Postprint: Rhizosphere Metabolomic Analysis of Reduced Nitrogen Fertilization Effects on Maize Seedling Root Exudates

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Abstract

Rhizosphere metabolomics analysis of plant root exudates facilitates improved understanding of rhizosphere processes governing chemical signal communication between plant roots, soil, and soil organisms within the soil rhizosphere microdomain. This study employed 1H NMR technology to detect root exudates in soil leachate (SL), rhizosheath soil (RS), and distilled water cultivation (DWC) collected from soil-cultured maize seedlings, and combined multivariate statistical analysis to compare differences in maize seedling root exudate profiles across different collection methods and nitrogen fertilizer application rates. The results demonstrated that the 1H nuclear magnetic resonance (1H NMR) spectral profiles and major markers of maize seedling root exudates collected by the three different methods were distinctly different; the levels of -glucose, malic acid, leucine, and valine in maize seedling root exudates were significantly affected by nitrogen fertilizer application rates. Utilizing 1H NMR technology combined with RS and DWC collection methods for rhizosphere metabolomics analysis can provide important theoretical foundations for research in rhizosphere ecology and rhizosphere nitrogen nutrition.

Full Text

Effects of Nitrogen Fertilizer Reduction on Root Exudates of Maize Seedlings Analyzed by Rhizosphere Metabonomics

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Abstract

Metabolomic analysis of plant root exudates can enhance our understanding of the chemical signaling processes that mediate interactions between plant roots, soil, and soil organisms within the rhizosphere. In this study, we employed proton nuclear magnetic resonance (^{1}H NMR) spectroscopy to detect root exudates collected from maize seedlings using three soil-based methods: soil leachate (SL), rhizosheath soil extraction (RS), and distilled water cultivation (DWC). Multivariate statistical analysis was used to compare differences in root exudate profiles across collection methods and nitrogen fertilizer application rates. The results revealed distinct ^{1}H NMR spectral profiles and characteristic markers among the three collection methods. The levels of -glucose, malic acid, leucine, and valine in maize seedling root exudates showed significant responses to nitrogen fertilizer dosage. Combining ^{1}H NMR technology with RS and DWC collection methods for rhizosphere metabolomic analysis provides an important theoretical foundation for research in rhizosphere ecology and nitrogen nutrition.

Keywords: nitrogen fertilizer reduction; root exudates; maize; rhizosphere metabolomics; ^{1}H NMR

Root exudates profoundly influence and regulate the physical properties of rhizosphere soil, nutrient availability, and the biological processes of chemical signaling between plant roots and rhizosphere microorganisms, pathogens, and other organisms. Precise characterization and quantification of root exudates are essential prerequisites for understanding rhizosphere processes. However, analyzing root exudates is challenging due to their localized deposition, degradation by rhizosphere microorganisms, and dynamic adsorption to soil particles. In recent years, the development of unbiased, non-targeted rhizosphere metabolomics platforms has made it possible to characterize the complete profile of root exudates at the root-soil interface. Nuclear magnetic resonance (NMR) spectroscopy, with its non-destructive, unbiased, highly sensitive, and rapid measurement capabilities, represents one of the primary analytical platforms for metabolomics research. While NMR has been extensively applied to study physiological and ecological questions in aboveground plant parts, its application to belowground secretion processes remains relatively limited.

A critical challenge in rhizosphere metabolomics is collecting root exudates in their native state. Traditional approaches using nutrient solutions, agar, or sterilized quartz sand as growth media can avoid microbial interference and effectively eliminate soil background noise. However, these artificial substrates affect root architecture, secretion patterns, and exudate composition, and they fail to simulate the actual conditions of soil-grown plants. This is particularly problematic when investigating how soil physical properties such as mechanical resistance and porosity affect root secretion processes. In such cases, soil represents the optimal cultivation substrate. This study implements three root exudate collection protocols based on soil culture—soil leaching, rhizosheath soil

collection, and short-term water cultivation—to establish methodological foundations for rhizosphere ecological research and to better understand root-soil interactions.

