isotopes close to the canopy could be alternative methods. However, even with a significant amount of ^{42}Ca at the foliage level, a long period may be necessary to homogeneously label the tree as this nutrient is not easily translocated from the foliage (Marschner 1995; Fife et al. 2008). An alternative could be to use radioisotopes (Brandtberg et al. 2004), but this approach has also environmental and technical constraints.

The results of the present in situ study obtained at the tree scale were in accordance with current knowledge on nutrient transport in plants, which is mainly based on annual plants or seedlings (see Marschner (1995) for a synthesis). This consistency suggested that the theory of transport of some nutrients as previously observed at the shoot scale in controlled conditions is likely to be valid at the tree scale. In this scheme, nutrients which are transported as anions (e.g. NO_3^- or PO_4^{2-}) are not be adsorbed in the sapwood and are be conducted in a very short time to the foliage (Ferguson and Bollard 1976; Göransson et al. 2006) by mass flow. Conversely, most of the upward transport of divalent cations (e.g. Ca^{2+} and Mg^{2+}) is accomplished by successive exchanges along the tree stem and branches.

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References

Augusto L, Ranger J (2001) Impact of tree species on soil solutions in acidic conditions. Ann For Sci 58:47–58

Augusto L, Meredieu C, Bert D, Trichet P, Porté A, Bosc A, Lagane F, Loustau D, Pellerin S, Danjon F, Ranger J, Gelpe J (2008) Improving models of forest nutrient export with equations that predict the nutrient concentration of tree compartments. Ann For Sci 65:808

Bakker MR, Jolicoeur E, Trichet P, Augusto L, Plassard C, Guinberteau J, Loustau D (2009) Adaptation of fine roots to

annual fertilization and irrigation in a 13-year-old *Pinus pinaster* stand. Tree Physiol 29:229–238

Bell CW, Biddulph O (1963) Translocation of calcium—exchange versus mass flow. Plant Physiol 38:610–614

Biddulph O, Nakayama FS, Cory R (1961) Transpiration stream and ascension of calcium. Plant Physiol 36:429–436

Brandtberg PO, Bengtsson J, Lundkvist H (2004) Distribution of the capacity to take up nutrients by *Betula* spp. and *Picea abies* in mixed stands. For Ecol Manage 198:193–208

Colin-Belgrand M, Ranger J, Bouchon J (1996) Internal nutrient translocation in chestnut tree stemwood: III. Dynamics across an age series of *Castanea sativa* (Miller). Ann Bot 78:729–740

Dail DB, Hollinger DY, Davidson EA, Fernandez I, Sievering HC, Scott NA, Gaige E (2009) Distribution of nitrogen-15 tracers applied to the canopy of a mature spruce-hemlock stand, Howland, Maine, USA. Oecologia 160:589–599

De Schrijver A, Staelens J, Wuyts K, Van Hoydonck G, Janssen N, Mertens J, Gielis L, Geudens G, Augusto L, Verheyen K (2008) Effect of vegetation type on throughfall deposition and seepage flux. Environ Pollut 153:295–303

Evans EC (1964) Polar transport of calcium in the primary root of *Zea mays*. Science 144:174–177

Ferguson I, Bollard EG (1976) The movement of calcium in woody stems. Ann Bot 40:1057–1065

Fife DN, Nambiar EKS, Saur E (2008) Retranslocation of foliar nutrients in evergreen tree species planted in a Mediterranean environment. Tree Physiol 28:187–196

Garten CT, Brice DJ (2009) Belowground fate of ^{15}N injected into sweetgum trees (*Liquidambar styraciflua*) at the ORNL FACE Experiment. Rapid Commun Mass Spectrom 23:3094–3100

Göransson H, Rosengren U, Wallander H, Fransson AM, Thelin G (2006) Nutrient acquisition from different soil depths by pedunculate oak. Trees 20:292–298

Heenan DP, Campbell LC (1981) Influence of potassium and manganese on growth and uptake of magnesium by soybeans (*Glycine max* (L.) Merr. CV. Bragg). Plant Soil 61:447–456

Jacoby B (1967) The effect of the roots on calcium ascent in bean stems. Ann Bot 31:725–730

Kozlowski TT, Hughes JF, Leyton L (1967) Movement of injected dyes in gymnosperm stems in relation to tracheid alignment. Forestry 40:207–219

Kuhn AJ, Bauch J, Schröder WH (1995) Monitoring uptake and contents of Mg, Ca and K in Norway spruce as influenced by pH and Al, using microprobe analysis and stable isotope labelling. Plant Soil 168–169:135–150

