Effects of Nitrapyrin on Functional Diversity of Soil Microbial Communities in Drip-Irrigated Cotton Fields: Postprint

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Abstract

It is known that the nitrification inhibitor chloromethylpyridine can effectively inhibit soil nitrification, reduce nitrogen leaching and nitrification-denitrification losses, and promote crop nitrogen uptake; however, its effects on soil microbial community functional diversity under drip irrigation conditions in arid regions remain unclear. This study investigated the effects of split application of urea with chloromethylpyridine (Nitrapyrin) via irrigation water on soil microbial carbon metabolism and community functional diversity in drip-irrigated cotton fields in arid regions. The experiment employed a randomized block design with three treatments: no nitrogen fertilizer [CK, 0 kg(N) · hm ^{2}], urea alone [Urea, 225 kg(N) · hm ^{2}], and urea with chloromethylpyridine [Urea+nitrapyrin, 225 kg(N) · hm ^{2}+2.25 kg(nitrapyrin) · hm ^{2}], with four replicates. The Biolog-ECO method was used to assess soil microbial carbon metabolism and functional diversity. The results showed that, compared with no nitrogen fertilizer (CK), both urea application and urea with chloromethylpyridine significantly enhanced the metabolic capacity (AWCD) and metabolic intensity (S) of soil microorganisms toward 31 carbon sources ($P<0.05$), increased soil microbial diversity and richness (Shannon index, Simpson index, McIntosh index, and Richness index), and improved the utilization capacity for various carbon sources. Following split application of urea with chloromethylpyridine via irrigation water, the soil microbial AWCD values, carbon metabolic intensity, Shannon index, Simpson index, McIntosh index, and Richness index were all greater than those in the urea-alone treatment, showing increases of 13.83%, 9.33%, 1.29%, 1.34%, 11.26%, and 11.79% compared with the urea treatment, respectively ($P>0.05$), while the evenness index was lower than that in the urea-alone treatment ($P>0.05$). PCA and cluster analysis results indicated that both urea application and urea with chloromethylpyridine exerted significant effects on soil microbial community functional diversity, though the difference between the urea with chloromethylpyridine treatment and the urea-alone treatment was

not significant. The addition of chloromethylpyridine enhanced the utilization of polymers, phenolic acids, carboxylic acids, amino acids, and amines by soil microorganisms, while reducing the utilization of carbohydrates (P>0.05). These findings demonstrate that in drip-irrigated cotton fields in arid regions, split application of urea with chloromethylpyridine via irrigation water can regulate the soil micro-ecological environment, enhance soil microbial metabolic capacity to a certain extent, increase microbial community functional diversity, and alleviate the decline in soil microbial activity caused by long-term application of inorganic nitrogen fertilizers.

Full Text

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Effect of Nitrapyrin on Functional Diversity of Soil Microbial Community in Drip-Fertigated Cotton Field*

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Abstract

As one of the nitrification inhibitors (NIs), nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine) effectively controls nitrification, reduces nitrogen leaching and nitrification-denitrification losses, and increases crop nitrogen uptake. However, information remains scant regarding the effect of nitrapyrin on the functional diversity of soil microbial communities under drip irrigation conditions in arid regions. This field trial employed a randomized block design to evaluate the impact of repeated nitrapyrin application with urea via fertigation on soil microbial carbon metabolism and community functional diversity in a drip-fertigated cotton field. Three treatments were established: CK [0 kg(N)·hm^{-2}], Urea [225 kg(N)·hm^{-2}], and Urea+nitrapyrin [225 kg(N)·hm^{-2} + 2.25 kg(nitrapyrin)·hm^{-2}], with four replications per treatment. Biolog-ECO technology was used to investigate soil microbial carbon metabolism and community functional diversity.

