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

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Abstract

It is known that the nitrification inhibitor nitrapyrin 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 experiment investigated the effects of split application of urea with nitrapyrin (Nitrapyrin) via drip irrigation on soil microbial carbon metabolism and community functional diversity in drip-irrigated cotton fields in arid regions. A randomized block design was adopted, with three treatments: no nitrogen fertilizer [CK, 0 kg(N) · hm 2], urea alone [Urea, 225 kg(N) · hm 2], and urea with nitrapyrin [Urea+nitrapyrin, 225 kg(N) · hm 2+2.25 kg(nitrapyrin) · hm 2], replicated four times. The Biolog-ECO method was used to study soil microbial carbon metabolism and functional diversity. The results showed that, compared with no nitrogen fertilizer (CK), both urea application and urea with nitrapyrin significantly increased the metabolic capacity (AWCD) and metabolic intensity (S) of soil microorganisms on 31 carbon sources ($P<0.05$), and enhanced soil microbial diversity and richness (Shannon index, Simpson index, McIntosh index, and Richness index) as well as the utilization capacity of various carbon sources. After split application of urea with nitrapyrin via drip irrigation, the soil microbial AWCD value, carbon metabolic intensity, Shannon index, Simpson index, McIntosh index, and Richness index were all greater than those in the urea alone treatment, with increases of 13.83%, 9.33%, 1.29%, 1.34%, 11.26%, and 11.79% compared with the urea alone 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 nitrapyrin had significant effects on soil microbial community functional diversity, but the difference between urea with nitrapyrin and urea alone was not significant; the addition of nitrapyrin enhanced the utilization of polymers,

phenolic acids, carboxylic acids, amino acids, and amines by soil microorganisms, and reduced the utilization of carbohydrates (P>0.05). Based on these research results, in drip-irrigated cotton fields in arid regions, split application of urea with nitrapyrin via drip irrigation can regulate the soil micro-ecological environment, enhance soil microbial metabolic capacity to a certain extent, increase microbial community functional diversity, and alleviate the reduction in soil microbial activity caused by long-term application of inorganic nitrogen fertilizers.

Full Text

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

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Abstract

As a nitrification inhibitor, nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine) effectively controls nitrification, reduces nitrogen leaching and nitrification-denitrification losses, and promotes crop nitrogen uptake. However, information remains scarce regarding its effects on soil microbial community functional diversity under drip irrigation 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 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 replicates per treatment. Biolog-ECO technology was used to analyze soil microbial carbon metabolism and community functional diversity.

The results showed that compared with CK, both urea and urea+nitrapyrin applications significantly enhanced the metabolic capacity (AWCD) and intensity (S) of soil microorganisms for 31 carbon sources ($P < 0.05$), increased microbial diversity and richness (Shannon, Simpson, McIntosh, and Richness indices),

and improved utilization of various carbon sources. The urea+nitrapyrin treatment increased AWCD, S, Shannon, Simpson, McIntosh, and Richness indices by 13.83%, 9.33%, 1.29%, 1.34%, 11.26%, and 11.79%, respectively, compared with urea alone ($P > 0.05$), while the Pielou evenness index decreased ($P > 0.05$). Principal component analysis and cluster analysis revealed that both urea and urea+nitrapyrin significantly affected soil microbial community functional diversity, though differences between urea+nitrapyrin and urea alone were not significant. Nitrapyrin addition increased microbial utilization of polymers, phenolic acids, carboxylic acids, amino acids, and amines while decreasing carbohydrate utilization ($P > 0.05$). These findings demonstrate that in arid-region drip-fertigated cotton fields, applying nitrapyrin with urea via split fertigation can regulate the soil micro-ecological environment, enhance soil microbial metabolic capacity, increase community functional diversity, and mitigate the decline in soil microbial activity caused by long-term inorganic nitrogen fertilizer application.

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

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

1.2 Experimental Design and Methods

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 was replicated four times, with a net plot area of 36 m^{2}. Cotton was sown on April 20 using plastic film mulching (film width 145 cm). Each film covered four cotton rows with two drip irrigation tapes laid underneath. Within-film row spacing was (30+55+30) cm, between-film row spacing was 55 cm, and plant spacing was 12 cm, resulting in a harvest density of 210,800 plants·hm ^{2}. The total nitrogen application rate during the growing season was 225 kg(N)·hm ^{2}, with nitrapyrin applied at 1% of

the pure nitrogen rate. The total phosphorus application rate was 90 kg(P O) · hm ². All nitrogen and phosphorus fertilizers were applied as topdressing via fertigation starting in June. The entire growth period included 10 irrigation events totaling 480 mm, with fertilizer applied in 8 of these events. Other management practices followed conventional field standards.

