Greater soil water and nitrogen availability increase C : N ratios of root exudates in a temperate steppe

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A B S T R A C T

A better understanding of how rates and stoichiometric signatures of root exudates respond to altered N deposition and rainfall patterns is critical for predicting future impacts of environmental changes on ecosystem function and services. Here, we conducted a field experiment examining the effects of rainfall reduction (-50% compared to the control with normal rainfall), rainfall addition ($+50\%$), N addition (~ 10 g N m^{-2} yr^{-1}), and their interactions on root C and N exudation rates and C : N ratios of root exudates from dominant species in a temperate steppe. We hypothesized that increasing soil water and N availability will increase C: N ratios of root exudates because plants will grow more and retain more N in their biomass, thus have lower root N exudation rates. We found greater changes in root N exudation rates (ranging from $-18\% \sim +11\%$) than C exudation rates (ranging from $-6 \sim +11\%$) in response to rainfall and N treatments. In addition, N addition and its interactions with rainfall treatments decreased root N exudation rates by 4–18% and therefore increased C : N ratios of root exudates by 8–27% compared to control; however, a contrasting pattern was found under rainfall reduction. Furthermore, changes in root C exudation rates in response to rainfall and N treatments were not related to changes in plant biomass. In contrast, root N exudation rates decreased with increasing plant biomass, resulting in an overall increase in C : N ratios of root exudates with increasing plant biomass. Overall, our results reveal the C : N stoichiometric plasticity of root exudates in response to resource modifications. These findings have important implications for understanding rhizosphere plant-soil-microbe interactions and ecosystem functioning under environmental changes.

1. Introduction

Anthropogenic activities, such as fossil fuel burning and agricultural fertilizer application, have led to an increase in global nitrogen (N) deposition (Reay et al., 2008). In the meantime, global rainfall patterns are predicted to change in the future, with subtropical and mid-latitude regions experiencing less precipitation while tropical and high-latitude areas receiving more (IPCC, 2018). Concurrent changes in N deposition and rainfall patterns can trigger complex interactive effects on plant growth and belowground carbon (C) allocation, altering plant-soil-microbe interactions and resulting in changes in C and nitrogen (N) cycling processes – with significant implications for ecosystem

functioning and the provision of ecosystem services (Knapp et al., 2002; Niu et al., 2009; Reichstein et al., 2013). Root exudation, the release of soluble organic compounds from roots to soils, is particularly relevant in this context, considering that root exudates can mediate the activity of soil microbes by providing C for their growth and can contribute significantly to soil organic matter formation (Badri and Vivanco 2009; Sokol et al., 2019). Despite this importance, however, effects of altered N deposition and rainfall patterns on root C and N exudation rates and C : N stoichiometric ratios of root exudates are not fully understood.

