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Original Article

Variation in fluxes estimated from nitrogen isotope discrimination corresponds with independent measures of nitrogen flux in *Populus balsamifera* L.

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ABSTRACT

Acquisition of mineral nitrogen by roots from the surrounding environment is often not completely efficient, in which a variable amount of leakage (efflux) relative to gross uptake (influx) occurs. The efflux/influx ratio (*E/I*) is, therefore, inversely related to the efficiency of nutrient uptake at the root level. Time-integrated estimates of *E/I* and other nitrogen-use traits may be obtainable from variation in stable isotope ratios or through compartmental analysis of tracer efflux (CATE) using radioactive or stable isotopes. To compare these two methods, *Populus balsamifera* L. genotypes were selected, *a priori*, for high or low nitrogen isotope discrimination. Vegetative cuttings were grown hydroponically, and *E/I* was calculated using an isotope mass balance model (IMB) and compared to *E/I* calculated using ^{15}N CATE. Both methods indicated that plants grown with ammonium had greater *E/I* than nitrate-grown plants. Genotypes with high or low E/I using CATE also had similarly high or low estimates of E/I using IMB, respectively. Genotype-specific means were linearly correlated ($r = 0.77$; $P = 0.0065$). Discrepancies in *E/I* between methods may reflect uncertainties in discrimination factors for the assimilatory enzymes, or temporal differences in uptake patterns. By utilizing genotypes with known variation in nitrogen isotope discrimination, a relationship between nitrogen isotope discrimination and bidirectional nitrogen fluxes at the root level was observed.

Key-words: isotope discrimination; flux analysis; nitrogen.

INTRODUCTION

As the interest in using woody biomass as a bioenergy feedstock increases, improving plant growth and yields while, at the same time, minimizing nitrogen fertilizer inputs presents a major challenge. One prospect for improving nitrogen-use efficiency of plants is thought to lie in increasing the efficiency of nitrogen acquisition from the soil environment. Because most, if not all, nitrogen requirements are supplied by roots, improving acquisition is not only dependent on increasing uptake but also increasing the ability of plants to assimilate and translocate nitrogen into sink tissues. The heterogeneity of nitrogen availability in the natural environment and the complex response of plants to external nitrogen cues suggest that traditional short-term measures of nitrogen response may not reflect uptake efficiencies on a greater time-scale. Therefore, integrated approaches to understanding nitrogen uptake and assimilation in plants would be helpful to integrate the complexity of nitrogen uptake-efficiency (Hirel *et al.* 2007). One of these approaches is to use nitrogen isotope discrimination as an integrated proxy-indicator of nitrogen fluxes between the root and substrate and fluxes within the plant (Comstock 2001; Evans 2001; Kalcsits and Guy 2013a; Kalcsits *et al.* 2014).

Nitrogen movement between the rhizosphere and roots is bidirectional. After uptake (influx), nitrate and/or ammonium ions may be assimilated or, alternatively, returned to the rooting medium (efflux) through leakage or excretion (Lee and Clarkson, 1986; Xu *et al.* 2012). Inorganic nitrogen may also reach the xylem for transport to the shoot. These processes, in combination, result in the homeostatic balance of cytoplasmic inorganic nitrogen in roots (Lee and Clarkson, 1986). The presence of significant efflux suggests some inefficiency in nitrogen acquisition, whereby the plant is unable to expeditiously assimilate available cytosolic inorganic nitrogen or to balance intracellular pH and ionic balance (Britto and Kronzucker, 2006). Efflux of inorganic nitrogen has been reported under dynamic and steady-state nitrogen conditions (Kronzucker *et al.* 1995; Hawkins and Robbins 2010), and is dependent on substrate concentration (Morgan *et al.* 1973; Kronzucker *et al.* 1995; Hawkins and Robbins, 2010), root maturity and plant nitrogen demand (Bloom *et al.* 2010; Hawkins and Robbins, 2010). Cycling of unassimilated, inorganic nitrogen between the substrate and the root is thought to reflect inorganic nitrogen source preference (Kronzucker *et al.* 1997) and has been shown to be associated with reduced nitrogen use efficiency (Chen *et al.* 2013). In some cases, high efflux/influx (*E*/*I*) can consume large amounts of energy indicated by increases in root respiration (Kronzucker *et al.* 2001; Scheurwater *et al.* 1999). At high ammonium

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concentrations, this can reduce growth and may cause damage or even death to the plant (Britto and Kronzucker, 2006).

Though important, characterizing nitrogen fluxes in roots can be difficult because of high spatial and temporal heterogeneity in actively growing plants (Alber *et al*. 2012; Bloom *et al*. 2012; Hawkins *et al*. 2014). Inorganic nitrogen uptake has also been shown to vary diurnally (Delhon *et al*. 1995; Gessler *et al*. 2002). Using traditional assays, diurnal variation in nitrogen uptake patterns may result in poor characterization of genotypic variation in nitrogen use. Fluxes within roots have previously been characterized using microelectrode measurements (Henriksen *et al*. 1990; Miller and Zhen, 1991; Hawkins *et al*. 2010) or compartmental analysis of tracer efflux (CATE) using ^{13}N radioisotope (Lee and Clarkson, 1986; Siddiqi *et al*. 1991; Kronzucker *et al*. 1995) or ^{15}N stable isotope (Macklon *et al*. 1990; Devienne *et al*. 1994) labelling, under steady state conditions. CATE, which measures the rate of exchange between different root compartments, provides tissue specific information that cannot be obtained using other methods. These include compartment-specific measures of half-life of exchange, nitrogen concentration and efflux (Lee and Clarkson, 1986; Min *et al*. 2002; Kronzucker *et al*. 2005). Microelectrode measurements have the benefit of being a calibrated measure of nitrogen flux that is tissue or even cell specific (Henriksen *et al*. 1992). Although fast and accurate, there is a need for careful and extensive replication to account for spatial and temporal modulation of nitrogen flux.

