REGULAR ARTICLE

Root expression of nitrogen metabolism genes reflects soil nitrogen cycling in an organic agroecosystem

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Abstract

Background and aims Roots in agroecosystems encounter spatially and temporally heterogeneous nitrogen (N) availability in soil. Understanding root physiological processes in concert with soil microbial N dynamics following spatially discrete N pulses under field conditions will aid in the management of agroecosystem processes for N use efficiency.

Methods This study examined the short-term response (<5 days) of tomato (*Solanum lycopersicum* L.) roots and soil N cycling to a pulse of inorganic N in an undisturbed soil patch on an organic farm using a novel combination of molecular and ^{15}N isotopic techniques.

Results Tomato roots rapidly responded to and exploited the N pulse via upregulation of key N metabolism genes (e.g. cytosolic glutamine synthetase GS1) that comprise the core physiological response of roots to patchy soil N availability. Strong root activity limited accumulation of soil NO_3^- despite high rates of gross nitrification. Roots out-competed soil microbes for the inorganic N, even on a short time scale, likely as a result of high plant N demand and microbial C limitation. The transient root gene expression response (absent by 4 days after the N pulse) underscored the sensitivity of root N uptake to local N availability.

Conclusions Root expression of genes such as GS1 could complement soil inorganic N pools and measurements of soil microbial activity to serve as integrative indicators of rapid plant-soil N cycling.

Keywords Root gene expression · Cytosolic glutamine synthetase · Isotope pool dilution · Tomato (*Solanum lycopersicum*) · Nitrogen cycling

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Introduction

Roots encounter spatially and temporally heterogeneous nitrogen (N) availability in soil, which can result in plant N limitation as well as losses of N and subsequent environmental degradation (Robertson and Vitousek 2009). Heterogeneous N supply in agricultural systems results from pulses of N inputs, such as additions of synthetic N fertilizer or incorporation of organic matter (e.g. manure or leguminous cover crops), which cause rapid increases in soil inorganic N and potential for N losses if plants and microbes do not quickly capture the added N. Much research has necessarily focused on improving management options to increase fertilizer N use efficiency in agricultural systems (e.g. Giller et al. 2004). Basic knowledge of rhizosphere interactions that improve the synchrony between N availability and plant N uptake is also crucial, such as regulation of root responses to soil N patchiness together with microbial N mineralization-immobilization dynamics (Jackson

et al. 2008; Grandy et al. 2012). But understanding of how root behavioral responses and microbial N dynamics interact following soil N pulses remains limited (Cahill and McNickle 2011), especially in soil under field conditions relevant to actual agroecosystem processes.

Roots acquire plant-available forms of N, ammonium (NH_4^+) and nitrate (NO_3^-), in the rhizosphere. Roots sense N–rich patches through environmental cues such as NO_3^- presence and exploit the available N by increasing physiological N uptake capacity and/or proliferation of new roots (Glass 2003; Miller and Cramer 2004; Schachtman and Shin 2007; Hodge et al. 2009). Regulation of root enzymes involved in NH_4^+ and NO_3^- transport and assimilation is one such physiological mechanism, much of which occurs at the transcriptional level for genes encoding these enzymes (Lauter et al. 1996; Masclaux-Daubresse et al. 2010). Transporters located in the plasma membranes of root cortical and epidermal cells are responsible for NH_4^+ and NO_3^- uptake and are encoded by genes in the AMT and NRT gene families, respectively (Loqué and von Wirén 2004; Tsay et al. 2007). High affinity transport systems take up NH_4^+ and NO_3^- when N availability is low and can be constitutive or inducible (Nacry et al. 2013). When N availability is high (>~0.5 mM), constitutive low affinity transport systems take up inorganic N from soil. Once inside the root, NO_3^- can either be transported directly to shoots or reduced in roots to nitrite (NO_2^-) and then NH_4^+ by the sequential action of NO_3^- reductase (NR) and NO_2^- reductase (Nii). Glutamine synthetase (GS) and glutamate synthase (GOGAT) form the GS-GOGAT pathway and assimilate intracellular NH_4^+ originating from either direct NH_4^+ uptake from soil or reduction of NO_3^- (Miller and Cramer 2004). The complex, dynamic, and coordinated regulation of this suite of genes allows roots to tailor N acquisition tactics to match external N availability and internal N needs.

Responses of roots and soil microbes to N pulses are often considered separately, such as the study of root activity in hydroponic systems (e.g. Wang et al. 2001; Lopes and Araus 2008) or microbial activity in soil without roots (e.g. Recous et al. 1990; Shi and Norton 2000). But capturing the complexity of ecological interactions in the rhizosphere requires quantitative and simultaneous assessment of root ecophysiology and plant-soil-microbial N cycling (Jackson et al. 2008). Root responses to N pulses (e.g. increase in N uptake rates or precision foraging) are determined in part by plant genotype but are also strongly contingent on other environmental cues (Cahill and McNickle 2011), including competition with microbes for N and soil microbial N transformations. For instance, when high rates of nitrification rapidly convert NH_4^+ to NO_3^-, as occurs in agricultural soils when N mineralization and nitrification are stimulated by added N (Burger and Jackson 2003; Booth et al. 2005; Lu et al. 2011), roots may need to quickly increase N uptake and assimilation capacity to capture the mobile NO_3^-. Under some conditions, soil microbes rapidly assimilate an N pulse and out-compete plants in the short-term (hours to days) but often this response is limited in duration and magnitude by the amount of available carbon (C) to support microbial growth (Jackson et al. 1989; Hodge et al. 2000; Harrison et al. 2007). Plants may also be strong competitors for N during periods of peak N demand (Hodge 2004).

Rapid plant and/or microbial N uptake can result in low soil NH_4^+ and NO_3^- concentrations even if microbial N mineralization/nitrification rates are high or if there was a recent N input (Jackson et al. 1989; Burger and Jackson 2003). When input and output N fluxes are high, soil inorganic N pools turnover quickly and plant-available N in soil becomes difficult to assess (Jackson et al. 1989; Frank and Groffman 2009) since soil NO_3^- concentration, a common indicator of N availability in agricultural systems (Magdoff et al. 1984), does not build up. While low NO_3^- is ideal to limit N losses via denitrification or leaching below the root zone, it presents challenges for understanding and managing plant-soil N cycling.

