Evaluating the importance of root abscission versus efflux to plant N-loss: consequences for plant N-isotope composition

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Thesis presented for the degree of Master of Science in the Department of Biological Sciences
University of Cape Town
November 2017

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Scanned roots of *Triticum aestivum*

I think, therefore I am

*René Descartes*
Declaration

I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own. The thesis is submitted for the degree of Master of Science in the Department of Biological Sciences, University of Cape Town. It has not been for examination at any other university.

Chapter 2, “The role of N efflux and root abscission in determining plant $\delta^{15}N$”, was published in Plant and Soil (accepted March 2017). I conducted the experiments, data collection and statistical analyses, and wrote the paper. My co-author and supervisor MD Cramer provided advice and comments on the manuscript.

Signed

Kirsten Packer
Abstract

The common observation that plant $\delta^{15}$N values are lower than those of associated soil is generally attributed to transporter-facilitated efflux of $^{15}$N-enriched N. N efflux tends to occur under specific conditions, for instance, when the external N concentration is high, when the external medium is acidic and when roots experience mechanical stress. While efflux is presumed to act as a regulator of cytoplasmic N concentrations, it is energetically costly for plants to take up N only to release it back into the rhizosphere. A link between root tissue loss (e.g. root turnover or rhizodeposition) and plant $\delta^{15}$N has not been suggested, although root abscission is likely to be more ubiquitous than N efflux. This thesis questions the extent to which N efflux and root abscission contribute to plant N-loss and plant $\delta^{15}$N values. I hypothesized that: (1) plants supplied with more N would have more negative $\delta^{15}$N relative to the source, and greater root abscission from a relatively larger root biomass (2) the aeration necessary for hydroponic culture can act as a mechanical stressor on roots, accentuating plant N-loss through root abscission and N efflux. Wheat was grown in sand with NO$_3^-$ supplied at five relative addition rates (RAR) and in hydroponics with three physical disturbance regimes (direct aeration, aeration constrained within a pipe and circulation of nutrient solution through sand). The $\delta^{15}$N of roots and shoots, as well as the plant-derived N accumulation in both growth mediums, were determined. When the N supply matched the plant N demand, as determined by the relative growth rate, there was no discrimination between plant and source $\delta^{15}$N. N-loss here, although negligible, was in the organic form, which implies root abscission. By contrast, when N supply exceeded plant N demand, plant $\delta^{15}$N values decreased (e.g. after 47 d, plant $\delta^{15}$N of RAR 0.075 d$^{-1}$ was 0.4‰ but was $\sim$4.1‰ at RAR 0.175 d$^{-1}$) because they lost $^{15}$N-enriched N. This N was largely inorganic and presumably lost through efflux. In disturbed hydroponic conditions (i.e. direct and pipe treatments), root ‘fragments’ were a major biomass- (six-fold greater than root dry weight) and N-loss (two-fold greater than plant net N uptake) pathway. Plants from all treatments lost more N within root fragments than through efflux, although the cumulative N-loss was significantly smaller from plants grown in relatively undisturbed hydroponic conditions (i.e. sand). This suggests that root abscission is likely to be an important N-loss pathway for plants and thus contributes to the global offset between plant and soil $\delta^{15}$N values. Moreover, efforts to improve nitrogen use efficiency of crop plants, though reduced efflux, need to take cognizance of root abscission because it is an unavoidable artefact of root growth.
Acknowledgements

Firstly, I would like to thank my supervisor Assoc Prof Mike Cramer for believing in me, as well as providing inspiration and guidance through the complicated nature of plant physiology. I deeply appreciate your encouragement to publish the work and to tackle R.

Thanks to the academic, technical and administrative staff in the Biological Sciences department for always going the extra mile. Samson Chimphango gave me his time, allowing me to come and chat to him about my work and reading through a manuscript for me. Dawood Hattas patiently lent his experience to my never-ending technical questions and I am grateful that his door was always open. Des Barnes steered me through the toils of glasshouse experiments, while Francois Majola and Daphne Meyer were always on hand when I required assistance. Edward Chirwa helped with the tedious grinding of samples and setting up the hydroponic experiments.

I would like to thank Ian Newton (Department of Archeometry, UCT) and Mike Butler (iThemba Labs) for conducting the mass spectrometer analyses. I am grateful to Bouwer Rall at Wesgraan (Kaap Agri) for providing the wheat seed used in my experiments, and to Brittany Arendse for translating in Afrikaans when I went to fetch them.

Many thanks to my fellow postgrads: Jacques Nel, Kervin Prayag and Zander Venter for stimulating conversations about all manner of topics; Hana Petersen for that chocolate when I most needed it; and Dunja MacAlister for your help with root scanning and for being a great friend. A special thank you to Simon Power, you exemplify scientific thinking and approach, I have learnt a great deal from you in this regard; thanks for the support and motivation over the years.

Finally, thank you to my parents Dave and Tessa for the love and care you provide, and for always showing an interest in my work. My appreciation of the natural world stems from the holidays you have taken me on throughout the Southern African wilderness, for which I am grateful.

This project would not be possible without financial support provided by the National Research Foundation (NRF), South Africa.
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Chapter 1

General Introduction

Nitrogen is an essential element for plant life, but is often the most limiting nutrient in terrestrial environments (Aerts and Chapin III 1999; Hawkesford et al. 2011). Plants take up inorganic (i.e. NO$_{3}^{-}$ and NH$_{4}^{+}$) and organic (e.g. amino acids) N from soil and the availability of each form depends on the interplay between flora, fauna, microbial activity (e.g. mineralization) and climate (e.g. seasonal temperature and soil water content; Miller and Cramer 2004). Atmospheric N$_2$ is only available to the relatively few plants – mostly legumes – that form symbiotic relationships with N$_2$-fixing bacteria, and consequently N-fertilizers are a major agricultural input applied to maximise crop yields. This supplementation has repercussions for natural ecosystems, with groundwater becoming eutrified from leached N and heightened deposition of atmospheric N caused by a rise in NH$_3$ and N$_2$O emissions from cultivated fields (Nacry et al. 2013, Denk et al. 2017).

N has two naturally occurring isotopes, the lighter, more abundant $^{14}$N and the heavier, rarer $^{15}$N (Robinson 2001). The natural abundances of these isotopes have been commonly used to trace global, ecosystem and individual plant N-fluxes, and yet complete understanding of the processes responsible for determining the abundances remain somewhat elusive (Handley et al. 1999; Amundson et al. 2003; Yoneyama et al. 2003; Kalcsits et al. 2014; Craine et al. 2015a). The δ$^{15}$N value of plants varies across landscapes because of access to a variety of N sources with diverse isotope ratios (i.e. $^{15}$N:$^{14}$N; Robinson et al. 1998; Craine et al. 2015a). For example, the range in foliar δ$^{15}$N is 35‰ at a global scale and 25‰ locally, but most values fall between -7.8 and 8.7‰ (Craine et al. 2015a). Nonetheless, globally, plants are generally more depleted in $^{15}$N than the soil in which they grow, and consequently have lower δ$^{15}$N values than the bulk soil (Amundson et al. 2003). Plant N is the end-product of a series of fractionating steps through the soil-plant N cycle, beginning with the breakdown of soil organic matter, that leave plants relatively $^{15}$N-depleted and soil relatively $^{15}$N-enriched (Craine et al. 2015a; Craine et al. 2015b).

The efflux of $^{15}$N-enriched N from plant roots is widely accepted as the primary mechanism causing the deviation between plant δ$^{15}$N and the δ$^{15}$N value of its N-source, which is referred to as plant isotopic discrimination (Evans 2001). Assimilation of inorganic N is the major fractionating point within plants; these enzyme-mediated reactions favour the incorporation of relatively more $^{14}$N into the organic product and leave residual unassimilated inorganic N relatively enriched in $^{15}$N, due to the
energetic costs associated with the heavier $^{15}$N isotope (Robinson 2001). If the entire inorganic N pool is assimilated, the system is deemed ‘closed’ and no fractionation is evident (Robinson 2001). By contrast, if assimilation is only partially completed, a pool of $^{15}$N-enriched inorganic N will accumulate in the cytosol, some of which may be stored in vacuoles. Provided that the assimilation occurred in the root, these inorganic N pools are then available for efflux from the root cytoplasm. Although many plant species can efflux organic N such as amino acids, inorganic N efflux is more prevalent (Warren 2015). Net N uptake by plant roots is a balance between N influx and efflux, both of which are enabled by various transporters (Segonzac et al. 2007; Wang et al. 2012; Nacry et al. 2013). Influx, however, usually predominates unless a plant experiences stress that temporarily induces a higher efflux rate (Segonzac et al. 2007). Efflux is presumed to function as a regulator of cytoplasmic inorganic N concentrations, but it remains a perplexing physiological process because it is energetically costly for plants to take up N only to release it back into the rhizosphere (Britto and Kronzucker 2006).

The N efflux conundrum is pertinent to plants from both natural and agricultural ecosystems. Agricultural research strives to produce crop plants with high N use efficiency (NUE), a measure dictated by net N uptake and assimilation, because this will ultimately allow for reduced fertilizer application with associated environmental benefits (Glass 2003; Xu et al. 2012). The efflux of a substantial fraction of N taken up, instead of assimilating that N and using it for growth, is inefficient. Moreover, it is puzzling that even wild plants would efflux what is usually a limiting nutrient. This leads to the question of whether there are alternative pathways by which plants lose N. Root rhizodeposition and root turnover are unavoidable artefacts of root growth and are substantial nutrient sinks because nutrients, including N, are not fully resorbed during tissue senescence (Lauenroth and Gill 2003; Wichern et al. 2008). Roots and root fragments abscised from plants may contain pools of $^{15}$N-enriched N, and could therefore be confused with measured efflux and play a role in the global depletion of plant δ$^{15}$N relative to soil.

### 1.1 Alternatives to efflux that generate the offset between plant and soil δ$^{15}$N

Evidence for a global occurrence of efflux derives from the global pattern of lower δ$^{15}$N values in plant tissue than in bulk soil. This offset, however, could result from plant-available N having a different δ$^{15}$N signal to that of bulk soil. Amundson et al. (2003) found that the divergence between plant and bulk soil δ$^{15}$N increased towards higher latitudes. In these cold ecosystems, mineralization and nitrification rates are slow, organic N is predominant and the mineral N pool is small (Handley et al.
1999; Craine et al. 2015b). While plants can take up organic N (Owen and Jones 2001), the majority at high latitudes is probably unavailable to plants, and the conversion to available forms must incur heavy fractionation, meaning that the plant-available δ\(^{15}\)N does not reflect that of bulk soil (Chapin III et al. 1993; Atkin 1996; Amundson et al. 2003). At lower, warmer latitudes, where microbial breakdown and conversion is faster, inorganic N is relatively more available in the bulk soil for plants to take up (Handley et al. 1999; Craine et al. 2015b). Although fractionation during microbial processing still occurs here, the plant-available δ\(^{15}\)N approaches that of the bulk soil because of the larger mineral N pool. Ultimately, plant N-loss can only be inferred if there is a δ\(^{15}\)N offset between a plant and its source N. While Craine et al. (2015a) showed little difference between foliar δ\(^{15}\)N and soil inorganic δ\(^{15}\)N from a limited number of studies, many hydroponic studies have found plant isotopic discrimination (Kohl and Shearer 1980; Evans 2001; Kolb and Evans 2003; Kalcsits and Guy 2013).

Plant acquisition of N through associated mycorrhizal fungi may contribute to plant \(^{15}\)N-depletion; this is illustrated by the often \(^{15}\)N-enriched fungal structures relative to the δ\(^{15}\)N of the host plant (Gebauer and Dietrich 1993; Handley et al. 1996; Högberg et al. 1996). Plant δ\(^{15}\)N values vary according to the type of mycorrhizal symbioses, which differ in the distance from the root that they can scavenge for nutrients and the exudates they use to acquire N (Craine et al. 2015a). For example, Craine et al. (2009) established that ericoid-mycorrhizal plants show the most foliar δ\(^{15}\)N depletion (-5.0‰), then ectomycorrhizal (-2.3‰) and arbuscular-mycorrhizal plants (-1.1‰), while non-mycorrhizal plants had the highest foliar δ\(^{15}\)N (0.9‰). When the direct availability of soil N to plants increases, they can reduce their dependency on mycorrhizal-N, which coincides with less plant \(^{15}\)N-depletion (Högberg et al. 2011). In colder ecosystems (e.g. arctic, boreal and heathland) plants depend appreciably on mycorrhizal uptake of organic N (Yano et al. 2010), and this association probably contributes to the larger plant-bulk soil δ\(^{15}\)N offset at higher latitudes. Considering that mycorrhizal symbioses are prevalent among all plant communities, and that a significant N flow occurs between the fungi and host plant, mycorrhizae probably contribute to the global offset between plant and soil δ\(^{15}\)N.

1.2 Why is N efflux energetically costly?

Futile cycling occurs when two metabolic pathways run simultaneously in opposite directions with no effect other than to dissipate energy (Schwender et al. 2004). Interestingly, there is evidence that these cycles exist in plant physiology despite the high energetic costs. For example, several intermediary C
metabolites (sucrose, UDP glucose, hexose-P and triose-P) are continually synthesized and broken down to allow the central C metabolic pathway to run at a stable rate (Rontein et al. 2002). Inorganic N ion flux (i.e. influx and concurrent efflux) across the plasma membrane of root epidermal cells also constitutes a futile cycle (Britto and Kronzucker 2006). Plasma membranes are polarized by ATP-dependent H+-ATPase pumps that exude H⁺ ions into the external medium (1H⁺ per ATP hydrolyzed) and generate a negative voltage inside a cell relative to the outside (Lambers et al. 2008). The anion NO₃⁻ must be actively transported across the plasma membrane into the negatively charged root cytoplasm, by coupling with 2H⁺ through NO₃⁻-specific symports (Hawkesford et al. 2011; Wang et al. 2012; Nacry et al. 2013). ATP is required for the uptake of NO₃⁻ across a wide range of concentrations (Glass et al. 1992). By contrast, the cation NH₄⁺ may enter the root passively by moving down the electrochemical gradient through NH₄⁺-specific uniports (Hawkesford et al. 2011; Nacry et al. 2013). From an energetic perspective, NO₃⁻ could exit the root passively whereas NH₄⁺ cannot, and thus for both forms of inorganic N the combination of influx and efflux is energetically demanding and is never truly passive (Britto and Kronzucker 2006). It has been proposed that susceptibility to NH₄⁺ toxicity at high external concentrations is caused by energy-sapping efflux of NH₄⁺ (Britto et al. 2001). These authors reported that root respiration in barley, a NH₄⁺-sensitive species, increased by 41% during NH₄⁺ efflux. Contrastingly, rice is tolerant of high-NH₄⁺ conditions because it can depolarize root plasma membranes, eliminating the gradient against which NH₄⁺ efflux must operate and therefore reducing the energetic impact of efflux (Britto et al. 2001).

1.3 How is N efflux enabled?

N efflux is transporter-mediated and there are several non-selective plasma membrane transporters, which convey a variety of ions, that could participate in N efflux (Miller and Cramer 2004). NO₃⁻ is probably excreted through inducible anion channels, while NH₄⁺ requires an antiport (cation-H⁺ exchange) to exit the root (Aslam et al. 1996a; White 2011). A role for aquaporins in the efflux of NH₃ has also been suggested (Coskun et al. 2013). Using a functional biochemical approach, Segonzac et al. (2007) identified a NO₃⁻ excretion transporter (NAXT1) in Arabidopsis. Under standard hydroponic conditions, however, naxt1 mutants (i.e. plants with impaired expression of NAXT1) showed no significant difference in root and shoot NO₃⁻ content and NO₃⁻ efflux compared to wild-type plants. NAXT1 was only induced when the root cytosol became acidified (Segonzac et al. 2007). For a transporter to play a role in N efflux it must be expressed in the plasma membrane of root epidermal cells in contact with the external medium. In situ hybridization followed by histochemical staining of
7-day old *Arabidopsis* seedlings revealed that NAXT1 was most common in secondary roots and younger parts of primary roots, but not in root tips, with a strong expression in cortical cells and to a lesser extent in epidermal cells (Segonzac et al. 2007). Many transporters convey N within and between cells (Wegner and Raschke 1994; Grouzis et al. 1997). For example, NRT1.5 is responsible for loading NO$_3^-$ into the xylem and members of the chloride channel (CLCa,b) family carry NO$_3^-$ into the vacuole through the tonoplast (Wang et al. 2012). Interestingly, *naxt-RNAi* plants (i.e. impaired expression of all potential members of the NAXT family) had 30% more root NO$_3^-$ and 26% less shoot NO$_3^-$ than wild-type plants, which suggests a possible role for this transporter family in moving NO$_3^-$ to the shoot (Segonzac et al. 2007). Transporters could very well aid symplastic movement of N through the root cortex and across the Casparian strip towards the xylem (Wegner and Raschke 1994), or into vacuoles for storage, rather than being present in epidermal cell plasma membranes for the exclusive reason of N efflux to the external medium.

