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# Nitrogen and phosphorus addition did not affect neutral sugars in plant but decreased them in soil in an alpine grassland on the Tibetan Plateau

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#### ARTICLE INFO

Keywords: Neutral sugars Plant- and microbial-derived N and P addition Alpine grassland

#### ABSTRACT

As crucial carbon (C) and energy sources for soil microorganisms, neutral sugars are essential component of the labile soil organic carbon (SOC) pool derived from plants and microorganisms and can directly affect the accumulation of SOC. However, the changes in labile SOC components with elevated soil nitrogen (N) and phosphorus (P) inputs remain unclear. Taking advantage of a 10-year field N and P addition experiment in an alpine grassland on the Tibetan Plateau, we analyzed the compositions and concentrations of neutral sugar in plant versus soil, coupled with soil nutrient properties and plant biomass, we assessed factors affecting the preservation of neutral sugars in soil. We found that the concentrations of neutral sugar were lower in plant shoots than those in roots. The concentrations of neutral sugar and the GM/AX ratio (GM: galactose and mannose; AX: arabinose and xylose) in shoots and roots were insensitive to nutrient addition. The concentrations of neutral sugar in the topsoil were significantly lower than those in the subsoil, and both significantly decreased with N alone and P alone addition. The GM/AX ratio was significantly higher in the topsoil than that in the subsoil and both increased with nutrient addition (except for NP addition in the topsoil). Additionally, SOC was negatively correlated with soil neutral sugar concentrations but positively correlated with the GM/AX ratio. This result emphasizes the important role of microbial transformation of neutral sugars in regulating the relative magnitude of the labile SOC pool versus the stable SOC pool, which is consistent with the concept of "microbial carbon pump" (MCP). This study demonstrates differential compositions and concentrations, as well as responses of neutral sugars to N and P addition in plant versus soil, providing new information to understand the effect of nutrient addition on the SOC pool in the plant-soil system.

#### 1. Introduction

Carbon (C) is more abundant in soil than in the combined atmosphere and vegetation, making soil the world's largest C reservoir (IPCC, 2014; Lehmann and Kleber, 2015). Even slight changes in this considerable soil organic carbon (SOC) stock would trigger intense terrestrial C-climate feedback (Jackson et al., 2017; Lehmann and Kleber, 2015; Wang et al., 2022a). As an important C sink that can affect global change, research on SOC stocks and the corresponding accumulation mechanisms has always been a hot concern (Bai and Cotrufo, 2022; Blonska et al., 2021; Li et al., 2021; Qi et al., 2016).

The SOC consists of a variety of components from different sources with varied chemical structures and stability, and the mean residence time ranges from days to years and millennia (Schmidt et al., 2011). Plants and microorganisms are vital contributors to SOC (Gunina and Kuzyakov, 2015; Kögel-Knabner, 2002). Plant is the primary source of SOC, which can continuously provide fresh organic matter to soil; some of them either serve as C substrates or are directly preserved in soil as

https://doi.org/10.1016/j.apsoil.2023.105028

Received 4 December 2022; Received in revised form 23 June 2023; Accepted 26 June 2023 0929-1393/© 2023 Published by Elsevier B.V.

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plant-derived compounds (Gunina and Kuzyakov, 2015). On the other hand, microorganisms are crucial drivers of SOC transformation, which convert plant-derived C into microbial C and accumulate in soil (Cotrufo et al., 2013; Liang et al., 2017). Previous studies on SOC accumulation have focused on stable plant- and microbial-derived SOC components and a growing body of evidence suggests that microbial necromass is important for SOC formation and accumulation (Liang et al., 2017; Zhu et al., 2020). Recent studies have confirmed that microbial necromass accounts for as much as >50 % of SOC in temperate agricultural (55.6 %) and grassland (61.8 %) soil (Bai and Cotrufo, 2022; Liang et al., 2020). It has been claimed that SOC formation is dominated by microorganisms with a "microbial carbon pump" (MCP), which is involved in two concurrent microbial metabolic pathways: ex vivo (extracellular) modification and in vivo pathways (Liang et al., 2017; Zheng et al., 2021). For ex vivo modification, microorganisms break down or modify macromolecules generated from plant by secreting extracellular enzymes, resulting in the build-up of plant-derived C in soil (Cui et al., 2016: Liang et al., 2017). Through in vivo turnover, small-molecular weight and labile plant-derived compounds undergo a microbial uptake-growth-death cycle and are ultimately incorporated into soil as microbial necromass and byproducts. Both pathways highlight the crucial role of microbial catabolism and anabolism in the transformation and sequestration of soil C. Under the contemporary understanding, the degradation or transformation of labile C components in plant can be described as a mechanistic "front gate" related to stabilizing SOC formation (Cui et al., 2016). Collectively, elucidating the dynamics of labile C may underpin the alterations and persistence of SOC stocks, which can provide a novel perspective to better understand the global C cycle.