Kurvits A, Kirby EA (1980) The uptake of nutrients by sunflower plants (*Helianthus annuus*) growing in a continuous flowing culture system, supplied with nitrate or ammonium as nitrogen source. Z Pflanz Bodenkunde 143:140–149

Loustau D, Bosc A, Colin A, Ogée J, Davi H, François C, Dufrene E, Déqué M, Cloppet E, Arrouays D, Le Bas C, Saby N, Pignard G, Hamza N, Granier A, Bréda N, Ciais P, Viovy N, Delage J (2005) Modeling climate change effects on the potential production of French plains forests at the sub regional level. Tree Physiol 25:813–823

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London, p 889

Meinzer FC, Clearwater MJ, Goldstein G (2001) Water transport in trees: current perspectives, new insights and some controversies. Env Exp Bot 45:239–262

Moreno-Marcos G, Gallado-Lancho JF (2002) Atmospheric deposition in oligotrophic *Quercus pyrenaica* forests: implications for forest nutrition. For Ecol Manage 171:17–29

Nadelhoffer KJ, Downs MR, Fry B (1999) Sinks for ^{15}N enriched additions to an oak forest and a red pine plantation. Ecol Appl 9:72–86

Orians CM, van Vuuren MMI, Harris NL, Babst BA, Ellmore GS (2004) Differential sectoriality in long-distance transport in temperate tree species: evidence from dye flow, ^{15}N transport, and vessel element pitting. Trees 18:501–509

Porté A, Bosc A, Champion I, Loustau D (2000) Estimating the foliage area of maritime pine (*Pinus pinaster* Ait.) branches and crowns with application to modelling the foliage area distribution in the crown. Ann For Sci 57:73–86

Porté A, Trichet P, Bert D, Loustau D (2002) Allometric relationships for branch and tree woody biomass of Maritime pine (*Pinus pinaster* Ait.). For Ecol Manage 158:71–83

Proe MF, Mead DJ, Byrne D (2000a) Effect of pruning on nitrogen dynamics within crowns of *Pinus radiata*. Tree Physiol 20:653–661

Proe MF, Midwood AJ, Craig J (2000b) Use of stable isotopes to quantify nitrogen, potassium and magnesium dynamics in young Scots pine (*Pinus sylvestris*). New Phytol 146:461–469

Ranger J, Turpault MP (1999) Input–output nutrient budgets as a diagnostic tool for sustainable forest management. For Ecol Manage 122:139–154

Ritson P, Sochacki S (2003) Measurement and prediction of biomass and carbon content of *Pinus pinaster* trees in farm forestry plantations, south-western Australia. For Ecol Manage 175:103–117

Schneider A (2003) Characterisation of soil potassium supply as derived from sorption–desorption experiments. Plant Soil 251:331–341

Seiter S, Horwath WR (1999) The fate of tree root and pruning nitrogen in a temperate climate alley cropping system determined by tree-injected ^{15}N. Biol Fertil Soils 30:61–68

Shear CB, Faust M (1970) Calcium transport in apple trees. Plant Physiol 45:670–674

Staelens J, Houle D, De Schrijver A, Neirynck J, Verheyen K (2008) Calculating dry deposition and canopy exchange with the canopy budget model: review of assumptions and application to two deciduous forests. Water Air Soil Pollut 191:149–169

Swanston CW, Myrold DD (1998) Evaluation of the stem injection technique and subsequent ^{15}N partitioning in red alder crowns. Plant Soil 198:63–69

Umebayashi T, Utsumi Y, Koga S, Inoue S, Shiiba Y, Arakawa K, Matsumura J, Oda K (2007) Optimal conditions for visualizing water-conducting pathways in a living tree by the dye injection method. Tree Phys 27:993–999

van Ieperen W, van Gelder A (2006) Ion-mediated flow changes suppressed by minimal calcium presence in xylem sap in *Chrysanthemum* and *Prunus laurocerasus*. J Exp Bot 57:2743–2750

Waisel Y, Liphschitz N, Kuller Z (1972) Patterns of water movement in trees and shrubs. Ecology 53:520–523

Weatherall A, Proe MF, Craig J, Cameron AD, Midwood AJ (2006) Internal cycling of nitrogen, potassium and magnesium in young Sitka spruce. Tree Physiol 26:673–680

Zwieniecki MA, Melcher PJ, Holbrook NM (2001) Hydrogel control of xylem hydraulic resistance in plants. Science 291: 1059–1062