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ORIGINAL ARTICLE

Enzymatic activities and microbial biomass in black soil as affected by azoxystrobin

Pengpeng Guo · Lusheng Zhu · Jinhua Wang · Jun Wang · Hui Xie · Dongdong Lv

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Abstract As a high-efficiency and broad-spectrum new fungicide, azoxystrobin has been widely applied on various crops. However, little is known about the effect of azoxystrobin on microbial biomass and enzymatic activities in black soils. A laboratory experiment was conducted to study the impact of azoxystrobin application at different concentration levels (0, 0.1, 1.0 and 10.0 mg kg^{-1}) on cultivable microbial biomass and soil respiration in black soil. Urease, protease, dehydrogenase and catalase activities were also monitored on days 7, 14, 21 and 28. The results showed that the populations of cultivable bacteria, fungi and actinomycetes were seriously inhibited when the incubation time was over 7 days. The variation in soil respiration showed similar inhibition tendencies on the 14th, 21st and 28th days. A significant increase in the activity of catalase in black soils in response to higher levels (1 and 10 mg kg^{-1}) of azoxystrobin was observed only on the 28th day. Azoxystrobin had similar significantly negative influences on the activities of urease, protease and dehydrogenase; however, the level of inhibition depended on the doses of azoxystrobin and the time after its application.

Keywords Fungicide · Soil respiration · Catalase · Urease · Protease · Dehydrogenase

P. Guo · L. Zhu (✉) · J. Wang (✉) · J. Wang · H. Xie · D. Lv
Resources and Environment, Key Laboratory of Agriculture Environment in Universities of Shandong, Shandong Agricultural University, Tai'an 271018, People's Republic of China
e-mail: lushzhu@sdau.edu.cn

J. Wang
e-mail: wjh@sdau.edu.cn

Introduction

Strobilurin fungicides represent one of the most important groups of pesticides that are currently used (Howell et al. 2014). Azoxystrobin (AZO) [methyl-E-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate], released in 1996 as the first strobilurin compound to control fungal crop pathogens, has been registered for use on 84 different crops in 72 countries (Sopeña and Bending 2013). Due to its bactericidal broad spectrum, long duration, high activity, low toxicity and safety to nontarget organisms (Bartlett et al. 2002; Dijksterhuis et al. 2011), AZO has a broad application prospects in Northeast China as a new fungicide. However, upon application, AZO is adsorbed by soil, leading to questions about the potential contamination of the soil (Bending et al. 2006; Bending et al. 2007; British Crop Protection Council 2009; Gosh and Singh 2009).

Due to their ability to provide information that integrates many environmental factors and the sensitive reaction to microenvironmental soil change, biological indicators of soil quality are widely used to evaluate the effects of pesticides on the soil ecosystem, serving as one of the best rapid indicators of soil pollution levels (Karlen et al. 2006; Nogueira et al. 2006). Additionally, microbial respiration is an ideal indicator for measuring microbial activity and mineralizable soil substrate (Jia et al. 2006). Simultaneously, soil enzymes catalyze an extensive number of biological processes in the soil and provide a unique assessment of soil function (Mijangos et al. 2006; Yang et al. 2014).

The influence of pesticides on soil microorganisms has received considerable attention in recent years (Cycoń et al. 2010; Stefani et al. 2012; Xiong et al. 2013). Previous studies have shown that AZO affected the soil microbiota

by inhibiting soil dehydrogenase activity and altering the structure of the soil fungal community, with no significant effect on the community of bacteria (Diédhiou et al. 2004; Bending et al. 2007; Adetutu et al. 2008; Gosh and Singh 2009). With the development of research on AZO and its application, it is becoming extremely urgent to obtain information related to the nontarget effects of AZO on total soil respiration and soil enzymes. Therefore, we studied the effect of AZO at different concentrations on soil respiration, soil enzymatic activities and microbial populations. The main objective of this work is to provide a reference for rational use of AZO and an assessment of the security of the soil environment.

Materials and methods

Azoxystrobin has been widely used in Northeast China leading to potential contamination of the soil environment. To study the effects of different concentrations of AZO on the ecosystem in black soil (Bending et al. 2006; Gosh and Singh 2009; Wang et al. 2010), three methods, including soil enzyme assays, plate counting and soil respiration rate determination, have been used.