### 1.4 When does N efflux occur?

A major determinant of the extent of inorganic N efflux is the external inorganic N concentration ([N$_i$]$_{ex}$) (Britto and Kronzucker 2006). Inorganic N influx into roots is governed by two transport systems, high-affinity (HATS) and low-affinity (LATS) (Britto and Kronzucker 2006; Hawkesford et al. 2011). HATS operates at low [N$_i$]$_{ex}$ and is saturable, while LATS is typically induced at an [N$_i$]$_{ex}$ of >1 mM and allows the influx rate, and in turn the cytoplasmic [inorganic N], to increase linearly with [N$_i$]$_{ex}$. The rise in root cytoplasmic [inorganic N] is correlated with a rise in the rate of efflux, for both NO$_3^-$ and NH$_4^+$ (Breteler and Nissen 1982; Teyker et al. 1988; Siddiqi et al. 1991; Jackson and Volk 1992; Wang et al. 1993; Volk 1997; Britto et al. 2001). For example, Teyker et al. (1988) lowered root cytoplasmic [NO$_3^-$] sequentially from 106 to 3 µmol g$^{-1}$ fresh weight (FW) by growing maize in an N-free solution for up to 72 h. The efflux rate ranged from 0.19 to 2.8 µmol g$^{-1}$ FW h$^{-1}$, depending on the root [NO$_3^-$], when the plants were subsequently transferred to 0.15 mM [NO$_3^-$]$_{ex}$ for 30 min (Teyker et al. 1988). Likewise, the NH$_4^+$ efflux rate in rice rose from 0.13 to 3.1 µmol g$^{-1}$ FW h$^{-1}$ when the [NH$_4^+$]$_{ex}$ was increased from 2 to 1000 µM (Wang et al. 1993), and was 18.6 µmol g$^{-1}$ FW h$^{-1}$ at 10 mM (Britto et al. 2001). Here, cytoplasmic [NH$_4^+$] was 3.7, 38.1 and 232 mM, respectively (Wang et al. 1993; Britto et al. 2001). Furthermore, under LATS conditions the ratio of efflux to influx (E/I) strengthens, in other words efflux rates approach that of influx (Presland and McNaughton 1986; Britto and Kronzucker 2006). These results provide evidence that efflux is strongly determined by root tissue [inorganic N] and may serve to regulate cytoplasmic inorganic N.
Slower-growing species tend to exhibit larger ratios of N influx to net uptake, in other words N efflux becomes more pronounced (Scheurwater et al. 1999; Mata et al. 2000; Glass 2003), even though they have substantially reduced influx rates compared to fast-growing species, and so the root cytoplasmic [inorganic N] is unlikely to be excessive. Ultimately, it is the plant demand for N relative to the N supply that drives efflux in these situations (Kalcsits et al. 2014). At low [N$_i$]$_{ex}$ and under high plant N demand (e.g. fast-growing plants) all the inorganic N taken up is likely to be assimilated. Conversely, at higher [N$_i$]$_{ex}$ or when plant demand for N is low (e.g. slow-growing plants), a plant may not be able to assimilate, or transport to the shoot, the inorganic N taken up at an accelerated rate, leading to a pool of inorganic N available for efflux. During assimilation, inorganic N is combined with carbon skeletons to form amino acids through reactions catalyzed by the enzymes nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamine:oxoglutarate aminotransferase (GOGAT) (Miller and Cramer 2004). The conversion of NO$_3^-$ to NO$_2^-$ is the rate-limiting step in this pathway, yielding an in vitro fractionation factor ($\Delta^{15}$N between the inorganic source and the organic product) of 15.4–22‰ (Ledgard et al. 1985; Carlisle et al. 2014), while GS yields an in vitro fractionation factor of 16.5‰ (Yoneyama et al. 1993). However, in vivo fractionation realized during the entire N assimilatory process in an intact plant could be less than these factors. Mariotti et al. (1982) measured low NR activity in young pearl millet seedlings, indicating that the enzyme was probably saturated at this growth stage. They suggested that an efflux-available inorganic N pool was likely to form in the root because the influx rate exceeded the assimilation capacity, and their result of increased discrimination between plant and source $\delta^{15}$N corroborated this (Mariotti et al. 1982). The growth rate of a plant therefore regulates the demand for N, which in turn controls the rate of N assimilation and the potential for efflux.

The net N uptake rate of plants declines under night-time conditions and this is sometimes due to an elevated efflux rate (Scheurwater et al. 1999; Mata et al. 2000). Although the influx rate slows down in the dark, NO$_3^-$ commonly accumulates in roots during the night because root NO$_3^-$ reduction decreases and lower night-time transpiration slows xylem transport of NO$_3^-$ to the shoot (Delhon et al. 1995). The accumulating cytoplasmic NO$_3^-$ could induce efflux to the external medium (Scaife 1989). For example, Scheurwater et al. (1999) found greater efflux at night (e.g. efflux was ca. 80% of influx at night and only ca. 30% during the day) in certain grass species (Deschampsia flexuosa and Festuca ovina) and Pearson et al. (1981) found the efflux rate in pearl millet was 1.3-fold higher at night. However, other results do not support this phenomenon. Pearson et al. (1981) did not find diurnal variation in the efflux rate of maize, while Macduff and Jackson (1992) measured a three-fold increase.
in \( \text{NO}_3^- \) efflux from Italian ryegrass after prolonged exposure to darkness, but a five-fold decrease in \( \text{NH}_4^+ \) efflux. Controls on diurnal variation of efflux probably depend on species-based night-time transpiration and the capacity for providing carbohydrates to N assimilation end-products (Pearson et al. 1981). Thus, although there is some evidence for a regulatory role of efflux at night, this is complex and certainly not a universal mechanism for controlling root N accumulation.

N efflux is heightened by plant stress such as: the mechanical disturbance of roots (e.g. transference between hydroponic solutions; Bloom and Sukrapanna 1990; Delhon et al. 1995; Aslam et al. 1996b; Steege et al. 1998), defoliation (Macduff and Jackson 1992), dramatic changes in \([\text{N}]_{\text{ex}}\) during flux experiments and the acidification of the external medium. For example, Aslam et al. (1995) found that the \( \text{NO}_3^- \) efflux rate of barley seedlings remained constant between pH 6 and 9 (ca. 1.23 µmol g\(^{-1}\) FW h\(^{-1}\)), but increased at pH 4 (1.79 µmol g\(^{-1}\) FW h\(^{-1}\)). Likewise, Segonzac et al. (2007) measured a rise in \( \text{NO}_3^- \) efflux and a decline in \textit{Arabidopsis} root \( \text{NO}_3^- \) content under acid load (pH 6, using 10 mM propionate) or hydroponic medium acidification (pH 3, generated with \( \text{H}_2\text{SO}_4 \)). From these experiments, Segonzac et al. (2007) suggested that efflux acts as a pH-stat, counteracting a drift of the cytosolic pH towards acidic conditions. When the external medium is acidic (e.g. acidic soils), the influx of \( \text{H}^+ \) into the root is heightened and the plasma membrane begins to depolarize; greater \( \text{H}^+\text{-ATPase} \) activity is then required to maintain polarization. The electroneutral efflux of \( \text{H}^+:\text{NO}_3^- \) across the plasma membrane could serve to reverse this cytosolic acid load (Segonzac et al. 2007). This would, however, require large amounts of cytoplasmic \( \text{NO}_3^- \), which is not as common as \( \text{NH}_4^+ \) in naturally occurring acidic soils (Hawkesford et al. 2011).

N efflux has never been demonstrated in the field because all studies reporting on it are ‘laboratory-based’, where plants are usually grown under hydroponic conditions for obvious practical reasons. It is presumed to occur in all plants because of the presence of N-efflux transporters, but the function(s) of these are ambiguous (see above). In the case of crop plants, the luxury consumption of fertilizer N (Aerts and Chapin III 1999), under LATS conditions, could allow for efflux-available N pools to develop in roots, and thus a high N influx rate could well be associated with concurrent high efflux rate. Quantifying N availability to plants in soil is challenging due to its heterogeneous spatial and temporal distribution (Marschner and Rengel 2011), but concentrations in natural soils are usually lower than in agricultural soils (Owen and Jones 2001, Britto and Kronzucker 2002), and are likely to be lower than the level at which LATS operates (Britto and Kronzucker 2006). Therefore, the relevance of N efflux in natural ecosystems is questionable, given that it is widely regarded as a physiological mechanism for regulating root cytoplasmic [inorganic N] but natural soils are likely to be relatively...
N-limited (Aerts and Chapin 1999). If N efflux is an intrinsic physiological trait, surely plants with lower inherent N efflux rates must have a selective advantage in natural ecosystems.

1.5 Are root turnover and rhizodeposition a significant N-loss pathway?

The idea that root turnover is an important N-loss pathway, and that the occurrence of N efflux is questionable, has been posed for at least sixty years. Butler and Bathurst (1956) reported that the “excretion of simple nitrogenous compounds” (i.e. what we term efflux today) from clover root systems is only likely to happen when the N requirements of the legume are exceeded and that the observed deposition of N to pasture soils is probably entirely due to decaying roots and nodules. The loss of root tissue to soil can be categorized into two different processes, namely rhizodeposition and root turnover. Roots proliferate into patches of nutrients and water but die back (i.e. turnover) once the transient resources are depleted (Lauenroth and Gill 2003). Roots will also die back if above-ground biomass is lost (e.g. by herbivory) because C flow to the roots is reduced (Dawson et al. 2000). As roots grow through soil they deposit debris into the rhizosphere (zone of soil surrounding the root that the root manipulates). For example, border cells and mucilage are released from the root cap zone to aid root elongation, while root hairs, epidermal and cortical cells may be sloughed off from frictional stress (Rovira 1956; Wichern et al. 2008). Rhizodeposition is also advantageous to a plant because it attracts beneficial soil microbes into the rhizosphere. Functionally separating rhizodeposited N from root turnover derived N (i.e. N lost from living or dying roots) is difficult in the field. As both mechanisms involve the natural detachment of plant parts, they are referred to here, collectively, as ‘root abscission’.

Rhizodeposition is commonly quantified in terms of carbon, for example 5–10% of net C fixed is lost in rhizodeposits (Jones et al. 2004), as the release of C into the rhizosphere is a major energy substrate for microbes (Newman 1985). The concomitant N deposition can, however, be significant. In a review on rhizodeposition, Wichern et al. (2008) found that rhizodeposited N from crop legumes was 4-71% of total plant N and was 4-56% for cereals (wheat and barley), and the medians were 16 and 14% respectively. Legumes tend to exhibit greater N-rhizodeposition because their symbiosis with N₂-fixing bacteria forms transient N-rich nodules. Research has focused on agronomic species and the factors affecting rhizodeposition in cultivated fields (e.g. soil texture, density and moisture) because, although it represents a mode of plant N-loss, it can be harnessed as an N-gain. For example, the root residue of pasture legumes becomes a natural fertilizer for the succeeding crop (Butler and Bathurst...
In natural ecosystems, the ‘luxury’ N legumes enjoy can also benefit coexisting non-legumes (Gubsch et al. 2011).

The potential contribution of roots to the terrestrial N cycle is determined by the size of the below ground biomass, the amount of N contained there, and the scale and rate at which it turns over. Fine roots (< 2 mm diameter) represent a considerable proportion of annual primary productivity because their global biomass is huge. Jackson et al. (1997) found that living fine root biomass ranges across biomes, from 0.13 (desert) to 0.95 (temperate grassland) kg m\(^{-2}\), and that the N pool tied up in global living fine roots is 4.8x10\(^8\) metric tons, which is approximately 7% of living plant biomass (Wang et al. 2010). Root [N] is determined by the physiological function of the root (e.g. water and nutrient acquisition, storage, transport or anchorage), which in turn is usually dependent on its diameter and order on a branch (Eissenstat et al. 2000; Pregitzer 2002). Generally, root [N] tends to increase as the diameter decreases, highlighting the importance of fine roots to the root N pool. For example, in a meta-analysis on fine root nutrient concentrations, Gordon and Jackson (2000) found a 1.7-fold decrease in [N] as root diameter class increased from < 2 mm to 2–5 mm. Furthermore, in natural ecosystems very fine roots (< 0.5 mm diameter) can cumulatively contain more nutrients than leaves (Gordon and Jackson 2000). This root N is only available to the N cycle if it is not reabsorbed before root senescence. Minimal N resorption is possible if a root is unnaturally excised (e.g. herbivory by rodents or nematodes), but re-translocation of some N can occur prior to programmed root senescence (John et al. 2002; Jones et al. 2004). For instance, Kunkle et al. (2009) found a decrease in [N] of up to 28% between live and dead fine roots of deciduous trees. Additionally, they found that N resorption from fine roots of various trees was never more than 50%, once data from the literature had been mass-loss corrected (Kunkle et al. 2009). Given that N in senescing roots is unlikely to be entirely resorbed, root turnover has the potential to be a significant N-loss pathway.

The scale of root turnover varies between biomes and with latitude because root turnover rates generally increase exponentially with temperature, brought about by rising root maintenance respiration (Gill and Jackson 2000). Root turnover rates also increase when elevated temperatures are combined with reduced growing season precipitation. Grasslands have higher turnover rates of whole root systems (53% annually, averaged across latitudes) than shrublands (34%) and forests (10%), most likely due to growth rate differences between plant functional types (Gill and Jackson 2000). Forest fine roots do, however, have large turnover rates (e.g. roots < 1 mm diameter = 120% annually and roots < 5 mm = 56%; Gill and Jackson 2000). A quarter to half the roots present in upper soil layers
are dead or dying (Newman 1985) and this is characterized in Jackson et al. (1997) analysis which found that on average, across biomes, ca. 50% of root stocks have already senesced.

There is an abundance of evidence suggesting root abscission is a significant pathway by which plants lose N, but does it contribute to the global offset between plant and soil δ\(^{15}\)N values? Root abscission will affect plant δ\(^{15}\)N if the abscised roots have a different δ\(^{15}\)N signal to the rest of the plant (i.e. are relatively \(^{15}\)N-enriched; Handley and Raven 1992). Remobilization of N from various plant organs to aid growth of new shoots, roots or reproductive parts is common, and probably contributes to distinct intra-plant variation in δ\(^{15}\)N because protein hydrolysis is a fractionating process (Kolb and Evans 2002). Roots participate in N remobilization to an extent (Bausenwein et al. 2001; Andersson et al. 2005), and so have the potential to be relatively \(^{15}\)N-enriched after N has been remobilized. Additionally, while N resorption from leaves before senescence does not seem to fractionate (Kolb and Evans 2002), there is no evidence regarding fractionation during N resorption from roots. Considering root abscission is ubiquitous across agricultural and natural ecosystems, and is an unavoidable phenomenon of being a plant, even if the \(^{15}\)N-enrichment of roots is small, the loss of root tissue is still likely to be a major contributor to plant-soil δ\(^{15}\)N divergence.

### 1.6 Hypothesis and thesis outline

In this thesis I evaluate the extent to which N efflux and root abscission contribute to plant N-loss and therefore to plant δ\(^{15}\)N values. I focus on the discrimination between plant and inorganic source δ\(^{15}\)N, rather than: the discrepancy between bulk soil and plant-available N, plants that access different soil N pools, or the influence of mycorrhizal symbioses. I hypothesize that the discrimination between plant and source δ\(^{15}\)N values is largely an artefact of root abscission and that N efflux is minimal under standard growth conditions. To test this hypothesis, I divided my thesis into two data chapters that each explore and manipulate a plant growth condition.

In Chapter 2, I investigate the relationship between plant δ\(^{15}\)N and rates of NO\(_3^-\) supply in wheat and how this relates to N efflux and root abscission. I hypothesized that plants supplied with more inorganic N would have more negative δ\(^{15}\)N relative to the source, as the ratio of N influx to assimilation increases, and that this isotopic discrimination would increase over time as the root biomass turns over. Furthermore, I expect root abscission to contribute to the N that accumulates in the growth medium and, correspondingly, to that lost from the plant. Wheat was grown in sand with NO\(_3^-\) supplied at five
relative additions rates (which correspond to incrementally increasing relative growth rates – RGR); the $\delta^{15}$N of roots and shoots, as well as the N accumulation in the growth medium, were periodically determined.