There is some evidence that root exudation rates are responsive to changes in soil water and N availability. For example, severe drought can reduce root exudation rates due to photosynthesis inhibition (de
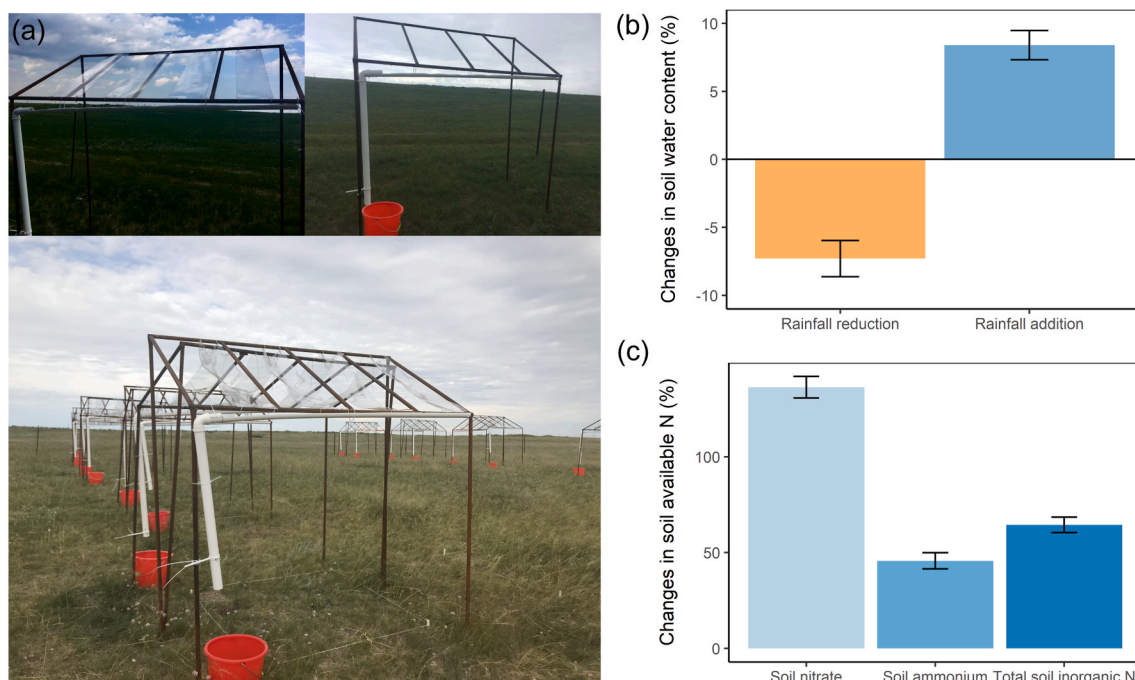
Fig. 1. A layout of the experimental design (a), changes in soil gravimetric water content (%) resulted from rainfall reduction and addition (b), and changes in soil available N (%) resulted from N addition in a temperate steppe in Inner Mongolia. Changes are compared to the control treatment and values are mean ± se. See figures S1, S3, and S4 for more details.

Vries et al., 2019; Williams and de Vries 2020); however, greater soil water and N availability can stimulate plant growth and productivity, which may allow plants to allocate more organic compounds for root exudation (Liljeroth et al., 1990; Ataka et al., 2020). Considering water and N are two major determinant factors of plant growth, it is possible that, as soil water availability increases, plant growth becomes more limited by soil N availability (Yang et al., 2011). Therefore, it is reasonable to predict that root exudation rates would increase when rainfall and soil available N both increase. Additionally, changes in soil water and N availability may not only alter the rate of root exudation, but also the quality of root exudates. Recent advances in metabolomics and chemical-analytical platforms allow a more comprehensive identification of compounds exuded into the rhizosphere by plant roots, leading to a better understanding of the potential roles that different metabolites are playing between plants and soil biota (van Dam and Bouwmeester 2016). However, the majority of previous studies on qualitative composition of root exudates focus on C-based organic compounds, which is presumably because root exudates are considered as the C-energy nexus for symbiotic microbial associations (Badri and Vivanco 2009; Phillips et al., 2011). Therefore, much less is known about how N concentrations and C : N ratios of root exudates respond to environmental changes (Grayston et al., 1998; Mergel et al., 1998; Zhang et al., 2016). Theoretically, concurrent rainfall addition and N fertilization can strongly enhance plant growth as well as N concentration in green leaves (Yang et al., 2011; Yuan and Chen 2015), potentially resulting in greater resorption of N and therefore lower root N exudation rates. As such, we expect that greater soil resource availability may increase C : N ratios of root exudates, though this has not been experimentally investigated.

The objectives of this study were: (1) to assess how root C and N exudation rates and C : N stoichiometric ratios of root exudates respond to altered soil water and N availability; and (2) to examine whether responses of root exudation rates are closely linked to changes in plant growth. We hypothesized that increasing soil water and N availability will increase C : N stoichiometric ratio of root exudates because plants will grow more and retain more N in their biomass, thus have lower root

N exudation rates. To test this hypothesis, we applied an *in-situ* method (Phillips et al., 2008) to collect root exudates for C and N analyses from two dominant species (*Stipa krylovii* and *Allium polyrhizum*) that have experienced rainfall reduction, rainfall addition, N addition, and their interactions in a temperate steppe in Inner Mongolia.