Chemicals and soils

Analytical-grade AZO (99 %) was obtained from Dr. Ehrenstorfer (Germany). The storage solution of AZO was prepared by dissolution in acetone. All other chemicals used were of analytical grade and obtained from Sigma Chemical Co. and Shanghai Sangon Biological Engineering Technology and Service Co. Soil samples (a depth of 0–15 cm), without AZO application for last 5 years, were collected from Jilin province (41.12′N, 126.18′E) and stored at 4 °C in the dark. Shortly before the study, the soil was kept in an incubator at 25 °C at 60 % maximum water-holding capacity (WHC) for 7 days (Hinojosa et al. 2004). The properties of the soil are presented in Table 1.

Table 1 Basic properties of the tested soil

Soil type	Black soil
pH	6.84 ± 0.11
TOC (g kg^{-1})	26.54 ± 1.60
Available N (mg kg^{-1})	124.33 ± 13.65
Available P (mg kg^{-1})	25.73 ± 1.26
Available K (mg kg^{-1})	132.73 ± 10.57
Clay (<0.002 mm %)	34.43 ± 2.33
Sand (>0.05 mm %)	37.14 ± 4.56
Silt (0.05–0.002 mm %)	28.43 ± 2.74
Water-holding capacity (WHC)	26.23 ± 1.98

Azoxystrobin application in soil

Laboratory-based experiments were conducted with the following treatments: 0, 0.1, 1.0 and 10.0 mg AZO per kg of soil. Bending et al. (2006) showed that the recommended field dose of AZO was 5 mg kg^{-1}. In the present study, the maximum dose is 10 mg kg^{-1} (double of the recommended field dose). Similarly, Howell et al. (2014) giving concentrations of 1, 5, 10 and 25 mg kg^{-1} soil to evaluate the impact of AZO on the community structure diversities of bacterial, fungal and nematode.

For each experiment, three replications each with 100 g of soil (dry basis) were maintained. Then, 1 mL of AZO stock acetone solution was thoroughly mixed with 100 g of soil in a tumbling mixture, after which the soils were placed in individual 125 mL of amber wide-mouth packers plugged with cotton pad (three replicates in each treatment, four sampling intervals). The moisture content was adjusted to 60 % of the maximum water-holding capacity and checked gravimetrically every 3 days.

Gosh and Singh (2009) have established that the half-life values of AZO were 107.47 and 62.69 days in aerobic and anaerobic soils, respectively. Wang et al. (2010) designed a field trial and reported that after 45 days in ginseng fields in Beijing and Jilin, the AZO soil concentration decreased from 9.01 mg kg^{-1} to less than 0.01 mg kg^{-1}. Therefore, the soil concentration of AZO changes most obvious within 1 month. In the present study, soil samples were incubated in the dark for 28 days to discard the effect of light at a controlled temperature of 25 °C. Samples from each bottle were collected on the 7th, 14th, 21st and 28th days for enzyme assays and to determine the soil respiration and cultivable microbial populations.

Analytical determinations

Soil microbial biomass

The effect of AZO on population of bacteria, fungi and actinomycete was isolated from the soil using the dilution plate method and determined using a method described by Lin (2010). During the experiment, 10.0 g of fresh soil sampled from each replicate of every treatment was mixed with 90 mL of sterile water in an Erlenmeyer flask (250 mL). After being oscillated at 200 rpm for 20 min, soil samples were dispersed in the diluted soil suspension. Conducting the dilution method of 10 times, 1 mL of the soil suspension was mixed with 9 mL of sterile water to prepare the appropriate soil suspension ratio. According to the number of microorganisms in the soil sample, 10^{-4} of the soil suspension was used for bacteria, 10^{-2} for fungi and 10^{-4} for actinomycete.