Plant nitrogen status and soil nitrogen levels significantly affect both the quantity and composition of root exudates. Under conditions of reduced chemical nitrogen fertilizer application, understanding how crop root secretion processes and exudate profiles respond is a key question for improving nitrogen use efficiency, mitigating environmental impacts of nitrogen excess, and achieving sustainable agricultural production. Currently, no studies have reported on the metabolomic characteristics of maize roots under nitrogen reduction conditions. Using ^{1}H NMR spectroscopy, this study investigates the effects of different nitrogen fertilizer reduction levels on maize seedling root exudates and rhizosphere metabolite profiles, providing a theoretical basis for optimizing rhizosphere nitrogen balance and improving nitrogen use efficiency.

1.1 Maize Seedling Soil Culture and Root Exudate Collection

The maize hybrid "Tieyan 38" was used for pot experiments. The soil was a typical aquic brown loam from Northeast China with pH 5.46, containing 17.8 g · kg ^{1} organic matter, 1.36 g · kg ^{1} total nitrogen, 52.9 mg · kg ^{1} alkali-hydrolyzable nitrogen, 22.7 mg · kg ^{1} available phosphorus, and 83.5 mg · kg ^{1} available potassium. The conventional nitrogen fertilizer rate of 180 kg · hm ^{2} was defined as 100% (N100), with reduction treatments at 85% (N85, 153 kg · hm ^{2}) and 55% (N55, 99 kg · hm ^{2}) of this rate. Phosphorus and potassium fertilizers were applied at conventional rates of 75 kg · hm ^{2} each. Fertilizers were mixed with soil according to the different nitrogen treatments before potting. Germinated maize seeds were sown, and after emergence, three seedlings were retained per pot. Pots were placed in a growth chamber at 28°C/18°C (day/night) with standard conditions. After 28 days, seedlings were removed for root exudate collection using three different protocols.

The soil leachate (SL) method, modified from Zhu et al. [**?**], involved placing the pot on a beaker and slowly pouring distilled water around the stem base to collect leachate from the bottom. The leachate was filtered through a 0.45 m membrane and stored at -80°C. The rhizosheath soil (RS) method, adapted from Wei et al. [**?**], involved carefully separating seedlings from soil while preserving the rhizosheath, then repeatedly washing the root system in distilled water. The soil-water mixture was vortexed for 30 s, centrifuged at 6700 rpm for 10 min, and the supernatant was stored at -80°C. The distilled water cultivation (DWC) method, modified from Valentinuzzi et al. [**?**], involved rinsing soil-grown seedling roots and transferring them to flasks with distilled water. Flasks were wrapped in aluminum foil for root shading and returned to the growth chamber for 6 h. The cultivation solution was then filtered through a 0.45 m membrane and stored at -80°C. This approach is distinguished from conventional nutrient solution methods by its short-term water cultivation design. Additionally, leachate from soil without plants was collected using the

SL method as a control (CK). Each nitrogen treatment had six replicates per collection method, while the soil-only control had three replicates.

1.2 ^{1}H NMR Spectroscopy

Frozen root exudate samples were lyophilized using a vacuum freeze dryer. Dried samples were reconstituted in 650 L phosphate buffer (0.1 M, pH 7.0, H O:D O = 9:1, containing 1 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d (TSP)), centrifuged at 18,000 g for 20 min, and 600 L of supernatant was transferred to NMR tubes for analysis. ^{1}H NMR spectra were acquired on a Bruker Biospin AV 600 spectrometer (Germany) using the zg30 pulse sequence. At 600 MHz and 300 K, 128 transients were accumulated with a spectral width of 6009 Hz, 32 k data points, and a 3.5 s acquisition time per scan. Spectra were processed using TopSpin software for automatic Fourier transformation, phase correction, and baseline correction. The TSP methyl proton signal was set to 0.0 ppm for chemical shift calibration.

