Effects of Different Phosphorus Supply Levels on Functional Diversity of Rhizosphere Microorganisms in Alfalfa (Postprint)

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Date: 2022-12-20T00:00:00+00:00

Abstract

Investigating the metabolic functional diversity of rhizosphere microorganisms in alfalfa under different phosphorus supply levels facilitates a deeper understanding of the efficient fertilizer utilization mechanisms of alfalfa. This study established five distinct phosphorus supply levels and, through field experiments, utilized the Biolog method to examine the metabolic characteristics of alfalfa rhizosphere microbial communities. The results indicated: (1) Phosphorus fertilizer application significantly enhanced the metabolic activity of alfalfa rhizosphere microorganisms; at a phosphorus supply level of P3 (300 $kg \cdot hm^{-2}$), the Simpson index and Richness index attained their maximum values, thereby improving microbial utilization of various categorized carbon sources. (2) Excessively high phosphorus fertilizer concentrations significantly reduced the Simpson index of the microbial community while concurrently decreasing the utilization rates of diverse carbon sources by microorganisms. (3) Principal component analysis revealed significant differences in the utilization rates of different carbon sources by rhizosphere microorganisms under varying phosphorus supply levels; at a phosphorus supply level of P4 (450 $kg \cdot hm^{-2}$), the differences in utilization of various carbon sources by the microbial community were minimal.

Full Text

Abstract

Exploring the metabolic functional diversity of *Medicago sativa* rhizosphere microorganisms under different phosphorus supply levels can further our understanding of the efficient fertilizer utilization mechanisms in alfalfa. This study established five different phosphorus supply levels and investigated the metabolic characteristics of the alfalfa rhizosphere microbial community using Biolog microplate technology through field experiments. The results showed

that: (1) Phosphorus fertilizer application significantly enhanced the metabolic activity of alfalfa rhizosphere microorganisms. The Simpson and Richness indices reached maximum values at a phosphorus supply level of 300 $kg \cdot hm^{-2}$ (P3), improving microbial utilization of different classified carbon sources. (2) When phosphorus fertilizer concentration was too high, it significantly reduced the Simpson index of the microbial community and decreased microbial utilization of various carbon sources. (3) Principal component analysis revealed significant differences in carbon source utilization by rhizosphere microorganisms under different phosphorus supply levels, with the smallest differences in microbial community carbon source utilization observed at 450 $kg \cdot hm^{-2}$ (P4). These findings demonstrate that appropriate phosphorus fertilization promotes rhizosphere microbial metabolic activity, while excessive phosphorus inhibits microbial functional diversity.

Keywords: *Medicago sativa*; phosphorus fertilizer; rhizosphere microorganisms; metabolic diversity

Introduction

Medicago sativa is a common perennial legume in China, known as the "king of forage crops," rich in nutrients, and represents an important resource for the country' s high-quality forage industry [**?**]. Since 2015, domestic alfalfa prices have continuously declined while planted area has gradually shrunk [**?**]. Developing the alfalfa industry has become imperative for improving comprehensive forage benefits, yet numerous limiting factors have slowed its progress in China [**?**]. Research indicates that one crucial factor restricting alfalfa industry development is the deficiency of plant-available phosphorus in most cultivated lands. Therefore, effective and rational addition of exogenous phosphorus to improve alfalfa yield and quality has become a key focus for researchers [**?**].

Phosphorus constitutes only 0.2%~0.4% of alfalfa biomass, yet it plays a vital role in physiological metabolism, directly affecting critical biological processes including cell nucleus formation, cell division, and genetic material synthesis [**?**]. Studies show that phosphorus fertilizer application is an important means to enhance soil nutrients and promote crop growth, with alfalfa requiring substantial phosphorus during growth [**?**]. Zhang [**?**] reported that single phosphorus fertilizer application significantly increased alfalfa hay yield ($P<0.05$). Lin [**?**] found that phosphorus application significantly affected alfalfa growth characteristics and soil fertility. Research on plant phosphorus absorption mechanisms reveals that *Medicago truncatula* SPX1 and SPX3 genes regulate plant phosphorus homeostasis, enhancing phosphorus starvation responses under low phosphorus conditions while suppressing them under high phosphorus conditions [**?**]. Under phosphorus-deficient conditions, alfalfa root systems secrete substances that decompose phosphorus from iron phosphates in low-phosphorus soils, making it available for plant uptake [**?**]. Root exudates directly influence plant resistance to low-phosphorus soils [**?**].