In Chapter 3, I determined the extent to which N efflux and root abscission contribute to plant N-loss in hydroponics, and the consequences for plant $\delta^{15}$N. I hypothesized that the aeration necessary for hydroponic culture can act as a mechanical stressor on roots, breaking off finer root components (e.g. root hairs) and accentuating plant N-loss. Additionally, vigorous hydroponic aeration that allows for constant mixing could also increase N-loss through efflux by strengthening diffusion gradients away from the rhizosphere. Wheat was grown in a hydroponic system which comprised three ‘stability’ treatments for root growth: direct aeration, aeration constrained within a pipe and circulation of nutrient solution through sand. The $\delta^{15}$N of root fragments and plant-derived N in solution were periodically measured; root and shoot $\delta^{15}$N were measured at harvest.

Each data chapter was written as an independent paper and thus there is a degree of repetition in the thesis.
Chapter 2

The role of N efflux and root abscission in determining plant $\delta^{15}$N

2.1 Abstract

I investigated the relationship between plant $\delta^{15}$N and rates of nitrate supply in wheat (*Triticum aestivum*; L. cv. SST015) and how this relates to N efflux and root abscission. Wheat was grown in sand with NO$_3^-$ supplied at five relative addition rates (RAR). I periodically determined the $\delta^{15}$N of roots and shoots, as well as the N accumulation in the growth medium due to efflux and root abscission. Plant $\delta^{15}$N values decreased when N supply exceeded plant demand; e.g. after 47 d plant $\delta^{15}$N of RAR 0.075 d$^{-1}$ was 0.4‰ but was -4.1‰ at RAR 0.175 d$^{-1}$. 23% of N taken up would need to be effluxed in order to explain the plant $\delta^{15}$N of RAR 0.175 d$^{-1}$. By contrast, the loss of only 2.3% of root biomass could explain this plant $\delta^{15}$N. Indeed, four-fold more N was lost from plants across all RAR’s than accumulated in the growth medium leachate. This excess N-loss was likely due to root abscission. Plant $\delta^{15}$N is influenced by a combination of efflux and root abscission but the proportion of each changes as N supply increases. The efflux of $^{15}$N-enriched inorganic N is more likely at high N supply, whereas with lower N supply plants probably lose more N in root fragments.
2.2 Introduction

Net N uptake and assimilation are two major controls on NUE (Xu et al. 2012). It is widely accepted that N influx is associated with concurrent concentration dependent efflux of N from plant roots, which can have adverse consequences for NUE and plant growth, because it may constitute a futile cycle (Britto and Kronzucker 2006). Futile cycling occurs when two metabolic pathways run simultaneously in opposite directions and have no overall effect other than to dissipate energy (Schwender et al. 2004). Efflux has, however, been suggested to regulate root cytoplasmic [inorganic N] and is enabled by various transporters (Miller and Cramer 2004; Britto and Kronzucker 2006; Segonzac et al. 2007). Nevertheless, it is energetically costly for plants because ATP is required for inorganic N uptake (Britto and Kronzucker 2006). Stable isotopes of N are commonly used to track the movement of N through the soil and into a plant. Isotope mass-balance rules dictate that plant δ^{15}N should match the source δ^{15}N, unless the plant preferentially loses ^{15}N (Robinson et al. 1998). Globally, plants are more depleted in ^{15}N than the soil in which they grow and consequently have more negative δ^{15}N values than bulk soil (Amundson et al. 2003). This pattern is mostly ascribed to efflux of ^{15}N-enriched N from plant roots (Kalcsits and Guy 2016).

The deviation of plant δ^{15}N from that of soil could result from bulk soil having a different δ^{15}N signal to plant-available N, due to soil processes including decomposition, mineralisation, nitrification and denitrification (Pörtl et al. 2007; Kahmen et al. 2008; Craine et al. 2015a). In general, however, plant δ^{15}N largely reflects bulk soil δ^{15}N (Kahmen et al. 2008). During N transport into the rhizosphere (mass flow and diffusion), the difference between ^{14}N and ^{15}N movement is negligible (Högberg 1997). Moreover, there is scant unequivocal evidence for fractionation during passive or active transport of N across root membranes (Handley and Raven 1992; Högberg 1997; Robinson 2001). Evans (2001) argued that there is no inherent fractionation during NO_3^- uptake because several studies show no isotopic discrimination between plant and N source across a range of [NO_3^-]_ev. Furthermore, unassimilated cytoplasmic NO_3^- and NH_4^+ in roots is actually enriched in ^{15}N relative to the source (Yoneyama and Kaneko 1989), indicating that most fractionation is subsequent to uptake.

The assimilation of inorganic N is a major fractionating point within plants. The fractionation favours the incorporation of relatively more ^{14}N into the organic product and leaves residual unassimilated inorganic N enriched in ^{15}N, due to the energetic costs associated with the heavier ^{15}N isotope (Robinson 2001). Although there is a range of fractionation factors for N-assimilatory enzymes, the most commonly cited factors are 15.4‰ for NR (Ledgard et al. 1985) and 16.8‰ for GS (Yoneyama
et al. 1993). Depending on the form of N and where it is assimilated, varying intra-plant δ¹⁵N patterns can arise. There is little intra-plant divergence with NH₄⁺ nutrition because the majority of NH₄⁺ is immediately assimilated in the root, leaving the δ¹⁵N of organic N throughout the plant relatively uniform (Yoneyama et al. 1991; Evans et al. 1996; Kalcsits et al. 2015). With NO₃⁻ nutrition, however, shoot δ¹⁵N can be 3 to 7‰ more positive than that of roots (Yoneyama and Kaneko 1989; Evans et al. 1996; Kalcsits et al. 2015). If a pool of NO₃⁻ is not entirely assimilated in the root the remaining ¹⁵N-enriched NO₃⁻ is transported to the shoot for further assimilation, leading to relatively more positive shoot δ¹⁵N. Andrews (1986) and Gojon et al. (1994) suggested that root assimilation predominates at low [NO₃⁻]ₑₓ while significant amounts of NO₃⁻ are only transported to the shoot when root NR activity is saturated, under high [NO₃⁻]ₑₓ.

These assimilation patterns explain intra-plant δ¹⁵N variability, but do not explain how plant δ¹⁵N becomes more depleted than soil δ¹⁵N. If a residual pool of ¹⁵N-enriched inorganic N is lost externally, the plant becomes depleted of ¹⁵N relative to the inorganic N source (Robinson 2001). The efflux of inorganic N is widely accepted as the principal mechanism causing this plant isotopic discrimination (Kalcsits and Guy 2016). Key determinants of root [N], and thus the amount of N effluxed, are the [Nᵢ]ₑₓ, the rate of influx versus the rate of root assimilation and the plant demand for N (Kalcsits et al. 2014). At low external concentrations, all inorganic N may be taken up and assimilated. Conversely, if the [Nᵢ]ₑₓ is higher, a plant may not be able to assimilate, or transport to the shoot, all the N taken up leading to a ¹⁵N-enriched pool available for efflux from the root. For example, Mariotti et al. (1982) found greater plant discrimination in pearl millet seedlings, which decreased as the plants matured. They measured low NR activity in young seedlings and suggested the enzyme was saturated at this stage, greatly increasing the ratio of influx to assimilation and allowing the efflux-available pool to form. The ratio of efflux to influx (E/I; Kalcsits et al. 2015), adapted from the model originally described by Mariotti et al. (1982), shows how well N supply meets plant demand and should be directly proportional to plant discrimination.

Apart from N efflux, plant δ¹⁵N may also become more depleted than soil δ¹⁵N if roots containing residual pools of ¹⁵N-enriched N are mechanically abscised. Roots proliferate into patches of water and nutrients but die back as soon as the transient resources are depleted. During this cyclical growth and death, roots deposit debris into the rhizosphere such as sloughed epidermal cells and roots hairs (Wichern et al. 2008). The scale of rhizodeposition is proportional to root biomass and increases as roots mature (Wichern et al. 2008). Root turnover can be substantial but depends on the size of root, for example, 56% of fine forest roots are replaced annually compared to 10% of the entire root system.
Root turnover and rhizodeposition are substantial nutrient sinks for plants (Lauenroth and Gill 2003). The life-span of roots is more dynamic than leaves, with some roots surviving for less than one week (Gordon and Jackson 2000). Roots can contain higher nutrient concentrations than leaves but the resorption of these nutrients during senescence can be less efficient (Gordon and Jackson 2000; Brant and Chen 2015). Kunkle et al. (2009) found a decrease in N concentration of up to 28% between live and dead fine roots of deciduous trees, which indicates that N in senescing roots is unlikely to be entirely resorbed. Thus, there is an opportunity for residual $^{15}$N-enriched inorganic N pools to be lost by root turnover and rhizodeposition. Although effluxed inorganic N can be termed a rhizodeposit, it is estimated that 90% of rhizodeposits are actually root debris and not low molecular weight substances (Wichern et al. 2008).

While efflux as a driver of plant $\delta^{15}$N, and root turnover as a nutrient sink are well known, the link between root abscission and plant $\delta^{15}$N has not been explored. Here, I investigated how the $\delta^{15}$N of wheat is affected by $[\text{NO}_3^-]_{\infty}$ over time and how this is related to efflux and/or root abscission. I hypothesized that plants supplied with more N would have more negative $\delta^{15}$N relative to the source, as the ratio of N influx to assimilation increases, and that this isotopic discrimination would increase over time as the root biomass turns over. Furthermore, I expect root abscission to contribute to the amount of N that accumulates in the growth medium, and correspondingly to that lost from the plant. Root and shoot $\delta^{15}$N were also measured because intra-plant variability is an important indicator of assimilation patterns, and therefore the possibility for $^{15}$N-enriched inorganic N pools to form in the root.

### 2.3 Methods and Materials

#### 2.3.1 Plant germination and growth conditions

Wheat plants (*Triticum aestivum* L. cv. SST015) were grown from seed that was soaked in aerated water for 24 h before being sown in trays of vermiculite. The trays were kept moist until the emerging seedlings were suitably established. After 7 d the seedlings were transplanted into 15 cm diameter pots, one seedling per pot, each containing 1.9 kg of pH-neutral acid-washed (0.1% HCl) silica sand. Plants were given a single daily 400 ml application to the sand surface (i.e. to field capacity) of a modified Long Ashton nutrient solution (Hewitt 1966) containing 1.1 mM MgSO$_4$, 2 mM K$_2$SO$_4$, 4 mM CaCl$_2$, 0.14 mM H$_3$BO$_3$, 20.8 µM MnSO$_4$, 2.3 µM ZnSO$_4$, 3.3 µM CuSO$_4$, 0.25 µM Na$_2$MoO$_4$, 90 µM Fe-EDTA and a phosphate buffer (pH 6.5) of 0.67 mM NaH$_2$PO$_4$ and 1.5 mM Na$_2$HPO$_4$. 
Nitrogen was supplied to the plants in the form of NaNO$_3$ by means of a relative addition rate (RAR), with the relevant concentrations added to the Long Ashton nutrient solution. To maintain a constant plant [N] during the exponential growth phase, N must be supplied at a range of exponentially increasing rates, each corresponding to a RAR (Ingestad 1982). The daily addition of N ($\Delta N$ mmol plant$^{-1}$) to plants was calculated according to Macduff et al. (1993): $\Delta N = N_{t+1} - N_t = N_t(e^{RAR} - 1)$, where $N_t$ and $N_{t+1}$ are the amount of N (mmol plant$^{-1}$) in the plant after $t$ and $t+1$ days, respectively. RAR is the relative addition rate of N (g g$^{-1}$ d$^{-1}$). The plant N content at $t = 0$ (7 d) was 0.0578 mmol, determined from seedlings grown prior to this experiment. Five RAR’s of NaNO$_3$ were selected: 0.075, 0.100, 0.125, 0.150 and 0.175 d$^{-1}$, and the $\delta^{15}$N value of the source was 0.904‰, which remained constant over the growth period.

The plants were grown for 40 d in a controlled environment at the University of Cape Town. The light period was 14 h with 10 h full irradiance and 2 h prior to and after the full irradiance having a gradual increase/decrease in light intensity. A photon flux rate of 300 µmol m$^{-2}$ s$^{-1}$ was provided by 21 high pressure sodium lamps (400W, Sunmaster), 14 metal halide lamps (400W, Sunmaster) and 24 incandescent lamps (60W, Osram). The temperature of the light period was 25°C and the night temperature was 18°C.

### 2.3.2 Plant harvest

Wheat seeds ($n = 10$) were dried and ground to a fine powder with a mortar and pestle. Seedlings ($n = 10$) were harvested directly from vermiculite after 7 d and once dried, the roots and shoots were cut into small pieces. Thereafter, plants were destructively harvested every 10 d (i.e. at 17, 27, 37 and 47 d) with 5 replicates taken from each RAR. The root systems were gently washed in water to remove sand after which the root was dipped into 2 mM CaSO$_4$ to rinse off any residual nutrients. Plants were separated into root, shoot and flower fractions (if present) and after drying, each fraction was ground to a fine powder using a ball mill (MM400, Retsch, Germany). All biomass was dried at 60°C for 48 h before dry weight (DW) was taken.

Xylem sap was collected from all RAR’s during each harvest, where bleeding occurred. To stimulate flow, pots were watered with the appropriate nutrient solution 1 h before excising the shoots. Shoots were severed just below the insertion of the first leaf; the stumps were washed with deionised water and blotted dry. Xylem sap exuding from the stump was collected with a Pasteur pipette for 2 h
following excision, the first drop being discarded to avoid contamination. The sap was collected onto ice and stored frozen (-18°C) until analysed. After 46 d, pots were flushed with 1 L deionised water and the resulting leachates were collected and freeze dried (30 ml) (Cryolizer B-65, New Brunswick Scientific, USA) to form powders representing ‘total N’. Fractions of each leachate powder were dry-ashed in a muffle furnace (440°C for 2 h) to burn off all organic compounds and allow for the proportions of inorganic and organic N to be determined. 2 M NaNO$_3$ stock solution (20 ml) and N-free (apart from chemical contamination) Long Ashton nutrient solution (40 ml) were also freeze dried to obtain source δ$^{15}$N. Both solutions were sampled at the start and end of the experiment for consistency.

2.3.3 Nitrogen isotope analysis

δ$^{15}$N and [N] of seeds, roots, shoots, flowers, leachates, 2 M NaNO$_3$ and N-free Long Ashton nutrient solution were determined using mass spectrometry. Approximately 1 µg of each sample was weighed into a tin capsule (5x9 mm Säntis Analytical, Switzerland). The samples were then combusted in a Flash 2000 organic elemental analyzer and the gases passed to a Delta V Plus isotope ratio mass spectrometer (IRMS) via a Conflo IV gas control unit (all from Thermo Scientific, Germany). Eight in-house standards and one International Atomic Energy Agency standard (USGS25) were used to calibrate the results. The δ$^{15}$N and [N] of xylem sap were also determined using mass spectrometry. According to the amount of xylem sap collected (which ranged from 0 to 245.6 µl) and the anticipated [N], portions of sap were pipetted into tin capsules and then dried in an oven at 40°C for 3 h before analysis.

2.3.4 Calculations and data analysis

Plant δ$^{15}$N was calculated as a mass-weighted average of root, shoot and flower (if present) δ$^{15}$N values, likewise for [N]. The source δ$^{15}$N (0.904‰) was calculated as an [N]-weighted average of the 2 M stock NaNO$_3$ δ$^{15}$N and N-free Long Ashton nutrient solution δ$^{15}$N from the start and end of the growth period. Efflux/influx was calculated using the approach of Kalcsits & Guy (2016): $E/I = (\Delta^{15}N_{plant})/(\Delta_{enz} \times P_{root})$, where the E/I ratio is a function of the change in isotope composition of the plant relative to the source ($\Delta^{15}N_{plant}$), the fractionation factor of NR ($\Delta_{enz}$) and the proportion of assimilation occurring in the root ($P_{root}$). $P_{root}$ is derived from the proportion of total plant N in the shoot, the difference in δ$^{15}$N between shoot and root and the fractionation factor of NR. I assumed a
fractionation factor of 22‰ for NR, as suggested by Kalcsits & Guy (2016), rather than the standard reference of 15.4‰ found by Ledgard et al. (1985).