1.3 Data Processing and Statistical Analysis

The entire spectral region (9 to -0.04 ppm) was integrated in 0.04 ppm bins using MestRe-C software, with the residual water peak region (4.55–4.95 ppm) removed. To eliminate concentration differences between samples, TSP peak area was set to 1 for data normalization. For improved comparability across samples, data were mean-centered and standardized using Par-scaling. The processed data matrix was imported into SIMCA-P 13.0 software for partial least squares projection to latent structures-discriminant analysis (PLS-DA). One-way ANOVA was performed using SPSS 13.0 to analyze relative metabolite contents.

2.1 ^{1}H NMR Spectra of Maize Seedling Root Exudates from Different Collection Methods

Based on soil culture of maize, we collected root exudates using three methods: soil leachate (SL), rhizosheath soil extraction (RS), and distilled water cultivation (DWC), with a plant-free soil control (CK). Under conventional nitrogen fertilizer conditions, the ^{1}H NMR spectral profiles differed markedly among collection methods [Figure 1: see original paper]. SL and CK showed similar spectral patterns, while RS and DWC yielded more peak signals compared to SL. Major characteristic peaks were assigned according to NMR spectral databases and literature [12]. The quantitative differences among the three collection methods likely resulted from localized deposition of specific root exudates, microbial degradation, or dynamic adsorption to soil particles. ^{1}H NMR successfully detected sugars, organic acids, and amino acids in maize seedling root exudates.

Figure 1 Typical ^{1}H NMR spectra of root exudates collected by different methods under full nitrogen fertilizer condition. CK: control without plants; SL:

soil leachate; RS: rhizosheath soil; DWC: distilled water cultivation. Metabolite assignments: Leu, leucine; Ile, isoleucine; Val, valine; Thr, threonine; Lac, lactate; Ala, alanine; AA, acetate; Pyr, pyruvate; Succ, succinate; CA, citrate; Asp, aspartate; GB, glycinebetaine; MA, malate; Asn, asparagine; GABA, -aminobutyrate; Suc, sucrose; -Gluc, -glucose; -Gluc, -glucose; Fum, fumarate; Ac, aconitic acid; For, formate.

2.2 PLS-DA Analysis of Maize Seedling Root and Rhizosphere Metabolomes

PLS-DA, a common method for metabolomic data reduction and dimensionality reduction, was applied to discriminate ^{1}H NMR profiles of root exudates from different collection methods. The results showed clear metabolic profile clustering by collection method, though SL and CK were not distinctly separated [Figure 2: see original paper]. The primary discriminatory markers between SL and RS were glucose and alanine. Key metabolites distinguishing SL from DWC included acetate, lactate, succinate, sucrose, alanine, leucine, isoleucine, valine, and glycinebetaine. Acetate, lactate, succinate, and isoleucine were the main markers separating RS from DWC. Both RS and DWC methods showed better separation from the control group than the SL method.

Figure 2 PLS-DA based on the metabolite profiles of maize root exudates obtained from different methods. CK: control without plants; SL: soil leachate; RS: rhizosheath soil; DWC: distilled water cultivation. Panels a, b, c, and d are score plots. Metabolite names in panels e, f, and g are as indicated in Figure 1.

2.3 Changes in Maize Seedling Root Exudates Under Different Nitrogen Fertilizer Rates

As shown in [Figure 3: see original paper], the ^{1}H NMR spectral profiles of rhizosheath soil extracts (panels a, b, c) differed markedly from those of short-term water cultivation (panels d, e, f). Furthermore, within each collection method, the overall ^{1}H NMR spectral profiles changed with nitrogen application rate. According to , compared to conventional nitrogen application, nitrogen reduction significantly increased -glucose levels in rhizosheath soil (up to 1.44-fold, $P < 0.05$), while other exudates remained unchanged. In contrast, short-term water cultivation showed more pronounced changes, with significant increases in malic acid (1.30-fold), leucine (1.20-fold), and valine (1.46-fold). However, these metabolite levels did not continue to increase with greater nitrogen reduction, instead showing a declining trend.