1.3 Soil Sample Collection and Analysis

At the cotton peak boll stage (August), soil samples were collected from the 5–15 cm layer at six random points directly below drip emitters within cotton rows in each plot. After removing debris and fine roots, the samples were mixed thoroughly, passed through a 2 mm sieve, and stored at 4 °C for analysis of soil microbial diversity.

Soil microbial metabolic functional diversity was assessed 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 differences in utilization degree. Specifically, the sieved fresh soil was stored at 4 °C for 4–6 days. Then, 5.00 g of soil was placed in a 50 mL sterile centrifuge tube with 50 mL of sterile phosphate buffer solution (PBS) and shaken vigorously at room temperature for 2 h. Next, 0.5 mL of the soil suspension was accurately measured and diluted to 50 mL with PBS buffer, shaken for 30 min, and allowed to settle. The supernatant was then inoculated onto Eco plates (ECO) at 150 L per well, with three replicates per soil sample. The inoculated plates were covered and incubated at 28 °C. Optical density at 590 nm was measured at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, and 144 h using a Biolog microbial analysis system to calculate 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 [11]:

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

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 (ECO plate, $n = 31$).

Soil microbial community functional diversity analysis: This study used AWCD values at 72 h 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 [12]:

$$S = \sum_{i=1}^{n} \frac{V_i}{t_i}$$

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 [13]:

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

Simpson diversity index (dominance) was calculated as [14]:

$$D = 1 - \sum_{i=1}^{n} P_i^2$$

where P_i represents the ratio of absorbance in the ith non-control well to the sum of absorbance in all non-control wells:

$$P_i = \frac{C_i - R}{\sum_{i=1}^{n}(C_i - R)}$$

McIntosh diversity index (Euclidean distance-based diversity in multidimensional space) was calculated as [15]:

$$U = \sqrt{\sum_{i=1}^{n} n_i^2}$$

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 [16]; in this study, it was the number of wells with $(C_i - R) > 0.2$.

Pielou evenness index was calculated as [17]:

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

where S is the total number of utilized carbon sources.

1.4 Data Processing

Data were processed and graphed using Microsoft Excel 2007. SPSS 19.0 was used for analysis of variance (ANOVA), and the LSD test was employed for significance testing of treatment means. Principal component analysis (PCA), cluster analysis, diversity index calculations, and plotting were performed using Canoco 5.0 software in combination with SPSS 19.0.

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

AWCD can characterize differences in the utilization of the same carbon source by different microorganisms, reflecting the carbon source utilization capacity of microbial communities and thereby indicating microbial community structural diversity from a functional metabolic perspective [18]. As shown in Figure 1a [Figure 1: see original paper], during the 144 h incubation period, the utilization rate of 31 carbon sources (AWCD values) by soil microorganisms in all treatments increased gradually with incubation time, with treatment differences becoming more pronounced from 36 h onward. From 36 h to 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 higher AWCD values than the Urea treatment (by 13.83%), the difference was not significant ($P > 0.05$).

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

2.2 Changes in Soil Microbial Utilization of Various Carbon Sources After Urea Plus Nitrapyrin Application

To clarify the effect of nitrapyrin on soil microbial utilization of different carbon sources, the 31 carbon sources utilized by microorganisms were categorized into six groups after 72 h 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 utilization capacity of soil microorganisms for these six carbon source categories under different treatments is presented in Figure 2 [Figure 2: see original paper]. The results showed that the utilization capacity for all six carbon source categories in the Urea and Urea+nitrapyrin treatments was greater than in CK. Compared with the Urea treatment, the Urea+nitrapyrin treatment increased microbial utilization of polymers, pheno-

lic 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 ($P > 0.05$).

2.3 Effects of Nitrapyrin Addition on Soil Microbial Community Functional Diversity

Principal component analysis (PCA) was performed on AWCD values when carbon metabolism showed exponential changes (at 72 h). Four principal components were extracted from the 31 carbon sources, with the first and second components contributing 48.2% and 22.1% of the variance, respectively, for a cumulative contribution of 70.3%. Therefore, the scores of the first two principal components were plotted to characterize the carbon source metabolic profiles of soil microbial communities under different treatments [19] (Figure 3a [Figure 3: see original paper]). In the PCA plot, smaller distances between sample scores indicate higher similarity in carbon source utilization capacity. In the PC1 direction, the four replicates of CK clustered most closely, indicating stable carbon source utilization by soil microorganisms under no-fertilization conditions. In contrast, the replicates of Urea and Urea+nitrapyrin treatments showed greater distances compared with CK, suggesting that urea and nitrapyrin addition altered the carbon source utilization characteristics of soil microbial communities and introduced greater variation. Cluster analysis results (Figure 3b [Figure 3: see original paper]) showed that CK treatment clustered into one group, while Urea and Urea+nitrapyrin treatments clustered into another group, indicating that although nitrapyrin addition affected soil microbial community metabolic functional diversity, the difference was not significant compared with urea alone (Figure 3 [Figure 3: see original paper]).