Combining molecular and isotopic tools in natural plant-soil systems, such as quantitative gene expression in conjunction with ^{15}N isotope tracer and pool dilution techniques (Burger and Jackson 2005; Ruzicka et al. 2010), may yield insight into rapid plant-soil-microbial interactions following an N pulse. For example, expression levels of root N metabolism genes may indirectly indicate plant available N in soil and provide a complement to indicators of soil N availability like soil NO_3^-. The current field study builds upon previous work that used transcriptomic approaches to identify a core set of differentially expressed genes in tomato (*Solanum lycopersicum* L.) roots in response to an enriched ^{15}N patch within 24 h in a greenhouse pot study with an organic farm soil and in a field study on an organic farm (Ruzicka et al. 2010, 2011). In these studies, root genes

most affected by an N pulse encoded NH_4^+ and NO_3^- transporters and enzymes involved in NO_3^- reduction and NH_4^+ assimilation (Suppl. Table 1), particularly cytosolic glutamine synthetase GS1 (Ruzicka et al. 2010, 2011). The overall objective in this study is to determine how root expression of N metabolism genes and root growth respond to an ephemeral pulse of inorganic N over time, and how this N pulse affects plant and microbial N uptake and gross soil N transformations. The context was the same organically-managed farm as in previous experiments so that root and microbial activity were relevant to agroecosystem processes. High rates of gross N transformations were also likely to occur (Burger and Jackson 2003). The timing of the N pulse corresponded to a period of high plant N demand, early anthesis (Jackson and Bloom 1990; Elia and Conversa 2012), when organic farmers consider adding additional highly-labile N at a similar application rate as the N pulses used here (i.e. 10 and 62 kg N ha^{-1}). To maximize root responsiveness, soil N pulses were confined to only a small portion of the root system and measured over the following 5-days.

Methods

Description of organic farm field

The experiment was conducted in a tomato field on an organically-managed farm, Durst Organic Growers, Inc., in Esparto, Yolo Co., California, USA. The farm has been organically-managed since 1988 and a tomato/leguminous cover crop/alfalfa rotation is typically employed. The soil type was a Tehama loam, a fine-loamy, mixed, superactive, thermic Typic Haploxeralfs. Soil properties are presented in Cavagnaro et al. (2006). Crop residue from winter barley had been incorporated 4 weeks before planting tomatoes, and no organic fertilizers or amendments were added prior to the experiment.

Seeds of *Solanum lycopersicum* L Cv. 76R (Barker et al. 1998) were germinated as described in Ruzicka et al. (2010). Seven-week old seedlings were transplanted 7 May 2010, 30 cm apart. Plants were irrigated using surface drip line with 1.9 L hour^{-1} emitters spaced 30 cm apart, centered between plants.

Experimental design and treatments

As an overview, the experiment consisted of three treatment levels of inorganic N added as a pulse to a small undisturbed soil patch in between tomato plants (Fig. 1) followed by measurement of soil inorganic N and root gene expression in the patch 48, 96, and 120 h later. Since previous studies showed strong root gene expression responses to an N pulse within 24 to 48 h (Ruzicka et al. 2010, 2011), we chose these sampling times to examine the duration of this response and to encompass the expected microbial response (Burger and Jackson 2005). Root biomass and root N content in the patch were measured at 48 and 120 h after fertilizer was added. At 96 h following the N pulses, a tracer amount of ^{15}N was added to calculate gross soil N transformations and N uptake during a 24-hour period.

The study was arranged in a completely-randomized design with 30 plots, which were divided among three N pulse treatments: low NH_4NO_3-N, high NH_4NO_3-N, and a water control (see below for details) with ten plots per treatment. Each plot was composed of four experimental plants with two buffer plants on each side, for a total of six plants per plot. An additional buffer plant separated each plot. Half of the 30 plots were sampled at 48 h and again at 96 h following the N pulses and half were used subsequently in the ^{15}N tracer and ^{15}N pool dilution experiment (see below) between 96 and 120 h following the N pulses. Thus, there were five experimental units ($n=5$) sampled for each of three sampling times. Low and high-N pulses consisted of 10 or 65 μg-NH_4NO_3-N g^{-1} soil (6.97 and 45.30 mg N plant^{-1}), respectively, applied via syringe to a 10 cm diameter area between each tomato plant, directly beneath the drip emitter for irrigation (Fig. 1). These concentrations were approximately 4 and 27 times greater than ambient total soil inorganic N (NH_4^+-N and NO_2^- + NO_3^--N) (see below). Controls received the same amount of ddI-H_2O. Thus, both sides of every experimental plant were injected (see below) for a total of five locations in every plot. A template, 10 cm in diameter with 13 evenly spaced holes, was used to guide syringe placement. Sprotte needles were used to inject a total of 39 mL of solution, 3 mL into each hole at three depths (5.5, 3.5, and 1.5 cm below soil surface). Initial tests established that this was sufficient to apply the solution evenly to a volume of soil 10 cm in diameter and 8 cm deep (i.e. 628 cm^3, or 697 g soil at a measured bulk density of 1.11 g cm^{-3}). The volume of solution increased
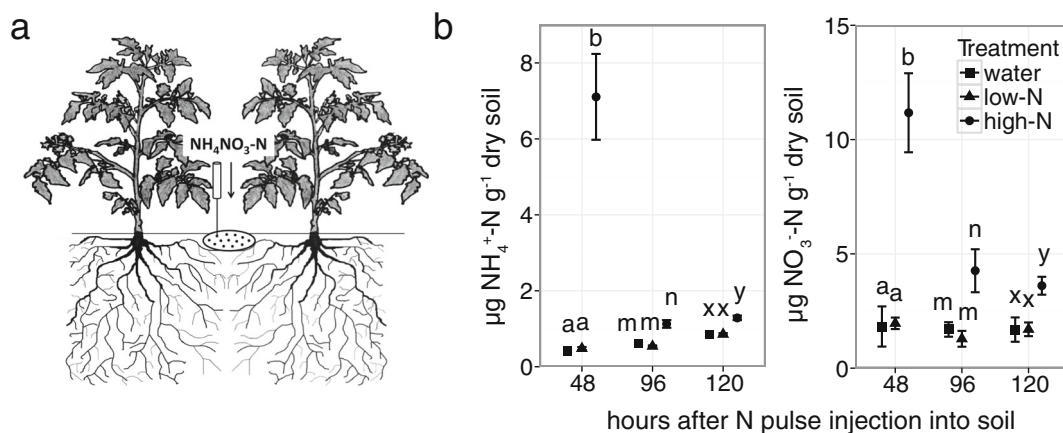
a

b

Fig. 1 Experimental setup (**a**) showing how a pulse of NH_4NO_3-N was injected in to a patch of undisturbed field soil in between two tomato plants at three levels (no N – water; 10 µg N g^{-1} soil – low N; and 65 µg N g^{-1} soil – high N) using a syringe and template to guide injections. The patch was 10 cm in diameter in the top 0–8 cm of soil. Soil cores of the same size were removed 48, 96, and 120 h following the N pulse to measure plant-soil N cycling, including expression of root N metabolism genes; Soil NH_4^+ and NO_3^- concentrations (**b**) at three times following the N pulses

gravimetric water content by ~6 %. Injection locations were carefully marked to facilitate subsequent soil sampling. The surface area of the N patch (i.e. 78.5 cm^2) represented <2 % of the area afforded each plant, so that the N pulses effectively simulated an increase in N availability available to only a small portion of the root system. The N treatments were applied to all plots on 4 July, 2010, during tomato anthesis, when tomato N demand is maximal (Elia and Conversa 2012).