2. Materials and methods

2.1. Study site and experimental design

In this study, we conducted a field experiment examining the interactive effects of N addition, rainfall reduction and addition on C and N concentrations of root exudates in a temperate steppe at the Hulun Lake Reserve in Inner Mongolia (113.21°E, 48.75°N), China. Mean annual rainfall and temperature of this region are 283 mm and −0.45 °C, respectively. Soil is classified as Calcic Luvisols (FAO, 1988). Soils are composed of 70% sand, 26% silt, and 4% clay, with a pH of 7.5 and soil bulk density of 1.1 g cm^{-3}. Vegetation is a typical steppe dominated by perennial species, including *Stipa krylovii* and *Allium polyrhizum*.

We used a complete factorial design with two N treatments (control and addition) and three rainfall treatments (control, reduction [-50%], and addition [+50%]) randomized within five blocks – resulting in thirty 2 m × 2 m plots that were established in June 2018. The N addition rate (in the form of NH$_4$NO$_3$) was 10 g N m^{-2} yr^{-1}, which was applied evenly in June, July, and August. The rainfall treatments were achieved with rainfall shelters, which consisted of a tubular steel frame supporting a clear plastic roof, rainfall collection pipes and storage buckets (Fig. 1). The plastic film covered half of the roof for the rainfall reduction treatment, with collected rainfall being evenly applied to the addition one for each rainfall event.

2.2. Field sampling and lab analyses

Root exudates were collected monthly from intact roots of *S. krylovii* and *A. polyrhizum* in July, August, and September of 2018, using a modified method from Phillips et al. (2008). Briefly, individuals with

(a)

(b)

(c)

Rainfall and nitrogen treatments
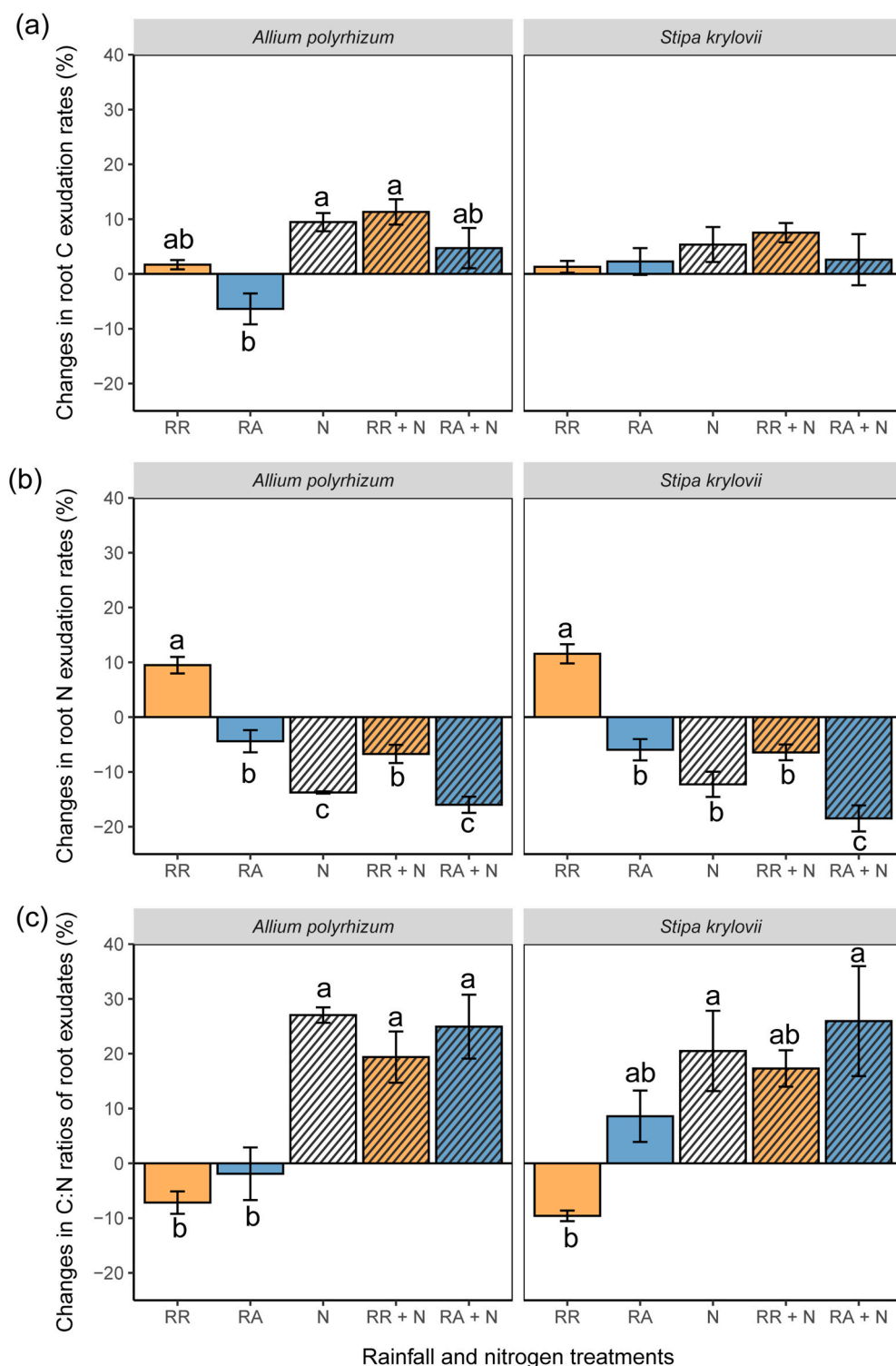
Fig. 2. Changes in root carbon (C) exudation rates (%) (a), nitrogen (N) exudation rates (%) (b), and C : N ratios of root exudates (%) (c) in response to different N and rainfall manipulative treatments in a temperate steppe. Values are mean (±se) over the growing season of 2018 (*i.e.*, July, August, and September, n = 3). See Figures S5-S7 for more details on monthly measurements of root exudation rates. Different letters indicate statistically significant differences between treatments following Tukey's HSD test ($p < 0.05$). RR, RA, N, RR + N, and RA + N indicate rainfall reduction, rainfall addition, N addition, rainfall reduction + N addition, and rainfall addition + N addition, respectively. Treatments include rainfall reduction and addition are highlighted with different colors while involve N addition are highlighted with stripes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