Rhizosphere microorganisms directly participate in and influence plant physiological processes, serving as indicators of soil fertility [?]. Microbial community metabolic activity and functional diversity are direct indicators of microbial community activity changes and important bases for explaining microbial ecosystem characteristics and environmental conditions [?]. In natural environments, scientific fertilization can effectively regulate soil microbial cycling and reuse of carbon sources, significantly increasing soil microbial community growth, metabolic activity, and functional diversity [?]. Xu et al. [?] found that microbial diversity in tiger nut rhizosphere soil significantly decreased with increasing fertilizer application. Shan [?] reported that phosphorus addition may inhibit oxygen content in marsh soils, possibly due to reduced microbial activity. Shan [?] found that phosphorus fertilizer significantly increased microbial populations in peony rhizosphere soil. Song et al. [?] demonstrated that exogenous phosphorus addition affects soil respiration in wetlands. Research shows that phosphorus fertilizer enhances respiratory system responses to carbon sources, and long-term phosphorus-potassium combined application significantly increases rhizosphere microbial metabolic activity and functional diversity [?, ?]. Therefore, studying phosphorus fertilizer effects on alfalfa rhizosphere microorganisms is practically significant for improving soil fertility, rhizosphere microbial metabolic activity, promoting nutrient uptake, and increasing alfalfa yield. Based on this, our study used different phosphorus supply levels as experimental variables, employing Biolog technology to analyze alfalfa rhizosphere microbial functional diversity and elucidate phosphorus fertilizer effects on rhizosphere microbial metabolic functional diversity, providing theoretical basis for improving alfalfa nutrient utilization and yield.

Materials and Methods

1.1 Experimental Materials

The tested alfalfa variety was "Xinmu No. 1," provided by the Forage Seed Breeding Base of Sanping Internship Farm, Xinjiang Agricultural University. Fertilizers used included monoammonium phosphate (P_2O_5 61%), urea (N 46%), and potassium sulfate (K_2O 40%).

1.2 Experimental Site Description

The experiment was conducted at the Forage and Grassland Experimental Station of Sanping Internship Farm, Xinjiang Agricultural University, located at the northern foothills of the Tianshan Mountains on the southern edge of the Junggar Basin (43°56 N, 87°35 E, altitude 580 m). The region has a mid-temperate continental climate with an average annual precipitation of 228.8 mm, characterized by aridity and low rainfall. The annual average maximum temperature is 25.7°C, minimum temperature -15.2°C, with large temperature variations. The experimental area is a typical alluvial piedmont plain with a native habitat of gravel desert, later reclaimed as farmland. Basic physicochemical properties of the topsoil (0–20 cm) were: available phosphorus 15.64 $mg \cdot kg^{-1}$,

alkali-hydrolyzable nitrogen 22.39 mg · kg^{-1}, organic matter 14.64 g · kg^{-1}, and available potassium 250.34 mg · kg^{-1}.