Plant and soil sampling

Soil and plant samples were taken 48, 96, and 120 h following the N pulse. At 48 h, patch soil (i.e. one of the locations of the N pulse) was removed from half the plots (i.e., $n=5$) using a PVC ring (10 cm in diameter, 8 cm in height). Roots extending below the ring were carefully severed. Three subsamples of roots (each 50 to 150 mg, fresh weight) were promptly removed from this soil core, rinsed in ddI-H_2O, patted dry, and flash frozen in liquid N_2 under minimized light conditions for subsequent RNA extraction (see below). In the field, 2 M KCl was added to a representative soil subsample at a liquid: soil ratio of 2.5:1. The remaining soil was placed on ice for several hours until laboratory processing. At 96 h following the N pulse, another soil core was removed from the same plots as those sampled at 48 h, two plants away from the initial sampling. Root and soil samples were taken in the same manner as above.

^{15}N tracer and pool dilution

Plant N uptake and gross soil N transformations were measured over a 24-hour period between 96 and 120 h following the N pulse via ^{15}N tracer and ^{15}N pool dilution techniques. This time period was chosen so as to avoid the rapidly changing conditions occurring immediately following the N pulses, when heterogeneity of soil NO_3^- could make calculation of gross nitrification and NO_3^- immobilization invalid (Burger and Jackson 2003). For both methods, 1 µg $^{15}NH_4^+$-N g^{-1} dry soil (as $^{15}NH_4Cl$, 99 % atom percent enriched [APE]; Cambridge Isotope Laboratories Inc., Tewksbury, MA) and 1 µg $^{15}NO_3$-N g^{-1} dry soil (as $K^{15}NO_3$, 99 % APE) were injected using the same procedure as the N pulse injections into two locations separated by two plants in half the plots (i.e. $n=5$; those plots not yet sampled), 96 h following the N pulses. This amounted to 697 µg ^{15}N $plant^{-1}$. The ^{15}N tracer was injected in the same patches as the prior unlabeled N pulse. An equivalent volume of water was injected into a third location in each plot. At 120 h following the fertilizer injection (i.e. 24 h following ^{15}N injections), three soil cores (patches treated with either $^{15}NH_4^+$, $^{15}NO_3^-$, or water) were removed and all experimental plants in these plots were harvested. Root samples were taken from these cores in the same manner as above for gene expression analysis and for biomass and subsequent ^{15}N analysis (see below). Three 40 g subsamples of well-mixed soil from each

core were extracted with 2 M KCl in the field. Remaining soil was kept on ice for several hours until processing for microbial biomass and extraction of roots (see below). The two plants adjacent to the $^{15}NH_4^+$ and $^{15}NO_3^-$ patches were cut at ground level to be assessed separately for ^{15}N uptake from either N source (see below).

The diffusion technique was used to analyze ^{15}N in KCl extractions of soil NH_4^+ and NO_3^-, as well as NO_3^- from persulfate digestions of non-fumigated and fumigated samples for microbial biomass (Stark and Hart 1996), using Devarda's alloy to reduce NO_3^-. The ^{15}N content of filter disks was determined on a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility, USA. Gross mineralization and nitrification were calculated according to Kirkham and Bartholomew (1954). Immobilization of NH_4^+ or NO_3^- ($i_{NH4,NO3}$) was calculated from ^{15}N recovery in microbial biomass according to Davidson et al. (1991), as:

$$i_{NH_4,NO_3} = \frac{v_t}{\left(\frac{y_0}{x_0}\right)\left(\frac{1-e^{-k}}{k}\right)}$$

where

$$k = -\ln\left(\frac{y_t x_0}{y_0 x_t}\right)$$

and where $v_t = \mu g\ ^{15}N\ g^{-1}$ soil of MBN at $t=1$ d; $y_0, y_t =$ initial and final pool sizes, respectively, for $^{15}NH_4^+$ (or $^{15}NO_3^-$) in $\mu g\ ^{15}N\ g^{-1}$ soil; and $x_0, x_t =$ initial and final pool sizes for $^{14+15}NH_4^+$ (or $^{14+15}NO_3^-$) in $\mu g\ N\ g^{-1}$ soil. Soil cores for measuring inorganic N concentrations at the time of ^{15}N injections were not taken from ^{15}N injection sites so as to avoid any root disturbance in the zone that would subsequently be sampled. Instead, initial ^{15}N pool sizes were calculated based on the quantity of ^{15}N added and the concentration of ambient soil NH_4^+ and NO_3^- measured in the patch receiving water (see above) in between the ^{15}N injection sites in every plot, which had also previously received the un-labeled N pulses. These estimates of initial $^{15}NH_4^+$ pool sizes were corrected for NH_4^+ fixation (Davidson et al. 1991). A parallel experiment at three locations chosen randomly in the 0.02 ha study site showed a mean

recovery of 61 ± 2 % of $^{15}NH_4^+$ at 15 min after ^{15}N injection using the same protocol as above.

Recovery of ^{15}N in plant shoots, patch roots, microbial biomass, and soil NH_4^+ and NO_3^- pools in the patch that was injected with ^{15}N, was calculated as:

$$^{15}N\ recovery\ (\%) =$$

$$\left(\frac{^{15}N\ in\ excess\ of\ nat.\ abun.\ in\ pool}{total\ ^{15}N\ added\ to\ patch}\right)*100$$

Recovery of $^{15}NO_3^-$-N in microbial biomass was less than zero so was considered to be not detectable.

Plant and soil analysis

Soil NH_4^+-N and NO_2^--N + NO_3^--N concentrations were determined from KCl extracts at all sampling times (see above) by colorimetry using modifications of Miranda et al. (2001) and Foster (1995). Chloroform fumigation extraction followed by UV-persulfate oxidation and alkaline persulfate oxidation were used to measure microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), respectively, 120 h after the N injections (Wu et al. 1990; Cabrera and Beare 1993). MBC and MBN were calculated as the total flush of C or N, respectively, from fumigated samples. A conversion factor for MBC and MBN (i.e. K_{EC} and K_{EN}) of 0.45 was applied to take into account incomplete extraction (Jenkinson et al. 2004). Unfumigated soil samples provided a measure of extractable organic carbon (EOC) and extractable organic nitrogen (EON) (Ros et al. 2009). Gravimetric moisture was determined at every sampling event, after drying soil subsamples at 105 °C for 48 h.

Roots from a ~300 g subsample of patch soil were collected at 48 and 120 h and were washed by wet sieving. Roots were segregated into two size categories (<1 and >1 mm in diameter) for separate analyses. Roots were oven dried at 60 °C, weighed, and ground to a fine powder, as were the shoots collected at 96 and 120 h. All dried plant material was analyzed for total N and $\delta^{15}N$ on a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility, USA.