The dynamic nature of inorganic nitrogen flux in time and space suggests that an integrated approach to assess nitrogen flux may better capture whole plant nitrogen-use. Differences in the natural abundance of stable nitrogen isotopes of plants grown under steady-state nitrogen conditions have potential to provide temporal and spatially-integrated information on plant and organ level nitrogen uptake and assimilation patterns. Kalcsits and Guy (2013a; 2014) proposed a mass balance isotope discrimination model that, in part, may account for the variation in plant and organ level isotopic composition. Although the model does not discern between cellular compartments at the tissue level, it can quantify whole-plant and organ-level nitrogen-use traits such as efflux/influx between the root and the substrate, allocation of nitrogen within the plant and partitioning of assimilation. For evaluating short-term responses to changes in nitrogen concentration or other treatments, CATE and/or microelectrode measurements are more appropriate. However, nitrogen isotope discrimination provides an integrated alternative for measuring nitrogen uptake and allocation in plants.

Nitrate and ammonium transport have been extensively studied in model plants such as *Arabidopsis thaliana* (L.) Heynh. and *Nicotiana tobacum* L. (Stitt and Feil, 1999; Glass *et al*. 2002). However, to date, few studies have investigated nitrate or ammonium transport in *Populus* spp. (Min *et al*. 2002; Selle *et al*. 2005; Couturier *et al*. 2007, Polle *et al*. 2013). As the interest increases for using *Populus* as a bioenergy source (Yemshanov and McKenney, 2008), it will be important to identify variability in nitrogen transport fluxes to improve nitrogen-use efficiency in new cultivars. A high degree of trait variability, particularly for adaptive traits such as phenology and photosynthesis, has been observed in the geographically wide-ranging balsam poplar (*Populus balsamifera* L.) and black cottonwood (*Populus trichocarpa* Torr. & A. Gray) (Soolanayakanahally *et al*. 2009; McKown *et al*. 2013). However, intraspecific variability in nitrogen flux within these species, or indeed, any woody plant, has not been explored. The objective of this study was to compare independent methods of estimating *E*/*I* and net nitrogen uptake using genotypes of balsam poplar selected, *a priori*, for having high or low nitrogen isotope discrimination grown on either nitrate or ammonium. More specifically, we sought to compare estimates of *E*/I using the isotope mass balance to estimates of *E*/*I* using CATE whereby *E*/*I* was estimated from ^{15}N isotope discrimination in the former, versus ^{15}N labelling in the latter. Developing alternative approaches to phenotyping nitrogen-use will allow researchers to better identify the controlling traits of nitrogen-use, particularly for woody plants such as poplar that will be used for biofuel in the future.

MATERIALS AND METHODS

Plant material and experimental design

Genotypes of balsam poplar from the Agriculture Canada Balsam Poplar (AgCanBaP) collection (Soolanayakanahally *et al*. 2009) were selected, *a priori* for either having high or low nitrogen isotope discrimination (Kalcsits 2013). First-year branches of these genotypes were obtained from the AAFC-AESB Agroforestry Development Centre, Indian Head, Saskatchewan, Canada and stored at 4 °C for approximately 3 months to fulfill chilling requirements. Two-node vegetative cuttings, approximately 6–8 cm long, were arranged in a randomized complete block design with three blocks consisting of two nitrogen treatments supplied as either 250μM Ca$(NO_3)_2$ or 250μM $(NH_4)_2SO_4$. Pre-existing nitrogen was accounted for by applying a correction (Kalcsits and Guy 2013b) that uses a mass balance model to quantify the amount of carry-over nitrogen remobilized from the cutting and then proportionately adjusts tissue $\delta^{15}N$ to only represent newly acquired nitrogen during the experiment.

Hydroponics system

The hydroponics system was comprised of six 1000L containers lined with black 45 mil rubber pond liner material (Firestone, Nashville, TN, USA) constructed in a greenhouse under ambient light conditions supplemented by sodium halide lighting providing a minimum PPFD of 600 $\mu mol\, m^{-2}\, s^{-1}$ and 18/6 h day/night photoperiod. Temperatures in the greenhouse were kept between 20 and 24 °C. Each container was fitted with a floating Perspex 'raft' that had a capacity of 32 plants. The hydroponics solution was a modified 1/10th strength Johnson's solution (Johnson *et al*. 1957) with either 500μM nitrate (as Ca$(NO_3)_2$; $\delta^{15}N = +60.3$ ‰) or ammonium (as $(NH_4)_2SO_4$; $\delta^{15}N = -0.96$ ‰). Final nutrient composition, excluding nitrogen, was: 200μM KH_2PO_4, 200μM K_2S0_4, 100μM $MgSO_4$, 100μM $CaSO_4$, and micronutrients: 5μM Cl, 2.5μM B, 0.2μM Mn, 0.2μM Zn, 0.05μM Cu and 50μM Fe^{2+} (EDTA complexed). A centrifugal pump, with a pumping capacity of approximately 20 L per minute, provided circulation and aeration of the solution for each container. The solution was monitored

periodically for oxygen levels, temperature and pH. Powdered calcium carbonate ($CaCO_3$) was added to buffer pH in the range of 6–7.5. Media NH_4^+ and NO_3^- concentrations were assayed using the phenol:hypochlorite (Solorzano 1969) and perchloric acid (Cawse 1967) methods, respectively. The solution was completely replaced every 14 days to ensure that there was no substantial decrease (>10%) in concentration of nitrate or ammonium over time that could increase the solution $\delta^{15}N$.