I calculated the weight of root required to be abscised from each RAR to give the respective observed plant δ¹⁵N value (i.e. potential root loss), using the difference between the expected and observed plant δ¹⁵N (See Appendix S1). In the absence of significant fractionation during uptake (Evans 2001) and other losses of ¹⁵N from the plant, I expected plant δ¹⁵N to equal source δ¹⁵N. Where plant δ¹⁵N was less than that of the source, ¹⁵N must have been lost from roots, and for this calculation (c.f. E/I) I assumed that this was through root abscission. The total [N] that accumulated in the growth medium of each RAR, over and above the N that was supplied, was calculated using the difference between the leachate δ¹⁵N and the source δ¹⁵N (See Appendix S1). Total [N] (i.e. leachate N, µg L⁻¹) thus represents N originating from plants. Based on the dry-ashed leachates, the inorganic and organic [N] accumulation in the growth medium was also calculated. A linear model comparing the total N lost from plants (µg plant⁻¹) to the leachate N (µg L⁻¹) was generated in R 3.2.3 (R Core Team 2015).

When comparing independent sample means, if 95% confidence intervals (CI) overlap by no more than half then p ≤ 0.05 and if 95% CI’s do not overlap then p ≤ 0.01 (Cumming and Finch 2005). The 95% CI’s here, displayed as ribbons, were calculated using non-parametric bootstrapping with 1000 replicates from the Hmisc package (Harrell and Dupont 2016) in R. I conservatively only state significance between treatments where CI’s do not overlap. Linear models relating root and shoot δ¹⁵N to their respective N content (mg) at 47 d were generated in R.

2.4 Results

2.4.1 Plant growth

The RAR of 0.075 d⁻¹ was the only treatment to match the expected RGR (Fig. 2.1). Although plant growth increased with RAR, the growth rates fell well below the identity line. For instance, plants expected to reach a RGR of 0.175 d⁻¹ only reached 0.108 d⁻¹. The shoot:root ratios of all treatments increased over time and with RAR (Appendix Fig. S2). The 0.075 d⁻¹ RAR consistently had the lowest shoot:root over the entire growth period (2.37 after 47 d) and 0.175 d⁻¹ had the highest (3.47 after 47 d). Contrary to my expectation, plant [N] of all treatments did not stay constant over the course of the experiment (Fig. 2.2). All RAR’s showed an initial drop in [N], after which only 0.075 d⁻¹ stayed constant at 32 mg g⁻¹ until 47 d, while the [N] of the other treatments increased after 17 d but declined
before 47 d to *ca.* 40 mg g$^{-1}$. Plant dry weights (g) and N contents (mg) for each RAR and at each harvest are available in Appendix Table S2.

**Fig. 2.1** Relative growth rates (RGR, measured from DW) of wheat determined from harvested plant biomass against the respective relative addition rates of N (RAR). The grey dashed line represents a 1:1 relationship
Fig. 2.2 Temporal change in plant N concentration (mg N g$^{-1}$ DW) of wheat given five relative addition rates of N (RAR d$^{-1}$: 0.075, 0.100, 0.125, 0.150 and 0.175). Nutrients were first supplied on day 7. Points represent means ($n = 5$) and ribbons represent the bootstrapped 95% confidence intervals.
2.4.2 Plant $\delta^{15}N$

Plant $\delta^{15}N$ generally decreased over time, but the magnitude of this change depended on the RAR (Fig. 2.3). Although plant $\delta^{15}N$ was significantly greater than the source $\delta^{15}N$ before nutrients were first supplied ($p \leq 0.01$), after 17 d the plant $\delta^{15}N$ of all treatments was comparable to the source. At the end of the growth period the amount of plant discrimination relative to the source was greater where more N was supplied; for example, after 47 d plant $\delta^{15}N$ of 0.075 d$^{-1}$ was 0.41‰ and 0.175 d$^{-1}$ was -4.10‰. Furthermore, the plant $\delta^{15}N$ values of all treatments were significantly different to each other ($p \leq 0.01$).

The $\delta^{15}N$ of roots (2.93‰) and shoots (2.81‰) were similar before nutrients were first supplied (Fig. 2.3). After 17 d, root and shoot $\delta^{15}N$ of all treatments had diverged from each other, for example, the 0.125 d$^{-1}$ RAR showed the greatest disparity of 3.48‰. Root $\delta^{15}N$ was significantly less than shoot and source ($p \leq 0.01$); while shoot $\delta^{15}N$ was significantly greater than source ($p \leq 0.01$). After 47 d, root and shoot $\delta^{15}N$ of all treatments had converged but the values they converged on depended on RAR; 0.075 and 0.100 d$^{-1}$ were comparable to the source, but 0.125, 0.150 and 0.175 d$^{-1}$ were well below the source, reflecting plant $\delta^{15}N$. Both root and shoot $\delta^{15}N$ were negatively correlated with their respective N content at the end of the growth period, but the former showed a steeper slope (Fig. 2.4). Flowers emerged after 37 d; those from the 0.075 d$^{-1}$ RAR were the least depleted (-3.07‰) and from 0.175 d$^{-1}$ were the most depleted (-8.16‰, Fig. 2.3). The flower $\delta^{15}N$ of all treatments was therefore significantly less than that of the source, shoot and root ($p \leq 0.01$). Although I collected small and variable sample sizes of xylem sap, the data show several noteworthy patterns (Fig. 2.5). Between 17 and 27 d, the xylem sap $\delta^{15}N$ of all treatments was enriched compared to the source, but after 37 d had dropped substantially closer to the source (Fig. 2.5C). Furthermore, there was a spike in N content and [N] around 37 d, particularly for the 0.075, 0.100 and 0.125 d$^{-1}$ RAR’s, which indicates that the volume of sap flowing to the shoot, as well as the [N], was important at this stage (Fig. 2.5A and B).
Fig. 2.3 The effect of N supply on wheat plant, root, shoot and flower $\delta^{15}$N (‰) over time. Each facet displays a relative addition rate (RAR d$^{-1}$) of N. Points represent means ($n = 5$), while ribbons and error bars represent the bootstrapped 95% confidence intervals. The dark-grey dashed line represents the $\delta^{15}$N (0.904‰) of the nutrient solution, first supplied on day 7.
Fig. 2.4 Correlation of root and shoot N content (mg plant⁻¹) with their respective δ¹⁵N (‰) of wheat. Plants were given five relative addition rates of N (RAR d⁻¹: 0.075, 0.100, 0.125, 0.150 and 0.175). p < 0.001 for the root and shoot model. The regression equations and coefficients of determination (R²) are displayed.
Fig. 2.5 The effect of N supply on the (A) N content (mg), (B) N concentration (mg g$^{-1}$) and (C) $\delta^{15}$N (‰) of xylem sap from wheat. Plants were given five relative addition rates of N (RAR d$^{-1}$: 0.075, 0.100, 0.125, 0.150 and 0.175). Points represent means and standard errors, while sample sizes are displayed in the legend. The dark-grey dashed line in (C) represents the $\delta^{15}$N of the nutrient solution (0.904‰).
2.4.3 Estimates of efflux/influx and root loss

The ratio of N efflux to influx (E/I) and potential root loss followed similar patterns throughout the growth period, both being proportional to the amount of N supplied (Fig. 2.6A and B). At 17 d the E/I and potential root loss of all treatments were minimal; the uptake of N here was far greater than efflux and it was unlikely that there had been notable root turnover or rhizodeposition before 17 d. Over the last 30 d of growth the E/I of 0.175 d$^{-1}$ RAR was significantly higher than any other treatment ($p \leq 0.01$) and at 47 d, the E/I ratios of all treatments were significantly different to each other ($p \leq 0.01$), reflecting plant $\delta^{15}$N. The 0.150 and 0.175 d$^{-1}$ RAR’s potentially lost significantly more root biomass between 37 and 47 d than any other treatment ($p \leq 0.01$). Furthermore, the total estimated root loss over the whole growing period was a tiny fraction of the final root biomass for every treatment (Table 2.1).

2.4.4 Leachate N

When more N was supplied, a larger amount of N originating from the plant was found in the growth medium (Fig. 2.7A; Table 2.1). Four-fold more N was lost from plants compared to that accumulated in the growth medium by 47 d, but the two were tightly correlated across RAR’s (Table 2.1). The leachate $\delta^{15}$N was greater than that of the source and the respective plant $\delta^{15}$N, indicating that the growth medium gained $^{15}$N (Fig. 2.7B). The inorganic and organic composition of the N originating from the plant depended on the RAR. Leachates of lower RAR’s contained mostly organic N, while higher RAR’s comprised largely inorganic N (Fig. 2.7A). Because a substantial proportion of the 0.075 and 0.100 d$^{-1}$ leachates were organic N, the $\delta^{15}$N signals after dry-ashing were below the mass spectrometer detection limit. The inorganic $\delta^{15}$N of the 0.125, 0.150 and 0.175 d$^{-1}$ leachates was significantly higher than the source and respective plant $\delta^{15}$N ($p \leq 0.01$) (Fig. 2.7B). In contrast, the organic $\delta^{15}$N was lower than the plant $\delta^{15}$N for 0.125 and 0.150 d$^{-1}$ and although higher than the plant for 0.175 d$^{-1}$, was still a negative value.
Fig. 2.6 Temporal changes in (A) efflux/influx ratios and (B) potential root loss of wheat given five relative addition rates of N (RAR d⁻¹: 0.075, 0.100, 0.125, 0.150 and 0.175). Potential root loss (mg g⁻¹ DW) is the amount of root lost between each harvest and is not cumulative. For example, points at day 17 represent the potential root lost between 7 and 17 d. Points represent means (n = 5) and ribbons represent the bootstrapped 95% confidence intervals
Fig. 2.7 (A) Relationship between N supply to plants and leachate [N] (µg L\(^{-1}\)) that originated from plants. [N] was log-transformed for visual purposes. Pots were flushed with 1 L deionised water on day 46 giving ‘leachates’ that were analysed using mass spectrometry. (B) Relationship between N supply (relative addition rate, RAR) to wheat and δ\(^{15}\)N (‰) of plants and leachates (total, inorganic and organic N). The dark-grey dashed line in (B) represents the δ\(^{15}\)N (0.904‰) of the nutrient solution.
Table 2.1 A comparison between N lost from wheat by efflux or root abscission after 47 d at each relative addition rate (RAR). The N of plant origin that accumulated in the growth medium (i.e. leachate N) is a measure of efflux, possibly of previously decomposed root fragments. The proportion of efflux (%) is leachate N relative to total N lost from plants, while the proportion of N-loss through root abscission (%) is estimated as the balance. The total estimated root loss was taken as a percent of the root dry weight (DW) after 47 d. Values are mean ± SE (n = 5), where indicated.

<table>
<thead>
<tr>
<th>RAR (d⁻¹)</th>
<th>Total N lost from plant (µg plant⁻¹) *</th>
<th>Leachate N (µg L⁻¹) *</th>
<th>% efflux</th>
<th>% root abscission</th>
<th>Root DW (mg)</th>
<th>Total estimated root loss (mg)</th>
<th>Root loss % of root DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075</td>
<td>10.4 ± 1.4</td>
<td>1.6 ± 0.3</td>
<td>16</td>
<td>84</td>
<td>176 ± 30</td>
<td>0.3 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>0.100</td>
<td>45.1 ± 2.7</td>
<td>12.4 ± 0.9</td>
<td>27</td>
<td>73</td>
<td>266 ± 19</td>
<td>1.2 ± 0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>0.125</td>
<td>181.3 ± 10.3</td>
<td>45.3 ± 2.3</td>
<td>25</td>
<td>75</td>
<td>302 ± 14</td>
<td>4.3 ± 0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>0.150</td>
<td>373.8 ± 23.8</td>
<td>94.4 ± 3.2</td>
<td>25</td>
<td>75</td>
<td>443 ± 28</td>
<td>9.1 ± 0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>0.175</td>
<td>504.0 ± 25.2</td>
<td>114.0 ± 11.8</td>
<td>23</td>
<td>77</td>
<td>504 ± 47</td>
<td>11.4 ± 1.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* correlation between these gave $R^2 = 0.904$, $y = 4.03x - 0.53$ and $P \leq 0.001$. 
2.5 Discussion

Consistent with previous results (Evans 2001) I found that the δ¹⁵N of wheat was strongly affected by the amount of N supplied (Fig. 2.3). The close match between plant and source δ¹⁵N at lower RAR’s of N (0.075 and 0.100 d⁻¹) indicates that these plants did not lose ¹⁵N, most likely because the supply closely matched the plant N demand. The inability of all plants, aside from RAR 0.075 d⁻¹, to reach their expected RGR’s (Fig. 2.1) resulted in an oversupply of N that consequently led to plants losing ¹⁵N from roots at higher RAR’s (0.125, 0.150 and 0.175 d⁻¹). This conforms to the theoretical models of Mariotti et al. (1982) and Kalcsits et al. (2014), where N supply exceeding demand leads to greater plant isotopic discrimination. That plant δ¹⁵N is dictated by ¹⁵N-loss from roots is evident from the sharper decrease in root δ¹⁵N with root N content compared to that of the shoot (Fig. 2.4). The fact that RAR did not match RGR is probably because some other factor(s) became limiting, with the consequence of tissue [N] accumulation (Fig. 2.2). For example, light intensity in the phytotron (300 µmol m⁻² s⁻¹) may have been too low to promote maximum growth or the required N:P:K ratio was not met, meaning that phosphorus or potassium became limiting. Plant discrimination has been shown to decrease (e.g. pearl millet, Mariotti et al. 1982; ryegrass and marigold, Kohl and Shearer 1980), remain constant (e.g. tomato, Evans et al. 1996) or increase marginally over time (e.g. non-nodulating soybean, Kohl and Shearer 1980). Unlike Mariotti et al. (1982), who fed their seedlings a constant 12 mM NO₃⁻ and found strong early discrimination (ca. 20‰), I did not find discrimination in seedlings (Fig. 2.3) because the N supply at this stage was low (due to the RAR method). Later in the experiment, the excess of N at higher RAR’s drove plant δ¹⁵N down. In all likelihood, an endogenous pool of ¹⁵N-enriched NO₃⁻ formed in the root when N supply exceeded demand (Robinson 2001), and this pool could subsequently be lost through efflux or root abscission.

Plants from all RAR’s lost considerably more N than the amount of N, from plant-origin, that accumulated in the growing medium (i.e. leachate N, Table 2.1). This deficit was presumably caused by root abscission because leachate N is a measure of direct efflux of organic and inorganic N and possibly of root fragments broken down through bacterial action into low molecular weight substances. Intact root fragments were not apparent in the leachate, having been filtered out by the sand medium. Given that I supplied N as NO₃⁻ and not NH₄⁺, the ¹⁵N-enrichment of the growth medium (Fig. 2.7B) due to the volatilisation of NH₃ was unlikely (Högberg 1997). Furthermore, substantial ¹⁵N-enrichment of the growth medium by partial denitrification was also unlikely. High rates of denitrification are usually found in finer-textured soils, with low [O₂] and high C availability, that are prone to waterlogging (Tiedje et al. 1983; Högberg 1997; Hofstra and Bouwman 2005); but the coarse sand in
this experiment was well-drained, lacking significant organic C content and had large pore sizes that were probably well oxygenated. The anticipated amount of N effluxed by the 0.175 d⁻¹ RAR increased from 0% to ca. 23% of the N influx, over 47 d (Figure 2.6A). On the other hand, only 2.26% (Table 2.1) of the entire 0.175 d⁻¹ RAR root biomass would need to be lost over the whole growth period to attain this plant δ¹⁵N value, and this percentage decreases for lower RAR’s. Therefore, a small degree of root abscission can substantially affect plant δ¹⁵N compared to the amount of N efflux needed to reach the same δ¹⁵N value.

While root turnover and rhizodeposition were likely to be constant contributors to plant δ¹⁵N during plant growth, it is also possible that the loss of fine roots during harvesting could have affected the plant δ¹⁵N signal. In fact, Janzen & Bruinsma (1993) found that small root fragments deposited in soils during growth and specifically harvesting of wheat represented up to 60% of the soil N that originated from plant roots. This is comparable to the maximum estimated root loss of ca. 75% (Table 2.1), but could indicate I overestimated root abscission to an extent. The fact that Janzen and Bruinsma’s (1993) study used sandy loam soil, whereas I used coarse sand, may have affected root abscission. Soil texture, bulk density and porosity influence rhizodeposition by altering the friction and mechanical impedance roots face while growing through soil, where finer-textured, dense soils give more resistance (Nguyen 2003; Gregory 2006). The N content of the root fragments in Janzen and Bruinsma’s (1993) study varied from 4.7 to 9.7 mg plant⁻¹, depending on the level of N supplied. This is substantially more than the entire amount of N (0.5 mg plant⁻¹) lost from my wheat plants even at the highest RAR (Table 2.1). Janzen and Bruinsma (1993) did, however, supply their plants with N concentrations that far exceeded ours and so the root biomass would have accumulated more N. Nevertheless, the meticulous freeing of roots from harvested soil is crucial to quantifying rhizodeposition.