Neutral sugars, also known as non-cellulosic carbohydrates, are primarily generated by plants and microorganisms and constitute 5-25 % of the total SOC (Cui et al., 2016; Feng, 2022; Kögel-Knabner, 2002; Zhang et al., 2021). They typically consist of 8 monosaccharides: xylose, arabinose, ribose, glucose, galactose, mannose, rhamnose, and fucose (Gunina and Kuzyakov, 2015; Kögel-Knabner, 2002). Pentose arabinose (A) and xylose (X) are exclusively produced by plants, whereas hexose galactose (G) and mannose (M) are derived mainly from microbes (Angst et al., 2021; Gunina and Kuzyakov, 2015; Oades, 1984). Hence, the hexose-to-pentose ratio GM/AX is usually used to estimate the relative contribution of microorganisms and plants to neutral sugars, which can more widely represent the degree of microbial transformation of labile SOC (Cui et al., 2016; Gunina and Kuzyakov, 2015; Oades, 1984; Xie et al., 2014). Therefore, it also serves as an indicator for evaluating the status of the accumulated SOC (Cui et al., 2016; Murata et al., 1999). Investigating the compositions and concentrations of neutral sugar is an effective approach for better understanding labile C pool and SOC sequestration.

Under current climate change, atmospheric nitrogen (N) and phosphorus (P) deposition have significantly increased since the industrial revolution, and consequently, their availability in soil has increased (Galloway et al., 2008; Pan et al., 2021; Wang et al., 2017). The N and P are essential nutrients that limit plant growth and microbial function in most ecosystems (Elser et al., 2007; Wieder et al., 2015). Numerous studies have shown that plant and microbial communities are sensitive to additional N and P inputs into the soil (Chen et al., 2014; Chen et al., 2022b; Han et al., 2019; Jeske et al., 2018). Enriched N and P enhance plant production, reduce plant diversity, and alter plant biosynthetic processes (Gao et al., 2019; Henning et al., 2021; Yoneyama et al., 2012). Similarly, it has been reported that additional N and P inputs affect microbial communities in terms of population abundance, species diversity, and functional activity (Ma et al., 2020; Sun et al., 2015; Wang et al., 2018b; Yang et al., 2022). The effects of N and P addition on microbial growth, composition, and enzyme activity vary, being neutral, stimulated, or suppressed (Jing et al., 2016; Liu et al., 2012; Wang et al., 2018a; Wang et al., 2008; Xiao et al., 2018; Zhang et al., 2018). The complex responses of plants and microorganisms to N and P addition make the compositions and concentrations of SOC components

unpredictable. Hence, it is crucial to probe the compositions and concentrations of neutral sugar with increasing N and P inputs to better understand the transformation and accumulation patterns of labile and stable SOC.

The Tibetan Plateau has been a net C sink, which stores 73.6 Pg C in the upper 3 m of soil (Chen et al., 2022a). In recent years, intensive human activities have enhanced the input of N and P in the alpine grasslands of the Tibetan Plateau (Chen et al., 2022a; Wang et al., 2022b). Increased N and P inputs to soil may exert significant impacts on C cycling in fragile Tibetan Plateau ecosystems (Liu et al., 2018; Wang et al., 2012). Thus, we conducted a 10-year N and P addition field experiment in an alpine grassland on the Tibetan Plateau to quantify the effect of N and P addition on the labile SOC pool. The objectives of this study were to (1) investigate the effects of N and P addition on the compositions and concentrations of neutral sugar in plant and soil and (2) assess the factors influencing the concentrations of neutral sugar in soil by combining plant biomass and soil nutrient properties with N and P addition. We hypothesized that (1) decreased concentrations of neutral sugar in plant with nutrient addition, (2) unchanged concentrations of neutral sugar in soil with nutrient addition due to enhanced growth of both plants and microorganisms, but increased the GM/AX ratio with nutrient addition due to increased microbial transformation, and (3) more concentrated neutral sugars with a lower GM/AX ratio accumulates in the topsoil than in the subsoil owing to direct plant inputs to the topsoil.

#### 2. Materials and methods

#### 2.1. Study area

The experiment was carried out on a typical alpine grassland at the Haibei Alpine Grassland Ecosystem Research Station  $(37^{\circ}29'-37^{\circ}45' \text{ N}, 101^{\circ}12'-101^{\circ}23' \text{ E}; 3220 \text{ m}$  above sea level), which is located northeast of the Tibetan Plateau in Qinghai Province, China. The research station experiences a typical continental monsoon climate, with an average annual temperature of  $-1.1 \,^{\circ}$ C and annual precipitation of 480 mm, 80 % of which occurs during the growing season from May to September. The soil is classified as a Mat-Gryic Cambisol with a clay loam texture (IUSS Working Group WRB, 2007). In the experimental site, the basic soil properties (at the depth of 10 cm) are: total C, 78.2 g kg<sup>-1</sup>; organic carbon (OC), 63.1 g kg<sup>-1</sup>; N, 5.75 g kg<sup>-1</sup>; P, 0.79 g kg<sup>-1</sup>; clay content, 65.3 %; bulk density, 0.75 g cm<sup>-3</sup>; pH, 7.85. The dominant native plant community consists of *Elymus nutans, Stipa aliena*, and *Gentiana straminea* (Ma et al., 2017).

#### 2.2. Experimental design

The N and P addition experiment on the Tibetan Plateau was established in May 2011, and N and P addition has been carried out so far. The field experiment was conducted using a randomized complete block design, with 6 blocks and 6 treatments. The treatments included no addition (CK, Control), 3 levels of N addition (N1, 25 kg ha<sup>-1</sup> yr<sup>-1</sup>; N2, 50 kg ha<sup>-1</sup> yr<sup>-1</sup>; and N, 100 kg ha<sup>-1</sup> yr<sup>-1</sup>), P addition (P, 50 kg ha<sup>-1</sup> yr<sup>-1</sup>), and combined N and P addition (NP, 100 kg N ha<sup>-1</sup> yr<sup>-1</sup> plus 50 kg P ha<sup>-1</sup> yr<sup>-1</sup>). Six blocks were set for each treatment as replicates with 36 plots (6 m × 6 m). To avoid interference, there were 1 m and 2 m buffer strips between the plots and blocks, respectively. At the beginning of June, July, and August, N fertilizer (urea) and P fertilizer (triple superphosphate) were separated into three equal portions and manually applied to the ground surface after sunset under conditions of high moisture levels and low temperature.