Sampling and natural abundance isotope analysis

After 45 days of growth, plants were randomly sampled for either nitrogen isotope mass balance or ^{15}N CATE (N = 3). For natural abundance isotope analysis, samples were separated into leaves, stems and roots. Samples were flash frozen in liquid nitrogen and stored at −80 °C until freeze drying. Roots, leaves and stems were weighed for dry mass and then ground to a fine powder using a mortar and pestle and then ball milled (Fritsch Laborgeratebau, Terochem Scientific). Subsamples of 3 ± 0.1 mg were weighed into tin capsules (Elemental Microanalysis Ltd., 8 × 5 mm, D1008) and analysed for $\delta^{15}N$ and nitrogen on a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (University of California Stable Isotope Facility, Davis, CA).

^{15}N efflux apparatus

Plants sampled for ^{15}N CATE analysis were transferred to a holding tank with the same nutrient composition as the growing solution. The holding tank was placed under fluorescent lighting (200 μmol m^{-2} s^{-1}) until plants were loaded with the ^{15}N solution. Each plant was then individually placed in 1000 mL of aerated, loading solution of 98 atom% ^{15}N for 60 min, after which the plant was removed, and roots were allowed to drain gravitationally for 30 s. Each plant was then secured to the side of a glass funnel, and the roots were rinsed with the eluate flowing into 15 pre-weighed glass beakers at intervals of 3 × 15 s, 3 × 30 s, 2 × 60 s and 7 × 120 s for a combined elapsed time of 18 min 15 s. Rinsing solution (identical to the original hydroponics solution) was supplied using a peristaltic pump and polyvinyl chloride tubing (Kuritec, Brantford, Ontario, Canada) at a rate of 4 mL s^{-1}. Eluate volumes were determined by mass difference between the dry beaker and beaker plus eluate. Acidified (pH 2.0) 15-mL aliquots were stored in disposable scintillation vials at 2 °C until processing for isotope analysis. Roots were weighed for fresh mass then oven-dried for 2 days at 60 °C to obtain dry mass.

^{15}N efflux isotope analysis

To determine the isotopic composition of the eluate samples, tin capsules (~1.5 mL) were prepared from foil squares (30 mm × 30 mm, Elemental Microanalysis UK). Eluate was then pipetted in 7 × 1 mL increments (~49 μg of N total) into the capsules, with drying at ~50 °C between each increment. Root samples were prepared and loaded into the tin capsules as above. Nitrogen isotope analysis was performed at the University of Saskatchewan Soil Science Stable Isotope Laboratory using a Costech ECS4010 elemental analyser coupled to a Delta V mass spectrometer with Conflo IV interface. Samples were run with IEAE isotope standards and in-house standards.

Calculations

Compartmental analysis of tracer efflux (CATE)

Compartmental analysis of tracer efflux was based on methods in Lee and Clarkson (1986) except release of excess ^{15}N from the root (μmol g fw^{-1} min^{-1}) was substituted for rate of ^{13}N release, as follows:

$$^{15}N\ efflux\ (nmol\ N) = \frac{(^{15}N\ Atom\%_{Eluate} - {}^{15}N\ Atom\%_{Rinse})}{S_0} \times N_{eluate}\ (nmol\ N) \quad (1)$$

where, $^{15}N\ Atom\%_{Rinse}$ was equal to 0.36654% and the specific activity of the loading solution, S_0, was equal to 98%. The rate of ^{15}N efflux, expressed on a per mass basis, was then calculated as:

$$^{15}N\ efflux\ rate\ (nmol\ N\ g\ fw^{-1}\ min^{-1}) = \frac{(^{15}N\ Efflux)}{m \times t} \quad (2)$$

where, m = fresh mass of root, and t = time of eluate sampling and represents the elapsed sampling time for each individual solution sample.

The ^{15}N efflux rate was log transformed and plotted against elapsed elution time. Then, segmental linear regression was performed in Graphpad Prism 6 (La Jolla, CA, USA) to determine breakpoints and segment slopes for corresponding compartments (Fig. 1). Segments represent nitrogen release from three compartments: the root surface, the apoplastic (intercellular) space and the cytoplasmic (intracellular) space (Lee and Clarkson, 1986). Vacuolar half-life was excluded from the

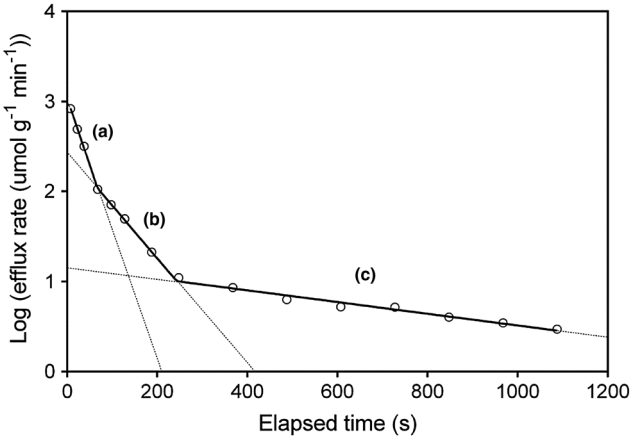

Figure 1. Representative segmental linear regression for determining half-lives of exchange and flux rates for the surface (a), apoplastic (b) and cytoplasmic (c) compartments. Each data point represents the log (efflux rate) at x-elapsed time (s).

analysis because of the long time to exchange, and this would not be measurable with a measured flux time of 18 min.