approximately same sizes for each species were selected at each plot. Fine roots linked to plants were carefully excavated, rinsed with deionized water, and placed into a 30-mL glass syringe filled with silica sand and sealed with a rubber septum (Figure S1). The syringe with fine roots and a small amount of C and N-free solutions (0.1 mmol/L KH$_2$PO$_4$, 0.2 mmol/L K$_2$SO$_4$, 0.2 mmol/L MgSO$_4$, and 0.3 mmol/L CaCl$_2$) was buried in soils for 24 h. After the 24 h equilibration period, solutions within the syringe were completely removed with a vacuum pump. Then, the same procedure was repeated and fine roots were incubated

for another 24 h. After the 24 h incubation, solutions within the syringe were collected, filtered (0.22 μm), and stored at −20 °C until analysis. Fine roots within the syringe were harvested for biomass. Total organic C and total N of collected solutions were determined on a TOC analyzer (Multi N/C 3000, Analytik, Jena, Germany). Root exudates were expressed based on root dry biomass. See Shen et al. (2020) for more details.

In addition, soils in the 0–10 cm depth interval were sampled biweekly from July to September of 2018 to measure soil water content

Table 1
Results (F and p values) of two-way ANOVA on the effects of species and treatment on changes in root C and N exudation rates and C : N ratio of root exudates in a temperate steppe during the growing season of 2018. *Df* indicates degree of freedom.