Root RNA purification and quantitative real-time
RT-qPCR

Root RNA was extracted using Trizol reagent
(Invitrogen, Carlsbad, CA) according to manufacturer's
guidelines followed by DNase digestion using RQ1
RNase-free DNase (Promega, Madison, WI). Total
RNA was purified using the RNeasy Plant Mini
Kit (Qiagen Sciences, Germantown, MD). RNA con-
centrations and quality were assessed using the Agilent
Nanodrop and the RNA 6000 Nano Assay (Bioanalyzer
2100, Agilent, Santa Clara, CA). Only RNA samples
with RNA integrity numbers (RIN) of at least 7.0 were
used for subsequent analyses. These RNA were used for
cDNA synthesis for qRT-PCR analysis. cDNA was syn-
thesized from 0.5 ug DNase-treated total RNA using the
Superscript III kit (Invitrogen, Carlsbad, CA).

Quantitative real-time RT-PCR (qRT-PCR) was per-
formed as described in Ruzicka et al. (2010, 2011),
using the primer pairs tested and reported therein, using
a StepOnePlus Real-Time PCR system (Applied
Biosystems, Foster City, CA). We focused on
genes involved in N uptake and assimilation iden-
tified via microarray analysis of roots in response
to an N pulse in the same tomato genotype and
organic farm soil (Ruzicka et al. 2010, 2011).
These genes included (Suppl. Table 1): high-
affinity NH_4^+ transporters AMT1.1 and AMT1.2
(XM_004247726; NM_001247324); high-affinity
NO_3^- transporter NRT2.1 (AF092655); nitrite reduc-
tase Nii (XM_004230772); cytosolic and plastidic glu-
tamine synthetases GS1 and GS2 (XM_004236638;
XM_010324491); NADH- and ferredoxin-dependent
glutamate synthases NADH-GOGAT and Fd-GOGAT
(XM_004234907; XM_004234782); glutamate dehy-
drogenase GDH (XM_010329382); and asparagine
synthetase AS (AY240926). The tomato actin gene
(XM_004235020) was used as the reference control
gene, as it did not exhibit differential expression among
N treatments in previous experiments (Ruzicka et al.
2010, 2011). Relative expression was analyzed accord-
ing to the ΔΔCt method, and standard error was com-
puted from the average of the ΔCt values for each
biological sample.

Statistical analysis

Treatment effects on plant and soil variables were ana-
lyzed using one-way analysis of variance (ANOVA)

within each sampling time. Root gene expression was
analyzed by analysis of covariance (ANCOVA) with
gravimetric water content as a covariate, since soil
moisture can strongly influence both microbial activity
and nutrient diffusion dynamics. The Shapiro-Wilk W
test for normal distribution and Levene's test for homo-
geneity of variance were used to test that data fulfilled
the ANOVA assumptions. For ANCOVA, the signifi-
cance of the interaction between treatment and the co-
variate was tested to ensure homogeneity of slopes. Data
were log transformed as necessary when assumptions
were not met. The Tukey-Kramer HSD test was used to
separate significantly different means at $p<0.05$. All
statistical analyses were performed using R.

Principal components analysis (PCA) was performed
using the *vegan* package in R (Oksanen et al. 2012) on
log transformed gene expression data. PCA was selected
because these data were normally distributed and the
relationships appeared linear. The *ordiellipse()* function
was used to generate 95 % confidence ellipses around N
treatments (i.e. water, low N, and high N).

Results

Soil inorganic N and microbial biomass

Injection of NH_4NO_3 into a localized patch of soil
adjacent to tomato plants caused significant increases
in soil NH_4^+ and NO_3^- concentrations in the high N
pulse (65 µg N g^{-1} dry soil added) but no changes in the
low N pulse (10 µg N g^{-1} dry soil added) relative to the
water control at 48 h following injections. At this time
soil NH_4^+ concentrations in the high N pulse were 17-
fold higher and soil NO_3^- concentrations were 6-fold
higher than the water control (Fig. 1b; overall treatment
effect, $p<0.0001$ for both NH_4^+ and NO_3^- at 48 h).
However, soil inorganic N (NH_4^+-N + NO_3^--N) in the
high N pulse at this time was only approximately 25 %
of the calculated amount of NH_4NO_3-N added 48 h
prior. This indicates rapid N uptake and/or losses of N
from the patch during this time period. By 96 h follow-
ing injections, soil NH_4^+ and NO_3^- concentrations in the
high N pulse soil patches had declined substantially and
were 2- and 2.5-fold higher than the water control,
respectively, and then remained similar at 120 h. They
remained significantly higher than the low N pulse and
the water control at both times (Fig. 1b; overall treat-
ment effect, $p<0.0001$ for NH_4^+ at 96 and 120 h; $p=$

0.003 and $p=0.01$ for NO_3^- at 96 and 120 h, respectively). In contrast, soil NH_4^+ and NO_3^- concentrations in the low N pulse patches were not significantly different from the water control at any sampling time.

MBC and MBN were not affected by N treatments at 120 h following N injections (Suppl. Table 2); means were 349 µg C g^{-1} soil and 33 µg N g^{-1} soil, respectively, across all treatments. The mean MBC:MBN ratio (10.6) was consistent across treatments indicating that no substantial microbial N uptake had occurred, which would have decreased this ratio. Thus, at ~75 days after incorporation of the barley straw, which visual inspection confirmed was largely decomposed, microbial N demand was low. Furthermore, EOC was only marginally affected by the N treatments ($p=0.07$), increasing from 24.4 µg C g^{-1} soil in the water control to 31.9 µg C g^{-1} soil in the high N treatment, with the low N pulse intermediate. EON did not differ among treatments and had a mean of 3.3 µg N g^{-1} soil (Suppl. Table 2).

Expression of root N uptake and N assimilation genes

Significant changes in expression of root N metabolism genes, including NH_4^+ transporters and N assimilation genes, were manifest 48 h following the NH_4NO_3 pulse (Fig. 2). Expression of high-affinity NH_4^+ transporter AMT1.2 was 4-fold higher in the high N pulse roots relative to the low N pulse roots, though similar to the water control roots ($p=0.03$). The high-affinity NH_4^+ transporter AMT1.1 showed 2-fold lower expression in the high N pulse roots compared to the low N pulse roots, which were also similar to the water control roots ($p=0.02$). In contrast, while expression of NRT2.1 was 3-fold higher in the high N pulse roots compared to the water control roots, this difference was not significant at 48 h ($p=0.095$) (Fig. 2).

Changes in expression levels were particularly pronounced for cytosolic glutamine synthetase GS1. Expression levels of this gene increased approximately 32-fold in the high N pulse and 3-fold in the low N pulse relative to the water control ($p<0.0001$) (Fig. 2). Chloroplastic glutamine synthetase GS2 and glutamate synthase NADH-GOGAT were significantly higher in the high N pulse roots relative to the water control roots ($p=0.004$ for GS2 and $p<0.001$ for NADH-GOGAT). Changes in expression of other N assimilation genes in the roots, including asparagine synthetase AS and nitrite reductase Nii, were not statistically significant.