Half-lives for compartmental exchange were calculated as:

$$t_{1/2}\ (half\text{–}time\ of\ exchange) = \frac{0.693}{k}\ \left(min^{-1}\right) \quad (3)$$

where, k is the rate constant and is equal to:

$$k\ = \frac{\beta}{2.303} \quad (4)$$

where, β is the slope of the regression line. Compartmental efflux can be calculated as:

$$Efflux\ \left(nmol\ g\ fw^{-1}\ h^{-1}\right) = \frac{R_0}{S_0} \quad (5)$$

where R_0 is the intercept of the regression line with the ordinate for each compartment (i.e. the ^{15}N efflux rate at time zero).

Net uptake was calculated as the excess ^{15}N remaining in the roots after rinsing and expressed as μmol N g fw^{-1} h^{-1}. Efflux/influx (E/I) was then calculated as:

$$E/I = \frac{Efflux}{(Efflux + Net\ Uptake)}. \quad (6)$$

Flux from the root to the shoot could not be calculated because of the possibility of direct contamination of leaves or stems by the highly enriched labelling solution during the loading process. Slight enrichment of stems and leaves was, however, observed (not shown).

Compartmental nitrate or ammonium content was calculated as the area under the exponential decay curve of a plot of time versus ^{15}N efflux rate (μmol h^{-1}) and was approximated as the total ^{15}N effluxed (μmol) during five half-lives:

$$\sum_{i=1}^{5} N_i = (t_i - t_{i-1}) \times \frac{(r_i - r_{i-1})}{2} \quad (7)$$

where $i = i^{th}$ half-life, t = time and r = rate of ^{15}N efflux at i^{th} half-life. r at the i^{th} half-life was calculated by multiplying the initial rate of ^{15}N efflux by 0.5^{i}. For the cytoplasm, this was then divided by the E/I similar to Siddiqi *et al.* (1991). Then, assuming that 5% and 10% of root volume was occupied by cytoplasmic and apoplastic space, respectively (Lee and Ratcliffe, 1983), nitrate and ammonium concentrations of these compartments were calculated.

Isotope mass balance flux calculations

Efflux/influx was calculated using a modified approach from Kalcsits *et al.* (2014) after correcting for carry-over of tissue nitrogen in stem cuttings (Kalcsits and Guy 2013b). In the model, efflux/influx is a function of overall plant discrimination against ^{15}N relative to the substrate, the discrimination factor of the enzyme (nitrate reductase or glutamine synthetase for nitrate or ammonium, respectively) and the localization of assimilation in roots versus leaves.

The assimilation-averaged net flux of inorganic nitrogen into the root is equal to the total plant nitrogen, after correcting for contaminating nitrogen from the original cutting, divided by the root biomass:

$$Net\ Uptake\ \left(\ \mu mol\ N\ mg\ dw^{-1}\right) = \frac{N_{total}}{Biomass_{root}}. \quad (8)$$

The proportion of total plant nitrogen in leaves (N_{leaf}/N_{total}) is:

$$\frac{N_{leaf}}{N_{total}} = \frac{Biomass_{leaf} \times N\ Concentration_{leaf}}{Biomass_{plant} \times N\ Concentration_{plant}}\ . \quad (9)$$

Partitioning of assimilation between root (P_{root}) and shoot ($1 - P_{root}$) is a function of N_{leaf}/N_{total}, the difference in δ^{15}N between leaves and roots and the discrimination factor of the enzyme (Δ_{enz}):

$$P_{root} = 1 - \left(\left(\frac{N_{leaf}}{N_{total}}\right) \times \frac{\left(\Delta^{15}N_{leaf} - \Delta^{15}N_{root}\right)}{\Delta_{enz}}\right). \quad (10)$$

The discrimination factor for glutamine synthetase is thought to be near 16.8‰ (Yoneyama *et al.* 1993). Ledgard *et al.* (1985) reported a Δ_{enz} of 15.4‰ for nitrate reductase.

From P_{root}, plant Δ^{15}N and the discrimination factor of either nitrate reductase or glutamine synthetase, the estimate of efflux/influx (E/I) was obtained as:

$$\frac{E}{I} = \frac{\Delta^{15}N_{plant}}{\Delta_{enz} \times P_{root}}. \quad (11)$$

Statistical analysis

To compare net uptake and E/I across the two methods, a three-way ANOVA in SAS 9.3 (SAS Institute, Cary, NC) was performed using the following model:

$$Y_{ijk} = \mu + \alpha_i + \tau_j + \rho_k + \beta_{ij} + \gamma_{ik} + \lambda_{jk} + \varepsilon_{ijk} \quad (12)$$

where, μ is the overall mean response, α_i is the effect because of the genotype, τ_j is the effect because of the nitrogen source, ρ_k is the effect because of method, β_{ij} is the effect because of any interaction between the genotype and nitrogen source, γ_{ik} is the effect because of any interaction between genotype and method, λ_{jk} is the effect because of any interaction between nitrogen source and method and ε_{ijk} is the effect because of any three-way interaction between genotype, nitrogen source and method. Geometric mean regression (Ricker 1984) was used to express the relationship between the two independent methods.