The results showed that compared with CK, both urea and urea plus nitrapyrin applications significantly enhanced the metabolic capacity of soil microorganisms for 31 carbon sources (AWCD) and carbon metabolism intensity (S) ($P < 0.05$), while increasing microbial diversity and richness (Shannon index, Simpson index, McIntosh index, and Richness index) and improving utilization of various carbon sources. The Urea+nitrapyrin treatment increased AWCD, S, Shannon index, Simpson index, McIntosh index, and Richness index by 13.83%, 9.33%, 1.29%, 1.34%, 11.26%, and 11.79% respectively compared with the Urea treatment ($P > 0.05$), while decreasing the Pielou evenness index ($P > 0.05$). Principal component analysis and cluster analysis indicated that urea and urea plus nitrapyrin applications significantly influenced soil microbial community functional diversity, though no significant difference was observed between the Urea and Urea+nitrapyrin treatments. Furthermore, nitrapyrin addition increased microbial utilization of polymers, phenolic acids, carboxylic acids, amino acids, and amines, but decreased carbohydrate utilization compared with the Urea treatment ($P > 0.05$). These results demonstrate that repeated nitrapyrin application with urea via fertigation can regulate the soil micro-ecological environment, enhance soil microbial metabolism and community functional diversity, and mitigate the reduction in soil microbial activity caused by long-term inorganic nitrogen fertilizer application in drip-fertigated cotton fields in arid areas.

Keywords: Nitrapyrin; Soil microbial; Carbon metabolism; Community functional diversity; Drip-fertigation; Cotton field

Introduction

Soil microorganisms constitute a vital component of farmland ecosystems, playing crucial roles in decomposing plant and animal residues, nutrient cycling, nitrogen fixation, maintaining soil structure and fertility, and pest control. Microbial diversity reflects the fundamental condition of agroecosystems to a certain extent, and preserving microbial ecological processes and diversity forms the foundation of sustainable agricultural production. Fertilization represents a critical agricultural practice affecting soil quality and sustainable utilization, altering soil microbial activity, abundance (microbial biomass carbon and nitrogen), and community structure, thereby influencing soil carbon and nitrogen transformation rates and pathways, soil nitrogen supply capacity, and carbon storage capacity, ultimately affecting soil quality. As an essential nutrient element for soil microorganisms, changes in soil available nitrogen content influence microbial nitrogen utilization and consequently microbial metabolism.

Nitrification constitutes a key transformation and transport pathway for nitrogen fertilizers in soils. In northern China' s arid and semi-arid regions, soil nitrification strongly affects nitrogen fertilizer utilization and soil nitrogen loss. In calcareous soils, crops primarily absorb nitrogen as NO -N, and even when

urea or ammonium nitrogen fertilizers are applied, they rapidly convert to NO - N within a short period. Nitrification inhibitors can suppress soil nitrification and reduce nitrogen loss by affecting the population structure and functional diversity of key microorganisms involved in nitrification-denitrification processes, thereby promoting crop nitrogen absorption. Previous studies have demonstrated that the nitrification inhibitor nitrapyrin significantly suppresses nitrification in various soil textures and promotes crop nitrogen uptake under drip irrigation conditions, substantially improving nitrogen use efficiency. However, research on the effects of such nitrification inhibitors on soil microbial community structure and functional diversity in calcareous soils remains limited, particularly regarding the impacts of nitrapyrin use on soil microbial diversity in drip-irrigated farmland ecosystems in arid regions. Since the quantity and size of soil microorganisms correlate positively with their carbon source utilization capacity (AWCD values), soil microbial community activity can be characterized by carbon source utilization strength. Therefore, this study employed the Biolog-ECO microplate method to investigate the effects of nitrapyrin co-applied with urea via fertigation on soil microbial community functional diversity, providing a theoretical basis for using nitrification inhibitors to regulate soil microbial flora, slow nitrification processes, reduce nitrogen loss, and promote efficient nitrogen utilization in drip-irrigated farmland in arid regions.

1. Materials and Methods

1.1 Experimental Site and Materials

The experiment was conducted at the experimental station of the College of Agriculture, Shihezi University. The previous crop was wheat (*Triticum aestivum*). The soil texture was medium loam, with the 0–20 cm topsoil containing organic matter 16.15 g · kg ^{1}, total nitrogen 0.92 g · kg ^{1}, available nitrogen 61.34 mg · kg ^{1}, available phosphorus 10.95 mg · kg ^{1}, available potassium 257.5 mg · kg ^{1}, and pH 7.76.

The test crop was upland cotton (*Gossypium hirsutum*) cultivar 'Xinluzao 45' . Nitrogen fertilizer was urea (purity 98.5%, N content 46.0%). Phosphorus fertilizer was potassium dihydrogen phosphate (KH PO , purity 98.0%, P O content 51.5%). The nitrification inhibitor was nitrapyrin emulsifiable concentrate [Nitrapyrin, 2-chloro-6-(trichloromethyl)pyridine, active ingredient content 24.0%], produced by Zhejiang Aofutuo Company.