Both the amount and composition of N lost from roots to the growth medium changed with the rate of N supply (Fig. 2.7A). Similar to my results, Janzen (1990) found a high proportion of organic root-derived N in low [N] soils occupied by wheat, but an equal amount of inorganic and organic root-derived N in high [N] soils. Although I could not obtain a signal for the organic δ¹⁵N in leachates from lower RAR’s, the amount of organic N lost from these roots was too small for it to strongly affect the plant δ¹⁵N, as evidenced by similarity between plant and source δ¹⁵N values (Fig. 2.3). Many plant species can efflux organic N with a variety of δ¹⁵N signals, but it usually does not impact plant δ¹⁵N because it is a negligible proportion of total plant N (Robinson et al. 1998; Werner and Schmidt 2002; Kalcsits et al. 2014; Warren 2015). The E/I ratios of lower RAR’s do suggest that efflux was minimal
(Fig. 2.6A). Alternatively, organic N may have originally been lost as root fragments that were subsequently broken down into lower molecular weight organic molecules. This could be a feasible pathway of N-loss at lower N supply because strong plant N demand dictates that all of the inorganic N taken up must be assimilated into organic N, which in turn must be utilized for growth (Kolb and Evans 2003). Root turnover and rhizodeposition are unavoidable artefacts of root growth (Lauenroth and Gill 2003; Wichern et al. 2008) but efflux of organic N is probably less likely at lower N supply. For plants receiving higher RAR’s, the loss of inorganic N, presumably by efflux (Fig. 2.6A), contributed more to the plant δ^{15}N than the loss of organic N. This is evidenced by the inorganic δ^{15}N value of the respective leachates, which was more positive relative to either plant or source δ^{15}N (Fig. 2.7B).

The difference between root and shoot δ^{15}N varied depending on the growth stage of the plant, with consequences for plant δ^{15}N (Fig. 2.3). The early divergence between root and shoot δ^{15}N of all RAR’s was probably a result of partial NO_{3}⁻ assimilation in the root followed by transport of the remaining heavily ^{15}N-enriched inorganic N to the shoot (Kolb and Evans 2003), given that xylem sap δ^{15}N here was heavily enriched compared to the source (Fig. 2.5C). At this stage, some of the enriched inorganic N pool must have been lost from roots of higher RAR’s, instead of being transported to the shoot, because plant δ^{15}N began to fall below that of the source. During the ear-forming stage (after 27 d), however, the root and shoot δ^{15}N of all RAR’s converged and the xylem transported N, of greater concentration and volume, that was closer to the source δ^{15}N value (Fig. 2.5). This convergence may have been caused by a shift in assimilation from the root to the shoot at the start of reproductive growth (Andrews 1986; Evans et al. 1996). Additionally, the transport of remobilised amino acids, which are ^{15}N-depleted compared to the residual protein pool (Kolb and Evans 2002), from the root to the shoot would induce an increase in root δ^{15}N towards that of the source, while the shoot δ^{15}N would gradually become more depleted (Fig. 2.3). Nevertheless, as root assimilation at this stage was probably minimal, plants from all the RAR’s, except 0.175 d⁻¹, did not lose ^{15}N between 27 and 37 d (Fig. 2.3) because no ^{15}N-enriched inorganic N pool would have been available for efflux or root abscission. The 0.175 d⁻¹ RAR could possibly have continued to lose ^{15}N either as a small amount of enriched NO_{3}⁻ that was cycled back to the root (Peuke 2010) or as abscised root fragments that contained relatively enriched residual protein pools.
2.5.1 Conclusion

The determinants of plant $\delta^{15}$N and variation of intra-plant $\delta^{15}$N values are complex, but overall, plants generally have more negative $\delta^{15}$N than the $\delta^{15}$N value of the inorganic N supplied (Evans 2001; Kalcsits et al. 2014; Craine et al. 2015a). I suggest that this isotopic discrimination is driven by a combination of efflux and root abscission, through which $^{15}$N-enriched N is lost from plants. Root abscission probably contributes strongly to determining plant $\delta^{15}$N because it is a persistent N sink and an unavoidable characteristic of root growth. It is likely an important determinant of plant $\delta^{15}$N in natural ecosystems and so future research into plant N isotopes should take root abscission into account. By contrast, efflux is likely more important when substrate N supply exceeds plant demand, particularly in agriculture where plants are fertilized.
Chapter 3
Quantifying the effect of N-loss through root abscission and efflux on wheat N budgets and δ¹⁵N values

3.1 Abstract

Lower plant δ¹⁵N values relative to soil are attributed to $^{15}$N efflux. I determined the extent to which root abscission contributes to plant N-loss and consequences for plant δ¹⁵N. Wheat (*Triticum aestivum* L. cv. SST015) was grown in hydroponics with direct aeration, aeration constrained within a pipe and circulation of nutrient solution through sand, representing three levels of stability for root growth. The δ¹⁵N of root fragments and plant-derived N in solution were periodically determined, as well as root and shoot δ¹⁵N. Plants in solution had significantly more negative δ¹⁵N (-8.9 and -9.2‰) than plants in sand (-6.9‰), suggesting greater $^{15}$N-loss, and root fragments were a major biomass- (six-fold greater than root dry weight) and N-loss (two-fold greater than plant net N uptake) pathway. These plants had more ephemeral roots and two-fold more root tips than the sand treatment. I estimated that root fragment loss decreased plant δ¹⁵N by at least -3.7, -2.6 and -1.0‰ in the direct, pipe and sand treatments, respectively. All plants lost more N (expressed as mg g⁻¹ d⁻¹) within root fragments (17.6, 18.8, 1.3) than through efflux (0.2, 0.2, 0.1). Root abscission and root turnover are likely to be important N-loss pathways in plants. Plant δ¹⁵N values are probably influenced by a combination of root abscission and N efflux.
3.2 Introduction

Net N uptake by plant roots is a balance between N influx and efflux (Nacry et al. 2013). The ratio of N efflux to influx (E/I) determines N uptake efficiency, and ultimately plant NUE, because it is energetically costly for plants to take up N only to release it back into the rhizosphere (Britto and Kronzucker 2006; Xu et al. 2012). Although this is considered a ‘futile cycle’, efflux is thought to function as a regulator of root cytoplasmic [inorganic N] and several efflux transporters have been identified (Miller and Cramer 2004; Britto and Kronzucker 2006; Segonzac et al. 2007; Wang et al. 2012). For example, plants that are prone to NH$_4^+$ toxicity at high external concentrations cannot properly regulate NH$_4^+$ influx, causing the ion to accumulate in the root cytosol (Britto et al. 2001). Consequently, an increased rate of NH$_4^+$ efflux counteracts the root’s inability to exclude NH$_4^+$, inducing greater root respiration and energy use. While inorganic N efflux is probably more prevalent, the efflux of organic N, such as amino acids, can occur by leakage down the steep concentration gradient across the plasmalemma (Nacry et al. 2013; Warren 2015). Corroborating the importance of N efflux are plant $\delta^{15}$N values that tend to be more negative than their source $\delta^{15}$N; the widely accepted reason for this offset is that the efflux of $^{15}$N-enriched N depletes plant $^{15}$N pools (Evans 2001; Craine et al. 2015a). Importantly, however, N influx normally predominates unless a plant experiences stressful conditions (Segonzac et al. 2007). Given the common offset between plant and source $\delta^{15}$N, and the specificity of N efflux, an alternative pathway of N-loss from roots may contribute to plant $\delta^{15}$N and N budgets.

Hydroponic culture is typically used for plant growth and physiological experiments (e.g. N uptake, metabolism and cycling) because it is easy to regulate (Breteler and Nissen 1982; Teyker et al. 1988; Cooper and Clarkson 1989; Yoneyama and Kaneko 1989; Siddiqi et al. 1991; Evans et al. 1996; Volk 1997). Hydroponics are, however, not without detractions. While a well-stirred solution prevents the rhizosphere from becoming N-depleted, the rhizosphere diffusion gradient may be eliminated by the constant mixing and soil microbes are lacking (Mackay and Barber 1984; Handley et al. 1998; Uren 2001). Although plant harvest is substantially easier and roots incur less damage than when extracted from soil, normal root architecture is altered under hydroponics due to the lack of mechanical resistance against root elongation (Groleau-Renaud et al. 1998; Handley et al. 1998). Thus, hydroponic culture could mechanically stress roots and lead to a shift in the ratio of efflux to influx. Gentle physical handling of roots can inhibit N influx, promote N efflux and injure roots by unnatural bending or rupturing of root hairs (Miller 1987; Bloom and Sukrapanna 1990; Steege et al. 1998). Investigating an extreme situation, Aslam et al. (1996b) found that mechanical perturbation (e.g. striking with a
glass rod) of barley roots inhibited net NO\textsubscript{3}\textsuperscript{-} uptake because efflux was stimulated. In contrast, Scheurwater et al. (1999) found no effect of transfer stress on influx rates of monocots and proposed that the effect of manipulation may be species specific. Nevertheless, the vastly different environment for root growth in hydroponic culture compared to a solid medium may have ramifications for plant $\delta^{15}$N and N budgets.

Efflux is not the only mechanism by which roots can lose N; root rhizodeposition and turnover – collectively termed root abscission – are also substantial nutrient sinks for plants (Lauenroth and Gill 2003). As roots proliferate through soil they naturally deposit debris, such as sloughed epidermal cells and root hairs, and when roots senesce their tissue nutrients, including N, are not fully resorbed (Wichern et al. 2008; Kunkle et al. 2009). The root N pool available for efflux is governed by the $[N_i]_{ex}$, the rate of inorganic N influx versus its assimilation into amino acids, the location of this assimilation (i.e. root or shoot) and the plant demand for N (Kalcsits et al. 2014; Packer and Cramer 2017). Over and above these conditions, N lost through root abscission should also be dependent on root longevity, including untimely excision through disturbance (e.g. herbivory), and the capacity for resorbing N from roots. If root N lost through efflux or root abscission is $^{15}$N-enriched – which commonly occurs because assimilation fractionates against $^{15}$N leading to residual $^{15}$N-enriched inorganic pools in the root cytosol – plant $\delta^{15}$N will become more negative relative to the source $\delta^{15}$N (i.e. plant isotopic discrimination results; Robinson 2001). Hence, plant $\delta^{15}$N values can indicate the extent of N-loss by these mechanisms (Packer and Cramer 2017). Kalcsits et al. (2015) grew aspen, spruce and pine in hydroponics and sand to investigate N source preference and found plant discrimination ranged from -1.65 to -5.94‰ in hydroponics, but no discrimination was found in sand-grown plants. While they suggest localized nutrient depletion in the sand medium resulted in a ‘Raleigh-model closed system’ (Robinson 2001), the differences in plant discrimination could also indicate that the hydroponic medium stimulated greater N-loss.

While plant N-loss is expected in both hydroponic and solid media cultivation, no studies have quantitively questioned the role of N efflux relative to N losses from root tissue loss in hydroponics. I hypothesized that the vigorous aeration necessary for hydroponic culture can act as a mechanical stressor on roots, breaking off finer root components (e.g. root hairs) and accentuating plant N-loss. Additionally, the constant mixing could also increase N-loss through efflux by strengthening diffusion gradients away from the rhizosphere. Plants grown in aerated hydroponics are therefore likely to have more negative $\delta^{15}$N values relative to the source $\delta^{15}$N than plants growing in a solid medium. Furthermore, I expect roots growing in solution without support to reflect more ephemeral
morphologies than those anchored in a solid medium. I tested these hypotheses by growing wheat in a hydroponic system that comprised three levels of stability for root growth. The natural abundance of the $^{15}$N stable isotope was used to indicate if plants lost N to the growing medium, and whether the N-loss was through efflux or root abscission.

3.3 Methods and materials

3.3.1 Plant germination, growth conditions and harvest

Wheat plants (*Triticum aestivum* L. cv. SST015) were grown from seed that was soaked in aerated water for 24 h before being sown in trays of vermiculite. The trays were kept moist until the emerging seedlings were suitably established. After 12 d, seedlings were transferred into individual 350 mL plastic bottles; the hypocotyls were inserted through lids and secured with foam rubber. These bottles comprised a hydroponic system of three stability treatments for root growth, with 10 replicates in each (Fig. 3.1). The first treatment (‘direct’) followed a traditional hydroponic set-up where the solution in each bottle was directly aerated, with the bubble stream causing disturbance around the roots. For the second treatment (‘pipe’), the aeration line and bubble stream were positioned within a 15 mm diameter irrigation pipe allowing the air to circulate upwards inside the pipe, thus limiting the disturbance of the roots. The bottles of the third treatment (‘sand’) were filled with silica sand (*ca.* 440 g, pH-neutral, acid-washed with 0.1% HCl). Here, the aeration line and bubble stream were also placed within a pipe and resulting upward flow from the bottom of the bottle aided solution circulation. The air flow of all treatments was regulated through an SMC air regulator (EAR2000, Japan) and 2 L h$^{-1}$ drippers (Netafim, Israel). The bottles were wrapped in thick black plastic to limit light penetration and prevent algal or bacterial growth which would, nevertheless, be limited due to frequent replacement of nutrient solutions.
The plants were supplied with a modified Long Ashton nutrient solution (Hewitt 1966) (1.1 mM MgSO$_4$, 2 mM K$_2$SO$_4$, 4 mM CaCl$_2$, 0.14 mM H$_3$BO$_3$, 20.8 µM MnSO$_4$, 2.3 µM ZnSO$_4$, 3.3 µM CuSO$_4$, 0.25 µM Na$_2$MoO$_4$, 90 µM Fe-EDTA and a phosphate buffer (pH 6.5) of 0.67 mM NaH$_2$PO$_4$ and 1.5 mM Na$_2$HPO$_4$) containing 3 mM NH$_4$NO$_3$. Nutrient solutions were replaced every second day and the partially-depleted solutions (henceforth referred to as ‘depleted solutions’) were collected and stored frozen (-18°C) until filtration. Measured solution N depletion was never greater than the recommended 10% (Kalcsits et al. 2014). Plants from the direct and pipe treatments were gently lifted out of their bottles before each solution was changed, while the solutions from the sand treatment were drained from a port in the bottom of the bottles. The plants were grown for 18 d in a glasshouse at the

Fig. 3.1 The hydroponic set-up of the experiment, with three stability treatments for wheat root growth. (A) The direct treatment followed a traditional hydroponic set-up, where the solution in each bottle was directly aerated causing disturbance around the roots. (B) The aeration line and bubble stream of the pipe treatment were positioned within 15 cm diameter irrigation pipe, allowing the air to circulate upwards inside the pipe and thus limiting the root disturbance. (C) The bottles were filled with sand; the aeration line and bubble stream were also placed within a pipe so that circulation throughout was created by an upward moving flow from the bottom of the bottle.
University of Cape Town with an average day-time temperature of 19.5°C and a day-time range of 12 – 25°C. The NH₄NO₃ stock solution (1 M, 20 mL) and fresh N-free Long Ashton nutrient solution (40 mL) were collected and freeze dried (Cryolizer B-65, New Brunswick Scientific, USA) to measure the source δ¹⁵N. Both solutions were sampled at the start and end of the experiment. The δ¹⁵N value of the source was -0.733‰, which remained constant over the growth period.

Plants were harvested after 18 d of growth in the hydroponic system and separated into root and shoot fractions. Roots were dipped in 2 mM CaSO₄ to rinse off residual nutrients. Plant tissue was dried at 60°C for 48 h, weighed and then ground to a fine powder using a ball mill (MM400, Retsch, Germany). During the sand treatment harvest, after the plants were removed, the sand was vigorously washed in deionised water to release root fragments that had potentially been trapped over the growth period, and a sample of this wash solution was kept for further analysis. At the time of transplant from vermiculite to hydroponics, seedlings (n = 10) were harvested, oven dried as above, and the roots and shoots cut into small pieces for analysis.