Nitrogen source and genotype fixed effects on biomass and on physiological variables unique to a particular method (i.e. root and leaf nitrogen concentrations, compartmental fluxes, half-lives of exchange and compartmental nitrogen concentrations) were tested using two-way ANOVA. The statistical model was as follows:

$$Y_{ij} = \mu + \alpha_i + \tau_j + \beta_{ij} \quad (13)$$

where, μ is the overall mean response, α_i is the effect because of

the genotype, τ_j is the effect because of the nitrogen source and β_{ij} is the effect because of any interaction between the genotype and nitrogen source. ANOVA were carried out using Graphpad Prism 6 (La Jolla, CA, USA) followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where $P < 0.05$.

RESULTS

Plant growth

Plant biomass was greater when grown with nitrate ($P < 0.05$) where mean biomass was 5.60 and 1.65 g under NO_3^- and NH_4^+, respectively (Fig. 2). There were no significant differences in biomass for genotypes that had high nitrogen isotope discrimination compared to genotypes with low nitrogen isotope discrimination. Root biomass was not significantly different among treatments where the root biomass was 0.31 g and 0.35 g for low and high NO_3^--grown plants, respectively and 0.18 g and 0.38 g for low and high NH_4^+-grown plants, respectively. Shoot biomass was greater for NO_3^--grown plants than NH_4^+-grown plants ($P < 0.05$). Mean shoot biomass was 2.84 g and 4.03 g for low and high NO_3^--grown plants, respectively and 0.89 g and 1.23 g for low and high NH_4^+-grown plants, respectively. Root:shoot ratios were more than two times greater under NH_4^+ (0.25) than under NO_3^- (0.10) (Fig. 3). Similar to biomass, there were no differences between genotypes with high or low nitrogen isotope discrimination. Root:shoot ratios were inversely proportionate to biomass where treatments with greater biomass (nitrate) had lower root:shoot ratios. Leaf and root nitrogen concentrations were not significantly different between nitrogen sources (Table 1). However, genotypes with high nitrogen isotope discrimination had significantly greater tissue nitrogen concentrations in both the roots and shoots. These differences were magnified in leaves. Root nitrogen concentration was approximately 0.5 mmol g dw^{-1} greater than, but also significantly correlated with, leaf nitrogen concentration ($r = 0.634$; $P = 0.002$). Mean root and leaf nitrogen concentrations were 2.46 and 1.94 mmol N g dw^{-1}, respectively.

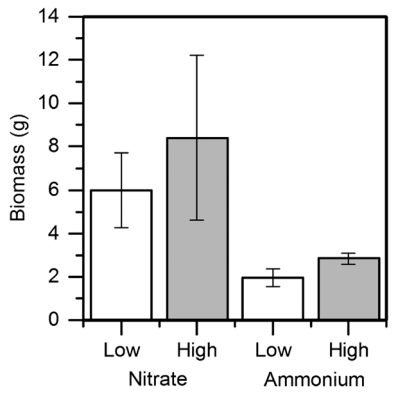

Figure 2. Total biomass (means ± SE) of *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically on either 500μM NO_3^- or NH_4^+ for 45 days.

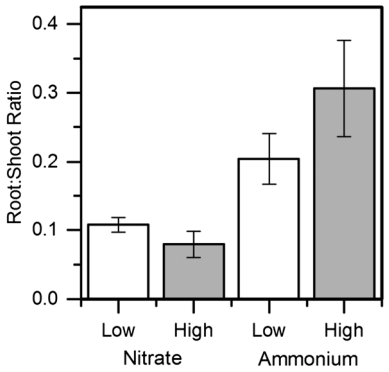

Figure 3. Root:shoot ratio (means ± SE) of *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically on either 500μM NO_3^- or NH_4^+ for 45 days.

Compartmental analysis of tracer efflux (CATE)

CATE indicated that surface flux was nearly two orders of magnitude greater than flux from the apoplast, which in turn was about one order of magnitude higher than the cytoplasmic efflux (Table 2). The surface flux represents exchange between the boundary layer and substrate. In contrast, the flux from the apoplast averaged 0.96 and 3.37 mmol g fw^{-1} h^{-1} for NO_3^- and NH_4^+, respectively ($P = 0.007$). Although the outward flux of NO_3^- from the apoplast was not significantly different between genotypes showing high and low nitrogen isotope discrimination, the flux of NH_4^+ from the apoplast was greater in genotypes that had high versus low nitrogen isotope discrimination. Similarly, cytoplasmic efflux averaged approximately twice as high ($P = 0.0078$) for NH_4^+ (0.27 mmol g fw^{-1} h^{-1}) than for NO_3^- (0.13 mmol g fw^{-1} h^{-1}). There were no significant differences in the outward fluxes of nitrogen from the cytoplasm among genotypes with differing nitrogen isotope discrimination.

Half-life of exchange ($t_{1/2}$) indicates the time taken for 50% of the inorganic nitrogen to be lost from a NO_3^- or NH_4^+ pool or compartment (Table 3). Although loss rates from the root surface were not different, the $t_{1/2}$ was significantly longer under NH_4^+ (13.8 s) than with NO_3^- (7.9 s). In contrast, the $t_{1/2}$ of the apoplastic space did not differ significantly among treatments. The overall apoplastic $t_{1/2}$ was 69.3 s, approximately six times that of the surface compartment. $t_{1/2}$ for the apoplast was not significantly different between genotypes with high or low nitrogen isotope discrimination when grown with nitrate but the $t_{1/2}$ was lower in genotypes with high nitrogen isotope discrimination when grown with ammonium (Table 3). Half-lives of exchange for the cytoplasmic space were approximately 32 and 12 min for NO_3^- and NH_4^+, respectively ($P = 0.0488$). Genotypes that had high nitrogen isotope discrimination had lower half-lives of exchange in the cytoplasm when grown with either NO_3^- or NH_4^+.

