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How do Earthworms in Soil Respond to Nitrification Inhibitor? - An investigation from A Microbial Aspect

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A laboratorial incubation experiment was conducted to test the response of earthworm (*Eisenia fetida*)-related microbes to the application of dicyandiamide (DCD), a nitrification inhibitor. The potential of ammonium oxidation in soil was inhibited during the 90 days cultivation. At day 90, it was observed the soil surface was drilled more severely by earthworms in DCD treatment than E treatment. Meanwhile, comparedwith E treatment, the results of soil community level physiological profiles indicated that the potential of carboxylic acids and carbohydrates utilization in soil was enhanced by DCD addition.The active microbial biomass was increased by 43% andthe polyphenol oxidase activity was enhanced by 14% in DCD treated soil than control. More importantly, the number of denitrifying bacteria in earthworm gut content at day 90 was markedlydeclined from 1.79×10^{10}g [fresh wt]$^{-1}$ in control to less than 1×10^{6} g [fresh wt]$^{-1}$in DCD treatment.These results implied that DCD application was anabiotic selection pressureon the activities of earthworms and themicrobes both in soil and in earthworm, which might led to a shift of interaction between earthworms and the microbes.

Key words*:* Earthworm; DCD; Microbe, soil quality.

Carbon and nitrogen dynamics in soil are intensively affected by earthworms[1], and agricultural management significantly influences the magnitude and direction of such effects[2]. It was reported that the symbiosis of earthworms and microbes can provide earthworms with some new metabolic capabilities[3].For instance, the microbes in earthworm digestive tract can play the part as plant growth promoters, free-living nitrogen fixers and phosphate solubilizers[3]. Due to the large amount of culturable denitrifying bacteria (DNB) in earthworm gut, denitrification activity was considerable in living earthworm[4]. Therefore, we assumed that if the DNB in earthworm gut were affected by some agronomic measures, e.g., the application of nitrification inhibitor, the association between DNB and earthworm would be changed, and the subsequent earthworm's effects (such as burrowing) on soil function would be influenced.

Dicyandiamide (DCD) is one of the commonly used nitrification inhibitor that act as an additive in slow-release fertilizers because of its long half-life time and degradation in soil[5]. It is well-known thatnitrification inhibitor can inhibit the activities of ammonia oxidizing bacteria in soil, and indirectly influence the denitrification[6-8]. For example, it was reported that nitrapyrin inhibited nitrification and consequently denitrification by restricting the supply of nitrate to the denitrifying organisms[7], and DCD affected nitrification and denitrification in a slurry amended soil[6].

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Fertilization would disturb on soil ecosystem, and would lead more severely influence on the epigeic earthworms (living in surface soil) than endogeic and anecic species. However, the response of earthworms to nitrification inhibitor is unknown, especially from a perspective of microbe in both soil and earthworm. Therefore, in this study, a laboratorial experiment was conducted to try to identify the influences of a nitrification inhibitor, DCD, on earthworms (*Eisenia fetida*, an epigeic earthworm) in soil.

MATERIALS AND METHODS

A controlled incubation experiment was conducted for 90 days using a farmland soil in presence of earthworm with and without DCD application.Each jar (2.5 L) was filled with two kg of air-dried soil (sieved by a 2 mm sieve). The soil was sampled from a maize field of the National Field Research Station of Shenyang Agroecosystems, Northeast China (41°322 N, 123°322 E). Before the initiation of this experiment, the soil was humidified to 20% (w/w) moisture and kept at 20±3°C for 7 days for pre-incubation. In this experiment, two treatments: i) *Eisenia fetida* (abbreviated toE) and ii) *Eisenia fetida*+ DCD (abbreviated toDCD), were incubated for 90 days at 17-23 °C. There were three replicates for each treatment. Additional 36 parallel jars were set up for the soil samplingat specific time intervals (day 7, 14, 21, 31, 49 and 64).

Urea-N concentration of the soil was adjusted to 200 µg kg^{-1} dry soil with urea solution, on day 1, and then soil water content was adjusted to 25% (w/w) moisture (about 60 water-filled pore space). The DCD, withan application rate of 2% (w/w) of applied urea-N, was mixed with the urea solution in the DCD treatment. Nine earthworms (*Eisenia fetida*, about 0.26 g per individual) were assigned to each jarand recorded the weight of earthworms. By determining the weight loss of jars, soil moisture was maintained to 25% by applying a water supplement twice a week during the 90 days cultivation.