According to the microbial growth needs, the agar plate matrix was combined with different nutrients to meet the microbial needs. Because different microbes require different nutrient condition, three specific agar plates were prepared for each microbial count: Luria–Bertani (LB) medium (Aagot et al. 2001; Hobel et al. 2004; Davis et al. 2005) was prepared for bacterial colonies; potato dextrose agar (PDA, Difco) (Royse and Ries 1978) was prepared for fungi; and Gauze's medium No. 1 (Lin 2010) was prepared for actinomycetes. The agar plates were inoculated with 0.10 mL of soil suspension and incubated at 30 °C for 36 h for bacteria, 48 h for fungi and 5 days for actinomycetes. Triplicate replicates were performed.

Soil respiration

To measure the soil respiration rate, a certain concentration of NaOH solution was used to absorb the CO_2 released from soil respiration. The content of CO_2 was calculated according to the consumption of the NaOH solution (Ge et al. 2009).

In a 2,500-mL sealable tank, we put two 100 mL tall beakers, one of which contained 35 mL 1 mol L^{-1} NaOH and the other contained 50 g soil sample (adjusted to 60 % of the WHC of soil) treated with different concentrations of AZO and 1 g glucose. The sealed tanks were incubated at 25 ± 1 °C for 7 days. The respiration activity of the soil was expressed as mg CO_2 g^{-1} dry soil 1 h^{-1}.

Soil enzymes

In the experiment, activities of catalase, urease, protease and dehydrogenase were determined on days 7, 14, 21 and 28, by the method described by others (Öhlinger 1996; Alef et al. 1998; Lin 2010; Baćmaga et al. 2014).

Catalase activity was assayed using the $KMnO_4$ titration method. During the experiment, 5 g of soil was put into an Erlenmeyer flask (150 mL) with 40 mL of distilled water and 5 mL of H_2O_2 (0.3 %). The flask was sealed, shaken and placed in a water bath at 37 °C for 30 min. Next, 5 mL H_2SO_4 (3 mol L^{-1}) was added to terminate the reaction. Then, 0.02 mol L^{-1} $KMnO_4$ was used to determine the amount of surplus H_2O_2 from the 25 mL of the filtrate.

Urease activity was expressed as mg NH^4+–N produced from kg^{-1} dry soil 24 h^{-1}. During the experiment, 5 g of soil was treated with 1 mL methylbenzene at room temperature for 15 min in a 50-mL volumetric flask. Five mL of 10 % urea and 10 mL of citrate buffer were added to the flask. The flask was sealed, shaken and gently placed in a water bath at 37 °C for 24 h. Next, the soil reaction solution was diluted with 38 °C distilled water to 50 mL. Subsequently, 1 mL filter liquor was placed in a 50 mL Erlenmeyer flask and then diluted with room temperature distilled water to 10 mL, 4 mL of sodium carbolate and 3 mL of sodium hypochlorite were added, and then the flask was sealed and shaken. The absorbance of the mixture was measured at 578 nm using an ultraviolet–visible spectrophotometer (Shimadzu, UV-2550, Japan).

Protease activity was assayed using the method described by Xiong et al. (2013). One gram of soil was weighed into a glass vial, and 2.5 mL of Tris buffer (0.2 mol L^{-1}, pH 8.0) and 2.5 mL of a 2 % Na-caseinate solution were added. The capped vials were incubated in a water bath at 50 °C for 2 h. After the incubation, the remaining casein was precipitated with 5 mL of 10 % trichloroacetic acid. Then, 1.5 mL of the solution was pipetted into a microcentrifuge tube and centrifuged at 13,000 rpm for 1 min. Subsequently, 0.5 mL of the clear supernatant was mixed with 0.75 mL Na_2CO_3 (1.4 mol L^{-1}) and 0.25 mL threefold diluted Folin–Ciocalteu reagent in a microcuvette. The tyrosine concentration was measured colorimetrically at 680 nm after exactly 5 min. For the controls, the same procedure was followed, but the Na-caseinate was added after the incubation and the addition of the trichloroacetic acid.