Table 1. Root and leaf nitrogen concentration (mmol N g dw^{-1}) (means ± SE) for *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically with either 500μM NO_3^- or NH_4^+ for 45 days

	NO_3^-		NH_4^+	
	Low	High	Low	High
Root (mmol N g dw^{-1})	2.30 ± 0.07	2.64 ± 0.06	2.24 ± 0.17	2.53 ± 0.126
Leaf (mmol N g dw^{-1})	1.72 ± 0.16	2.29 ± 0.10	1.56 ± 0.21	1.97 ± 0.23

Table 2. Outward fluxes (μmol N g fw^{-1} h^{-1}) of nitrate (NO_3^-) and ammonium (NH_4^+) (means ± SE) as estimated from compartmental analysis of tracer efflux (CATE) for *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically with either 500μM NO_3^- or NH_4^+ for 45 days

	NO_3^-		NH_4^+	
	Low	High	Low	High
Surface	69.22 ± 28.85	73.93 ± 30.85	39.31 ± 15.95	31.61 ± 5.25
Apoplastic Space	0.99 ± 0.31	1.01 ± 0.59	2.50 ± 0.78	5.46 ± 1.43
Cytoplasm	0.11 ± 0.04	0.14 ± 0.09	0.27 ± 0.04	0.29 ± 0.05

Table 3. Half-lives of exchange ($t_{1/2}$) in seconds for NO_3^- and NH_4^+ of root surface film, apoplastic space and cytoplasm (means ± SE) estimated from compartmental analysis of tracer efflux (CATE) for *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically with either 500-μM NO_3^- or NH_4^+ for 45 days

	NO_3^-		NH_4^+	
	Low	High	Low	High
Surface	8.47 ± 1.48	6.78 ± 2.09	12.83 ± 2.02	15.15 ± 1.64
Apoplastic space	66.06 ± 5.78	65.90 ± 6.13	88.94 ± 15.34	51.06 ± 10.23
Cytoplasm	1578.3 ± 411.8	1185.9 ± 188.8	783.5 ± 121.9	483.4 ± 34.8

Estimated apoplastic NH_4^+ concentrations were approximately four to five times higher than NO_3^- concentrations ($P < 0.0012$) but did not vary between genotypes with high or low nitrogen isotope discrimination. Mean apoplastic concentrations were 0.28 mM and 0.83 mM for NO_3^- and NH_4^+, respectively. In contrast, cytoplasmic NO_3^- concentrations were significantly greater than NH_4^+ concentrations.

Independent estimates of net uptake and efflux/influx are correlated

Irrespective of method, *E*/*I* was significantly higher for NH_4^+ than NO_3^-. Genotypes that had low nitrogen isotope discrimination had lower estimates of E/I using CATE (Fig. 4). CATE indicated mean efflux/influx ratios of 0.4 and 0.22 for NH_4^+ and NO_3^-, respectively, whereas isotope mass balance modelling gave flux ratios of 0.65 and 0.45. Despite the relative differences in estimates of *E*/*I*, there was a significant positive relationship between the two measures of efflux/influx ($r = 0.77$; $P = 0.0065$). Generally, genotypes that had the highest *E*/*I* using the isotope mass balance approach also had the highest *E*/*I* using the CATE method (Fig. 4). However, the difference between the two estimates increased as efflux/influx increased (Fig. 5). The difference between the two measures was relative and was approximately 35%.

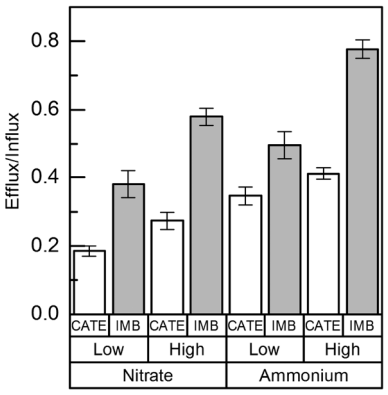

Figure 4. Estimates of efflux/influx (mean ± SE) using two approaches, isotope mass balance (IMB) and compartmental analysis of tracer efflux (CATE) for *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically grown on either 500μM nitrate or ammonium for 45 days.

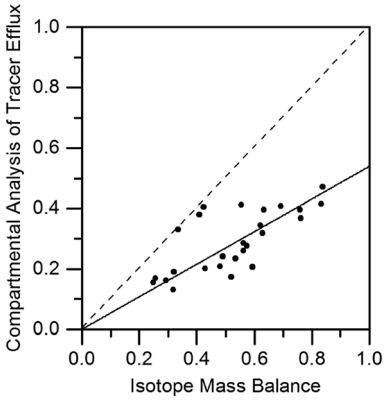

Figure 5. Coherence of estimates of efflux/influx by compartmental analysis of tracer efflux (CATE) and nitrogen isotope discrimination for *Populus balsamifera* L. genotypes grown hydroponically for 45 days. Each point represents clonal replicates from each hydroponic container used for either isotope mass balance or CATE analysis. The solid line indicates significant geometric mean regression through the origin ($P = 0.0065$). The dotted line shows the position of a 1:1 relationship.