#### 2.3. Plant sampling and analysis

Plants were sampled on 25–26 August 2020, during the peak growing season. For plant aboveground biomass, shoots were clipped at

the ground level in one randomly selected  $25 \times 25$  cm quadrat within 6 treatments of 6 blocks (6 replicates, 36 plots in total), and each quadrat was collected once and sorted into species. Correspondingly, plant roots (mixed) were obtained from each soil layer at 10 cm intervals between 0 and 30 cm, using a root drill with a diameter of 7 cm. Finally, the above- and belowground plant samples were oven-dried at 65 °C until they reached constant weight.

Plant samples from CK, N, P, and NP in 3 blocks were used for subsequent analysis. The mixed roots from each treatment were selected from 3 blocks (3 replicates). For plant shoots, the dominant 9 species that contributed 80 % of the total aboveground biomass were first identified and then separated from the corresponding treatment. Finally, 8 species in the CK, 7 species in the N, 8 species in the P, and 5 species in the NP were selected from the 3 blocks. The plant shoots of a single species from 3 blocks of the corresponding treatment were mixed. We then classified the shoots into grasses, sedges, legumes, and forbs, based on plant functional groups (Fig. S1).

The concentrations of OC and total nitrogen (TN) in plant were measured using an elemental analyzer (Elementar vario EL cube, Germany). The TP of plant was extracted by digestion of samples in  $H_2SO_4$ -HClO<sub>4</sub> and analyzed using an intermittent chemical analyzer (AMS Smarchem450, Italy).

#### 2.4. Soil sampling and analysis

Soil samples from the 6 treatments in 6 blocks (6 replicates, 36 plots in total) were collected on 24 August 2020. Four treatments (CK, N, P, and NP) and 3 blocks (3 replicates each) were selected for this study. For each studied plot (4 treatments, 3 replicates, 12 plots in total), 3 soil cores were obtained from the top- (0–10 cm) and subsoils (20–30 cm). Following the removal of grass roots and stones, the soil samples were mixed completely to create one composite sample for the top- and subsoils and then passed through a 2-mm sieve. The sieved soil samples were then immediately transported to the laboratory on ice and freezedried in vacuum for further analysis.

The concentrations of OC and TN in soil were measured using an elemental analyzer (Elementar vario EL cube, Germany), with inorganic C eliminated using 1 M hydrochloric acid before SOC determination. The soil total P (TP) was extracted by digestion of samples in  $H_2SO_4$ -HClO<sub>4</sub> and analyzed using an intermittent chemical analyzer (AMS Smarchem450, Italy).

#### 2.5. Extraction and analysis of neutral sugars

Neutral sugars were extracted using a modified procedure, based on the methods described by Amelung et al. (1996) and Liu et al. (2019). Briefly, samples containing approximately 4 mg of OC were hydrolyzed using 10 mL of 4 M trifluoroacetic acid (TFA) at 105 °C for 4 h. Then, the mixture was evaporated to dryness at 45 °C using a rotary evaporator after 100 µg adonitol was introduced as the quantitative internal standard. The residue was dissolved in ultrapure water and centrifuged at 3000 rpm for 10 min after using potassium hydroxide and TFA to obtain a pH of 6.6-6.8, after which the supernatant was dried once more with a rotary evaporator. After removing the added anhydrous methanol under a stream of N2, 1 mL of ultrapure water and 100 µg of myo-inositol (recovery of the internal standard) were added. These ingredients were then transferred to 3 mL of Reacti-Vial™ and freeze-dried for at least 8 h. The freeze-dried sample was added with 300  $\mu$ L derivative reagent (32 mg mL<sup>-1</sup> hydroxylamine hydrochloride, 40 mg mL<sup>-1</sup> 4dimethylamino-pyridine in 4:1 ( $\nu/\nu$ ) pyridine-methanol) and heated for 30 min at 75 °C. After the sample was cooled, 1 mL of acetic anhydride was added, and the mixture was heated for 20 min at 75  $^\circ$ C. The phase was separated with 1.5 mL dichloromethane once the derivatization was completed. Furthermore, 1 mL of hydrochloric acid and 1 mL of ultrapure water were successively added to remove impurities and extract the excess derivative reagents completely.

Neutral sugars were determined using gas chromatography (GC) coupled to a single quadrupole mass spectrometer (Shimadzu QP2020, Japan) with a DB-5 fused silica capillary column (30 m  $\times$  0.25 mm  $\times$  0.25 µm). The GC temperature program was as follows: a column temperature of 100 °C was first kept for 3 min, after which it was increased by 3 °C/min to 230 °C for 2 min and then by 8 °C/min to 300 °C for 5 min. Adonitol served as the internal standard for the GC measurements of neutral sugars, which included 8 monosaccharides in one sample. They can be divided into pentose (arabinose, xylose, and ribose) and hexose (glucose, galactose, mannose, rhamnose, and fucose). Analytical errors of recovery efficiency were typically <15 % based on repeated analyses of the plant and soil samples. The concentrations of neutral sugar were determined as the sum of 8 monosaccharides. The hexose-topentose ratio GM/AX (GM: sum of galactose and mannose; AX: sum of arabinose and xylose) was calculated.