Sources	Df	F	p
Changes in C rates			
Species	1	0.037	0.85
Treatment	4	5.05	0.004
Changes in N rates			
Species	1	0.002	0.97
Treatment	4	76.8	<0.0001
Changes in C : N ratio			
Species	1	0.001	0.98
Treatment	4	16.2	<0.0001

by oven-drying at 105 °C for 48 h. In August of 2018, additional soils from the same depth interval were collected, air-dried, and passed through a 2 mm sieve, which were further used to analyze soil available N. Briefly, 10 g air-dried soils were used to extract the available N with 50 mL of 2 M KCl, and the filtered extract was measured for nitrate and ammonium by a continuous flow analyzer (Futura, Alliance Instruments, Frépillon, France). At the peak of the growing season of 2018 (*i.e.,* the early August), the aboveground biomass was determined by clipping all the plants using a 0.5 × 0.5 m quadrat within each plot. Within the clipped area, a soil core (10 cm in diameter and 20 cm in depth) was collected, and roots were separated from soils by washing to determine belowground biomass. All plant materials were oven-dried at 85 °C for 48 h to obtain the dry weight of biomass.

2.3. Data analysis

We calculated treatment effects on root C and N exudation rates and C: N ratios of root exudates based on monthly mean values. The effect sizes (%) were calculated as:

$$100 \times \frac{T - control}{control}$$

and averaged across three monthly measurements, where *T* represents each treatment. We used a two-way ANOVA to detect the effect of species and treatment on these effect sizes. Significant differences between treatments were then detected using Tukey's HSD test. In addition, the same equation was used to calculate changes in plant aboveground, belowground, and total biomass in response to different

nitrogen and rainfall manipulative treatments. Changes in root C and N exudation rates and C: N ratios of root exudates were correlated with changes in plant biomass. All statistical analyses were performed using R 3.6.1 software (R Core Team 2019).

3. Results

Mean daily temperature and total rainfall from July to September of 2018 were 18.4 °C and 135.2 mm, respectively (Figure S2). Compared to the control treatment (9.62 ± 0.08%, n = 60), rainfall reduction (8.95 ± 0.09%, n = 60) reduced soil gravimetric water content in the 0–10 cm soil depth by 7.0% while rainfall addition (10.50 ± 0.08%, n = 60) increased it by 9.1%, irrespective of N treatment (Fig. 1 and S3). Compared to the control treatment (nitrate = 2.01 ± 0.05 mg/kg, ammonium = 7.57 ± 0.14 mg/kg, and total inorganic N = 9.58 ± 0.16 mg/kg, n = 15), N addition increased soil nitrate (4.72 ± 0.10 mg/kg, n = 15), ammonium (10.97 ± 0.24 mg/kg, n = 15), and total inorganic N (15.70 ± 0.30 mg/kg, n = 15) in the 0–10 cm soil depth by 134.8%, 44.9%, and 63.9%, irrespective of rainfall treatments (Fig. 1 and S4).

Though root C and N exudation rates and C : N ratios of root exudates were species-specific (Figure S5-S7), their responses to N and rainfall treatments did not differ significantly between two dominant species (i. e., *Allium polyrhizum* and *Stipa krylovii*) (Fig. 2 and Table 1), potentially indicating consistent effects of these treatments on root exudation rates across species in this temperate steppe. Compared to the control treatment, all N and rainfall treatments increased root C exudation rates for both species, except for the rainfall addition treatment on *Allium polyrhizum* (Fig. 2a and Table 1). Rainfall reduction significantly increased root N exudation rates for both species while other treatments decreased them (Fig. 2b and Table 1). Concurrently, rainfall reduction significantly reduced C : N ratios of root exudates for both species while other treatments increased them, except for rainfall addition on *Allium polyrhizum* (Fig. 2c and Table 1).