Differential expression of N uptake and assimilation genes was no longer apparent by 96 and 120 h following the NH_4NO_3 pulse, indicating a rapid but transitory response of root gene expression to the pulse of N in the soil patch (Fig. 2).

PCA of these gene expression data showed pronounced effects of the N pulse treatments, including the low N treatment; NRT2 and Nii were included in this multivariate analysis as well because they were affected by NH_4^+-N pulses of similar magnitude as this study in previous experiments using the same tomato genotype (Ruzicka et al. 2010, 2011). Clusters showed clear separation of the N pulse treatments and water control along the first principal component axis (Fig. 3). Axis 1 accounted for the majority (58.0 %) of the variation in gene expression. Expression of all genes except high affinity NH_4^+ transporter AMT1.1 increased in the direction of the high N pulse. GS1 was most strongly associated with this axis and contrasted in direction with the expression of AMT1.1.

Root and shoot biomass and N content

Roots showed stronger responses to the N treatments than shoots over the course of the 5-day experiment. Shoot biomass did not change between 96 and 120 h following the N treatments nor did it differ among N treatments at either sampling time; the mean value was 113 ± 2.8 g $plant^{-1}$, i.e., 246 ± 6.2 g m^{-2} (data not shown). For roots in the patch that were <1 mm in diameter, biomass increased from 0.17 to 0.32 mg roots g^{-1} patch soil between 48 and 120 h across all N treatments ($p<0.0001$), but without treatment differences (Fig. 4a). However, when roots >1 mm in diameter were included, root biomass in the high N pulse was significantly higher at 120 h (0.45 mg roots g^{-1} patch soil) than biomass in the low N pulse and water control (0.29 and 0.35 mg roots g^{-1} patch soil, respectively, $p=0.008$; data not shown).

A rapid response occurred in the N concentration of roots <1 mm in diameter in the patch soil in the high N treatment, which increased ~0.5 % at 48 h after the N pulse, and was significantly higher than the low N treatment and water control at both 48 and 120 h (Fig. 4b; overall treatment effect: $p=0.01$ at 48 h, $p=0.0002$ at 120 h). The total N in the <1 mm root biomass in the patches was affected by the N treatments at both 48 and 120 h ($p=0.03$ at 48 h, $p=0.004$ at 120 h) (Fig. 4c). Thus, the higher total N content of small
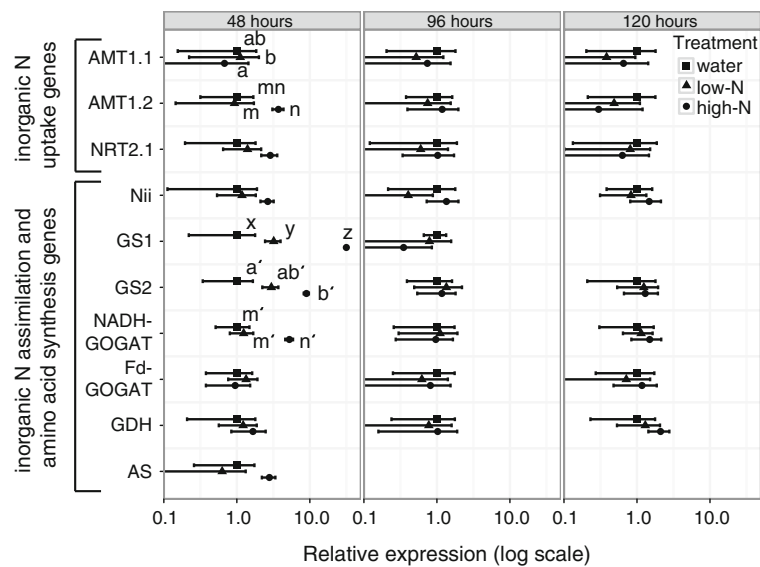
Fig. 2 Expression levels of high-affinity NH_4^+ transporters AMT1.1 and AMT1.2, high-affinity NO_3^- transporter NRT2.1, nitrite reductase Nii, cytosolic glutamine synthetase GS1, chloroplastic/plastidic glutamine synthetase GS2, glutamate synthase NADH-GOGAT, glutamate synthase Fd-GOGAT, glutamate dehydrogenase GDH, and asparagine synthetase AS in roots harvested from undisturbed organic farm soil 48, 96, and 120 h after high N (65 µg N g^{-1} soil), low N (10 µg NH_4^+-N g^{-1} soil), or water control treatments in the top 0–8 cm of soil. Relative quantity was calculated using the $\Delta\Delta CT$ method with actin as the reference control, and the water control group normalized to 1. For a given gene, means (± SE) followed by different *letters* are significantly different from one another at $p<0.05$ (ANCOVA)
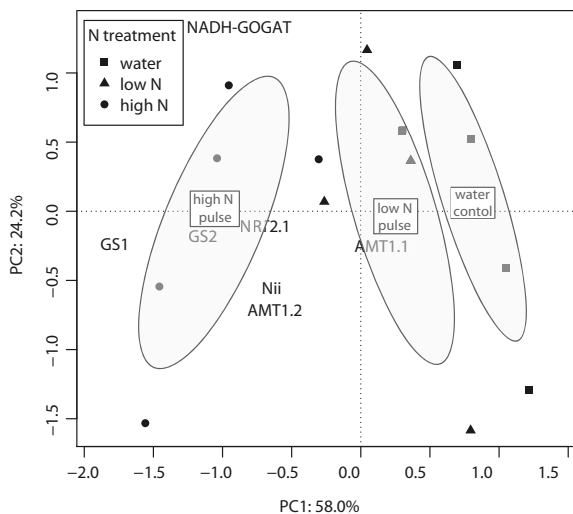

Fig. 3 Principal components analysis biplot showing expression of genes significantly affected by the N treatments in roots 48 h after an N pulse in a discrete soil patch in this study or previous work (Ruzicka et al. 2010, 2011). Shaded areas show 95 % confidence ellipses for each treatment: high N (65 µg N g^{-1} soil), low N (10 µg NH_4^+-N g^{-1} soil), or water control. Figure 2 shows responses of individual genes at three sampling times following the soil N pulse

(<1 mm diameter) roots in the patch soil in the high N pulse at 120 h was driven by increases in the N concentration of roots rather than from increasing root biomass. Percent total N in the shoots was 2.47 % across all treatments and did not differ among treatments at either sampling time (data not shown).