1.3 Experimental Design

A single-factor (different phosphorus supply levels) five-level completely randomized block design was adopted. The five phosphorus fertilizer gradients were: 0 kg · hm^{-2} (P0), 150 kg · hm^{-2} (P1), 300 kg · hm^{-2} (P2), 450 kg · hm^{-2} (P3), and 600 kg · hm^{-2} (P4), based on the phosphorus gradient design of Chen et al. [**?**]. Each treatment had three replicates, with a plot area of 4.5 m × 0.5 m and 0.5 m intervals between plots. All plots were arranged in a completely randomized block design. Sowing occurred on April 15, 2021, using plump, uniformly colored seeds at a rate of 3 kg · hm^{-2} with row spacing of 0.3 m. Drip irrigation using groundwater was employed. Fertilizer application followed the P4 treatment standard: 120 kg · hm^{-2} urea and 60 kg · hm^{-2} potassium sulfate as base fertilizers, with nitrogen applied as topdressing. All plots except phosphorus levels were managed uniformly for watering, weeding, and thinning.

1.4 Soil Sampling and Processing

During alfalfa full-bloom stage, rhizosphere soil samples were collected using the five-point sampling method. After excavating plants, soil 0–2 cm from the root zone was removed, and soil tightly adhering to the root surface was collected. Samples were placed in sterile ziplock bags, labeled, immediately transported to the laboratory, ground, passed through a 2 mm sieve, and stored at -80°C.

1.5 Determination of Rhizosphere Microbial Metabolic Characteristics

Biolog ECO microplate method was used to analyze rhizosphere microbial metabolic characteristics. Sample preparation and measurement followed Cui et al. [**?**] and Garland [**?**]. Biolog ECO microplates (USA) were used, with absorbance measured at 590 nm using a multifunctional microplate reader.

Average Well Color Development (AWCD) describes microbial community metabolic activity intensity, calculated as:

$$AWCD = \sum_{i=1}^{31}(C_i - R)/31$$

where C_i is the absorbance value of each carbon source well and R is the absorbance of the control well. Negative $(C_i - R)$ values were recorded as zero.

Microbial metabolic functional diversity indices included:

Shannon-Wiener index (H), the most widely used indicator for species population distribution, where higher values represent greater microbial community functional diversity:

$$H = -\sum_{i=1}^{31} P_i \ln P_i$$

where P_i is the ratio of the absorbance value of well i to the total absorbance of the entire plate.

Richness index (S) represents the total number of utilized carbon sources in the microplate, calculated as the number of wells where $(C_i - R) > 0.25$.

Pielou evenness index (E) indicates the level of microbial utilization of all carbon sources in the microplate, with higher values indicating greater carbon source utilization efficiency:

$$E = H / \ln S$$

Simpson index (D_s), sensitive to dominant populations, provides a concentrated assessment of microbial community diversity, with higher values indicating greater diversity:

$$D_s = 1 - \sum_{i=1}^{31} P_i^2$$

Calculation methods followed Zhang et al. [**?**].

1.6 Data Statistical Analysis

Data were organized using WPS Excel. SPSS 26.0 was used for one-way ANOVA and multiple comparisons at significant levels. Origin 2018 was used for figure production.

Results

2.1 Metabolic Activity and Functional Diversity of Alfalfa Rhizosphere Microorganisms

Based on AWCD calculations, microbial metabolic activity under different phosphorus levels gradually increased with incubation time, following typical microbial growth patterns. During the incubation period, AWCD values showed an increasing trend. In the first 48 h, microbial growth rates were slow as microorganisms had not fully adapted to the Biolog microplate carbon source environment, resulting in flat growth curves. Calculations showed that during the 48–72 h period, the AWCD curve slope was maximal, indicating exponential growth and peak microbial activity. After 120 h, carbon source utilization curves gradually plateaued. Microbial growth curves at all phosphorus levels

during the 168 h incubation period were consistent with conventional microbial growth curves [Figure 1: see original paper].

Diversity indices were analyzed during the 72 h period when microbial carbon source utilization diversity was maximal. Rhizosphere microbial community diversity indices varied under different phosphorus levels (Table 1). The Richness index reached maximum at P3 (300 $kg \cdot hm^{-2}$), significantly higher than P1 ($P<0.05$), increasing by 66.7%. The Simpson index peaked at P3, significantly higher than P4 ($P<0.05$), increasing by 12.5%. The Shannon-Wiener index was highest at P3, 14.6% higher than P0. The Pielou index was highest at P3, 18.3% higher than P0, with no significant differences among treatments.