DISCUSSION

As shown here using rooted balsam poplar cuttings, the natural abundance isotope mass balance method presented in Kalcsits and Guy (2013a) yields estimates of *E*/*I* that are comparable, although not identical, to CATE. CATE by isotope labelling has, for some time, been the only method used for calculating whole root *E*/*I*, but provides a more complete picture of ion flux in and out of roots. Using nitrogen isotope discrimination to estimate *E*/*I* provides a reliable, time-integrated, alternative approach to calculate cycling between the root and substrate.

Compartmental analysis of tracer efflux using ^{15}N

Based on the methods for CATE modified from Clarkson and Lee (1986) for ^{13}N, balsam poplar roots were loaded with ^{15}N-labelled nitrate and ammonium to measure net flux characteristics under steady-state conditions. Although the ^{13}N radioisotope has more frequently been used in such studies (e.g. Siddiqi *et al.* 1991; Kronzucker *et al.* 1995*a*, 1995*b*; Min *et al.* 2002), similar precision was achieved using ^{15}N. Stable isotope labelling with ^{15}N has also been successfully applied to CATE in *Triticum aestivum* L. (Devienne *et al.* 1994) and *Allium cepa* L. (Macklon *et al.* 1990). To the best of our knowledge, the present study is the first to report ^{15}N CATE of a woody plant species.

Half-lives of exchange for nitrate as compared to ammonium were longer for the cytoplasm and shorter at the root surface and the apoplast (Table 3). Rates of efflux from the apoplast and cytoplasm were greater for ammonium. Although differences in substrate concentrations in previous work limit direct comparison to our results, the fluxes and half-lives of exchange in balsam poplar were within the same order of magnitude as other tree species, including white spruce (*Picea glauca* (Moench) Voss), lodgepole pine (*Pinus contorta* Dougl.), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and trembling aspen (*Populus tremuloides* Michx.) (Kronzucker *et al.* 1995a, 1995b; and Min *et al.* 2002).

Observed greater biomass for nitrate-grown plants indicates that balsam poplar has a general preference for nitrate but will use both nitrate and ammonium. Nitrate concentrations were more than two times higher than ammonium concentrations in the cytoplasm, but were lower in the apoplast (Table 4). For white spruce, which has an ammonium preference (Kronzucker 1995*a*, *b*), nitrate concentrations in the cytoplasm were lower than ammonium, but the same was also true of trembling aspen (Min *et al.* 2002). In the apoplast, mean nitrate concentration was approximately half the concentration of the substrate (Table 4). In contrast, in spruce, the apoplastic nitrate concentrations were approximately equal to the substrate (Kronzucker *et al.* 1995*a*, *b*; Min *et al.* 2002). For balsam poplar, the mean ammonium concentration in the apoplast was greater than the substrate, although not to the degree reported for spruce and aspen. Kronzucker *et al.* (1995*a*, *b*) attributed differences between apoplastic nitrate and ammonium concentrations to the Donnan equilibrium, where the negative charge of the cell wall increases the binding capacity for cations (NH_4^+) but not for anions (NO_3^-).

The compartmental concentrations we report are lower than those observed by Kronzucker *et al.* (1995a; 1995b) and Min *et al.* (2002). These differences may in part be because of uncertainties in estimating compartmental volumes or a species-level difference. Based on Laties (1959), Lee and Clarkson (1986) suggested that 10% of the total root volume is apoplastic free space and 5% is cytoplasm, estimates commonly used in CATE. However, volume fractions may vary with species or genotype and are known to be affected by root development and environment (McGarry, 1995). Variations in compartmental volume could account for differences in compartmental inorganic nitrogen concentrations calculated here. The vacuole can accumulate large amounts of nitrate whereas for ammonium, the accumulations are much smaller (REF).

Table 4. Apoplast and cytoplasm nitrate and ammonium concentrations (means ± SE) as estimated by compartmental analysis of tracer efflux (CATE) for *Populus balsamifera* L. genotypes selected, *a priori*, for either high or low nitrogen isotope discrimination grown hydroponically with either 500-μM NO_3^- or NH_4^+ for 45 days

	NO_3^-		NH_4^+	
	Low	High	Low	High
Apoplastic space (mM)	0.24 ± 0.07	0.23 ± 0.12	0.81 ± 0.12	0.86 ± 0.26
Cytoplasm (mM)	27.68 ± 7.52	15.73 ± 2.50	7.26 ± 1.17	4.07 ± 0.29

Few, if any, studies have used CATE to examine within species variation in nitrogen efflux, half-life of exchange and compartmental nitrogen concentration. Here, we show that intraspecific variability exists for traits measured using CATE. Intraspecific variability in half-life of exchange, particularly in the cytoplasm, was high. The reasons underlying these variations are not entirely clear. However, they could be related to physiological or morphological variations regulating nitrogen uptake and/or demand at the root level and would be worthwhile to investigate further.

Compartmental analysis of tracer efflux and isotope mass balance estimates of *E/I*

Expressed relative to each other, rates of influx and efflux not only describe bidirectional movement of inorganic nitrogen between the root and rhizosphere but are also indicative of the efficiency of uptake. This measure is useful because it reflects species-specific source preference (Kronzucker *et al.* 1997), possible ammonium toxicity (Kronzucker *et al.* 2001; Britto and Kronzucker, 2006), and how well external nitrogen supply meets internal demands (Pritchard & Guy 2005). Although there was a significant relationship between *E/I* calculated from CATE and the isotope mass balance approach, using CATE, *E/I* was lower by approximately 33–50% (Fig. 4). There are several possible reasons why these two measures may be different. First, the two methods operate over different scales in time and, to some degree, space. Second, there are uncertainties in discrimination factors for the assimilatory enzymes. Neither of these is mutually exclusive.