#### 2.6. Statistical analysis

Before statistical analysis, all data sets were tested for normality using the Shapiro-Wilk test. If normality was not observed, as in most cases, non-parametric analysis was performed. Two-way analysis of variance (ANOVA) was used to test the effects of nutrient addition and plant organs or soil depths on concentrations of neutral sugar and the GM/AX ratio, with treatments, plant organs, or soil depths as independent variables. The effects of nutrient addition on plant properties, concentrations of neutral sugar, and the GM/AX ratio in plant shoots and roots were examined using a one-way ANOVA. Similarly, the effects of nutrient addition on soil properties, concentrations of neutral sugar, and the GM/AX ratio in the top- and subsoils were examined using a one-way ANOVA. Duncan's multiple range test (DMRT) was used to examine the variations among nutrient addition treatments. When the homogeneity of variances was unequal, Tamhan's T2 was used to assess the variations among the nutrient addition treatments. The differences in the concentrations of neutral sugar between plant shoots and roots or topsoil and subsoil and the differences in neutral sugars between pentose and hexose for each plant functional group were examined using the t-test or Mann-Whitney U test. Additionally, the block effects on soil and plant root parameters were considered, and the results showed that the block effects were not significant for the soil and plant root variables (p >0.05). All statistical analyses were performed using SPSS 24.0 (IBM SPSS Statistics 24.0, Chicago, IL).

Pearson's correlation was performed to examine the effect of plant biomass (above- and belowground) and soil nutrient properties on concentrations of soil neutral sugar and the GM/AX ratio with R software (version 4.1.3). Soil nutrient properties (TN was excluded because of its strong collinearity with SOC) showing significant correlations with the investigated parameters were selected for multiple stepwise regression to further elucidate the most important factors using SPSS 24.0 (IBM SPSS Statistics 24.0, Chicago, IL). Pearson's correlation and multiple stepwise regression analyses were performed using log10transformed data. The significance level was set at 0.05 for all statistical methods.

#### 3. Results

#### 3.1. Plant biomass and nutrient properties

The below ground biomass (1123.2  $\pm$  157.7 g m<sup>-2</sup>) was 3.4 times higher than that of a boveground biomass (334.4  $\pm$  37.0 g m<sup>-2</sup>; Fig. 1). Compared with CK, the a boveground biomass increased with NP addition (p < 0.05); however, the below ground biomass decreased with N alone and NP addition (p < 0.05; Fig. 1).

For plant shoots, the concentrations of OC decreased with N alone addition but increased with P alone addition (p < 0.05; Table 1). The concentrations of TN increased with N alone and NP addition and the concentrations of TP increased with P alone and NP addition (p < 0.05).



**Fig. 1.** Effects of N and P addition on above- and belowground biomass. Bars indicate mean  $\pm$  SE (n = 3). Bars with different capital letters indicate significant differences between above- and belowground biomass (p < 0.05). Bars with different lowercase letters indicate significant differences among nutrient addition treatments (p < 0.05).

Additionally, opposite changes were observed between the OC and TN: TP ratio, TN and OC:TN ratio, and TP and OC:TP ratio with the corresponding treatments mentioned above. For plant roots, the concentrations of OC, TN, and the OC:TN ratio did not respond significantly to nutrient addition. The concentrations of TP and the TN:TP ratio in roots showed a consistently increasing trend, whereas the OC:TP ratio showed a decreasing trend with N alone and NP addition (Table 1).

#### 3.2. Soil nutrient properties

In the topsoil, compared with CK, the concentrations of SOC were higher with N alone and NP addition (p < 0.05), but no distinct change was found with P alone addition (p > 0.05; Table 1). There were no changes in TN and the SOC:TN ratio in response to N and P addition (p > 0.05). With the addition of P alone or NP, the concentrations of TP increased (p < 0.05). The SOC:TP and TN:TP ratios both decreased by 21.1–27.2 % with the addition of N alone and 67.7–71.9 % with the addition of P alone and NP (p < 0.05; Table 1). In the subsoil, nutrient addition did not affect the aforementioned parameters (p > 0.05;

#### Table 1).

#### 3.3. Neutral sugars in plant

The concentrations of neutral sugar in plant shoots and roots were  $453.1 \pm 28.2 \text{ mg g}^{-1}$  OC on average, with no significant responses to different nutrient addition (Fig. 2a). In comparison, the concentrations of neutral sugar were higher in plant roots than those in shoots (p < 0.05; Fig. 2a). Among these, xylose was the most abundant neutral sugar monosaccharide, followed by glucose in shoots, arabinose in roots, and then galactose in shoots and roots, which together accounted for >90 % of the neutral sugars in shoots and roots (Fig. 3). The concentrations of the 8 neutral sugar monosaccharides showed no marked differences with N and P addition (p > 0.05), nor did those of pentose and hexose (p> 0.05; Fig. 3). The GM/AX ratio was 0.33  $\pm$  0.05 in shoots and 0.27  $\pm$ 0.03 in roots on average (Fig. 2b). No difference was found between the plant shoots and roots, and no significant difference was found in the GM/AX ratio among the different treatments (Fig. 2b). Furthermore, neutral sugars in plant were investigated based on plant functional groups, including the concentrations of neutral sugar and the GM/AX ratio in plant shoots (Fig. S1).