For the dehydrogenase assay, soil samples (6 g) were incubated at 37 °C for 24 h with 3 mL of colorless 2,3,5-triphenyltetrazolium chloride (TTC) solution (3 % by weight). The TTC was reduced by dehydrogenase enzymes to become red water-insoluble triphenyl formazan (TPF), which was extracted with 5 mL of methanol. The samples were mixed on a vortex for 10 s and centrifuged at 4,000 rpm for 2 min. The procedure was repeated twice. The intensity of the red color of the supernatant was measured using an ultraviolet–visible spectrophotometer (Shimadzu, UV-2550, Japan) at 485 nm and converted to 1 g TPF g^{-1} dry soil day^{-1}.

Statistical analysis

Each result shown in this paper was replicated three times. All the data were analyzed by SPSS software (version 17.0) and performed as mean ± standard deviation (SD). One-way ANOVA and least significant difference (LSD) analysis were used to determine significant differences ($p < 0.05$) among treatments at each time point.

Results

The cultivatable microbial populations, soil respiration and enzymatic activities were monitored at 7, 14, 21 and 28 days following the application of AZO in black soil. The data represent the analytical result of the AZO treatment concentrations (Figs. 1, 2, 3, 4, 5, 6).

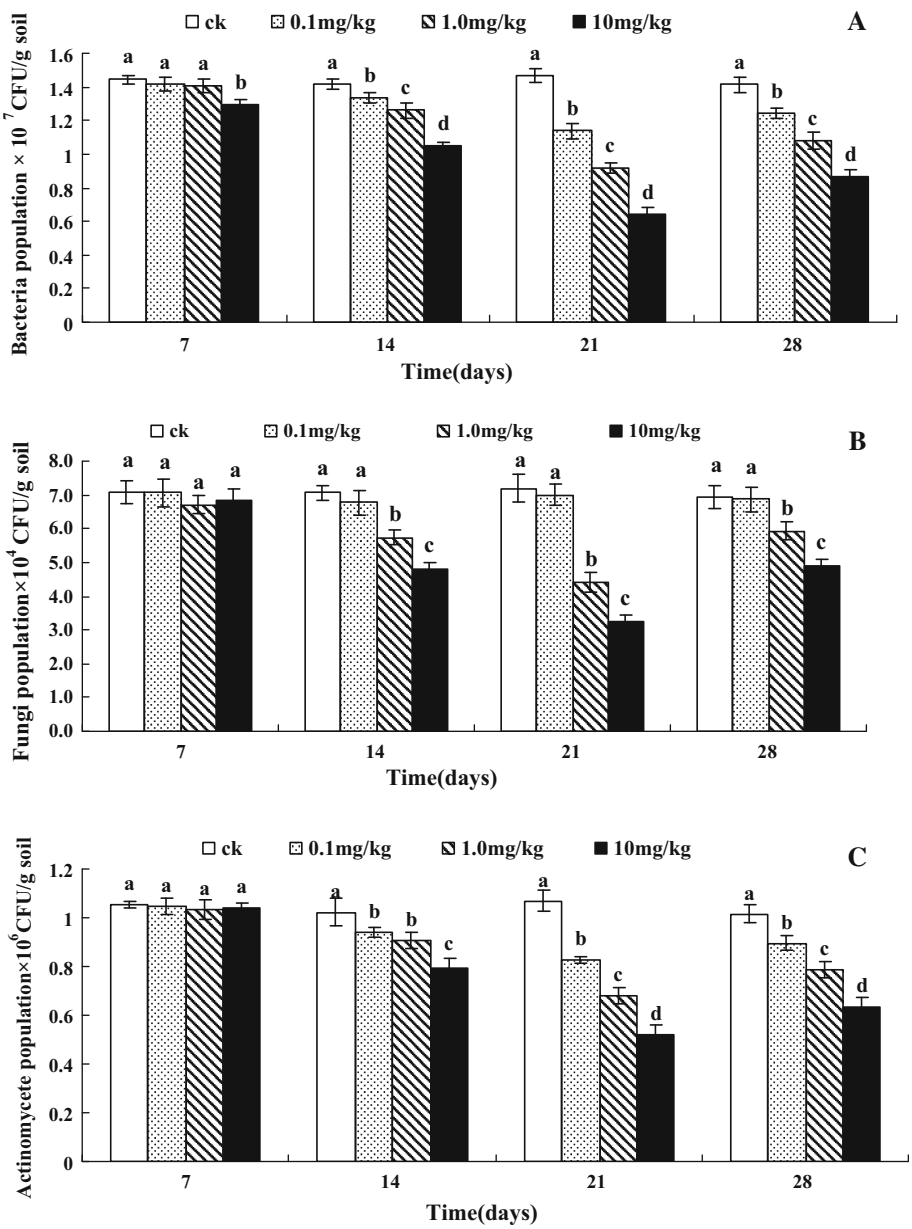

Fig. 1 Effects of azoxystrobin on the number of bacteria (**A**), fungi (**B**) and actinomycete (**C**) in soil. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