1.2 Experimental Design and Management

A single-factor randomized block design was employed with three treatments: CK [0 kg(N) · hm ^{2}], Urea [225 kg(N) · hm ^{2}], and Urea+nitrapyrin [225 kg(N) · hm ^{2} + 2.25 kg(nitrapyrin) · hm ^{2}]. Each treatment had four replications, with a net plot area of 36 m^{2}. Cotton was sown on April 20 using plastic film mulching (film width 145 cm), with four rows of cotton per film and two drip irrigation lines laid underneath. Row spacing was (30+55+30) cm within the film and 55

cm between films, with plant spacing of 12 cm and a harvest density of 210,800 plants · hm ².

The total nitrogen application rate during the growth period was 225 kg(N) · hm ², with nitrapyrin applied at 1% of the pure nitrogen rate. Total phosphorus application was 90 kg(P O) · hm ². All nitrogen and phosphorus fertilizers were applied as topdressing via fertigation starting in June. The field received irrigation 10 times totaling 480 mm, with fertigation applied 8 times. Other management practices followed conventional field standards.

1.3 Soil Sampling and Analysis

During the cotton peak boll stage (August), six sampling points were randomly selected within each plot at the dripper position directly below the drip line between cotton rows. Soil samples were collected from the 5–15 cm layer, mixed after removing debris and fine roots, passed through a 2 mm sieve, and stored at 4 °C for microbial diversity analysis.

Soil microbial metabolic functional diversity was determined using the Biolog-ECO method, which is based on the reduction of tetrazolium dye by free electrons produced during microbial carbon source utilization, with color intensity reflecting the degree of carbon source utilization. Specifically: fresh soil passed through a 2 mm sieve was stored at 4 °C for 4–6 days. Then, 5.00 g of soil sample was placed in a 50 mL sterile centrifuge tube, 50 mL of sterile phosphate buffer solution (PBS) was added, and the mixture was shaken vigorously on a reciprocating shaker at room temperature for 2 hours. Subsequently, 0.5 mL of soil suspension was accurately measured and diluted to 50 mL with PBS buffer solution, shaken on a reciprocating shaker for 30 minutes, then left to stand. The supernatant was inoculated onto ECO plates at 150 L per well, with three replicates per soil sample. Inoculated plates were covered with sterile ECO plate lids and incubated at 28 °C. Optical density values at 590 nm were measured using a Biolog microbial automatic analysis system at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, and 144 hours of incubation to calculate the average well color development (AWCD) as an indicator of microbial metabolic functional diversity.

The average well color development (AWCD) was calculated according to the formula:

$$AWCD = \frac{\sum(C_i - R)}{n}$$

where C_i is the absorbance value of the ith non-control well, R is the absorbance value of the control well, and n is the number of carbon source types in the medium (ECO plate, $n = 31$).

Soil microbial community functional diversity analysis: AWCD values at 72 hours were used to analyze soil microbial community functional diversity,

calculating carbon metabolic intensity (S), Shannon-Wiener index (H'), Simpson index (D), McIntosh index (U), Richness index (R), and Pielou evenness index (J) using the following formulas:

Carbon metabolic intensity (S) was estimated using curve fitting:

$$S = \sum \frac{V_i}{t_i}$$

where t_i is the ith hour and V_i is the AWCD value at time i.

Shannon-Wiener diversity index (richness) was calculated as:

$$H' = -\sum P_i \ln P_i$$

Simpson diversity index (dominance) was calculated as:

$$D = \sum P_i^2$$

where P_i represents the ratio of the absorbance value in the ith non-control well to the sum of absorbance values in all non-control wells, calculated as:

$$P_i = \frac{C_i - R}{\sum (C_i - R)}$$

McIntosh diversity index (a diversity index based on Euclidean distance in multidimensional community species space) was calculated as:

$$U = \sqrt{\sum n_i^2}$$

where n_i is the relative absorbance value of the ith well ($C_i - R$).

Richness index refers to the total number of utilized carbon sources, which in this study was the number of wells with ($C_i - R$) values greater than 0.2.

Pielou evenness index was calculated as:

$$J = \frac{H'}{\ln S}$$

where S is the total number of utilized carbon sources.

1.4 Data Processing

Data were processed using Microsoft Excel 2007 for chart preparation. SPSS 19.0 was used for analysis of variance, with LSD test for significance testing of treatment means. Canoco 5.0 software combined with SPSS 19.0 was used for principal component analysis (PCA), cluster analysis, diversity index calculations, and plotting.