Compared to the control treatment, rainfall reduction decreased plant aboveground, belowground, and total biomass, but other treatments (*e.g.*, rainfall addition, N addition, rainfall addition + N addition) increased these parameters (Figure S8 and S9). Changes in root C exudation rates were not related to changes in plant biomass in response to N and rainfall treatments (Fig. 3 and S10). However, increases in plant biomass were associated with decreases in root N exudation rates while an opposite pattern was found between changes in plant biomass and in C : N ratios of root exudates (Fig. 3 and S10).
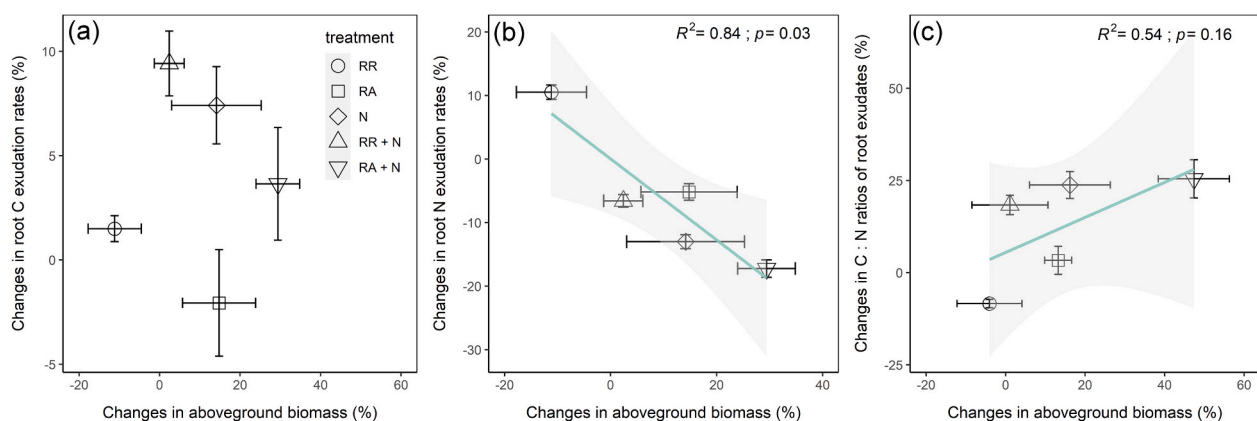
Fig. 3. Relationships between changes in plant aboveground biomass (%) and changes in root carbon (C) exudation rates (%) (a), nitrogen (N) exudation rates (%) (b), and C : N ratios of root exudates (%) (c) in a temperate steppe. Changes in root C and N exudation rates and C : N ratios of root exudates in response to different N and rainfall treatments were averaged overall two species and three measurements of the growing season in 2018 (n = 6). Changes in aboveground biomass were measured in August of the growing season and were averaged overall five blocks (n = 5). See Figures S8-S10 for more details on changes in plant aboveground, belowground, and total biomass and their relationships with changes in root C and N exudation rates and C: N ratios of root exudates. RR, RA, N, RR + N, and RA + N indicate rainfall reduction, rainfall addition, N addition, rainfall reduction + N addition, and rainfall addition + N addition, respectively.

4. Discussion

Our measurements on soil water content and total inorganic N concentration provided robust evidence that rainfall and N treatments have effectively altered soil water and N availability in this *in-situ* experiment (Figures S3 and S4). These changes in soil resource availability have substantially modified root C and N exudation rates and C : N ratios of root exudates across two dominant species in this temperate steppe (Figures S5-S7). However, rainfall and N treatments on root exudation rates did not differ significantly between these two examined species (Fig. 2 and Table 1). Therefore, our following discussion centered on treatment effects only.

Though variations existed across treatments, we found greater changes in root N exudation rates than C exudation rates in response to altered soil water and N availability (Fig. 2 and Table 1). We speculate that changes in resource availability have altered the composition of root exudates in this temperate steppe, especially for the relative proportion of organic compounds containing N, such as amino acids and proteins. However, rainfall reduction increased root N exudation rates while other treatments decreased (Fig. 2b). Though there are limited empirical studies on metabolites of root exudates in response to environmental changes (van Dam and Bouwmeester 2016), Gargallo-Garriga et al. (2018) found that roots of *Quercus ilex* exuded greater concentrations of abscisic acid, choline, terpenoids, proline, aspartate, leucine, acacetin and maleic acid under water stress, in which a majority of them are N-rich amino acids. In addition, plant roots under water stress have been observed to release more enzymes (protein-based compounds) to the rhizosphere for nutrient acquisition (Song et al., 2012; Preece and Peñuelas 2016), which might be extended to mycorrhizal constituents, but no studies have investigated this yet.