Microbial biomass

As shown in Fig. 1, the number of bacteria, fungi and actinomycetes in soil spiked with AZO had no significant variation compared with the control after 7 days of incubation. However, from 14 days on, the number of bacteria and actinomycetes significantly decreased ($p < 0.05$) in AZO-treated soil, and the effect of this inhibition became stronger with increased concentrations of AZO. The cultivable number of fungi also significantly decreased in soil spiked with 1.0 and 10 mg kg^{-1} of AZO, similar to the behavior of bacteria and actinomycetes in 1.0 and 10 mg kg^{-1} AZO treatments. The fungi populations in soil spiked with 0.1 mg kg^{-1} of AZO were not significantly different from those of the control throughout the incubation period.

Soil respiration

After 7 days of incubation, the soil respiration in AZO treatments exhibited no significant ($p < 0.05$) differences compared with the control group. In the period of 14–21 days, the soil respiration intensity gradually decreased with the incubation and the increase in AZO treatment concentrations. The soil respiration intensity gradually returned to undifferentiated with the control at

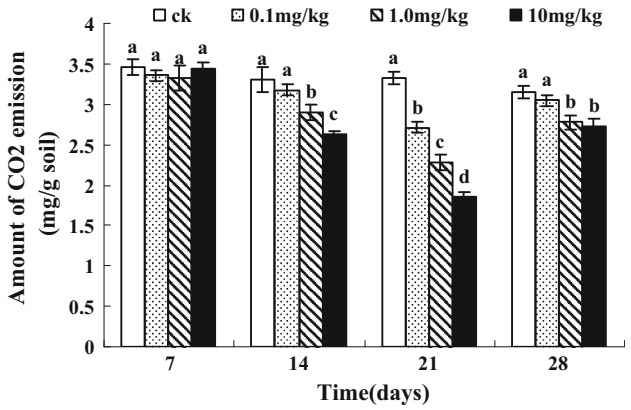

Fig. 2 Soil respiration as influenced by azoxystrobin at different concentrations. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

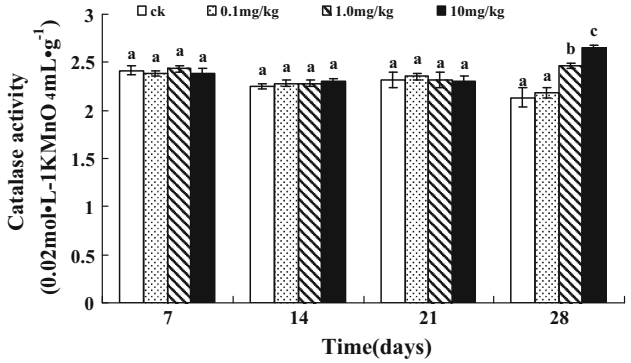

Fig. 3 Soil catalase activity as influenced by azoxystrobin at different concentrations. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

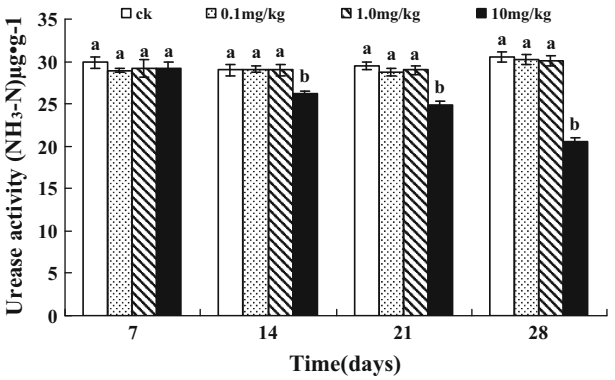

Fig. 4 Soil urease activity as influenced by azoxystrobin at different concentrations. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