Soil subsamples were taken by hand from jars (protecting earthworms against mechanical damage) at specific time intervals (day 7, 14, 21, 31, 49, 64 and 90) for the ammonia oxidation potential's determination which was followed the procedure of Belser *et al*.[9]. Soil subsamples at day 90 were taken for determining soil characteristics and analyzing the community level physiological profiles (CLPP) of the soil microbes. The measurements of polyphenol oxidaseactivity, acid phosphatase activity and liable organic carbonwere conducted according to the procedures of Hu *et al*.[10], Li *et al*.[11] and Blair *et al*.[12]. Soil ammonium and nitrate nitrogen were extracted by 1M KCl solution in a ratio of 1:2.5(soil:KCl solution). Flow analyser(Future, Alliance, France) determine the concentration of ammonium and nitrate nitrogen.The CLPP was determined with a micro-respiration system (MicroResp™ plates, The James Hutton Institute, Scotland)[13]. Microbial diversity was estimated using Shannon-Weaver diversity index (H') which was calculated as follows:

$$H' = -\Sigma Pi\ (\ln Pi),$$

where the summation is over all substrate-induced CO_2 evolution *i* and *Pi* is the relative abundance of CO_2 evolution *i*. Active microbial biomassin soil was measured according to the procedure of Alvarez *et al*.[14]. At day 90, the earthworms from each jar were hand-sorted. The earthworms were then washed, placed in the moistened paper, weighted and put into a sterile petri dish. The earthworm was sacrificed in bottle with ether (earthworm did not contact with ether liquid), its surface was sterilized by 70% alcohol, and thenearthworm gut content homogenate was prepared using sterilized phosphate buffer and glass bead. The population of DNB in earthworm gut content in the E treatment and DCD treatment at day 90 were determined with the most probable number method (MPN)[4]. Each earthworm gut content homogenate was incubated into 3 Hungate tubes.

Statistical analyses were performed by using two-tails T-test, and difference between the two treatments were regarded as statistically significantly if $p < 0.05$.

RESULTS

After 90days'cultivation, all of the earthworms in the treatments survived, and their weights were not significantly different between E treatment (mean weight 0.111 ± 0.003g) and DCD treatment (mean weight 0.115± 0.005g). As expected, it was observed the soil surface was drilled more severely by earthworms in DCD

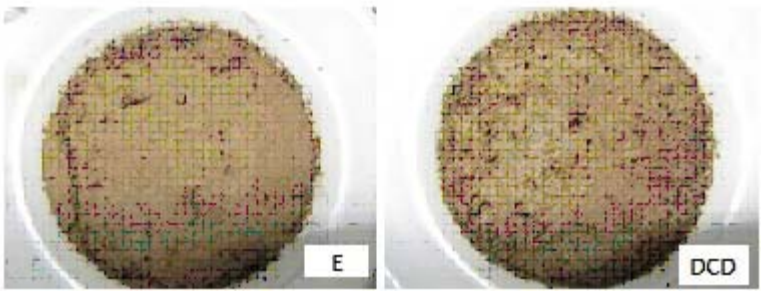

Fig. 1. The surface of soil in E treatment and DCD treatment after 90 days'cultivation. E and DCD represented E treatment and DCD treatment

treatment than E treatment at day 90 (Fig. 1).

It was observed that the numbers of the DNB in earthworm gut content were 1.79×10^{10} g [fresh wt]$^{-1}$ in the E treatment and less than 1×10^{6} g [fresh wt]$^{-1}$ in DCD treatment (n=3, p<0.05). This result showed that DNB in the earthworm gut content could be indirectly influenced by the reduced soil nitrification under DCD treatment.

During the period of cultivation, it showed that ammonia oxidation potential of soil was inhibited by DCDover the 90-day cultivation except day 49 and 64 (Fig. 2).