2. Results

2.1 Effects of Nitrapyrin on Soil Microbial Carbon Source Utilization and Metabolic Intensity

AWCD characterizes the differential utilization of the same carbon source by different microorganisms, reflecting the carbon source utilization capacity of microbial communities and thereby demonstrating community structural diversity from a functional metabolic perspective. As shown in [Figure 1: see original paper], during the 144-hour incubation period, the utilization rate of 31 carbon sources (AWCD values) by soil microorganisms in all treatments increased gradually with incubation time, with inter-treatment differences becoming more pronounced from 36 hours onward. From 36 hours until the end of incubation, AWCD values in the Urea and Urea+nitrapyrin treatments remained significantly higher than in the CK treatment ($P < 0.05$). Although the Urea+nitrapyrin treatment showed 13.83% higher AWCD values than the Urea treatment, the difference was not significant ($P > 0.05$).

The trend in soil microbial carbon metabolic intensity mirrored that of AWCD values. From 36 hours of incubation, carbon metabolic intensity in the Urea and Urea+nitrapyrin treatments exceeded that of CK ($P < 0.05$). While the Urea+nitrapyrin treatment showed greater carbon metabolic intensity than the Urea treatment, the difference was not significant, with a 9.33% increase over the Urea treatment at the end of incubation ($P > 0.05$) ([Figure 1: see original paper]).

2.2 Changes in Soil Microbial Utilization of Different Carbon Sources

To clarify the effects of nitrapyrin on soil microbial utilization of different carbon sources, the 31 carbon sources utilized by microorganisms were categorized into six groups after 72 hours of incubation: carbohydrates (12 types), phenolic acids (2 types), carboxylic acids (5 types), amines (2 types), amino acids (6 types), and polymers (4 types). The analysis revealed that the Urea and Urea+nitrapyrin treatments enhanced microbial utilization of all six carbon source categories compared with CK. Compared with the Urea treatment, the Urea+nitrapyrin treatment increased utilization of polymers, phenolic acids, carboxylic acids, amino acids, and amines, with significant increases observed for carboxylic acids and amino acids ($P < 0.05$), while carbohydrate utilization decreased slightly ($P > 0.05$) ([Figure 2: see original paper]).

2.3 Effects of Nitrapyrin on Soil Microbial Community Functional Diversity

Principal component analysis was performed on AWCD values during the exponential phase of carbon metabolism (72 hours). Four principal component factors were extracted from the 31 carbon sources, with the first and second principal components contributing 48.2% and 22.1% respectively, accounting for a cumulative contribution of 70.3%. Therefore, scores for the first two principal components were plotted to characterize carbon source metabolic features of different microbial communities ([Figure 3: see original paper]).

In PCA plots, smaller distances between sample scores indicate higher similarity in carbon source utilization capacity. Along the PC1 axis, the four replicates of CK treatment clustered most closely, indicating stable carbon source utilization by soil microorganisms under no-fertilization conditions. The Urea and Urea+nitrapyrin treatments showed greater distances between replicates compared with CK, suggesting that urea and nitrapyrin addition altered soil microbial community carbon source utilization characteristics and generated greater variation. Cluster analysis results ([Figure 3: see original paper]) showed that CK treatment formed one group, while Urea and Urea+nitrapyrin treatments clustered together, indicating that although nitrapyrin addition affected soil microbial community metabolic functional diversity, the difference compared with urea alone was not significant.

2.4 Effects of Nitrapyrin on Soil Microbial Diversity Indices

Using optical density values at 72 hours, Shannon, Simpson, and McIntosh diversity indices, richness, and evenness indices were calculated for different treatments (). The McIntosh index in the Urea and Urea+nitrapyrin treatments was significantly higher than in CK ($P < 0.05$), while Shannon index, Simpson index, and richness index were higher than CK but not significantly ($P > 0.05$). Evenness index decreased slightly compared with CK ($P > 0.05$). The Urea+nitrapyrin treatment showed greater Shannon index, Simpson index, McIntosh index, and richness index than the Urea treatment, with increases of 1.29%, 1.34%, 11.26%, and 11.79% respectively, though differences were not significant ($P > 0.05$). Evenness index was lower than in the Urea treatment ($P > 0.05$). These results indicate that applying urea with nitrapyrin at 225 kg(N)$\cdot$ hm ^{2} via repeated fertigation increased soil microbial community diversity and richness to some extent while decreasing evenness.