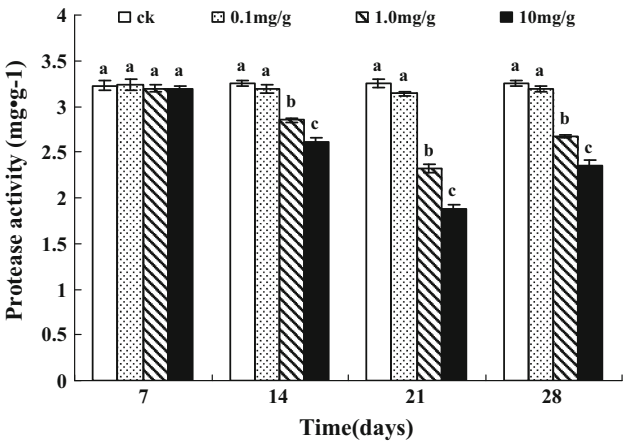

Fig. 5 Soil protease activity as influenced by azoxystrobin at different concentrations. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

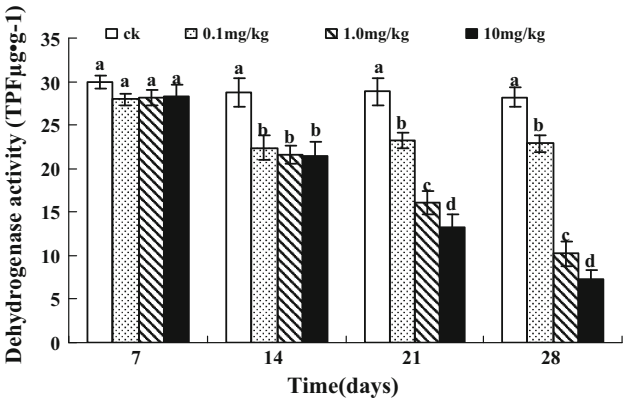

Fig. 6 Soil dehydrogenase activity as influenced by azoxystrobin at different concentrations. *Error bars* represent the standard deviation (SD). *Different letters* in columns indicate significant differences at $p < 0.05$ between treatments in the same sample time

28 days. Overall, high concentrations of AZO treatments had an inhibitory effect on soil respiration in a short term; with time, this inhibition was likely to disappear.

Enzymatic activity

The activity of soil catalase is shown in Fig. 3. From 7 to 21 days, the soil catalase activity was comparable with the control. On day 28, the soil catalase activity was significantly higher in the soils treated with 1.0 and 10.0 mg kg^{-1} of AZO. In addition, the stimulation effect gradually became stronger with increased concentrations of AZO.

The statistical analysis of soil urease activity is shown in Fig. 4. Compared with the control, the urease activity in

low-concentration (0.1, 1.0 mg kg^{-1}) treatments of AZO did not change significantly during the incubation period. However, for the 10.0 mg kg^{-1} AZO treatment, the soil urease activity was significantly decreased after 14 days, and the effect of this inhibition became stronger with time.

The protease activities of different AZO-treated soils are listed in Fig. 5. At low concentrations, AZO had little effect on soil protease activity. Nonetheless, the effects of AZO increased with increasing concentrations and incubation days. Compared with the control, the 0.1 mg kg^{-1} treatments had almost no effect on soil protease activity during the whole period. However, the 1.0 and 10 mg kg^{-1} treatments significantly inhibited ($p < 0.05$) soil protease activity at 14, 21 and 28 days.

As shown in Fig. 6, dehydrogenase activity in black soil was significantly affected by the concentration of AZO applied after 14-day incubation. On the 7th day, soil dehydrogenase activity exhibited no significant difference from the control. The effects of AZO on dehydrogenase activity generally decreased compared with those of the control groups on day 14. However, there were no significant differences of dehydrogenase activity with different concentration of AZO. After 21 days, the inhibition increased with increasing concentrations and the concentration-dependent decreased lasted on day 28.