CLPP is a method to indicate microbial potential utilization of organic carbon in soil. The CLPP of soil microbes in E treatment and DCD treatment at day 90 show significantly difference. The base respiration of soil in the DCD treatment (1.44±0.09 µg CO_2-C g h^{-1}) was 26% higher than that in the E treatment (1.14±0.17 µg CO_2-C g h^{-1}) (n=3, p<0.05). The catabolism of the carboxylic acids and carbohydrates were 13.25% and 13.18% higher in the DCD treatment than in the E treatment (Fig. 3) (n=3, p<0.05). Particularly, the induced-CO_2evolution of α-ketoglutaric acid was 16% higher in the DCD treatment (12.29±0.83 µg CO_2-C g h^{-1}) than in the E treatment (10.27±0.99µg CO_2-C g h^{-1}) (n=3, p<0.05). But the utilization of the other 14 organic carbon substrates did not show significantly different between the E treatment and the DCD treatment. However, the Shannon-Weaver index for microbial diversity was decreased by 5% in the DCD (2.13±0.05) treated soil than E treatment (2.25 ±0.03)(n=3, p<0.05).

At day 90, the activity of polyphenol oxidase in the DCD treatment (0.115±0.006 Gallic acidic mg g $^{-1}$soil h $^{-1}$) was observed 14% higher than in the E treatment (0.101± 0.005 Gallic acidic mg g $^{-1}$soil h $^{-1}$), whereas the activity of acid

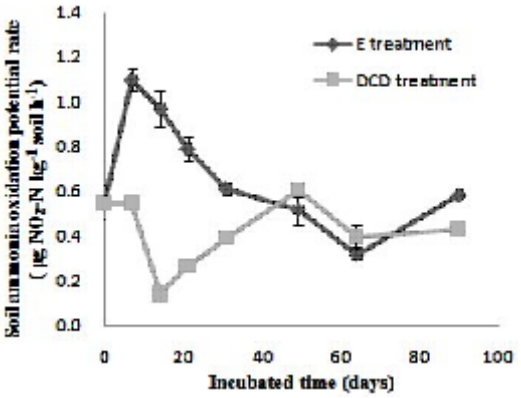

Fig. 2. Values of ammonia oxidation potential in the E treatment and the DCD treatment during cultivation. Error bars represent the standard error of the mean of three replicates

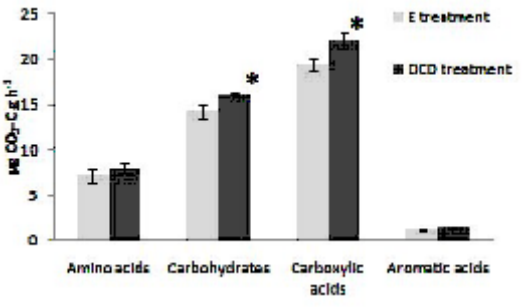

Fig. 3. Utilization rates of the four carbon source groups in the test soil in the E treatment and the DCD treatmenton day 90. Error bars represent the standard error of the mean of three replicates.The"*" indicates a significant difference (P<0.05)

phosphatase was 13% lower in the DCD treatment (0.552 ±0.02 µg *p*-nitrophenol released·h^{-1}·g^{-1} soil) than E treatment(0.478 ± 0.05µg *p*-nitrophenol released·h^{-1}·g^{-1} soil) (n=3, $p<0.05$). Nitrate concentration in E treatment and E+DCD treatment (210.5 ± 10.1 and 185.3 ± 10.6 mg kg^{-1} soil) were no significant differences.

In addition, at day 90, there was 43% more active microbial biomass in the DCD treatment (0.58± 0.06mg biomass kg^{-1} soil wet weight) than in the E treatment (0.40± 0.08mg biomass kg^{-1} soil wet weight)(n=3, $p<0.05$). The liable organic carbon content of soil in the DCD treatment (1.92± 0.15 mg C kg^{-1} soil) was 17% higher than that in the E treatment (2.25± 0.31 mg C kg^{-1} soil) (n=3, $p<0.05$).

DISCUSSION

Weisker *et al*. [8] reported that DCD and 3,4-Dimethylpyrazole phosphate not only inhibited N_2O emissions by an average of 26% and 49%, but also reduced the release of CO_2 by an average of 7% and 28% in a three-year field study. This showed that nitrification inhibitors can influence processes of carbon and nitrogen cycles in agroecosystems. In this study, the lower potential of soil nitrification in the DCD treatment than control (Fig. 2) indicated that the activities of ammonia oxidizing bacteria was inhibited by DCD application.