3.3.2 Solution filtration

The depleted solutions were filtered through 1.6 µm glass-fibre filters (MGA, Ahlstrom Munktell, Finland) under vacuum (Little Giant 13156, Gelman Instrument Company, USA) to collect root fragments. Prior to filtration, the filters were pre-combusted at 400°C for 8 h to remove contaminants and then weighed. Blank pre-combusted filters did not register a δ¹⁵N signal. After filtration, the filters were dried at 60°C for 24 h and re-weighed to determine the amount of sample collected. Root fragments collected from the direct and pipe treatments were scraped off the filters for N isotope analysis. Filters from the sand treatment were analyzed intact for δ¹⁵N and [N] as there was no visible root debris (referred to as ‘sand – filters’ in the results). The wash solution from the sand treatment harvest was also filtered, as above. Root fragments scraped off these filters (referred to as ‘sand – fragments’ in the results) and the remaining intact filters (which represent the last data point of ‘sand – filters’) were individually analyzed for δ¹⁵N and [N]. A portion of each filtered depleted solution was freeze dried and the resulting powders sent for N isotope analysis to determine plant-derived N content in solution.
3.3.3 Nitrogen isotope analysis

The $\delta^{15}$N and [N] of roots, shoots, root fragments, depleted solution powders, NH$_4$NO$_3$ stock solution and N-free Long Ashton nutrient solution were determined using mass spectrometry at the University of Cape Town. Approximately 1 μg of each sample was weighed into a tin capsule (5x9 mm Säntis Analytical, Switzerland). The samples were then combusted in a Flash 2000 organic elemental analyzer and the gases passed to a Delta V Plus isotope ratio mass spectrometer (IRMS) via a Conflo IV gas control unit (all from Thermo Scientific, Germany). Four in-house standards and one IAEA standard (USGS25) were used to calibrate the results. The $\delta^{15}$N and [N] of the sand treatment filters were determined using mass spectrometry at iThemba Labs (Gauteng, South Africa). The filters were halved and placed into tin capsules before combustion in a Flash HT Plus elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer by a Conflo IV gas control unit. One in-house standard and one Urea Working Standard (IVA Analysentechnik e.K., Germany) were used to calibrate the results.

3.3.4 Root morphology

Seven additional plants per treatment were grown with the same conditions as described above to determine what effect the disturbance/stability had on root morphology. Once harvested, all root biomass was stored refrigerated (4°C) in a 10% ethanol solution. Roots were rinsed with distilled water and stained by submersion in a warmed 2% solution of gentian violet for 5 min (Maistry et al. 2015). Total root length (m), mean root diameter (mm), the proportion root length in two diameter classes (0 to 0.5 mm; 0.5 to 1 mm) and the number of root tips were measured using a STD4800 scanner and WinRHIZO version 2013a program (Regent Instruments, Canada). Roots were then dried at 60°C for 48 h before DW was taken. Specific root length (SRL, m g$^{-1}$) was calculated as the total root length (m) divided by the root DW (g). To assess root hair traits, root segments ($n = 5$) of 1 cm were collected from each root system during harvesting. These segments were refrigerated (4°C) in 200 μM CaCl$_2$ to preserve cell integrity until measurement. The root segments were mounted on microscope slides with Hoyer’s solution and were examined under a DM500 Leica compound microscope where root hair density (abundance per mm root) and length (mm) were measured at 40x and 100x magnification.

3.3.5 Calculations and data analyses

Plant $\delta^{15}$N and [N] were calculated as mass-weighted averages of root and shoot values. The source $\delta^{15}$N (-0.733‰) was calculated as an [N]-weighted average of the stock NH$_4$NO$_3$ and N-free Long
Ashton nutrient solution $\delta^{15}N$ values from both the start and end of the growth period. The amount of plant-derived N in the growth medium, in other words N that accumulated in solution over and above what was supplied, was estimated using the difference between the source $\delta^{15}N$ and the $\delta^{15}N$ values of depleted solutions collected every 2 d (See Appendix S1) and difference in volumes were corrected for.

All statistical analyses were performed in R 3.2.3 (R Core Team 2015). A one-way Analysis of Variance (ANOVA) followed by a post-hoc Tukey test was used to assess significance between treatments (direct, pipe, sand) of root morphology traits (total root length, SRL, proportion of root length in two diameter classes, mean root diameter, the number of tips per mg of root, root hair density and root hair length), root and shoot DW’s and the shoot:root ratio. The post-hoc Tukey tests were determined using the agricolae package (de Mendiburu 2016). Total root length and the number of tips per milligram of root were both log transformed, while the proportion root length in two diameter classes were logit transformed, to improve homogeneity of variances and normality of data. A one-way ANOVA and a post-hoc Tukey test were also used to determine if significant differences in plant $\delta^{15}N$ existed between the direct, pipe and sand treatments. The root $\delta^{15}N$ and shoot $\delta^{15}N$ within each treatment were compared using a Welch two sample t-test, due to unequal variances (both root $\delta^{15}N$ and shoot $\delta^{15}N$ variances were more than 4x the variance difference). When comparing independent sample means, if 95% confidence intervals (CI) overlap by no more than half then $p \leq 0.05$ and if 95% CI’s do not overlap then $p \leq 0.01$ (Cumming and Finch 2005). Here, the 95% CI’s displayed as ribbons were calculated using non-parametric bootstrapping with 1000 replicates from the Hmisc package in R (Harrell and Dupont 2016).

3.4 Results

3.4.1 Plant growth and root morphology

Plants from the sand treatment had significantly more shoot biomass than the direct and pipe treatments, leading to a higher shoot:root ratio for these plants (Fig. 3.2A and B; Appendix Table S3.1). Root biomass from the pipe treatment ($30.2 \pm 1.4$ mg, mean $\pm$ SE) was intermediate between the direct ($27.9 \pm 1.6$) and sand ($32.8 \pm 0.8$) treatments (Fig. 3.2A). This was the only root trait where the pipe treatment was intermediate; the direct and pipe treatments (henceforth collectively referred to as ‘solution treatments’) were not significantly different across all other root traits (Fig. 3.3). Roots from solution treatments were significantly longer and thinner, with more tips and higher SRL, than roots
from the sand treatment (Fig. 3.3A, B, E, F; Table S3.1). Correspondingly, a majority of the solution root biomasses were less than 0.5 mm in diameter, compared to the sand root biomass of which almost a third was greater than 0.5 mm (Fig. 3.3C and D). Root hair density was not significantly different across all treatments, but roots from the direct treatment had significantly longer root hairs than the other two treatments (Table S3.1).
Fig. 3.2 The effect of hydroponic treatment on plant growth; (A) root and shoot dry weights and (B) shoot:root ratios. The boxes correspond to the interquartile range (IQR: 25th and 75th percentiles), while the horizontal line represents the median \((n = 7)\). The whiskers extend to the lowest value within \(1.5\times\text{IQR}\) and open circles represent outliers. The letters represent significant differences in plant growth traits between the three treatments, determined from one-way ANOVAs.
Fig. 3.3 Variability in root morphology traits between three hydroponic treatments. The boxes correspond to the interquartile range (IQR: 25th and 75th percentiles), while the horizontal line represents the median \((n = 7)\). The whiskers extend to the lowest value within 1.5*IQR and open circles represent outliers. The letters represent significant differences in root morphology between the three treatments, determined from one-way ANOVAs.
3.4.2 Plant $\delta^{15}N$

Plants from all treatments were depleted in $^{15}N$ relative to the source, indicating that plants discriminated against $^{15}N$ (Fig. 3.4). Furthermore, the plant $\delta^{15}N$ values of each treatment were more negative than the initial seedling $\delta^{15}N$. However, the sand treatment showed significantly less plant isotopic discrimination than did the solution treatments, which did not differ significantly from each other (Fig. 3.4; $F = 33.76$, df$_1$/df$_2 = 2/27$, p < 0.001). Root $\delta^{15}N$ was not significantly different to shoot $\delta^{15}N$ in the solution treatments, but in the sand treatment the root $\delta^{15}N$ was significantly more negative than that of the shoot $\delta^{15}N$ (Fig. 3.4; Appendix Table S3.2).

Fig. 3.4 Wheat plant, root and shoot $\delta^{15}N$ from the three hydroponic treatments relative to the seedling $\delta^{15}N$ values and the source $\delta^{15}N$ (-0.733‰, grey dashed line). The boxes correspond to the interquartile range (IQR: 25th and 75th percentiles), while the horizontal lines represent the median ($n = 10$). The whiskers extend to the lowest value within 1.5*IQR and open circles represent outliers. The letters represent significant differences between plant $\delta^{15}N$ of the three treatments, determined from a one-way ANOVA. The star represents a significant difference between the root and shoot $\delta^{15}N$ of the sand treatment (NS states non-significance, for the direct and pipe treatments), determined from Welch two-sample t-tests.
3.4.3 Root fragment loss and plant-derived N in solution

Root fragments and other plant-derived N were recovered from depleted solutions of all three treatments, but the amount of each and respective $\delta^{15}$N values varied depending on the treatment. Plants from the solution treatments lost ca. 14-fold more root fragment biomass cumulatively over the growth period than did plants grown in sand (Table 3.1). Additionally, this root fragment biomass was ca. six-fold greater than the respective root DW’s of solution treatments (Table 3.1). In contrast, the total sand root fragment biomass (including debris on filters and fragments from final wash solution) was only 40% of the root DW (Table 3.1). There was a marginal decline in root fragment production from solution treatment plants over the growth period, while root debris from sand treatment plants, which was collected on filters, remained constant (Fig. 3.5A). Due to the sampling technique from the sand treatment (i.e. draining the bottles from a port with minimal disturbance) root fragments no doubt accumulated in the sand and were recovered during the final harvest (Fig. 3.5A). Root fragments from the solution treatments contained ca. 11- to 13-fold more total N than that of the sand root fragments, and this N was two-fold greater than the net N taken up by the plants (Table 3.1). Conversely, the total N of the sand root fragments was only 15% of the plant net N uptake (Table 3.1). Plant-derived N in the direct and pipe depleted solutions was estimated to be 3.0 ± 0.3% (mean ± SE, n = 7) and 2.7 ± 0.3%, respectively, of net N taken up by plants, while in the sand treatment it was only 1.2 ± 0.06%. Although this is an estimate of plant-derived N, it is likely that considerably less N was lost in solution than in root fragments, for all treatments.

$\delta^{15}$N values of root fragments from the solution treatments were comparable to the source $\delta^{15}$N at the first nutrient change (14 d), but became progressively more negative than the source (Fig. 3.5B). In contrast, root fragments from the sand treatment had $\delta^{15}$N values that were significantly ($p \leq 0.01$) more positive than the source throughout the growth period. After 30 d, the $\delta^{15}$N of the pipe root fragments was not significantly different to that of the plant, but direct and sand root fragment $\delta^{15}$N values were significantly ($p \leq 0.01$) more enriched relative to their plant $\delta^{15}$N (Fig. 3.5B). Nevertheless, the difference between the plant and root fragment $\delta^{15}$N for the sand treatment was 11‰, whereas it was only 2.5‰ for the direct treatment. Also, root fragments recovered during the sand harvest had a $\delta^{15}$N value that was comparable to the respective sand filter $\delta^{15}$N (Fig. 3.5B). $\delta^{15}$N values of all depleted solutions were more positive than the source $\delta^{15}$N, and were thus significantly ($p \leq 0.01$) more enriched than plant $\delta^{15}$N values of each treatment (Fig. 3.6). While the $\delta^{15}$N of the direct and pipe solutions remained reasonably constant over time, $\delta^{15}$N became progressively more positive in the sand solution (Fig. 3.6).
Table 3.1: Root fragment loss from three hydroponic treatments, which had differing levels of stability for wheat roots. The root fragment biomass lost over the entire growth period was compared to the root biomass at harvest, while the cumulative N in root fragments was compared to net plant N uptake. Root debris from the sand treatment was collected on ‘Filters’ over the growth period whereas ‘Fragments’ trapped in the sand were recovered at the end of the experiment, during the harvest. The net N uptake of plants is the plant N content at harvest (30 d) minus the seedling N content (12 d). Values are mean ± SE (n = 10).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Direct</th>
<th>Pipe</th>
<th>Sand Filters</th>
<th>Sand Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant N content (mg)</td>
<td>4.9 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td>6.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Plant net N uptake (mg)</td>
<td>4.1 ± 0.3</td>
<td>4.5 ± 0.3</td>
<td>5.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Cumulative N content (mg) lost in root fragments</td>
<td>8.5 ± 0.4</td>
<td>10.0 ± 0.5</td>
<td>0.62 ± 0.03</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Root fragment N % of plant net N uptake</td>
<td>220 ± 20</td>
<td>231 ± 19</td>
<td>12 ± 1</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Root dry weight (mg)</td>
<td>28 ± 2</td>
<td>30 ± 1</td>
<td>33 ± 1</td>
<td></td>
</tr>
<tr>
<td>Cumulative root fragment dry weight (mg)</td>
<td>180 ± 6</td>
<td>191 ± 8</td>
<td>9 ± 0.6</td>
<td>4 ± 0.3</td>
</tr>
<tr>
<td>Root fragment DW % of root DW</td>
<td>672 ± 51</td>
<td>643 ± 37</td>
<td>29 ± 2</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>
Fig. 3.5 Temporal changes in (A) the amount of root fragments lost and (B) the root fragment δ¹⁵N values from wheat plants grown in three hydroponic treatments. Open symbols represent the mean plant δ¹⁵N of the three treatments and of the initial seedling (n = 10), for visual reference. Root debris from the sand treatment was collected on filters over the growth period; fragments potentially trapped in the sand were recovered at the end of the experiment, during the harvest. The grey dashed line in (B) is the δ¹⁵N of the source (-0.733‰). Points represent means (n = 6 to 10); ribbons and error bars represent the bootstrapped 95% confidence intervals.
Fig. 3.6 Change in the $\delta^{15}$N over time of depleted solutions collected every 2 d from three hydroponic treatments, relative to the initial source (-0.733‰, grey dashed line). Points represent means ($n = 7$) and ribbons represent the bootstrapped 95% confidence intervals.
3.5 Discussion

I found that wheat plants lost a significant amount of biomass as root fragments, which translated into this being the major pathway for N-loss (Fig. 3.5A; Table 3.1). The hydroponic medium, whether directly or indirectly aerated, resulted in high rates of root fragment loss far exceeding that from plants growing in a solid medium. I intended for the three treatments to provide variation in the degree of stability around the root system, to ascertain whether aeration acts as a mechanical stressor on roots. Plants from the solution treatments (i.e. direct and pipe) differed only in their root biomass and root hair length, and not in any other root morphology traits, plant $\delta^{15}$N or total root fragment biomass lost (Fig. 3.2A, 3.3, 3.4; Table 3.1). In contrast, plants grown in the sand treatment had considerably different root morphology and less plant isotopic discrimination than the other two treatments (Fig. 3.3 and 3.4). Aeration intensity per se was therefore probably not the driver of root abscission. Rather, root fragment loss may have been indirectly affected through the effect of the growth medium (solution versus solid) on root morphology (e.g. Groleau-Renaud et al. 1998).

The root morphology of the solution treatments tended to be ephemeral compared to roots in sand, with high SRL’s and thinner diameters (Fig. 3.3B and E; Eissenstat et al. 2000). While ephemeral roots may be more susceptible to abscising, the number of root tips was probably a noteworthy trait because the root cap zone is active in releasing mucilage and border cells (Hawes et al. 2003). These aid root elongation though soil and contain proteins that function to attract or repel various microbes to the rhizosphere, and thus could constitute a form of N-loss (Brigham et al. 1995). A relatively consistent number of border cells is released per tip depending on the species; for example, wheat root tips can release between 1100 and 1500 cells in 24 h (Hawes et al. 2003). While border cells are known to be continually released from immersed roots (Griffin et al. 1976; Hawes and Lin 1990), it is likely that considerably more were released in the solution treatments because the plants had twice the number of root tips than the sand treatment plants (Fig. 3.3F). The marginal decline in fragment production from the solution treatments (Fig. 3.5A) might have resulted from the roots maturing and thickening over time. Also, endophytic bacteria colonization may decrease root border cell production (Hawes et al. 2003). I observed some root fragments greater than 3 mm in solution, but the root border cells probably constituted a large component of what I termed ‘root fragments’.