^{15}N tracer and pool dilution

The mean rate of gross N mineralization at 96 to 120 h after the N pulse was injected was 4.06 µg N g^{-1} soil d^{-1} in the control and 5.28 µg N g^{-1} soil d^{-1} in the high N treatment, with the low N treatment intermediate (Fig. 5); this trend was marginally significant ($p=$ 0.065). These rates exceeded the size of the mean NH_4^+ pool, corresponding to a mean NH_4^+ turnover rate of 3.6 times per day across all treatments. The mean gross nitrification rate was 4.81 µg N g^{-1} soil d^{-1}, quite similar to the gross mineralization rates, indicating high potential for nitrification of newly mineralized N. Recovery of 92 % of the $^{15}NH_4^+$ tracer remaining in the inorganic N pool as $^{15}NO_3^-$-N illustrated the high nitrification rates. Gross nitrification rates were more
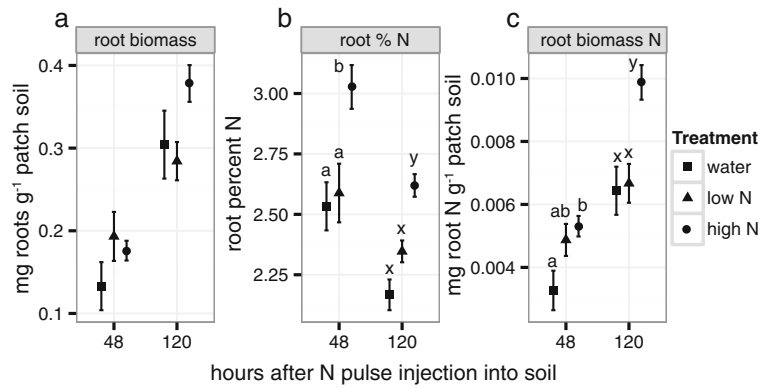
Fig. 4 Root biomass (**a**), root N concentration (**b**), and root biomass N (**c**) in roots <1 mm in diameter from a soil patch at two times (48 and 120 h) after high N (65 μg N g^{-1} soil), low N (10 μg NH$_4^+$-N g^{-1} soil), or water control treatments in the patches. The patch was 10 cm in diameter in the top 0–8 cm of soil. For a given sampling time, means (± SE) followed by different *letters* are significantly different from one another at $p<0.05$ (one-way ANOVA)

variable than gross mineralization rates and were not significantly different among treatments ($p=0.23$).

Across all treatments, ^{15}N tracer recovery in microbial biomass was 6.1 %, indicating little microbial N demand (Table 1). Microbial NH$_4^+$ and NO$_3^-$ immobilization were only 34 and 5 %, respectively, of gross mineralization; no significant differences occurred among treatments (Fig. 5). Microbes recovered more ^{15}NH$_4^+$ than ^{15}NO$_3^-$, reflecting the higher NH$_4^+$ immobilization than NO$_3^-$ immobilization. This indicates very little NO$_3^-$ assimilation by microbes. Surprisingly, even the zero N control, which had received no prior N, showed the same recovery of ^{15}N in the microbial biomass as the low and high N pulses.

Plants (shoots + patch roots) recovered ~5-fold more of the ^{15}N tracer than did microbial biomass in the patch (Table 1). This reinforces the prior indications of very rapid N uptake by roots in this small patch around a drip emitter. No treatment differences occurred in recovery of tracer ^{15}N in plant biomass, neither in shoots nor in the roots in the patch of soil that received the N pulse, regardless of the source of N (i.e. ^{15}NH$_4$-N or ^{15}NO$_3$-N) (Table 1). Thus, between 96 and 120 h after the N pulse, plants appear to have no lingering differential N uptake responses to the high, low, or zero N pulses. More ^{15}N was recovered in shoots than in the roots in the ^{15}N-labeled patch (24.5 % vs. 6.6 % across all treatments), indicating rapid translocation of ^{15}N from roots to

Fig. 5 Soil gross N mineralization and nitrification rates 4 days following an N pulse in a discrete soil patch at three different levels (water, low N, and high N) from an organic farm soil in the 0–8 cm depth. Shown are means ± SE. No significant differences were found among treatments
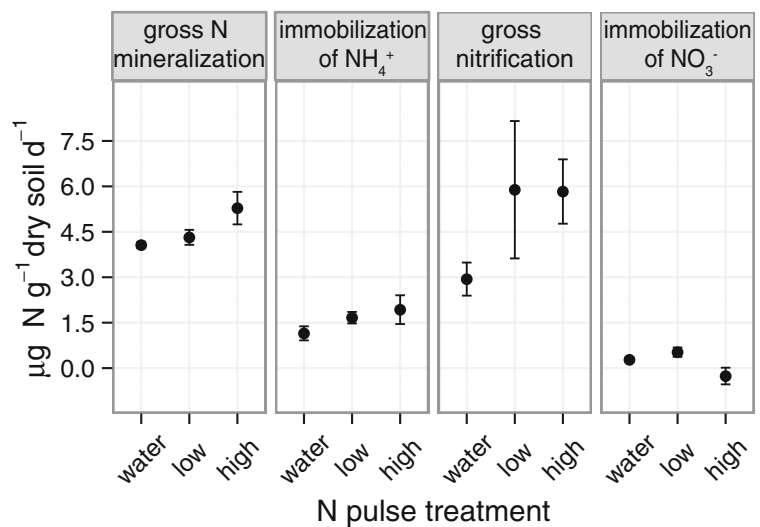
Table 1 Percentage of ^{15}N tracer (1 µg-N g^{-1} added, or 0.64 mg-N plant^{-1} as 99 % atom percent excess [APE]) soil that was recovered after 24 h in plant, microbial and soil NH_4^+ and NO_3^- pools depending on ^{15}N source and N pulse treatment

N pulse treatment	^{15}N tracer source	Patch roots		Total shoots		Microbial biomass		Left in soil NH_4^+-N pool		Left in soil NO_3^--N pool		Total recovery	
zero N	$^{15}NH_4^+$	8.5	±0.9	17.5	±0.8	2.2	±2.6	2.0	±0.2	36.7	±4.5	66.9	±3.7
low N	$^{15}NH_4^+$	6.8	±0.6	23.0	±1.1	8.7	±2.4	2.4	±0.1	37.2	±5.4	78.1	±5.4
high N	$^{15}NH_4^+$	7.1	±0.8	20.8	±1.5	8.1	±5.5	2.9	±0.5	34.5	±3.7	72.4	±3.2
zero N	$^{15}NO_3^-$	6.3	±1.1	27.8	±2.2	n.d.		0.8	±0.2	18	±1.9	55.1	±2.0
low N	$^{15}NO_3^-$	4.9	±0.1	28.7	±2.7	n.d.		0.9	±0.1	16.5	±4.5	49.4	±4.2
high N	$^{15}NO_3^-$	5.8	±0.8	29.2	±4.2	n.d.		0.5	±0.1	20.4	±6.5	56.3	±4.6
overall		6.6	±0.4	24.5	±1.2	6.1	±1.9	1.8	±0.2	27.2	±2.4	62.0	±2.2

The ^{15}N tracer was applied 96 h following the N pulse treatments. Shown are means ± SE ($n=5$). There were no significant differences among N pulse treatments

shoots over the 24 h period. This was particularly evident for shoot recovery of the $^{15}NO_3^-$-N vs. $^{15}NH_4^+$-N tracers, which was 28.6 % vs. 20.4 %, respectively ($p<0.001$). The opposite pattern was evident in roots, with slightly greater root ^{15}N recovery when ^{15}N was derived from $^{15}NH_4^+$-N vs. $^{15}NO_3^-$-N (7.5 vs. 5.6 %, $p<0.01$).