Previous research showed that earthworm-microbial interaction increased soil carbon evolution, soil nutrient availability, and microbial activity, but earthworms can reduce microbial biomass (earthworms feed on microbes) [15]. In this study, the variation of CLPP, which quantified by the potential respiration of 15 organic carbon substrates, indicated a markedly response of microbial physiology to DCD application. At day 90, the increased utilization of carboxylic acids and carbohydrates in the DCD treatment (Fig. 3) at day 90 of incubation, as well as the higher base respiration (1.44 and 1.14 µg CO_2-C g h^{-1} in E and DCD treatment, see result), implied that DCD application led to the higher potential of catabolism in DCD treated soil. Coincidently, in this study, the higher potential of catabolism in the DCD treatment might be partly illustrated by the 43% higher active microbial biomass (the heterotrophic microbes) in the DCD treated soil. Brown *et al*. [16] suggestedtheeffects of drilosphere (the part of the soil influenced by earthworm secretions and castings) on microbial activity and organic matter decomposition can be completely different (and even opposite) depending on the spatio-temporal scale of observation. Because earthworm led to different drilosphere on soil surface (Fig.1), our observations of more active microbial biomass and higher catabolism potential in the DCD treatment may be due to the DCD-disturbed influences of earthworm on soil microbes. In this study, the more active microbes might also lead to higher production of secondary metabolites, such as soil enzyme (the polyphenol oxidase), which were excreted into DCD treated soil.

Furthermore, in this study, the changes of soil characteristics resulted from the application of DCD might not only influence the nitrification and some carbon cycles processes, but also might indirectly influence the denitrification in earthworm gut. For example, in this study, the markedly decreased number of DNB in earthworm gut content by DCD applicationsuggested the significant changes of earthworm gut flora by DCD. It would also speculate that the association of earthworm and microbe would be influenced by DCD application. Considering the plenty of nitrate nitrogen in soil at day 90, the decreased number of DNB in earthworm gut content was partly a consequence of the adaption to the altered interaction between earthworm and soil microbe under DCD treatment.

A field study found that the DCD application affected on the individual numbers of nematodes (bacterivores, fungivores, plant parasites, omnivore-predators), which were higher in growing season and lower in ripening season in DCD treatment compared to control [17]. It deduced that the influence of DCD on the individual numbers of nematodes might be related to their foraging and food supply in DCD treated soil. The different performance of earthworms in E treatment and DCD treatment indicated earthworm foraging was influenced by DCD (Fig. 1). Base on the fact that earthworm could feed on microbes, in this study, the earthworm foraging strategy (following the concept of optimal foraging theory [18]) might be changed along with the interaction between earthworm and soil microbe under DCD treatment. Therefore, the foraging strategies of soil faunas, such as earthworms and nematodes, might be

changed following the alteration of soil microbes in DCD-treated soil. In addition, considering that the competition of nutrient and energy among the heterotrophic organisms (i.e., soil faunas and heterotrophic microbes) in soil, we infer that the returning crop straw or the application of manure can be a good supplement of the nutrients and energy materials to the soil with soil fauna under DCD application.

Canfield *et al*.[19] inferred that"humans can do something about managing the nitrogencycle, microbial processes will ensure that a new balance in the cycle will be reached". We deduced that the microbial processes would rebalance the nitrogen cycle affected by nitrification inhibitor.However, the duration of this rebalancing process remains unknown. Rajbanshi *et al*.[5] observed that DCD disappeared within 7 days in the sterilized and reinoculated treatment. However, in this study, the influences of DCD on microbial physiology both in soil and earthworm gut content were observed. Therefore, because of that DCD application resulted in the occurrence of a non-natural, unpredicted selection pressures on earthworm, the potential risk of DCD application on earthworm should be evaluated.

CONCLUSION

This study demonstrated that DCD application resulted in the activities of earthworm in soil, as well as the alteration of microbes both in soil and earthworm gut content. This should be derived from some types of selective pressures which were probably related to nutrient and energy supply to soil microbes and earthworms.

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