2.4 Effects of Nitrapyrin Addition on Soil Microbial Diversity Indices

This study used optical density values at 72 h to calculate Shannon, Simpson, and McIntosh diversity indices, richness, and evenness index for different treatments (Table 1). The McIntosh index of soil microorganisms 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 also higher than CK but not significantly ($P > 0.05$). The evenness index decreased slightly compared with CK ($P > 0.05$). The Urea+nitrapyrin treatment showed greater Shannon, Simpson, McIntosh, and richness indices than the Urea treatment, with increases of 1.29%, 1.34%, 11.26%, and 11.79%, respectively, though these differences were not significant ($P > 0.05$). The evenness index was lower than in the Urea treatment ($P > 0.05$). These results indicate that applying 225 $kg(N) \cdot hm^{2}$ nitrogen fertilizer with nitrapyrin via fertigation in split applications increased soil microbial community diversity and richness to some extent while decreasing the evenness index.

Discussion

Soil microorganisms are among the most active components in soil and drive soil material cycling. Soil microbial functional diversity represents the chemical reaction activity of soil enzymes and microorganisms involved in nutrient transformation and cycling, serving as an important manifestation of soil function. The effects of fertilization on microorganisms can be beneficial, detrimental, or neutral depending on fertilizer type and amount, baseline soil fertility, and duration of fertilization [20]. Early research found that rhizosphere microbial quantity and diversity increased with nitrogen application rates up to a maximum threshold, beyond which they were suppressed [21]. Xia et al. [22] reported that nitrogen fertilizer application enhanced soil microbial community carbon source utilization, increasing microbial richness and functional diversity, with medium nitrogen rates (120 kg · hm ^{2}) yielding the highest AWCD values, richness indices, and Shannon indices. Other studies, however, indicated that nitrogen fertilizer alone reduced carbon source utilization in brown soils [23] and that long-term nitrogen application decreased soil microbial activity [24]. Conversely, Sarathchandra et al. [25] found no significant effects of inorganic nitrogen and phosphorus fertilizers on soil microbial communities. The present study, conducted under a nitrogen rate of 225 kg · hm ^{2} (only 75% of the conventional rate for local drip-irrigated cotton fields) with urea applied in small amounts via multiple fertigation events, demonstrated that soil microbial community metabolic functional diversity and carbon source utilization were higher than in unfertilized soil, consistent with previous findings that appropriate nitrogen application increases microbial diversity.

Some reports suggest that nitrification inhibitors suppress soil nitrification by releasing toxic compounds that directly affect nitrifying bacterial communities and nitrification activity [26]. Subsequent research has shown that nitrification inhibitors primarily suppress nitrification by competing with ammonia monooxygenase (AMO)—the key enzyme in ammonia oxidation—for substrates, chelating AMO active sites, or inhibiting other proteins through products oxidized by AMO [27]. One study demonstrated 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 [28]. Nitrapyrin is known to inhibit nitrification through its oxidation products binding non-selectively to membrane proteins, affecting AMO activity and thereby inhibiting ammonia-oxidizing microorganisms, though its effects on other soil microorganisms remain unclear. The present study found that applying nitrapyrin with 225 mg · kg ^{1} nitrogen via split fertigation increased soil microbial carbon metabolic capacity and diversity indices compared with nitrogen alone, with AWCD values and Shannon indices increasing by 13.8% and 1.29%, respectively—lower than reported in previous studies, possibly due to differences in inhibitor type, soil type, and fertilization method. Although ni-

trapyrin primarily inhibits ammonia-oxidizing microorganisms, this study found that the carbon metabolic capacity and functional diversity of culturable microorganisms in soil increased overall. This may be because nitrapyrin addition maintained lower soil NO -N content under the same nitrogen application rate, mitigating the adverse effects on soil microorganisms caused by rapid NO -N accumulation from inorganic nitrogen fertilizer application and strong nitrification in calcareous soils. Furthermore, diversity indices, PCA, and cluster analysis all indicated that while nitrapyrin addition enhanced soil microbial metabolic functional diversity, the difference was not significant compared with urea alone, confirming the "specificity" of nitrapyrin in regulating soil microorganisms [27]—that is, it affects only ammonia-oxidizing microorganisms without significantly inhibiting other microbial populations.

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

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

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