#### 3.4. Neutral sugars in soil

The concentrations of neutral sugar in the topsoil were  $169.5 \pm 21.8$ mg g  $^{-1}$  SOC on average, lower than those in the subsoil (496.1  $\pm$  43.0 mg g<sup>-1</sup> SOC; p < 0.05; Fig. 5a). Compared with CK, the concentrations of neutral sugar decreased notably with N alone and P alone addition (p <0.05), whereas no difference was found with NP addition in both the topand subsoils (p > 0.05; Fig. 4a). Furthermore, the concentrations of monosaccharides all notably decreased with the addition of N alone and P alone (except glucose in the subsoil), showing trends consistent with the total concentrations of neutral sugar (p < 0.05; Fig. 5). Among the neutral sugar monosaccharides, glucose (30.0 %) was the most abundant, followed by galactose (19.6 %) and mannose (15.5 %) (Fig. 5). Consequently, hexose accounted for 75.8  $\pm$  1.0 % and 68.3  $\pm$  1.5 % of the total concentrations of neutral sugar in the top- and subsoils, respectively (p < 0.05; Fig. 5). The GM/AX ratio in soil (1.5  $\pm$  0.08; Fig. 4b) was higher than that in plant (0.3  $\pm$  0.04; Fig. 2b), with higher values in the topsoil (1.8  $\pm$  0.10) than in the subsoil (1.3  $\pm$  0.06; p <0.05; Fig. 4b). Compared with CK, the GM/AX ratio was higher with nutrient addition (p < 0.05, except for the NP treatment in the topsoil;

#### Table 1

Concentrations of organic carbon (OC), total nitrogen (TN), total phosphorus (TP), and the ratio of OC:TN, OC:TP, TN:TP in plant and soil. Mean and standard errors are shown.

Sample	Treatment	OC (mg $g^{-1}$ )	TN (mg $g^{-1}$ )	TP (mg $g^{-1}$ )	OC:TN	OC:TP	TN:TP
Plant							
Shoot	CK (n = 8)	$385.2\pm9.7~\mathrm{b}$	$17.2\pm1.3~\mathrm{c}$	$4.8\pm0.4~\text{b}$	$23.3\pm1.7~\mathrm{a}$	$83.4\pm8.5~a$	$3.6\pm0.4~\text{b}$
	N (n = 7)	$357.2\pm5.1~\mathrm{c}$	$25.2\pm1.3$ a	$4.8\pm0.4~\text{b}$	$14.5\pm0.8~c$	76.5 $\pm$ 5.7 ab	$5.4\pm0.4~\text{a}$
	P (n = 8)	$410.7\pm2.4~\mathrm{a}$	$18.7\pm1.6~bc$	$8.1\pm0.7~a$	$22.9 \pm 1.8 \text{ ab}$	52. 6 $\pm$ 3.7 bc	$2.2\pm0.2~c$
	NP (n = 5)	$397.9 \pm 14.5 \text{ ab}$	$22.6 \pm 1.7$ ab	$7.6\pm0.3$ a	$18.2\pm1.8~\mathrm{bc}$	$52.8\pm3.9~\mathrm{c}$	$2.9\pm0.1\ bc$
Root	CK (n = 3)	$\textbf{326.4} \pm \textbf{59.7}$	$10.8\pm1.9~\text{ab}$	3.5	$31.1\pm 6.6$	121.2	2.7
	N (n = 3)	$309.6\pm35.7$	$12.0\pm1.7~\mathrm{ab}$	3.7 (+7.9 %)	$26.0 \pm 1.7$	82.2 (-32.2 %)	3.1 (+12.6 %)
	P (n = 3)	$270.0\pm91.4$	$6.2\pm0.6~b$	n.a.	$\textbf{42.4} \pm \textbf{11.8}$	n.a.	n.a.
	NP (n = 3)	$397.2\pm4.5$	$15.4\pm2.5~\text{a}$	6.5 (+88.2 %)	$\textbf{27.0} \pm \textbf{3.8}$	60.6 (-50.0 %)	3.1 (+14.1 %)
Soil $(n = 3)$							
Topsoil (0–10 cm)	CK	$62.3\pm3.8~\mathrm{b}$	$5.5\pm0.3$ ab	$0.3\pm0.04~c$	$11.3\pm0.2$	$211.6\pm14.3~\mathrm{a}$	$18.8\pm1.5~\mathrm{a}$
	Ν	$73.3\pm0.8~\text{a}$	$6.0\pm0.2$ ab	$0.4\pm0.02\ c$	$12.2\pm0.5$	$167.0\pm9.4~\mathrm{b}$	$13.7\pm0.2~b$
	Р	$58.1\pm3.4~b$	$5.1\pm0.3~\mathrm{b}$	$0.9\pm0.07~b$	$11.3\pm0.3$	$68.3\pm4.5~\mathrm{c}$	$6.0\pm0.2\ c$
	NP	$74.8\pm4.3~a$	$6.2\pm0.4$ a	$1.2\pm0.07~a$	$12.0\pm0.2$	$63.5\pm1.3~\mathrm{c}$	$5.3\pm0.2~c$
Subsoil (20-30 cm)	CK	$\textbf{28.9} \pm \textbf{2.8}$	$3.0\pm0.2$	$0.6\pm0.03$	$9.5\pm0.4$	$\textbf{48.7} \pm \textbf{6.7}$	$5.1\pm0.5$
	Ν	$\textbf{27.4} \pm \textbf{0.9}$	$2.9\pm0.1$	$0.6\pm0.02$	$9.6\pm0.3$	$\textbf{45.5} \pm \textbf{1.9}$	$\textbf{4.8} \pm \textbf{0.4}$
	Р	$31.3\pm4.7$	$3.1\pm0.5$	$0.6\pm0.08$	$10.1\pm0.1$	$53.8 \pm 6.4$	$\textbf{5.3} \pm \textbf{0.6}$
	NP	$28.6\pm0.3$	$\textbf{2.9} \pm \textbf{0.1}$	$0.6\pm0.04$	$\textbf{9.9}\pm\textbf{0.4}$	$\textbf{45.5} \pm \textbf{5.2}$	$\textbf{4.7} \pm \textbf{0.7}$