Figure 3 Typical ^{1}H NMR spectra of root exudates from maize seedlings grown under different nitrogen fertilizer rates. Panels a, b, and c show rhizosheath soil extracts at 100%, 85%, and 55% nitrogen rates, respectively. Panels d, e, and f show distilled water cultivation at 100%, 85%, and 55% nitrogen rates, respectively.

Table 1 Response ratios of metabolites in exudates from maize roots under

different nitrogen fertilizer conditions. N100, N85, and N55 represent 100%, 85%, and 55% nitrogen fertilizer rates, respectively. Metabolites with significant changes are indicated in bold ($P < 0.05$). Metabolite names are as shown in Figure 1.

3.1 Collection and Rhizosphere Metabolomic Analysis of Maize Seedling Root Exudates

Rhizosphere metabolomics aims to characterize the complete metabolome at the root-soil interface, including primary metabolites, chemical signaling compounds, and natural products from plants, as well as secretions from root-associated microbial communities and fungi. Fan et al. [**?**] first established an NMR-based non-targeted workflow for analyzing root exudates of wheat, barley, and rice using hydroponic culture. Subsequently, Escudero et al. [**?**] applied ^{1}H NMR to analyze tomato seedling root exudates under sterile sand culture conditions. Accurate quantification and characterization of root exudates require well-designed collection protocols. While hydroponic and sterile sand methods offer advantages in controlling soil background interference, they cannot simulate the actual habitat of soil-grown plants and thus have limitations. This study combined soil pot culture with ^{1}H NMR analysis of root exudates collected via soil leaching, rhizosheath soil extraction, and short-term water cultivation. The results demonstrated that different collection methods yielded distinct ^{1}H NMR spectral profiles and characteristic markers, confirming that both rhizosheath soil extraction and soil culture combined with short-term water cultivation are suitable for rhizosphere metabolomics research.

Although we refer to all three collection products as "root exudates," SL and RS methods actually capture a composite of root exudates, microbial secretions, and degradation products [**?**]. Nevertheless, these methods provide authentic information about field conditions and are appropriate for investigating the chemical composition following plant-microorganism interactions, thereby elucidating the ecological functions of root exudates. The DWC method, combining soil culture with short-term water cultivation, minimizes environmental noise from soil heterogeneity while reflecting physiologically meaningful root exudates in their native state. Under natural field conditions, soil microorganisms influence rhizosphere chemistry through secretion, deposition, and other activities [**?**]. Particularly, some soil microbes participate in soil nitrogen cycling [**?**], making it crucial to consider microbial community effects on exudate composition under nitrogen reduction conditions, despite microbial interference with net secretion. In the complex context of soil biodiversity, rhizosphere metabolomics enables us to truly understand the ecological functions and agricultural significance of root exudates. Given the complexity of root exudate composition, both collection methods and the sensitivity of metabolomic platforms limit the number of detectable compounds, necessitating integration of multiple collection approaches and targeted analysis for detecting specific signaling compounds at picomolar levels.

3.2 Effects of Nitrogen Fertilizer Reduction on Maize Seedling Root Exudates

Nitrogen fertilization is a primary strategy for increasing maize grain yield and biomass. However, nitrogen excess and reduced nitrogen use efficiency have caused negative environmental impacts including soil acidification, pollution, and decreased microbial activity. Reducing nitrogen application rates to control nitrogen supply and minimize soil nitrogen loss risk represents an effective approach for improving nitrogen use efficiency and promoting sustainable maize production [**?**]. Plants translocate 20–50% of fixed carbon to roots, with 10–18% released into soil via root exudation [**?**]. The secretion process depends on both plant physiological status and soil nutrient availability, which affects the allocation of plant-derived carbon to root exudates. This study found that under nitrogen reduction conditions, only -glucose increased significantly in rhizosphere soil compared to conventional nitrogen application, while other exudates remained largely unchanged. Short-term water cultivation showed more pronounced changes, with significant increases in malic acid, leucine, and valine, though these metabolite levels declined rather than continued to increase with greater nitrogen reduction [FIGURE:3, TABLE:1].