The incomplete ^{15}N recovery (particularly $^{15}NO_3^-$) was partly due to measuring only patch roots for ^{15}N content; the vast majority of the root system, to which some ^{15}N was likely transported, was not feasibly sampled. Another challenge was re-sampling the precise area where ^{15}N injection was injected, since no soil ingrowth ring was used in order to minimize soil disturbance. No irrigation occurred following the ^{15}N tracer injections and water-filled pore space was ~25 % so ^{15}N losses via leaching or denitrification were probably small.

Discussion

N-limited tomato roots rapidly exploited an ephemeral N pulse in a discrete patch of undisturbed soil under field conditions via increased expression of N uptake and assimilation genes, resulting in higher root N concentrations and rapid declines in soil inorganic N over the 5-day experiment. The transient gene expression response underscored the sensitivity of root N uptake to local N availability. As will be discussed in more detail below, roots outcompeted microbes for tracer ^{15}N apparently as a result of net mineralization rather

than net immobilization, microbial C limitation, and high plant N demand during the anthesis period. Tight synchronization between plant N demand and soil N availability curtailed soil NO_3^- accumulation even though gross nitrification rates were high, thereby reducing potential for future N loss, such as via denitrification and leaching during irrigation or rainfall events. The sensitivity of the suite of root N metabolism genes, and in particular cytosolic glutamine synthetase GS1, to higher N in ephemeral soil patches also demonstrates how roots modulate N uptake capacity without root proliferation, a strategy that would be less costly than investment in new root structures with associated maintenance costs.

Root transcriptional responses to N pulses

The N pulse occurred during a period of high plant N demand when plants were likely N-limited. Ambient soil inorganic N (NH_4^+-N+NO_3^--N, 2.2 µg g^{-1} soil) was ~5-fold lower than typical soils in organic Roma-type tomato production in this area (Smukler et al. 2010; Bowles et al. 2014), perhaps in part because of N immobilization following the incorporation of barley residue, which typically has a C:N ratio of ~40 (Jensen 1997). The average shoot N concentration (2.47 %) was also lower than threshold sufficiency values for Roma-type tomatoes (~3.4 % for plants of this size) (Hartz and Bottoms 2009), indicating N deficiency. To address N deficiency, organic tomato farmers often add a highly-labile source of N at a similar rate as the N pulse used here. Thus, the N pulse was functionally similar to what

might occur on-farm, albeit confined to slightly smaller portion of the root system than would occur using farm implements.

The transcriptional response of tomato roots to the high N pulse represents a core suite of transcriptionally-regulated genes that underpin increases in root N uptake and assimilation capacity when roots encounter a patch of N, especially when the rest of the root system is experiencing low N availability (Miller and Cramer 2004; Schachtman and Shin 2007). Contrasting regulation of two NH_4^+ transporters, AMT1.1 and AMT1.2, in patch roots in the high N pulse (i.e. downregulation of AMT1.1 vs. the upregulation of AMT1.2) confirms the hypothesized role of AMT1.1 and AMT1.2 in NH_4^+ uptake in soil with low vs. high N availability, respectively (von Wirén et al. 2000a). AMT1.1 is induced by N deficiency (Gazzarrini et al. 1999; von Wirén et al. 2000b) while AMT1.2 is induced by NH_4^+ and NO_3^- resupply following N deprivation in tomato as well as other plants (Nacry et al. 2013; von Wirén et al. 2000b). The upregulation of N assimilation genes in roots in the soil patches receiving a high N pulse, including glutamate synthase NADH-GOGAT, chloroplastic/plastidic glutamine synthetase GS2, and in particular cytosolic glutamine synthetase GS1, was responsible for rapid incorporation of NH_4^+ into amino acids, the former derived from either direct uptake of NH_4^+ or reduction of NO_3^-, thus avoiding harmful accumulation of NH_4^+ in roots (Evans et al. 1996; Hirel and Lea 2001). The significant upregulation of GS1 in roots at 48 h after the low soil N pulse suggests that this gene was highly sensitive to even this small soil N pulse, which was so transient it did not increase soil NH_4^+ and NO_3^-, despite the pulse being ~4-fold higher than ambient inorganic N.

Cytosolic glutamine synthetase GS1 plays a fundamental role in plant N metabolism across plant species, integrating both environmental cues and plant N status to regulate N assimilation (Bernard and Habash 2009; El Omari et al. 2010). Since the GS1 gene showed the greatest difference between the low N and zero N control (Figs. 2 and 3), it is a promising target gene for use as an indicator of plant-soil N cycling in the local root environment. For instance, upregulation of GS1 could indicate high root N uptake due to high N fluxes or recent N additions, even if soil N pools are low. Although regulation of GS1 (and other N metabolism enzymes) can occur at several levels beyond transcription (Cren and Hirel 1999; Nacry et al. 2013), higher transcript levels of GS1 in sorghum roots following increased N availability were linked to higher glutamine synthetase activity and accumulation of protein, suggesting that transcription is linked to enzyme activity (El Omari et al. 2010). Glutamine synthetase activity in leaves is also a good indicator of plant N status (Kichey et al. 2006), reinforcing the central role of GS1 in plant N metabolism. Regression analysis revealed a significant positive relationship between GS1 expression in roots and gravimetric water content in the soil patches across all treatments (p=0.022; data not shown). This suggests that expression of GS1 may be particularly fine-tuned to N availability in the local root environment, since soil water content influences microbial activity as well as N diffusion and mass flow to roots (Moldrup et al. 2001).

More dynamic and ephemeral responses apparently occurred for NO_3^- than for NH_4^+ transporter and assimilation genes in roots. Despite a rapid decrease in soil NO_3^- concentrations, no significant effects on expression of NO_3^- transporter NRT2.1 or nitrite reductase Nii were present at 48 h after the soil N pulse. Thus, soil NO_3^- availability from the NH_4NO_3 pulse likely peaked prior to the 48 h sampling time. Roots had apparently already downregulated the inducible high affinity NO_3^- uptake/assimilation response, in contrast to Ruzicka et al. (2010, 2011), in which all additional NO_3^- had to be nitrified from the pulse of NH_4^+-N. Rapid induction of NRT2.1 in roots in response to NO_3^- re-supply followed by downregulation as NO_3^- supply is maintained or diminished has been shown in *Arabidopsis thaliana* grown in solution (Zhuo et al. 1999; Tsay et al. 2007). Such rapid fluctuations in NRT2.1 expression in very short time frames suggest that it is a good indicator of NO_3^- availability, but not for the time frames useful for field testing on farms.