The cumulative N associated with root fragments was considerably more than the estimated total amount of plant-derived N (presumably effluxed N) recovered in solution, for all treatments (Table 3.1). Moreover, root fragments were a major N-loss pathway because the N constituted a large
proportion of the net plant N uptake, especially in the solution treatments. In a review on cereal (wheat and barley) rhizodeposition in solid media across a range of experimental conditions, Wichern et al. (2008) found between 4.3 and 56% of total plant N (i.e. including the rhizodeposited N) was lost as rhizodeposits, and the median was 13%. These data, while not elucidating the nature of the deposited N, are comparable with root fragment N-loss from the sand treatment plants, which was ca. 15% of net plant N uptake (Table 3.1) or equivalent to ca. 13% of gross plant N uptake (i.e. total N influx). Studies conducted in hydroponics have focused on N efflux/influx quantification rather than N in rhizodeposition or root fragments, and have shown that as the \([N_i]_{ex}\) increases above 1 mM, the ratio of efflux to influx approaches 1 (Britto and Kronzucker 2006, and references therein). In a similar manner to E/I ratios, here extensive N-loss in root fragments from the solution treatments was ca. 68% of total N influx. For example, Jackson et al. (1976) found efflux of 2 to 7.6 mg g\(^{-1}\) d\(^{-1}\) in wheat, grown at 1 to 15 mM NO\(_3\)\(^-\), and I found root fragment N-loss of 17.6, 18.8 and 1.3 mg g\(^{-1}\) d\(^{-1}\) from the direct, pipe and sand treatments respectively, at 3 mM NH\(_4\)NO\(_3\). In comparison, the estimated plant-derived N in solution was ca. 0.2, 0.2 and 0.1 mg g\(^{-1}\) d\(^{-1}\), respectively. This estimate was based on \(\delta^{15}\)N of the depleted solutions, which includes unknown quantities of plant-derived \(^{14}\)N. Nevertheless, even if the assumed \(\delta^{15}\)N values of plant-derived N are extreme (e.g. \(\delta^{15}\)N in the range +10 or -10‰) this would only alter the N content marginally. Considering the difference in root fragment N-loss between the solution and sand treatments, it is likely that N-loss was accentuated through the effect of hydroponics on root morphology.

Root fragment loss can only promote plant isotopic discrimination if the fragments contain a pool of relatively \(^{15}\)N-enriched N, which most likely originates from root N assimilation (Kalcsits et al. 2014). NH\(_4^+\) from the NH\(_4\)NO\(_3\) supplied was probably readily and entirely assimilated in the root, with little accompanying potential for fractionation (Yoneyama et al. 1991; Evans et al. 1996; Miller and Cramer 2004; Kalcsits et al. 2015). NO\(_3^-\) may be fully or partially assimilated in the root depending on the N supply rate versus plant demand (Robinson et al. 1998), and if partially, the remaining pool of \(^{15}\)N-enriched NO\(_3^-\) is either transported to the shoot or stored in root vacuoles from where it could be lost to the rhizosphere. Moreover, maximum fractionation will occur here when the proportion of NO\(_3^-\) assimilated approaches 100% (Robinson 2001). Although the total N content in sand bottles was lower than in solution bottles due to the sand volume, I replaced nutrient solutions frequently enough to maintain stable N supply and limit N depletion. Nevertheless, the sand treatment plants may have experienced lower N supply than the solution treatments because they had fewer root tips and so less root hair zones for N uptake (Fig. 3.3F). Also, the greater shoot:root biomass ratio of the sand treatment plants could have induced higher plant N demand (Fig. 3.2B). This supply-vs-demand dynamic may
have contributed to greater fractionation during assimilation in sand treatment plants, and to greater $^{15}$N-enrichment of the residual $\text{NO}_3^-$ pool, explaining why the sand treatment root fragments were heavily enriched (Fig. 3.5B). Additionally, if root fragments were largely border cells, the proteins they produced would be relatively enriched if they were derived from $^{15}$N-enriched residual root $\text{NO}_3^-$ pools.

Plants from all treatments were depleted in $^{15}$N relative to the source (Fig. 3.4). It is unlikely this discrimination occurred during N uptake because there is no unequivocal evidence for fractionation during passive or active transport of N across root membranes (Handley and Raven 1992; Högb erg 1997). Furthermore, plant $\delta^{15}$N can match source $\delta^{15}$N over a range of $[\text{NO}_3^-]_{\text{ex}}$ (Evans 2001). Therefore, all plants must have lost $^{15}$N-enriched N during the growth period. I estimated that N-loss in root fragments drove plant $\delta^{15}$N down by at least $-3.7 \pm 0.3\%o$, $-2.6 \pm 0.3\%o$ and $-1.0 \pm 0.2\%o$ (mean ± SE, $n = 10, 10 & 20$) for the direct, pipe and sand treatments, respectively. This was determined by adding the cumulative total $^{15}$N and $^{14}$N content of the root fragments to the $^{15}$N and $^{14}$N content of the plants, and this $^{15}$N/$^{14}$N ratio then gave an estimated $\delta^{15}$N. Although root fragments from the sand treatment were heavily enriched relative to the other two treatments, they did not influence plant $\delta^{15}$N as much because of their substantially smaller biomass, consistent with isotope mass balance theory (Robinson 2001). The $^{15}$N-enrichment of the solution (Fig. 3.6) was probably due to plant-derived N, but the difference between direct N efflux from roots and potential bacterial decomposition of root fragments in solution could not be ascertained. Direct efflux of $^{15}$N-enriched N from roots would drive plant $\delta^{15}$N down (Evans 2001). That my estimation of plant $\delta^{15}$N, only taking root fragment loss into account, did not match the actual measured plant $\delta^{15}$N suggests a role for efflux of $^{15}$N-enriched N. Alternatively, decomposition of root fragments could result in the underestimation of root fragment loss, and could therefore contribute to the underestimation of root fragment influence on plant $\delta^{15}$N.

3.5.1 Conclusion

Attributing plant discrimination against $^{15}$N largely to biochemical efflux of $^{15}$N-enriched inorganic or organic N needs reconsideration. The turnover of whole roots and the loss of root cells are well known and unavoidable. Here, I have provided evidence that this abscised root biomass is an additional pathway for N-loss, alternative to N lost through efflux, and that both processes can influence plant $\delta^{15}$N. This is particularly the case for plants grown in hydroponics where rhizodeposition rates are high, but is also pertinent to plants growing in soil. As with efflux, root tissue loss can explain why N-loss from roots is responsive to rhizosphere [N] and plant growth rates. It also explains why N losses
occur even when access to N is limited, resulting in plant $\delta^{15}$N generally being lower than bulk soil $\delta^{15}$N globally (Craine et al. 2015a).
Chapter 4

General Discussion and Synthesis

4.1 Implications of root abscission as an alternative to N efflux, from laboratory to ecosystem

I explored two growth conditions, altering N supply to plants and disturbing root stability, to determine if plants lost significant N through root abscission and if this contributed to the offset between plant and source δ^{15}N values (i.e. plant isotopic discrimination). Researchers commonly attribute plant N-loss, represented by a reduction of net N uptake or increasing plant discrimination, to N efflux from roots and schematic models of within-plant N cycling represent this (e.g. see Fig. 2 in Liu et al. 2014). My results from Chapter 2 and 3 suggest that a schematic model should incorporate root tissue loss concurrent with the outward flux of N from roots (Fig. 4.1). Furthermore, based on my results I question how uptake experiments performed under hydroponic culture have been interpreted. At an ecosystem level, Handley et al. (1999) recognised that “organic and inorganic N leakage from plant roots” and “below-ground plant litter decomposition” are separate plant N losses in their flow diagram (see Fig. 2 in Handley et al. 1999). However, there were no fractionation factors listed for these two pathways, meaning that their effect on plant and ecosystem δ^{15}N values are unknown. Roots turned over, root fragments lost and any N effluxed could become a source of N for future root growth, adding to plant-available N in soil. Craine et al. (2009) proposed that the unresolved relationships between foliar δ^{15}N and soil N availability, climate or mycorrhizal symbioses, hinders the use of foliar δ^{15}N to infer plant-soil N cycling patterns at ecosystem and global levels. Here, I comment on these relationships with reference to my results, using laboratory experiments as a starting point for gaining insight into plant physiological processes in agricultural and natural ecosystems.

4.2 Hydroponic uptake experiments, should they be re-interpreted?

The net N uptake of plants is determined by the interplay between N fluxes into and out of roots (Nacry et al. 2013). A reduced net N uptake rate, relative to the influx rate, is generally ascribed to an increasing efflux rate (Aslam et al. 1994; Scheurwater et al. 1999; Glass 2003). I found, however, that a reduction in net N uptake may largely result from root tissue loss rather than from N efflux, even over a period of hours (Chapter 3). Despite this evidence for a shift in the mode of N-loss, measured net N uptake remains the same as plants have still lost N that was taken up.
Fig 4.1 Simplified schematic diagram highlighting plant N-loss through N efflux and root tissue loss. The source N (whether inorganic N from soil or hydroponic solution) is designated purple. Red colours indicate that an N pool is $^{15}$N-enriched, while blue colours indicate an N pool is $^{15}$N-depleted. Assimilation of inorganic N to organic N discriminates against $^{15}$N, leaving ON depleted relative to the residual IN. Different transporters are involved in influx, efflux and translocation to vacuole or xylem.
While root abscission (i.e. rhizodeposition and root turnover) does occur in soil-grown plants (Lauenroth and Gill 2003; Wichern et al. 2008), hydroponic culture may stimulate root abscission and contribute to an exaggerated reduction of net N uptake. This is evidenced by the significantly greater root tissue loss, and associated N-loss, when roots were not anchored in a solid medium (e.g. similarity of results between hydroponic sand treatment, Table 3.1, and pot experiment 0.175 d⁻¹ RAR, Table 2.1, compared to hydroponic solution, Table 3.1). Additionally, increasing discrimination between plant and N source δ¹⁵N can indicate greater plant N-loss (Robinson 2001; Kalcsits and Guy 2013), and plants from all hydroponic treatments showed considerably greater isotopic discrimination than any of the pot experiment plants (Fig. 3.4 and 2.3). Enhanced root abscission in hydroponic culture may have been interpreted by other authors to mean an increased N efflux rate but this implies that efflux is probably overestimated, which highlights the potential conflation of root abscission with efflux. Studies of N uptake efficiency, and affiliated NUE, require a true representation of a plant’s net N uptake (Glass 2003; Xu et al. 2012) that hydroponic culture may underestimate.

Considering that hydroponic culture has been adopted as an agricultural practise for its efficiency, farmers should take heed of the effect of solution flow on root dynamics. Measures to anchor roots in solution (e.g. rockwool) could reduce root tissue loss and concomitant N-loss, leading to greater N retention in plants (e.g. higher plant N content of hydroponic sand treatment, Table 3.1). Furthermore, estimating plant N demand over time and altering the N supply accordingly (Chapter 2) may go some way towards ameliorating the hydroponic effect on N efflux, root tissue loss and ultimately net N uptake.

4.3 Complexity of soil versus simplicity of hydroponic culture, regarding plant N availability

The use of foliar δ¹⁵N as a proxy to indicate the availability of N for plant uptake relies on the fact that foliar δ¹⁵N tends to increase with mineralisation rates (in situ or potential rates; Craine et al. 2009 and references therein). Furthermore, foliar δ¹⁵N also increases with foliar [N] (Craine et al. 2009). These are opposite to the patterns I found in Chapter 2, where shoot δ¹⁵N decreased and, more importantly, plant isotopic discrimination increased with an increasing NO₃⁻ supply (Fig. 2.3). In addition, root and shoot δ¹⁵N both decreased when their respective N content increased (Fig. 2.4). My data are consistent with results found in other hydroponic studies that measured the effect of N supply on plant δ¹⁵N values (Kohl and Shearer 1980; Evans 2001; Kolb and Evans 2003; Kalcsits and Guy 2013). This suggests that foliar δ¹⁵N of wild plants may be a legacy of fractionating process happening in bulk soil
and that direct delivery of an inorganic N source to the rhizosphere, as in hydroponic culture, renders a different outcome for plant δ\textsuperscript{15}N. The relative fluxes of depolymerisation, ammonification, nitrification, immobilisation and gaseous N losses from soil (i.e. soil fractionating processes) determine the relationship between bulk soil and plant-available δ\textsuperscript{15}N (Hobbie and Högberg 2012). Mineralisation of labile, complex organic N into monomeric organic N molecules and NH\textsubscript{4}\textsuperscript{+} progressively discriminates against \textsuperscript{15}N. Subsequent nitrification leaves NO\textsubscript{3}\textsuperscript{-} pools more \textsuperscript{15}N-depleted than NH\textsubscript{4}\textsuperscript{+} and microbial immobilisation of NH\textsubscript{4}\textsuperscript{+} promotes the enrichment of the organic N pool relative to NO\textsubscript{3}\textsuperscript{-} (Högberg 1997; Boddey et al. 2000; Denk et al. 2017). Nonetheless, when soil N availability and mineralisation rates are higher, gaseous losses that fractionate strongly – such as NH\textsubscript{3} volatilisation and denitrification – come into play, driving the \textsuperscript{15}N-enrichment of soil inorganic N pools and producing the above-mentioned foliar δ\textsuperscript{15}N pattern (Craine et al. 2015a; Craine et al. 2015b; Denk et al. 2017). This soil N cycling and attendant fractionations seemingly mask within-plant processes, such as N-loss from plants, that contribute to plant δ\textsuperscript{15}N.

Mineralisation rates are, however, not true measures of plant-available [N]. Rather, they imply that N is becoming increasingly more available in the form that plants require for uptake. Several studies have quantified the correlation between actual soil [inorganic N] and foliar δ\textsuperscript{15}N. Tateno et al. (2005) and Takebayashi et al. (2010) established that leaves of Fagus crenata (Japanese beech) and Chamaecyparis obtuse (Hinoki cypress) were more \textsuperscript{15}N-depleted at sites with high soil [inorganic N]. They assigned this to a shift in plant N source from NH\textsubscript{4}\textsuperscript{+} to NO\textsubscript{3}\textsuperscript{-}, given that NO\textsubscript{3}\textsuperscript{-} derived from nitrification is relatively \textsuperscript{15}N-depleted, rather than to loss of \textsuperscript{15}N-enriched N from plants. By contrast, Högbom et al. (2002) found that clear-felling of forested sites in Sweden resulted in a flush of soil NO\textsubscript{3}\textsuperscript{-}, with an associated increase in foliar δ\textsuperscript{15}N of the grass Deschampsia flexuosa, while in China, Cheng et al. (2010) measured decreased discrimination between foliar and mineral soil δ\textsuperscript{15}N with increasing [NO\textsubscript{3}\textsuperscript{-}], but the opposite for [NH\textsubscript{4}\textsuperscript{+}]. Again, the explanation for these foliar δ\textsuperscript{15}N patterns involved soil fractionation processes and shifts in plant N source. That these results are not conclusively similar to those from hydroponics (e.g. Chapter 2) may be ascribed to the complex nature of soil. For example, the studies mentioned here measure δ\textsuperscript{15}N signals or mineralisation rates in the top 50 cm of soil, but mature wild plants could be accessing N from deeper soil. Soil δ\textsuperscript{15}N becomes progressively enriched further down the soil profile because it has encountered more microbial processing (Hobbie and Ouimette 2009), and this implies that foliar δ\textsuperscript{15}N might reflect the use of N from deeper soil layers as well as the soil N cycling status.
4.4 Isotopic discrimination during root abscission

The majority of N losses reported in Chapter 2 and 3, whether by N efflux or root abscission, were $^{15}$N-enriched relative to the whole plant. Consequently, in the field these N-loss pathways must work to decrease plant $\delta^{15}$N values relative to bulk soil, as they augment the labile soil organic and inorganic N pools. Under the most controlled growth conditions (e.g. when N supply matched plant demand – 0.075 and 0.100 d$^{-1}$ RAR, or when roots were anchored in a solid medium – sand hydroponic treatment) root tissue loss was estimated to decrease plant $\delta^{15}$N by between -0.5 and -1.0‰, which is biologically significant (Handley and Raven 1992). This discrimination factor is likely to increase when plants experience more stress, as evidenced by the increasing offset between plant and source $\delta^{15}$N values (Fig. 2.3 and 3.4) when plants experienced an oversupply of N, or root systems were disturbed. The discriminating power of N efflux lies in the balance between influx and assimilation, which also contributes to the $\delta^{15}$N value of lost root tissue. Yet $\delta^{15}$N of abscised roots is also dependant on root longevity, including untimely excision through disturbance (e.g. herbivory), and the N resorption capacity from senescing roots. For instance, the full root system of perennials turns over several times throughout their life compared to annuals, but perennials may be more efficient at resorbing N and maintaining within-plant N cycling (Booth et al. 2003). Defining the range of possible isotope discrimination factors for root abscission and N efflux would allow the addition of these processes into ecosystem N cycling models such as the one proposed by Handley et al. (1999). However, since the fractionation caused by root abscission is determined by an even more complex suite of factors than N efflux, this is always likely to be highly variable.