Discussion

Soil microorganisms represent the most active components of soil and the primary drivers of soil material cycling. Soil microbial functional diversity characterizes the chemical reactivity of soil enzymes and microorganisms involved in nutrient transformation and cycling, representing an important manifestation of soil functions. The effects of fertilization on microorganisms vary depending

on fertilizer type and amount, baseline soil fertility, and fertilization duration, showing beneficial, detrimental, or neutral effects. Early studies found that increasing nitrogen fertilizer rates enhanced rhizosphere microbial quantity and diversity, but suppressed them when nitrogen application reached maximum threshold levels. Xia et al. reported that nitrogen application improved soil microbial community carbon source utilization rates and increased community richness and functional diversity, with medium nitrogen rates (120 kg · hm ^{2}) showing the highest AWCD, richness index, and Shannon index. Other reports indicated that nitrogen application alone reduced carbon source utilization in brown soils and decreased microbial activity with long-term nitrogen fertilization. Conversely, Sarathchandra et al. found that inorganic nitrogen and phosphorus fertilizers had no significant effect on soil microbial communities. The present study, conducted at 225 kg · hm ^{2} (only 75% of conventional nitrogen rates for local drip-irrigated cotton) with urea applied in small amounts multiple times via fertigation, demonstrated that soil microbial community metabolic functional diversity and carbon source utilization exceeded those of unfertilized soil, consistent with previous findings that appropriate nitrogen application increases microbial diversity.

Previous reports suggested that nitrification inhibitors suppress soil nitrification by releasing toxic compounds that directly affect nitrifying bacterial communities and nitrification activity. Later studies revealed that nitrification inhibitors primarily suppress nitrification by competing with ammonia monooxygenase (AMO) for substrates, chelating AMO active sites, or inhibiting other proteins through products oxidized by AMO. One study showed that when nitrogen application increased from 0 to 400 mg·kg ^{1}, soil microbial AWCD values and Shannon indices decreased by 78.4% and 22.3% respectively, but increased by 23.9% and 7.8% after DMPP addition. Nitrapyrin is known to inhibit non-selectively by binding to membrane proteins through its oxidation products, affecting AMO activity and thereby inhibiting ammonia-oxidizing microorganisms, though its effects on other soil microorganisms remain unclear. The present study found that at 225 mg · kg ^{1} nitrogen application with nitrapyrin applied via repeated fertigation, soil microbial carbon metabolic capacity and diversity indices increased compared with nitrogen alone, with AWCD and Shannon index increasing by 13.8% and 1.29% respectively—lower than previous findings, possibly due to differences in inhibitor type, soil type, and fertilization method. Although nitrapyrin primarily inhibits ammonia-oxidizing microorganisms, the overall culturable microbial carbon metabolic capacity and functional diversity increased in this study, possibly because nitrapyrin maintained lower soil NO -N content under the same nitrogen rate, mitigating adverse effects on soil microorganisms from rapid NO -N accumulation associated with inorganic nitrogen use and strong nitrification in calcareous soils. Additionally, diversity indices, PCA, and cluster analysis all indicated that nitrapyrin addition enhanced microbial metabolic functional diversity but did not differ significantly from urea alone, confirming the "specificity" of nitrapyrin in regulating soil microorganisms—specifically targeting ammonia-oxidizing microorganisms without significantly

inhibiting other microbial populations.

Under field conditions, factors influencing soil microbial communities are complex. The Biolog microplate method only reflects the metabolic capacity and functional diversity of culturable microorganisms and cannot define changes in key microorganisms during nitrification-denitrification processes. Therefore, future studies should integrate molecular biological techniques such as real-time PCR, isotope probing, and key gene cloning and sequencing to deeply elucidate the regulatory mechanisms of such nitrification inhibitors on soil microbial flora in drip-irrigated farmland.

In conclusion, in drip-irrigated cotton fields in arid regions, applying urea with nitrapyrin at 1% of the nitrogen rate (225 kg(N) · hm ^{2}) via repeated fertigation increased soil microbial AWCD values, carbon metabolic intensity, diversity indices, and richness index to some extent, enhanced microbial utilization of polymers, phenolic acids, carboxylic acids, amino acids, and amines, and improved the soil micro-ecological environment.

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