Another possible explanation for substantial changes in root N exudation rates in response to rainfall and N treatments may be related to corresponding changes in plant growth. Rainfall addition, N addition, and their interaction substantially increased plant total biomass by 13%, 16%, and 47%, respectively, while rainfall reduction decreased it by 4% (Figure S8 and S9). Greater plant growth requires more growth-limiting nutrients, which can be fulfilled by root acquisition from the external soil environment and/or enhanced internal distribution, cycling, and retention of these nutrients (Perring et al., 2008). In addition, multiple meta-analyses have indicated that N addition can increase foliar N content by 7–27% (Lu et al., 2011; Yuan and Chen 2015; Zhang et al., 2018). Therefore, it is conceivable that increased plant growth in response to rainfall and N addition leads to greater plant N retention, which results in a reduction in root N exudation rates. This inference is supported by our result that changes in root N exudation rates decreased with increasing plant biomass in response to rainfall and N treatments (Fig. 3 and S10).

Changes in root C and N exudation rates resulted in altered C : N ratios (range from 5–9.0) (Fig. 2c and Table 1), indicating the stoichiometric plasticity of root exudates in response to changes in resource availability. Existing limited evidence suggest that C : N ratios of root exudates can range from 0.2–18 but generally fall between microbial and soil C : N ratios (Mergel et al., 1998; Uselman et al., 1999; Zhang et al., 2016). It is worth noting that we selected a 0.22 μm filter in accordance with Phillips et al (2008), which may filter out some large sizes of dissolved organic carbon (<0.45 μm) and potentially reduce C : N ratios of root exudates. Despite this, such changes in C : N ratios of root exudates have significant implications on soil C and N cycles. For example, both warming and elevated CO_2 increased C : N ratios of root exudates, which in turn stimulate microbial N mineralization to meet nutrient demands for soil microbes, and to a lesser extent, for enhanced plant growth (Grayston et al., 1998; Zhang et al., 2016). This mechanism may also apply to our site where plant growth was promoted by increased soil resource availability (Figure S8-S9). Increased soil microbial activity for N acquisition due to greater C : N ratios of root exudates may also prime the decomposition of otherwise persistent organic C (Kuzyakov 2002; Xiao et al., 2015; Liu et al., 2020), potentially offsetting the contribution of root derived-C to soil organic matter formation (Sokol et al., 2019). However, the evidence for stimulated C and N cycles due to increased C : N ratios of root exudates is still limited, additional work on evaluating changes in soil microbial activities and processes related to C and N mineralization are required.

5. Conclusion and perspectives

Overall, our results provide robust evidence showing that C : N stoichiometric ratios of root exudates are sensitive to changes in soil water and N availability in this temperate steppe, indicating shifts in chemical signatures of root exudates. The experiment and dataset presented here are preliminary, but offer a template for further work that will expand this first result. Future work should integrate the advances in metabolomics to comprehensively identify changes in the composition of root exudates in response to resource disturbances (van Dam and Bouwmeester 2016), and take the advantages of metagenomic sequencing to link changes in metabolites to soil microbial community composition in the rhizosphere (Sasse et al., 2018). A better understanding of the potential roles that different metabolites are playing in the interactions between plants and soil biota is critical to study soil C and N cycles in response to environmental changes. In addition, although our results found consistent effects of rainfall and N treatments on root C and N exudation rates across two dominant species in this temperate steppe, it is worth exploring how other plant functional traits (e.g., N-fixer vs non-N-fixer, endo-vs. ecto-mycorrhizae) may quantitatively and qualitatively affect root exudates against abiotic stresses (Narula et al., 2012; Coskun et al., 2017). Lastly, our experiment examined short-term responses (*i.e.,* overall a growing season) of root C and N exudation rates to changes in soil water and N availability, it remains largely unknown about long-term responses and how the composition of root exudates may change with plant adaptation and acclimation to environmental changes. However, a mechanistical understanding of how changes in the composition of root exudates affect plant-soil-microbe interactions and ecosystem nutrient cycles still remains a challenge. This knowledge is necessary to understand responses of natural ecosystems to anticipated environmental changes.

Author contributions

C.X. designed this study; C.L., L.L., L.Z, and Y.Y. conducted the study; Y.Z. drafted the manuscript with inputs from C.L. and R.M.M.; all authors contributed to revisions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108384.

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