CK, control; N, N-alone addition; P, P-alone addition; NP, N and P together addition. n.a., not available. Different lowercase letters indicate significant differences among nutrient addition treatments (p < 0.05).



**Fig. 2.** Effects of N and P addition on neutral sugar concentrations (a) and GM/AX ratio (b) in plant shoots and roots. The effects of plant organs (O; shoots and roots), nutrient addition treatments (T), and their interaction (O  $\times$  T) are shown. Bars indicate mean  $\pm$  SE (shoots: n = 8 in CK and P treatments, n = 7 in N treatment, n = 5 in NP treatment; roots: n = 3). Bars with different capital letters indicate significant differences between shoots and roots. The absence of letters on the error bars indicates no significant differences among the nutrient addition treatments. n.s., not significant. GM/AX: (galactose and mannose)/ (arabinose and xylose). CK, control; N, N alone addition; P, P alone addition; and NP, N and P together addition.

#### Fig. 4b).

#### 3.5. Factors affecting concentrations of neutral sugar in soil

Pearson's correlation analysis was conducted to determine whether the concentrations of neutral sugar in soil were driven by plant biomass or soil nutrient properties (Fig. 6). The results showed no correlation between above- or belowground biomass and the concentrations of neutral sugar, pentose, hexose, or GM/AX ratio (p > 0.05; Fig. 6). In comparison, the concentrations of SOC and TN and the SOC:TN, SOC:TP, and TN:TP ratios were all negatively correlated with the concentrations of neutral sugar, pentose, and hexose in soil (p < 0.05; Fig. 6). The concentrations of SOC and TN and the ratios of SOC:TN and SOC:TP were positively correlated with the GM/AX ratio (p < 0.05; Fig. 6). An additional multiple stepwise regression analysis confirmed that SOC was the most important factor affecting the concentrations of neutral sugar, pentose, and hexose in soil ( $R^2 = 0.68$ , 0.66, and 0.69, respectively), whereas the SOC:TN ratio was the dominant factor affecting the GM/AX ratio ( $R^2 = 0.44$ ; p < 0.05; Table 2).

#### 4. Discussion

## 4.1. Effects of N and P addition on compositions and concentrations of neutral sugar in plant

Plant contributes a large proportion to neutral sugars in soil; however, there have been few studies on the concentrations of neutral sugar in plant in an alpine grassland on the Tibetan Plateau (Guan et al., 2018). We found the concentrations of neutral sugar in plant were 413.5  $\pm$  25.9 mg g  $^{-1}$  OC in shoots and 545.3  $\pm$  66.6 mg g  $^{-1}$  OC in roots on average, respectively (Fig. 2). The concentrations of neutral sugar in shoots and roots were close to those in shoots of herbs (437.5  $\pm$  4.6 mg  $g^{-1}$  OC) and in roots of *Picea abies* (494 mg  $g^{-1}$  OC) in the Bavarian Limestone Alps, respectively (Prietzel et al., 2013). On average, the GM/ AX ratio was  $0.33 \pm 0.05$  in shoots and  $0.27 \pm 0.03$  in roots (Fig. 2b). The GM/AX ratio in plant indicates the compositions of plant neutral sugar and can affect the compositions of neutral sugar in soil. In other words, the high GM/AX ratio in soil may be induced by high concentrations of hexose in plant but not by the high contribution of microorganisms (Gunina and Kuzyakov, 2015). We found that the GM/AX ratio in shoots and roots was lower than that in herbs (0.41-0.52) and trees (0.37-1.48) in an alpine grassland and a tropical and subtropical forest, respectively (Cui et al., 2016; Guan et al., 2018), but was close to that in trees (0.21-0.60) in German forests with a continental climate (Spielvogel et al., 2007). The differences in the GM/AX ratio of plant suggest that considering the GM/AX ratio in plant is important when applied to different treatments and ecosystems. Additionally, we evaluated the differences in neutral sugars in shoots among plant functional groups, the details of which are presented in the Supplementary Material (Fig. S1). These results appended a database on plant neutral sugar compositions and concentrations in the alpine grassland on the Tibetan Plateau and provided a reference for further studies exploring the labile C component in soil.