In the first instance, CATE monitors nitrogen flux over a period of several minutes whereas the isotope mass balance approach integrates over growing time (i.e. several weeks). With the latter, developmental and diurnal variation in *E*/*I* would be averaged according to when the nitrogen was acquired. Peuke *et al.* (2013) observed diurnal variation in nitrogen isotope discrimination for many nitrogen-containing organic compounds in plants suggesting that a discrepancy between integrated and time-point measurements should be expected. Using other approaches, increased efflux during night was observed in pearl millet (*Pennisetum glaucum* (L.) R.Br) with nitrate nutrition (Pearson *et al.* 1981), and in Italian ryegrass (*Lolium perenne* L.) with ammonium (MacDuff and Jackson 1992). Using ^{15}N labelling, net uptake rates decrease during the night for both ammonium and nitrate, but are species dependent (Delhon *et al.* 1995; Gessler *et al.* 2002). Kumar *et al.* (2003) demonstrated changes in the transcript abundance of ammonium transporters and a subsequent decrease in ammonium uptake at night in rice (*Oryza sativa* L.). Decreased uptake rates combined with increased efflux rates at night increases *E*/*I* at night (Scheurwater *et al.* 1999). Because CATE did not account for *E*/*I* during the night period, this could, in part, explain lower estimates of *E*/*I* compared to the isotope mass balance approach.

Spatially, the two techniques differ inasmuch as the isotope mass balance method accounts for the translocation of inorganic nitrogen from the root to the shoot, whereas we were not able to measure this flux using CATE. However, the inclusion of xylem flux using CATE could only lead to an increase in net uptake and a further decrease in the estimate of *E*/*I*. We acknowledge that not accounting for xylem flux likely underestimates the net uptake using the CATE approach. However, it cannot account for the discrepancy between the two methods because estimates of E/I were lower using CATE than the isotope mass balance approach and adding nitrogen flux to the shoot to the model would further accentuate these differences.

Underestimation of enzymatic discrimination factors for the assimilatory enzymes could result in an increase in the estimated *E*/*I* using the nitrogen isotope mass balance approach. Data in Figs. 4 and 5 assume discrimination factors of 15.4‰ for nitrate reductase (Ledgard *et al.* 1985) and 16.8‰ for glutamine synthetase (Yoneyama *et al.* 1993). However, more recent work has suggested that the discrimination factor of nitrate reductase is closer to 22–26‰ (Needoba *et al.* 2004; Tcherkez and Farquhar, 2006; Karsh *et al.* 2012), or even higher (Carlisle *et al.* 2014). If *E*/*I* is recalculated using 22‰ for both enzymes, *E*/*I* using the isotope mass balance decreases and the similarity between the two methods increases. Although discrimination by glutamine synthetase has not been similarly reassessed, the improved coherence of *E*/*I* on both nitrate and ammonium indicates that the discrimination factors of both assimilatory enzymes may indeed be underestimated to some unknown degree.

Advantages of the isotope mass balance approach

Considering all of the above, it is not surprising that there are differences in flux estimates and *E*/*I* as determined by either CATE or by isotope mass balance. The concurrence we do observe, however, at the treatment level and within species, helps validate estimates of *E*/*I* using the isotope mass balance approach, which has some significant advantages over CATE.

Although CATE provides detailed cell or tissue specific nitrogen flux information at the root level, CATE requires the use of either radioactive ^{13}N or stable ^{15}N. Specialized equipment and training is required to use ^{13}N for CATE and access to ^{13}N ($t_{1/2} = 9.97$ min) is limited to just a few labs worldwide. Using stable ^{15}N in the loading solution is equally effective. However, as with ^{13}N, at least 15 samples must be taken per assay to have enough data for segmental linear regression, and this is labour intensive and costly. On the other hand, establishing proper growth conditions for the isotope mass balance approach requires considerable care and attention and is also quite labour intensive. However, advantages of the isotope mass balance method include reduced costs for isotope analysis (two to four samples per assay; i.e. starting material, leaves, stems and roots) and opportunities for scaling up to the simultaneous assay of large numbers of plants under a variety of environmental conditions. Additionally, it provides time-integrated estimates of several other plant and organ level nitrogen-use traits (Kalcsits *et al.* 2014) that are not estimated using CATE.

CONCLUSION

Here, we report variation in nitrogen use traits between genotypes selected, *a priori*, for having low and high nitrogen isotope discrimination that correspond to estimates of E/I measured

using compartmental analysis of tracer efflux. The information reported here provides support to both the isotope discrimination model and the use of compartmental analysis to estimate nitrogen fluxes within roots. Regardless of the approach taken to study the flux of inorganic nitrogen across the root plasmamembrane, the information obtained is crucial for understanding whole-plant nitrogen dynamics. Increasing the confidence in our measurements of nitrogen fluxes and increasing our understanding of the relationship of the fluxes to other nitrogen-use processes contributes to an increased understanding of nitrogen uptake and translocation. Through reduced isotopic sampling and more universal access, and because experiments can be scaled up to better evaluate genetic variation in flux and nitrogen-use traits, the isotope mass balance approach provides a widely applicable method for measuring nitrogen fluxes in plants that is complementary to information obtained from using compartmental analysis of tracer efflux.

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