Roots may have quickly downregulated N uptake and metabolism genes due more to local negative feedback pathways than systemic (i.e. whole plant) pathways indicating plant N sufficiency (Gansel et al. 2001; Alvarez et al. 2012). First, local exogenous signals (e.g. soil NO_3^-) that induce N uptake (Zhuo et al. 1999) decreased rapidly in the N patches. Second, endogenous organic N assimilates (e.g. glutamine) that locally repress N uptake and assimilation genes (Glass et al. 2002; Schachtman and Shin 2007) may have increased via the activity of GS1 and GS2. Local signals are particularly important for regulation of NH_4^+ uptake,

while whole plant signals affect NO_3^- uptake to a greater extent (Gansel et al. 2001; Alvarez et al. 2012). Third, shoots remained N deficient during the experiment, so systemic signals would not likely have communicated N sufficiency to downregulate N uptake and assimilation genes.

Plant-soil N cycling

Rapid consumption of inorganic N by roots was surprisingly effective at depleting the soil pulse of NH_4NO_3. One reason may be the high root biomass near a drip emitter where tomato roots tend to accumulate (Machado et al. 2003) and where moisture enhances microbial activity and the diffusion of inorganic N. Roots efficiently scavenged a small amount of N (much lower than total daily plant demand) from a small patch (only 2 % of the total area afforded each plant). The high N pulse was only ~10 % of the potential daily plant N demand during anthesis, when maximal N uptake approaches 400–500 mg-N m^{-2} day^{-1} in tomatoes (Jackson and Bloom 1990; Hartz and Bottoms 2009). The actual rate of root N uptake from the N pulse is not known, but it probably exceeded 170 mg-N m^{-2} day^{-1} or 1.33 mg-N day^{-1} from the roots in the patch. This is based on calculating ^{15}N recovery in the plants and average enrichment of the soil N pool at 96 to 120 h after the high N pulse when the strong root transcriptional response had already subsided. Thus, specific conditions (i.e., plant N limitation, favorable microsite, high root biomass, and small soil N pulse size) favored very high N recovery by plants.

The delay in root proliferation, which was detected at 120 but not at 48 h after the N pulse, suggests that a threshold level of soil N availability regulates root growth in an N-rich patch (van Vuuren et al. 1996; Hodge 2004). The C costs of building and maintaining new roots may be greater than the benefits derived from increased nutrient uptake at lower levels of N availability (Jackson and Caldwell 1989). In previous studies with the same soil and tomato genotype, increased expression of a transcription factor regulating root developmental responses to N, ANR1 (Zhang and Forde 1998) followed a high but not a low soil N pulse (Ruzicka et al. 2010, 2011). Thus, even in these N-limited plants, rapid physiological adjustment of N metabolism genes may be a more efficient way to respond to an ephemeral N pulse.

The soil patch was a hotspot of root activity and soil N transformations, driven by the heterogeneity of N and water availability in the soil. Higher recovery of the ^{15}N tracer in the plant vs. microbes confirmed that plants were stronger competitors for soil inorganic N a few days after the N pulse. This is surprising given that microbes usually outcompete plants for inorganic N on a short time scale (≤24 h) (Jackson et al. 1989; Hodge et al. 2000; Harrison et al. 2007). One hypothesis is that labile C may have limited microbial growth and activity, given the lack of increase in microbial biomass following the N pulse (Schimel and Weintraub 2003). MBC, though similar to typical organic Roma-type tomato fields in this same landscape (Bowles et al. 2014), may have become increasingly C-limited following the decomposition of the barley residue incorporated ~3 months prior to the sampling. Moreover, both soluble C and its lability likely declined in the soil patch directly underneath the drip emitter where wet/dry cycles are frequent (Lundquist et al. 1999). As a consequence, microbial demand for N would have also declined. Carbon limitation restricts the ability of heterotrophic microbes to utilize NO_3^- in particular (Hodge et al. 2000; Burger and Jackson 2005; Inselsbacher et al. 2010), since NO_3^- requires much more energy to assimilate than NH_4^+, (Recous et al. 1990; Shi and Norton 2000; Geisseler et al. 2010), which was the preferred N source for microbes in this study.

Nitrifiers were the strongest competitors for NH_4^+, since more $^{15}NH_4^+$ in the soil patches was recovered as $^{15}NO_3^-$-N rather than in plants or microbial biomass, which is common in labile C-limited systems (Booth et al. 2005). Nitrifier populations were apparently already active at the beginning of the experiment, since the high nitrification rates were not stimulated by the N pulse (i.e. no differences among N pulse treatments). Nitrifier activity was likely supported by the high gross N mineralization rates in the patch. Heterotrophic denitrification was likely limited, since water-filled pore space did not exceed 40 % at any time and was predominately closer to 25 % (Linn and Doran 1984; Weier et al. 1993), but gaseous N losses from NH_3 oxidation pathways, which predominate at lower soil moisture levels (Zhu et al. 2013), may have occurred considering the rapid NH_4^+ transformations. Leaching of NO_3^- out of the patch is also possible since a small amount of irrigation occurred following the application of the soil N pulse (see Methods).

These dynamics illustrate the central role of C in controlling soil N cycling dynamics by regulating heterotrophic microbial N demand. If the N pulse had not been synchronized with plant N demand, then NO_3^- would likely have accumulated and been much more susceptible to losses, as when N inputs are poorly-timed with plant N demand in agricultural systems. Thus, sustaining an active soil biota with sufficient C resources is an important strategy for limiting NO_3^- accumulation and subsequent losses at times other than peak plant N demand (Drinkwater and Snapp 2007).

Conclusions: implications for sustainable N management

Synchronization between soil N availability and plant N demand is a crucial yet challenging goal for maintaining agricultural productivity while limiting harmful N losses (Grandy et al. 2012). Plant roots rapidly responded to and exploited an N pulse in an undisturbed soil patch via upregulation of key N metabolism genes that comprise the core physiological response of roots to patchy soil N availability. Strong root activity limited accumulation of soil NO_3^- despite high rates of gross nitrification. Roots out-competed soil microbes for the inorganic N, even on a short time scale, likely as a result of high plant N demand and microbial C limitation. This creates a window of management opportunity that takes advantage of the high physiological capacity of plant roots to exploit pulses of available N and to limit N losses. During such a time period, innovative approaches to assessing soil N availability are required, since NO_3^- pools - one of the most common metrics of soil N availability - may not build up. Our results suggest that root expression of genes such as glutamine synthetase GS1 could complement soil inorganic N pools and measurements of soil microbial activity to serve as integrative indicators of soil N availability and plant N status when plant-soil N cycling is rapid. Increasing reliance on biological processes and root-soil-microbial interactions for N synchrony in agroecosystems will support agricultural productivity while improving environmental quality.

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