The observed enzymatic activities at different AZO concentrations in this study are shown in Figs. 3, 4, 5 and 6. Dehydrogenase and protease activities decreased with increasing AZO concentrations compared with those of the control groups on days 14, 21 and 28. However, changes in urease activity in soils with the 10 mg kg^{-1} treatments decreased on the 28th day. Conversely, changes in catalase activity increased on the 28th day for only the 10 mg kg^{-1} treatments.

Discussions

Microbial biomass

The responses of soil microbial populations to different fungicides depend on the structure (Abboud 2011), the applied dose (Cycoń et al. 2010) and the soil types (Bending et al. 2007). In our study, significant decreases in the cultivable microorganism populations were observed in all treatments in the middle and later experiment periods (14, 21 and 28 days), with a proportional concentration-dependent effect. However, the control treatment without AZO application exhibited no significant changes, indicating no time-dependent relationship. During the incubation, the microbial populations in the soil samples with AZO were not supplied with exogenous food or energy. Therefore, this variation may be attributed to the effect of AZO. The mechanism of AZO inhibits the mitochondrial respiration by disrupting the production of ATP (Bartlett et al. 2002). Therefore, the population of some soil microorganisms may decrease because of suppressed respiration. This also means that the application of AZO would screen the indigenous microflora. As is reported by Riah et al. (2014), the disturbance by fungicides may have a harmful impact on microbial communities and activities of fungi in soil. Similar results of decreasing microbial biomass with time in soils were also demonstrated with the application of metalaxyl (Monkiedje et al. 2002), penconazole (Abboud 2011), butachlor (Yen et al. 2013), mancozeb and dimethomorph (Cycoń et al. 2010). However, Sukul (2006), Sukul et al. (2008) showed that metalaxyl had a significant stimulatory effect on bacterial and actinomycetes populations but a significant inhibitory effect on fungal populations. Due to the different properties of different soil types, such as organic matter content and the size of the microbial community (Bending et al. 2007), it is difficult to predict the effects of different fungicides on the population of cultivable microorganisms in different soil types.

Soil respiration

Soil respiration is considered an important indicator for evaluating the security of the soil microenvironment (Jia et al. 2006). Many studies have shown the influence of pesticides on soil respiration. However, the responses of different pesticides varied. In the present study, soil respiration was inhibited after 14–28 days following the application of AZO at doses of 1.0 and 10.0 mg kg^{-1}, which was the same as the populations of culturable microorganisms and the activities of protease and dehydrogenase. This inhibition tendency was also observed in the soil treated with 51 mg kg^{-1} benomyl, 125 mg kg^{-1} captan, 37 mg kg^{-1} chlorothalonil and 5–500 mg kg^{-1} tebuconazole (Chen et al. 2001; Muñoz-Leoz et al. 2011). Tebuconazole inhibits ergosterol biosynthesis, and chlorothalonil and AZO inhibit mitochondrial respiration (Lo 2010). According to the results shown in Fig. 2, no significant differences were observed at an AZO level of 0.1 mg kg^{-1} during the incubation period. This result was similar to the study by Xiong et al. (2013) that the application pyrimorph (0.5–150 mg kg^{-1}) inserted produced no significant effect on respiration over 14 days. It seems that low concentrations of AZO did not generate any toxicological threats to the respiration of black soil.

Enzymatic activity

Soil enzyme activities are considered an essential indicator for assessing contamination level responses to different

pesticides (Xiong et al. 2013; Baćmaga et al. 2014). Additionally, studies have illustrated that the responses of different soil enzymes to different pesticides varied.

Catalase is an intracellular enzyme that is in all aerobic bacteria and most facultative anaerobes in the soil (Khan et al. 2007). Our results showed increased catalase activity 28 days after the addition of AZO and were in agreement with those of Yan et al. (2011). Similarly, Xiong et al. (2013) showed that catalase activity significantly increased in soils treated with pyrimorph for 35 days. However, the application of validamycin at 240 mL mg^{-1} caused a significant decrease (14 %) in soil catalase compared with the control (Qian et al. 2007). The increase in catalase activity is contrast to the increase in populations of culturable microbial organisms in the present study. Microorganisms in the soil may change their metabolic activity in response to the contamination of fungicide in the soil ecosystem (Tejada 2009).