Continuous 10-year N and P addition did not significantly change the compositions and concentrations of neutral sugar in plant shoots and roots (Figs. 2-3). As neutral sugars can act as structural compounds that link cellulose and lignin, they can sustain plant shape and form a barrier for plant cell walls through the cell wall matrix (Du et al., 2022; Kögel-Knabner, 2002; Scheller and Ulvskov, 2010). When the external environment changes, neutral sugar synthesis is regulated to maintain physiological functions (Fernandes et al., 2013; Zhu et al., 2012). Kaakinen et al. (2004) found that concentrations of neutral sugar in hydroponic spruce seedlings decreased with a 6-month higher N addition, owing to the deposition of neutral sugars in plant secondary cell wall. Plant has evolved strategies to respond to changing environments and guarantee their growth and development to survive and reproduce (Ding et al., 2019; Gong et al., 2020). The similar concentrations of neutral sugar in response to nutrient addition in plant in this study indicated that the synthesis of plant cell walls was not affected, which may be the consequence of plant adaptation to 10-year N and P addition, but further verification is required. The vegetation in the studied alpine grassland is dominated by perennials (Ma et al., 2017), their roots could survive in soil for more than two years, and the 10 years of continuous nutrient addition seemed to be periodic fluctuations for plant accompanied by growth rhythm. Therefore, inconsistent with our first hypothesis, the concentrations of neutral sugar did not change with N and P addition.

## 4.2. Effects of N and P addition on compositions and concentrations of neutral sugar in soil

Inconsistent with our second hypothesis, we found that the concentrations of neutral sugar significantly decreased with N alone and P alone addition in the top- and subsoils (Fig. 4a). N and P are considered the most important limiting elements for plant (Hou et al., 2020; LeBauer and Treseder, 2008; Vitousek and Howarth, 1991), especially in the Tibetan Plateau (Liu et al., 2018). In the concept of stoichiometry,



**Fig. 3.** Effects of N and P addition on neutral sugar monosaccharide concentrations in plant shoots and roots. The effects of plant organs (O; shoots and roots), nutrient addition treatments (T), and their interaction (O  $\times$  T) are shown. Bars indicate mean  $\pm$  SE (Shoots: n = 8 in CK and P treatments, n = 7 in N treatment, n = 5 in NP treatment; Roots: n = 3). The absence of letters on the error bars indicates no significant differences among the nutrient addition treatments. n.s., not significant. CK, control; N, N alone addition; P, P alone addition; and NP, N and P together addition.



**Fig. 4.** Effects of N and P addition on neutral sugar concentrations (a) and the GM/AX ratio (b) in the top- and subsoils. The effects of soil depths (D), nutrient addition treatments (T), and their interactions (D  $\times$  T) are shown. Bars indicate mean  $\pm$  SE (n = 3). Bars with different capital letters indicate significant differences between the top- and subsoil (p < 0.05). Bars with different lowercase letters indicate significant differences among nutrient addition treatments. n.s., not significant. GM/AX: (galactose and mannose)/(arabinose and xylose). CK, control; N, N alone addition; P, P alone addition; NP, and N and P together addition.

when only the two nutrients are considered, the nutrient limitation is simply a result of the relative demand for one nutrient versus another (Agren et al., 2012; Čapek et al., 2018). It has been proved that the N alone addition can aggravate P limitation (Li et al., 2016), and prompt soil microorganisms to consume more C from neutral sugars to obtain enough energy (Cui et al., 2016; Smemo et al., 2021). A similar explanation can be applied to the P alone addition (He and Dijkstra, 2015; Vitousek et al., 2010; Zhan et al., 2017). The insignificant changes in the concentrations of neutral sugar with NP addition confirmed our interpretation. It is worth mentioning that the concentrations of neutral sugar decreased more in the subsoil when N and P were added alone (Fig. 4a). This may be caused by the high nutrient requirement and strong C limitation of microorganisms in the subsoil (indicated by the low concentrations of TN, TP, and OC:TN ratio in the subsoil; Table 1). In addition, the GM/AX ratio can be used to determine the extent of the microbial conversion of plant-derived neutral sugars (Cui et al., 2016; Gunina and Kuzyakov, 2015). We also found a consistently increasing GM/AX ratio with nutrient addition in both the top- and subsoils, suggesting that nutrient addition could enhance the microbial transformation of labile SOC components. Neutral sugars can quickly compensate for the additional C and energy demands caused by

stimulated microbial activities and nutrient demands with N and P addition (Cui et al., 2016; Gunina and Kuzyakov, 2015; Kirkby et al., 2013; Waldrop and Firestone, 2004). Therefore, all nutrient amendments increased the microbial transformation of plant-derived pentose to microbial-derived hexose, resulting in a higher GM/AX ratio.

On average, the concentrations of neutral sugar were 169.5  $\pm$  21.8 mg g  $^{-1}$  SOC in the topsoil and 496.1  $\pm$  43.0 mg  $\bar{g}^{-1}$  SOC in the subsoil, indicating it was more concentrated in the subsoil, but the GM/AX ratio was lower in the subsoil than in the topsoil (p < 0.05; Fig. 4). This result was opposite to our last hypothesis and those who observed an increased proportion of hexose with soil depth across various ecosystems (Cui et al., 2016; Folsom et al., 1974; Jia et al., 2022; Spielvogel et al., 2007). They attributed the increased relative hexose proportion to an abundance of incompletely decomposed plant litter in the topsoil (Folsom et al., 1974; Jia et al., 2022) or the preferential stabilization of microbial-derived neutral sugars by mineral interactions or incorporation into aggregates in the subsoil (Kiem and Kögel-Knabner, 2003; Rumpel et al., 2010). However, we detected a lower OC:TN ratio (p < p0.05; Table 1) and lower microbial biomass (Ma et al., 2023) in the subsoil, accompanied by a constant low temperature, which would jointly constrain microbial degradation and transformation of neutral