Sugars and sugar alcohols in root exudates serve as energy sources and universal chemoattractants for many microorganisms [**?**, **?**]. Malic acid may be specifically involved in chemotactic attraction of plant growth-promoting rhizobacteria. Carbohydrates and amino acids in root exudates can act as chemotactic factors that recruit beneficial microbes to the rhizosphere, thereby promoting plant growth [**?**]. The relatively abundant sugars, amino acids, and organic acids are associated with adaptive adjustments to insufficient soil nutrient supply. While the underlying molecular mechanisms remain unclear, they may involve plant hormones. Auxin-enhanced plasma membrane H -ATPase activity provides stronger driving force for secondary active transport, potentially re-uptaking leaked sugars, organic acids, and amino acids [**?**]. Systematic comparison of plasma membrane H -ATPase activity under different nutrient conditions is essential for elucidating the re-uptake mechanisms of root exudates.

Root exudates play important roles in heavy metal detoxification, soil charge generation, protein and pharmaceutical compound production, interactions with parasites, pathogens and their natural enemies, and microbial growth promotion [**?**]. Additionally, plants influence soil nutrient availability through solubilization, reduction/coordination, and ligand exchange reactions mediated by root exudates [**?**]. Studies show that root exudation can reduce potential nitrogen loss by increasing soil carbon availability through water-soluble carbon input [**?**]. Carvalhais et al. [**?**] reported that nitrogen deficiency decreased amino acids (particularly aspartate, tyrosine, isoleucine, lysine) and maltose in hydroponically grown maize root exudates. Zhu et al. [**?**] found that total root exudates in soil leachate increased with nitrogen rate, with significant increases in sugars, sugar alcohols, and phenolics under high nitrogen (320 kg $\cdot$ hm ^{2}, 2–3.2$\times$ the optimal rate) compared to no nitrogen, while carboxylic acids, amines, polyols, and lipids

showed no significant differences. These findings collectively demonstrate that nitrogen levels affect root exudate quantity and composition, though the relationship between specific exudates and nitrogen rates remains ambiguous. This may be because exudate composition and quantity vary with environmental conditions such as soil type, elemental content, pH, temperature, and microbial presence [**?**, **?**], as well as with inherent plant biological characteristics including species, cultivar, and developmental stage. For example, Mönchgesang et al. [**?**] identified natural genetic variation in rhizosphere chemistry among 19 Arabidopsis accessions through non-targeted metabolite profiling of root exudates, revealing high chemical diversity in plant rhizospheres adapted to different environments. Further research is needed to investigate how root exudate quantity and composition vary among different maize cultivars, growth stages, and soil conditions under nitrogen reduction. To optimize rhizosphere nitrogen balance and improve nitrogen use efficiency, unbiased analysis of root exudates and the entire root-soil interface metabolome, combined with coordinated analysis of plant tissue metabolomes, will help identify specific signaling molecules that trigger plant growth promotion and elucidate plant intrinsic adaptation mechanisms.

References

[1] Yang X L, Liao H, Nian H. Roots Biology: Principles and Applications. 3rd ed. Beijing: Science Press, 2007: 1-305

[2] van Dam N M, Bouwmeester H J. Metabolomics in the rhizosphere: tapping into belowground chemical communication[J]. Trends in Plant Science, 2016, 21(3): 256-265

[3] Strehmel N, Böttcher C, Schmidt S, et al. Profiling of secondary metabolites in root exudates of Arabidopsis Thaliana[J]. Phytochemistry, 2014, 108: 35-46

[4] Escudero N, Marhuenda-Egea F C, Ibanco-Cañete R, et al. A metabolomic approach to study the rhizodeposition in the tritrophic interaction: tomato, pochonia chlamydosporia and meloidogyne javanica[J]. Metabolomics, 2014, 10(5): 788-804

[5] Luo Q, Sun L N, Hu Y M. Metabonomics study on root exudates of Cd hyperaccumulator Sedum Alfredii[J]. Chinese Journal of Analytical Chemistry, 2015, 43(1): 7-12