TABLE:1 Diversity indices of *Medicago sativa* rhizosphere microbial community at 72 h

Treatment ($kg \cdot hm^{-2}$)	Shannon-Wiener	Pielou	Richness	Simpson
P0 (0)	2.972$±0.107a	1.023±0.041b	19.0±4.4ab	0.942±0.004ab
P1(150)	2.976±0.090a	1.227		

Note: Different letters indicate significant differences among phosphorus levels for the same index ($P<0.05$).

2.2 Carbon Source Utilization Capacity of Alfalfa Rhizosphere Microorganisms

As shown in [Figure 2: see original paper], under different phosphorus levels, alfalfa rhizosphere microorganisms showed significant differences in utilization of polymer carbon sources ($P<0.05$). Compared with P0, amino acid carbon source utilization increased by 27.9% at P2 and 30.8% at P3. Phenolic acid carbon source utilization was highest at P3, with utilization ranking: P0 > P3 > P2 > P4 > P1. No significant differences were observed in amine carbon source utilization among treatments ($P>0.05$). Carboxylic acid carbon source utilization showed significant differences ($P<0.05$), with utilization ranking: P3 > P0 > P2 > P1 > P4. Carbohydrate carbon source utilization also differed significantly ($P<0.05$), ranking: P3 > P0 > P2 > P1 > P4.

2.3 Principal Component Analysis of Carbon Source Utilization

Based on principal component analysis, two principal components were extracted from 31 carbon sources across different phosphorus treatments (Table 2). The first principal component (PC1) had an eigenvalue of 21.73, explaining 70.10% of variance. PC1 showed high loading factors for 25 carbon sources, including 9 carboxylic acids, 7 carbohydrates, 6 amino acids, and 3 polymers, with β-methyl-D-glucoside showing the highest loading factor (0.987). The second principal component (PC2) had an eigenvalue of 6.51, explaining 21.00% of variance, with high loading factors for 6 carbon sources including 2 carboxylic acids, 2 amino acids, 1 carbohydrate, and 1 polymer, with cyclodextrin showing

the highest loading factor (0.988). The cumulative contribution rate of the two principal components reached 91.15%, indicating they explain most of the variation. Therefore, PC1 and PC2 were used to analyze carbon source utilization characteristics.

TABLE:2 Initial eigenvalues and cumulative contribution of principal components

Component	Eigenvalue	Variance (%)	Cumulative (%)
PC1	21.73	70.10	70.10
PC2	6.51	21.00	91.10
PC3	1.23	3.96	95.06
PC4	0.58	1.87	96.93
PC5	0.36	1.16	98.09
PC6	0.23	0.74	98.83
PC7	0.15	0.48	99.31
PC8	0.11	0.35	99.66
PC9	0.06	0.19	99.85
PC10	0.03	0.10	99.95
PC11	0.01	0.03	99.98
PC12	0.00	0.01	99.99
PC13	0.00	0.01	100.00
PC14	0.00	0.00	100.00

Correlation coefficients between PC1, PC2 and categorical carbon sources are shown in Table 3. The PCA score plot [Figure 3: see original paper] showed that different phosphorus treatments were distributed across positive and negative axes. P0 and P2 scores were mainly distributed on the negative axis, with P0 in the range of -1.65 to -4.11 and P2 in -0.96 to -2.6. P1 and P4 were distributed on the positive axis, with P1 in 2.72–7.66 and P4 in -0.29 to -2.41. P3 was distributed in both positive and negative directions, mainly on the positive axis. Spatially, P0 was mainly in Quadrant III, P1 in Quadrants I and II, P2 in Quadrant II, P3 in Quadrants I and IV, and P4 in Quadrants I and III. These results indicate that phosphorus levels significantly affect microbial carbon source utilization.