Urease is considered a key component in the nitrogen cycle in soil for the hydrolysis of urea to ammonium. In the present study, higher doses of AZO significantly decreased the activity of urease after 14 days of exposures. This is consistent with the variation of the populations of bacteria and fungi in the AZO-treated soil, supported by the theory that urease exists in a large number of soil bacteria and fungi (Sarathchandra et al. 1984). Other studies have demonstrated similar results, showing that the fungicide such as prochloraz (Tejada et al. 2011), carbendazim (Yan et al. 2011), benomyl and captan (Chen et al. 2001) had a positive influence on soil urease activity. Cycoń et al. (2010) and Muñoz-Leoz et al. (2011) reported a significant decrease in urease activity in soils treated with mancozeb, dimethomorph and tebuconazole. However, the urease activity in soil with pyrimorph treatments at doses from 5.0 to 150.0 mg kg^{-1} showed significant increased in the first 60 days of incubation tested by Xiong et al. (2013).

Soil proteases are hydrolytic enzymes, which hydrolyze proteins and produce amino acid, and are known to be important in soil nitrogen cycling (Kamimura and Hayano 2000; Geisseler et al. 2010). Overall, soil contamination with AZO at 1.0 and 10.0 mg kg^{-1} had an inhibitory effect on the activity of soil protease after 7 days of incubation. However, Rasool and Reshi (2010) reported that protease activity was stimulated after treatment with mancozeb within 21 days of incubation and exhibited a sharp decline after 28 days.

The pesticide residuals in the soil could be used as a resource for some microorganisms. However, some sensitive microorganisms in the soil can be lethally inhibited due to the application of pollutants (Cycon et al. 2010). As a microbial intracellular enzyme, dehydrogenase responds to the physiological state of microorganisms more sensitively. In the present study, the activity of dehydrogenase proved to be sensitive to treatment of AZO treatments after 14 days of incubation, corresponding to the decreased populations of soil cultivable microorganisms. Monkiedje et al. (2002) also observed a significant inhibitory effect on soil dehydrogenase activity in the soil polluted by mefenoxam and metalaxyl and that among dehydrogenase, b-glucosidase and phosphatases, dehydrogenase was most sensitive to contamination by AZO. However, Bending et al. (2007) reported that dehydrogenase activity significantly decreased in low organic matter/biomass soils but was stable in high organic matter/biomass soils within 2 months of incubation. Different responses of dehydrogenase to fungicides in different soils most likely depend on a variety of factors, such as the structure of the microbial community, the concentration of the fungicides and different soil types (Bending et al. 2007).

Soil enzymes are controlled or related to all biochemical transformations in soil. Riah et al. (2014) drew the conclusion that fungicides seem to induce an overall negative response of enzymatic activities by analyzing numbers of recent research results. The application of fungicide may directly shift the biosynthesis of enzymes by induction or repression phenomena (Cycon et al. 2010; Lo 2010). Incidentally, the negative response of enzymatic activities also indicated the imbalance effects of AZO on microbial populations.

Conclusions

The soil ecological toxicity of AZO was associated with soil microbial properties, including populations of cultivable soil microorganisms, soil respiration and the activities of soil enzymes. Research on the effects of AZO on indigenous microbial communities and enzymes in soils remains limited, particularly for the black soil in Northeast China. This study filled the information gap by assessing the impacts of AZO application on biological properties of in the black soil through a series of laboratory experiments. We concluded that:

1. The populations of cultivable bacteria, fungi and actinomycetes were all inhibited after 7 days of exposure to the AZO, indicating that they were involved in the similar inhibition tendency of soil respiration in the middle of the incubation period.
2. Higher levels of AZO significantly increased in the activity of catalase after 28 days of the application of AZO. The activity of urease, protease and dehydrogenase showed similar negative responses to the AZO treatments via a clear dose–response relationship.

The practical implication of the study is that the application of AZO might cause a potential threat to the black

soil environment. Future research involving trails is needed to evaluate the actual condition of enzymatic activities and microbiomass in the field in response to the spraying of AZO on crops and soil.

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