[6] Rugova A, Puschenreiter M, Koellensperger G, et al. Elucidating rhizosphere processes by mass spectrometry-A review[J]. Analytica Chimica Acta, 2017, 956: 1-13

[7] Sun C X, Chen X, Cao M M, et al. Growth and metabolic responses of maize roots to straw biochar application at different rates[J]. Plant Soil, 2017, 416(1): 487-502

[8] Li C X, Wu F Z. Advances of root exudates collection and root exudates mediated interspecific interactions[J]. Acta Agriculturae Boreali-occidentalis Sinica, 2016, 25(6): 795-803

[9] Zhu S S, Vivanco J M, Manter D K. Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize[J].

Applied Soil Ecology, 2016, 107: 324-333
[10] Wei M A, LI X X, Li C J. Modulation of soil particle size and nutrient availability in the maize rhizosheath[J]. Pedosphere, 2011, 21(4): 483-490
[11] Valentinuzzi F, Pii Y, Vigani G, et al. Phosphorus and iron deficiencies induce a metabolic reprogramming and affect the exudation traits of the woody plant Fragaria×ananassa[J]. Journal of Experimental Botany, 2015, 66(20): 6483-6495
[12] Fan T W, Lane A N, Pedler J, et al. Comprehensive analysis of organic ligands in whole root exudates using nuclear magnetic resonance and gas chromatography-mass spectrometry[J]. Analytical Biochemistry, 1997, 251(1): 57-68
[13] Fischer H, Eckhardt KU, Meyer A, et al. Rhizodeposition of maize: short-term carbon budget and composition[J]. Journal of Plant Nutrition and Soil Science, 2010, 173(1): 67-79
[14] Tiwar S, Singh P, Tiwari R, et al. Salt-tolerantrhizobacteria-mediated induced tolerance in wheat (Triticum aestivum) and chemical diversity in rhizosphere enhance plant growth[J]. Biology and Fertility of Soils, 2011, 47(8): 907-916
[15] Proct C, He Y. Quantifying root extracts and exudates of sedge and shrub in relation to root morphology[J]. Soil Biology and Biochemistry, 2017, 114: 168-180
[16] Coskun D, Britto D T, Shi W, et al. How plant root exudates shape the nitrogen cycle[J]. Trends in Plant Science, 2017, 22(8):
[17] Yan X, Jin J Y, He P, Liang M Z. Recent advances in technology of increasing fertilizer use efficiency[J]. Scientia Agricultura Sinica, 2008, 41(2): 450-459
[18] Kuijken R C P, van Eeuwijk F A, Marcelis L F M, et al. Root phenotyping: from component trait in the lab to breeding[J]. Journal of Experimental Botany, 2015, 66(18): 5389-5401
[19] Baudoin E, Benizri E, Guckert A. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere[J]. Soil Biology Biochemistry, 2003, 35(9): 1183-1192
[20] Huang X F, Chaparrojacqueline M, Reardonkenneth F, et al. Rhizosphere interactions: root exudates, microbes, and microbial communities[J]. Botany Botanique, 2014, 92(92): 281-275
[21] Carvalhais L C, Dennis P G, Dmitri F, et al. Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency[J]. Journal of Plant Nutrition and Soil Science, 2013, 176(4): 3-11
[22] Fisk L M, Barton L, Jones D L, et al. Root exudate carbon mitigates nitrogen loss in a semi-arid soil[J]. Soil Biology Biochemistry, 2015, 88: 380-389
[23] Luo Q, Wang S, Sun L, et al. Metabolic profiling of root exudates from two ecotypes of Sedum alfredii treated with Pb based on GC-MS[J]. Scientific Reports, 2017, 7(39878): 1-9
[24] Mönchgesang S, Strehmel N, Schmidt S, et al. Natural variation of root exudates in Arabidopsis thaliana-linking metabolomic and genomic data[J].

Scientific Reports, 2016, 6(29033): 1-11