TABLE:3 Correlation coefficients of PC1, PC2 and categorical carbon sources

Carbon Source	PC1	PC2
Pyruvic acid methyl ester	0.982	0.152
Glucosaminic acid	0.985	0.151
D,L-α-glycerol	0.987	0.142
Galacturonic acid	0.986	0.138
Hydroxybenzoic acid	0.984	0.145

Carbon Source	PC1	PC2
Hydroxybenzoic acid	0.983	0.147
Asparagine	0.981	0.149
Phenylalanine	0.980	0.150

Discussion

This study investigated rhizosphere microbial functional diversity in alfalfa under five phosphorus levels through field experiments. The results showed that phosphorus fertilizer application effectively increased rhizosphere microbial metabolic activity and carbon source utilization. However, excessive phosphorus concentration inhibited microbial community growth and metabolism, reducing metabolic functional activity and decreasing the Simpson index and carbon source utilization efficiency. This indicates that soil phosphorus concentration regulates rhizosphere microbial metabolic activity, which is essential for phosphorus cycling in the alfalfa rhizosphere microecosystem.

Microbial community metabolic functional diversity can be described using diversity indices. Wu [**?**] reported that changes in soil nutrient content can regulate soil microbial community composition, and phosphorus fertilizer effectively increases targeted bacterial community richness. Luo [**?**] found that with increasing phosphorus levels, soil microbial communities in camellia forests first increased then decreased, peaking at 900 g phosphorus per plant annually; beyond 900 g, total microorganisms decreased. Liao et al. [**?**] showed that phosphorus fertilizer increased soybean microbial populations, with bacteria, actinomycetes, and fungi in 0–20 cm soil increasing by 56.97%, 44.34%, and 37.28%, respectively. Our results showed that Richness and Simpson indices peaked at P3 (300 $kg \cdot hm^{-2}$), significantly higher than P1 and P4, respectively, indicating that excessive phosphorus concentration may disrupt the original soil ecological environment.

Principal component analysis provides intuitive understanding of microbial carbon source utilization differences. Liu [**?**] reported that soybeans preferentially utilized carbohydrates, amino acids, and carboxylic acids, with weak amine utilization efficiency. Our PCA results showed that carbon sources with high correlation coefficients included carbohydrates (β-methyl-D-glucoside), polymers (Tween, cyclodextrin), carboxylic acids (D,L-α-glycerol, galacturonic acid), and amino acids (asparagine, phenylalanine) for PC1; and carbohydrates (erythritol), polymers (glycogen), carboxylic acids (itaconic acid), and amino acids (L-serine, glycyl-L-glutamic acid) for PC2. This indicates that alfalfa rhizosphere microorganisms mainly utilized these carbon source types, similar to findings by Zhang et al. [**?**].

The spatial distribution of different phosphorus treatments showed greater dispersion than non-phosphorus treatments, indicating significant effects of phosphorus levels on carbon source utilization. Overall, phosphorus-treated rhi-

zosphere microorganisms showed greater carbon source utilization than non-treated controls, likely because phosphorus application alters soil physicochemical properties, changing root exudate composition and consequently the quantity and types of available carbon sources for microorganisms.

Conclusion

Phosphorus fertilizer application effectively increases the metabolic activity of alfalfa rhizosphere microbial communities, enhances utilization of different carbon sources, and significantly increases microbial community metabolic diversity indices. However, excessive phosphorus concentration inhibits microbial community growth and metabolism, reducing metabolic functional activity and decreasing the Simpson index and carbon source utilization efficiency. Soil phosphorus concentration regulates rhizosphere microbial metabolic activity, which is essential for phosphorus cycling in the alfalfa rhizosphere microecosystem. Therefore, appropriate phosphorus application (300 $kg \cdot hm^{-2}$) positively affects rhizosphere microbial metabolism, representing the optimal phosphorus rate for promoting rhizosphere activity and improving alfalfa nutrient utilization, providing a theoretical basis for alfalfa agricultural production.

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Note: Figure translations are in progress. See original paper for figures.