**Fig. 5.** Effects of N and P addition on neutral sugar monosaccharide concentrations in the top- and subsoils. The effects of soil depths (D), nutrient addition treatments (T), and their interactions (D  $\times$  T) are shown. Bars indicate mean  $\pm$  SE (n = 3). Bars with different lowercase letters indicate significant differences among the nutrient addition treatments (p < 0.05). n.s., not significant. CK, control; N, N alone addition; P, P alone addition; and NP, N and P together addition.

sugars (Amelung et al., 1999; Zhao et al., 2021). Therefore, the concentrations of neutral sugar were higher in the subsoil with a decreased proportion of hexose in the alpine grassland of the Tibetan Plateau. A lower GM/AX ratio also occurs in the subsoil of permafrost (Dao et al., 2022). This discrepancy embodied the divergence in microbial degradation preferences in various ecosystems with diverse climatic and nutrient conditions.

#### 4.3. Potential driving factors for neutral sugar concentrations in soil

We found that the concentrations of neutral sugar, pentose, hexose, and the GM/AX ratio in soil were not significantly correlated with either aboveground biomass or belowground biomass (Fig. 6). These results are contrary to those of a previous study that concluded the dominant role of aboveground biomass in determining the concentrations of neutral sugar in soil in an unfertilized forest ecosystem (Zaher et al., 2020). They attributed this to the inaccessibility of neutral sugars to microbial degradation in soil (Zaher et al., 2020). However, in our study, the significantly increased GM/AX ratio in both the top- and subsoils after 10-year N and P addition highlighted enhanced microbial transformation and degradation in both the top- and subsoils (Fig. 4b).

The concentrations of neutral sugar, pentose, and hexose in soil were negatively correlated with SOC, TN, SOC:TN, SOC:TP, and TN:TP ratios (except pentose with TN:TP ratio), but the GM/AX ratio was positively correlated with SOC, TN, and SOC:TN ratio (p < 0.05; Fig. 6). Microorganisms are thought to transform plant-derived labile C into chemically or physically stable compounds by interacting with soil minerals (Liang et al., 2017; Spielvogel et al., 2007). Thus, SOC concentration

increased with a decrease in neutral sugars and an increase in the GM/ AX ratio (Fig. 6). This result emphasizes the important role of the microbial transformation of neutral sugars in regulating the relative magnitude of the labile SOC pool versus the stable SOC pool, which is consistent with the concept of MCP. Under the concept of MCP, a considerable proportion of the stable SOC pool is converted from labile SOC pool, and this conversion is largely driven by soil microorganisms (Liang et al., 2017; Zheng et al., 2021; Zhu et al., 2020). Hence, the mechanisms regulating microbial transformation and accumulation of neutral sugars in soil with increased atmospheric N and P deposition warrant further investigation.

#### 5. Conclusions

Our research on the compositions and concentrations of neutral sugar in plant and their responses to N and P addition expands the scant information on plant neutral sugars in the alpine grassland of the Tibetan Plateau. We speculated that the constant compositions of neutral sugar in shoots and roots may be a result of plant adaption, but more thorough research is required to support this view. In contrast to plant, the significant response of neutral sugars in soil highlights the important role of microbial transformation, rather than plant property, in regulating the compositions and concentrations of labile SOC. In addition, our findings indicates that labile SOC components are a transition SOC pool for synthesizing stable SOC pool through microbial conversion and transformation. By tracking the correlation between neutral sugars and SOC in the context of N and P addition over longer time scales, our findings may contribute to predicting variations in SOC from the



**Fig. 6.** Correlations between soil neutral sugar, pentose, hexose, GM/AX ratio and plant biomass, and soil nutrient properties. The scale color of the filled squares and the numerical size show the strength of the linear Pearson correlation coefficients (r). Only significant correlations with p < 0.05, either positive (blue) or negative (red), are exhibited. Boxes with a blank indicate insignificant correlation. NS: neutral sugar; GM/AX: (galactose and mannose)/ (arabinose and xylose); AGB: aboveground biomass; BGB: belowground biomass; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus.

#### Table 2

Multiple stepwise regression analysis of neutral sugar, pentose, hexose, and GM/ AX ratio with soil nutrient properties.

Index	$R^2$	<i>p</i> -Value	Beta coefficient	Significant variables
Neutral sugar	0.684	0.000	-0.827	SOC
Pentose	0.666	0.000	-0.812	SOC
Hexose	0.687	0.000	-0.829	SOC
GM/AX	0.435	0.000	0.660	SOC:TN

SOC, soil organic carbon; TN, total nitrogen; GM/AX, (galactose and mannose)/ (arabinose and xylose).

perspective of neutral sugars. This makes it feasible to precisely predict long-term SOC sequestration under increasing N and P inputs.

#### 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.

#### Data availability

Data will be made available on request.

#### Acknowledgments

This study was supported by the Natural Science Foundation of Gansu Province (21JR7RA500) and Lanzhou University's "Double First-Class Initiative" Guided Project Team Building-Funding-Research Startup Fee (561119221). We are grateful to the Haibei National Field Research Station in the Alpine Grassland Ecosystem for maintaining this experimental platform. We thank Binjie Liu, Lin Deng, Kaining Liu, Huaide Zhu, and Ruihan Xu for their assistance with the biomarker extraction of plants. We would like to thank KetengEdit (www.keten gedit.com) for linguistic assistance during the preparation of this manuscript.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.105028.

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