4.5 Temperature, precipitation and herbivory, how would root turnover respond?

I considered two conditions by which root abscission could influence plant $\delta^{15}$N, namely N supply and root disturbance, but there are several other factors of ecosystem level importance that could be explored in small-scale experiments, for instance: temperature, water availability and herbivory. I expect plant/foliar $\delta^{15}$N to decrease at higher temperatures, given that root turnover increases globally with mean annual temperature (Gill and Jackson 2000). That Craine et al. (2009) found the opposite pattern is, perhaps, another example of soil fractionating processes masking the contribution of root turnover to plant $\delta^{15}$N. The response of root turnover to water availability is probably more intricate than the broad prediction of mean annual precipitation, where foliar $\delta^{15}$N decreases with increasing MAP (Craine et al. 2009). While root turnover is likely to be highest in tropical, warm and wet ecosystems where growth is constant year-round (Gill and Jackson 2000), seasonal environments (e.g.
temperate deciduous forests or sclerophyllous shrublands) may induce considerable turnover, as flushes in above- or below-ground growth correspond to fluctuating temperature and/or precipitation. Hence, future experiments should test the response of root turnover, and plant/foliar δ\textsubscript{15}N, to covarying temperature and water availability (e.g. by using percentages of field capacity).

Climate is labelled as the overarching driver of ecosystem N cycling, but above- and below-ground herbivory inevitably have repercussions on root turnover in many ecosystems. Above-ground herbivory has the potential to stimulate root turnover and potentially deplete plant δ\textsubscript{15}N, as loss of photosynthetic area decreases C flow to roots (Dawson et al. 2000). For example, Frank and Evans (1997) found that plants from grazed sites of Yellowstone had consistently lower δ\textsubscript{15}N than those from ungrazed sites, although they attributed this pattern to increased NO\textsubscript{3}– uptake rather than a grazing effect on root turnover. Moreover, below-ground herbivory through the removal of root tissue by root feeding insects (e.g. beetles, flies and roaches) or nematodes is known to reduce standing fine root stocks and thus increase fine root turnover (Stevens and Jones 2006). A compelling consequence of root herbivory is that it may stimulate N efflux from the excised root ends (Aslam et al. 1996b). Root herbivory can therefore contribute to plant isotopic discrimination through root turnover and N efflux, provided that the N lost is \textsuperscript{15}N-enriched.

4.6 Mycorrhizae, a significant player in the plant-soil δ\textsubscript{15}N narrative

The contribution of mycorrhizal symbioses to the global plant-bulk soil δ\textsubscript{15}N offset, as demonstrated by Craine et al. (2009), is attributed to the transferral of \textsuperscript{15}N-depleted N compounds from mycorrhizae to the host plant. Whether mycorrhizae take up inorganic or organic N from the soil (Read and Perez-Moreno 2003), several conversions of the N take place before it becomes available to the plant (Hobbie and Högberg 2012). For example, inorganic N taken up by arbuscular mycorrhizae is assimilated in the extraradical mycelium (hyphae external to the host root), transported to the intraradical mycelium (hyphae inside a root) as arginine, and then broken down into NH\textsubscript{4}+ again before passing to the plant root via ammonium channels (Govindarajulu et al. 2005). The purpose of N assimilation and subsequent break down is firstly for efficient transport and secondly so that C is retained by the fungi during N transfer to the host plant (Govindarajulu et al. 2005), but through each step the product of these enzyme-mediated reactions becomes progressively more \textsuperscript{15}N-depleted, none more so than the end-product which is transferred to the plant (Robinson 2001). Interestingly, mycorrhizae make use of the same N-assimilatory enzymes as plants (e.g. NR and GS-GOGAT) and so the fractionation factors
may be comparable (Govindarajulu et al. 2005). This fractionation establishes the $\delta^{15}$N discrepancy between host plant and mycorrhizae, but mycorrhizal turnover undoubtedly sustains it.

The ubiquity of mycorrhizal symbioses and importance to the plant-soil N cycle is of no doubt. Mycorrhizae are associated with 82% of angiosperm species and all gymnosperms (Brundrett 2002). Infections range between 2 (arbuscular Festuca ovina) and 200 (ericoid Calluna vulgaris) points per millimetre of root (Lambers et al. 2008). The fungi are probably shorter-lived than most roots; the extraradical mycelium of arbuscular mycorrhizae live for 5 to 6 days (Staddon et al. 2003), while the formation of arbuscules (highly branched structures for nutrient exchange) must be continual for colonisation to progress through a root because they degenerate on a weekly basis (Gadkar et al. 2001). I argue that this rapid turnover of fungal structures, which are likely to be $^{15}$N-enriched relative to the roots of the host plant, may contribute to the global plant-bulk soil $\delta^{15}$N offset. I observed colonisation of wheat roots by mycorrhizae while determining root hair densities under the microscope for Chapter 3. Although I did not quantify if the extent of colonisation differed between the hydroponics treatments, or determine whether the hyphae observed were arbuscular mycorrhizae or pathogenic, mycorrhizal $^{15}$N-enrichment and turnover could have contributed to the plant isotopic discrimination found across all three treatments (Fig 3.4). Given that arbuscular mycorrhizae are N-rich (3–5% N or 10-fold more than Plantago lanceolata root N), turnover of the fungi must significantly contribute to global ecosystem N cycling (Hodge and Fitter 2010). Ultimately, mycorrhizal turnover may contribute to plant isotopic discrimination without the need for N losses from plants, an important implication of my thesis, which questioned the mechanism and extent of plant N-loss as indicated by plant $\delta^{15}$N.

4.7 Conclusion

Root abscission, incorporating rhizodeposition and root turnover, is a substantial N-loss pathway from plants, however, the implications of it for global plant $\delta^{15}$N values need to be distinguished from soil N cycling and attendant fractionating processes. The fact that mycorrhizae are intrinsically connected to roots should indicate that their concurrent turnover is influential to the global question of why plant $\delta^{15}$N is lower than that of soil. The use of plant $\delta^{15}$N and time-integrated N isotope discrimination could assist programmes screening for N use efficient crop genotypes. Efforts to improve NUE of crop plants by reducing N efflux should take heed that plants could lose more N through inherent and unavoidable root tissue loss than through N efflux. We are aware that hydroponic culture does not accurately represent the complex relationship between roots and soil, but studies conducted in
hydroponics should gauge its effect on the physiological process in question, especially if it regards root functioning. It is exciting to think that we have not discovered all there is to know about physiology at the level of the plant, and that focusing on ‘-omics-level’ studies may blinker undiscovered plant-level processes.
References


Frank DA, Evans RD (1997) Effects of native grazers on grassland N cycling in Yellowstone


Kahmen A, Wanek W, Buchmann N (2008) Foliar $\delta^{15}$N values characterize soil N cycling and reflect
nitrate or ammonium preference of plants along a temperate grassland gradient. Oecologia 156:861–870.


Appendix

S1: Expanded description of calculations in data chapters

In Chapter 2, potential root loss (Fig. 2.6B) and total N lost from plants (Table 2.1) were calculated using the difference between observed and expected plant $\delta^{15}$N. Furthermore, leachate N that originated from plants (Table 2.1) was determined from the difference between leachate $\delta^{15}$N and source $\delta^{15}$N. In Chapter 3, plant-derived N in hydroponic depleted solutions (see Results) was estimated from the difference between the $\delta^{15}$N values of the source and the depleted solutions collected every 2 d. The basic calculation of each of these components remained the same and so to avoid repetition certain formulas will be listed once with descriptions relevant to each component.

To determine the proportion of $^{15}$N in a component, atom percent was calculated using the following formula (GG Hatch Stable Isotope Laboratory, University of Ottawa, personal communication):

$$15N \text{ atom} \% = \frac{100 \times AR \times (\delta^{15}N/1000+1)}{1+AR \times (\delta^{15}N/1000+1)} \hspace{1cm} [1]$$

AR is the absolute ratio of $^{15}$N in air, which is 0.0036764 (Coplen et al. 2002), and $\delta^{15}$N is the value to be converted into atom percent.

If no $^{15}$N was lost from a plant (Chapter 2), we would expect the plant $\delta^{15}$N to equal the source $\delta^{15}$N, and therefore plant and source atom % would also be equal. We assumed that on day 7, when nutrients were first supplied to the plants, the expected $^{15}$N content was equal to the observed $^{15}$N content, as the plants had experienced the same conditions until then. If no $^{15}$N originating from the plant was found in the leachate (Chapter 2) or the depleted solutions (Chapter 3), we would expect each of these component $\delta^{15}$N values to equal the source $\delta^{15}$N, likewise for atom %.

Observed $^{15}$N content was then equal to the proportion of $^{15}$N in a component multiplied by the N content (mg) of that component:

$$\text{observed }^{15}N \text{ (mg)} = (\text{atom} \%_{\text{component}} \times N \text{ content}_{\text{component}}) / 100 \hspace{1cm} [2]$$

The expected $^{15}$N content of each component was calculated as the proportion of $^{15}$N in the source multiplied by the N content (mg) of the component:
**expected** \( ^{15}N \) (mg) = \( (\text{atom} \%_{\text{source}} \times N \text{ content}_{\text{component}}) / 100 \) \[3\]

To determine how the observed \(^{15}\)N content of a plant deviated from what we expected, observed \(^{15}\)N content was subtracted from expected \(^{15}\)N content (Eq. 4), given that plants were \(^{15}\)N-depleted relative to the source (Chapter 2). By contrast, to determine the extent that observed leachate or depleted solution \(^{15}\)N deviated from what was originally supplied, the expected \(^{15}\)N content of each was subtracted from their observed \(^{15}\)N content (Eq. 5), because leachates (Chapter 2) and depleted solutions (Chapter 3) were both \(^{15}\)N-enriched relative to the source.

\(^{15}\)N deviation (mg) = expected \(^{15}\)N − observed \(^{15}\)N \[4\]

\(^{15}\)N deviation (mg) = observed \(^{15}\)N − expected \(^{15}\)N \[5\]

**Leachate N of plant origin; plant-derived N in solution**

The final step for calculating leachate N that originated from plants (Eq. 6) and plant-derived N in depleted solutions (Eq. 7) was to use their respective atom % values to quantify the amount of N that comprises the deviation:

\[
\text{Leachate N of plant origin (\(\mu g\ L^{-1}\))} = (^{15}N \text{ deviation/atom} \%_{\text{leachate}}) \times 10^5
\]

\[
\text{Plant derived N (mg)} = (^{15}N \text{ deviation/atom} \%_{\text{depleted sol}}) \times 100
\]

Plant-derived N is an estimate because the atom % of the depleted solutions, as a whole, may not accurately represent the proportion of \(^{14}\)N in plant-derived N. This atom % represents a mixture of source and plant-derived \(^{15}\)N values, and therefore the plant-derived N content might have been underestimated (Chapter 3).

**Potential root loss and plant N-loss**

To determine how much \(^{15}\)N might have been lost from plants between each harvest (Chapter 2), the average deviation in \(^{15}\)N (for each RAR) at the previous harvest was subtracted from each sample \((Ht\) denotes harvest number):

\[
\Delta^{15}N \ (mg) = ^{15}N \text{ deviation}_t^{Ht+1} - ^{15}N \text{ deviation}_t^{Ht}
\]

\[8\]
Root atom % of each sample at Ht+1 was used to determine how much root N contained the amount of $^{15}$N lost (Eq. 9). The root N concentration was used to determine how much root mass contained the amount of root N lost between each harvest, on a dry weight basis (Eq. 10). Finally, this mass value was converted to a per-gram unit, dividing by the original root dry weight at Ht+1, to give potential root loss (Eq. 11).

$$\text{Root } N_{\text{loss}} (mg) = (\Delta^{15}N / \text{atom } \%_{\text{root}}) \times 100 \quad [9]$$

$$\text{Root biomass}_{\text{loss}} (mg DW) = (\text{Root } N_{\text{loss}} /[N]_{\text{root}}) \times 1000 \quad [10]$$

$$\text{Potential root loss } (mg g^{-1} DW) = \text{Root biomass}_{\text{loss}} / \text{Root DW} \quad [11]$$

Total estimated root loss (mg, Table 2.1) was calculated as the cumulative of the root biomass lost between each harvest. The total N lost from plants (µg plant$^{-1}$, Table 2.1) was calculated as the cumulative of the root N content lost between each harvest.

**Fig. S2** Temporal change in shoot:root ratios of wheat given five relative addition rates of N (RAR d⁻¹: 0.075, 0.100, 0.125, 0.150 and 0.175). Nutrients were first supplied on day 7. Points represent means (n = 5) and ribbons represent the bootstrapped 95% confidence intervals.
Table S2: The effect of increasing N supply, by relative addition rate (RAR), over time on the total dry weight (DW) and N content of wheat plants. The values are mean ± SE.

<table>
<thead>
<tr>
<th>Day</th>
<th>Plant DW (g)</th>
<th>RAR (d⁻¹)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.075</td>
<td>0.100</td>
<td>0.125</td>
<td>0.150</td>
<td>0.175</td>
</tr>
<tr>
<td>7</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>0.13 ± 0.02</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0.35 ± 0.03</td>
<td>0.43 ± 0.03</td>
<td>0.53 ± 0.06</td>
<td>0.74 ± 0.04</td>
<td>0.78 ± 0.05</td>
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</tr>
<tr>
<td>47</td>
<td>0.68 ± 0.09</td>
<td>1.19 ± 0.06</td>
<td>1.53 ± 0.05</td>
<td>2.15 ± 0.09</td>
<td>2.47 ± 0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plant N content (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.49 ± 0.05</td>
<td>0.49 ± 0.05</td>
<td>0.49 ± 0.05</td>
<td>0.49 ± 0.05</td>
<td>0.49 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.33 ± 0.12</td>
<td>1.55 ± 0.24</td>
<td>1.68 ± 0.28</td>
<td>2.24 ± 0.31</td>
<td>2.76 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>3.87 ± 0.38</td>
<td>6.53 ± 0.38</td>
<td>7.96 ± 0.42</td>
<td>12.37 ± 0.67</td>
<td>12.92 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>11.01 ± 0.91</td>
<td>19.46 ± 1.03</td>
<td>24.67 ± 2.74</td>
<td>36.58 ± 2.28</td>
<td>39.45 ± 1.74</td>
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<tr>
<td>47</td>
<td>21.98 ± 2.46</td>
<td>46.05 ± 2.39</td>
<td>62.47 ± 2.21</td>
<td>91.85 ± 4.51</td>
<td>101.46 ± 6.73</td>
<td></td>
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</table>
### Table S3.1
Summary statistics of the one-way ANOVAs used to assess significance of plant growth and root morphology between hydroponic treatments (direct, pipe, sand). Bold p-values indicate statistical significance, df$_1$ = 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>F-value</th>
<th>df$_2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total root length (m)</td>
<td>115.3</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SRL (m g$^{-1}$)</td>
<td>129.4</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>% Root length (0 to 0.5 mm diameter)</td>
<td>41.32</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>% Root length (0.5 to 1 mm diameter)</td>
<td>41.18</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean root diameter (mm)</td>
<td>131.1</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No. of root tips / mg root</td>
<td>124.2</td>
<td>18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Shoot dry weight (g)</td>
<td>22.25</td>
<td>27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>3.695</td>
<td>27</td>
<td>0.0382</td>
</tr>
<tr>
<td>Shoot:root ratio</td>
<td>30.91</td>
<td>27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Root hair density (abundance / mm root)</td>
<td>1.557</td>
<td>102</td>
<td>0.216</td>
</tr>
<tr>
<td>Root hair length (mm)</td>
<td>3.95</td>
<td>102</td>
<td>0.0223</td>
</tr>
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</table>

### Table S3.2
Summary statistics of the Welch two-sample t-tests used to compare root δ$^{15}$N and shoot δ$^{15}$N within each hydroponic treatment. Bold p-values indicate statistical significance.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SE Root δ$^{15}$N</th>
<th>Mean ± SE Shoot δ$^{15}$N</th>
<th>t-value</th>
<th>Degrees of freedom</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td>-9.20 ± 0.24</td>
<td>-8.81 ± 0.30</td>
<td>-1.018</td>
<td>17.038</td>
<td>0.3227</td>
</tr>
<tr>
<td>Pipe</td>
<td>-9.29 ± 0.20</td>
<td>-9.27 ± 0.19</td>
<td>-0.078</td>
<td>17.958</td>
<td>0.9384</td>
</tr>
<tr>
<td>Sand</td>
<td>-7.36 ± 0.14</td>
<td>-6.87 ± 0.14</td>
<td>-2.466</td>
<td>17.988</td>
<td>0.0239</td>
</tr>
</tbody>
</table>