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ORIGINAL ARTICLE

Effects of validamycin on some enzymatic activities in soil

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Abstract There have been few investigations of the possible effects of validamycin on the enzymatic activities in soil. The objective of this present work was to study the short-term influence of enzymatic activity in validamycin treated soils. The results showed that high dose of validamycin introduced into soil had an significant effect on soil enzymatic activities, the degree of inhibition or promotion of enzymatic activities was not only related to the concentration of validamycin, but also to the remain time. Compared with the control (non treatment with validamycin), 240 ml·mg^{-1} validamycin treatment caused a significant decrease (14%) of soil catalase activity; validamycin also inhibited urease activity to 67.3%, but subsequently, validamycin stimulated its activity higher than that of the control. Only the highest dose stimulated acid phophatase activities by 29.7%, and other concentration treatments had no effects on these enzymatic activities. The above results indicated that validamycin had effects on soil enzyme, but the effective time was short. It is also interesting to speculate that validamycin can be easily used by the soil microorganism as carbon source and energy.

Keywords Validamycin · Catalase · Urease · Phophatase

1 Introduction

Validamycin is a non-systemic antibiotic with fungicide action. It is obtained by fermentation of *Streptomyces hygroscopicus* var. limoneus as a mixture of validamycin A, validoxylamine A and further minor constituents (Meister, 1994; Thomson, 1982). It is manufactured as an agricultural biological fungicide and controls sheath blight of rice caused by *Rhizoctonia solani* in rice, potatoes, vegetables, and others as well as damping off diseases in vegetable seedlings, cotton, sugar beets, rice and other plants on a large scale (NIOSH, 1993; Thomson, 1982). Validamycin A is taken up into the cell of the fungus *Rhizoctonia solani*, hydrolyzed therein yielding validoxylamine A which is a potent inhibitor of trehalase.

Validamycin has ever been considered as low toxic matter, which can be degraded easily, but it was proved only by using especial bacterium line (*Flavobacterium*

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saccharophilum) to metabolize validamycin in the lab (Asano *et al.*, 1984). As we knew, there had so far received little attention about whether the validamycin or its degraded products influenced the agroecosystem. Bt was another kind of biological pesticide, which also regarded as non-toxic pesticide several decades ago, but following the rising of Bt-transgenic plants engineering, some reports showed that the Bt toxins could accumulate in the soil and pose a potential risk to the soil biological processes including microorganism biomass and soil enzyme (Donegan *et al.*, 1995; Tapp and Stotzky, 1995; Wu *et al.*, 2004a,b). In essence, biological pesticide is one kind of chemical substance possessing definite molecular weight, and will also cause environmental disturbance, just as the chemical pesticide (Shen, 1997). So it is necessary to assess the effect of validamycin on agroecosystem risks.

Biological and biochemical properties of the soil, including soil respiration, microbial biomass, nitrogen mineralization capacity and the activities of soil enzymes, have been proposed as indicators of soil quality and health (Dick, 1994, 1997; Dick *et al.*, 1996; Nannipieri, 1994), while soil enzyme activities are especially significant because they are intimately involved in catalyzing reactions necessary for organic matter decomposition, nutrient cycling, energy transfer, environmental quality and crop productivity (Dick, 1994; Tabatabai, 1994). Enzymatic activities also reflect the overall microbial activity of soil, and as such are sensitive to variations induced by natural and man-made factors (Gianfreda and Bollag, 1996). Accordingly, enzymatic activities are thus well suited to measure possible impacts of validamycin on soil health (Schinner *et al.*, 1996; van Beelen and Doelman, 1997). The objective of the present study was to evaluate the effect of validamycin on the soil enzymatic activity of soil.

2 Materials and methods

Soil

A yellow loamy soil collected from 0–20 cm layer from the Botanical Garden (Zhejiang University, Hua-jia-chi Campus, Hangzhou, China) was used in this investigation. Soil samples were air-dried at room temperature; sieved at 2 mm to remove plant materials, soil macrofauna and stones; homogenized in a rotary cylinder. The soil samples contained 1.41% total organic C content, and 115.8 mg kg^{-1} available N, 25.2 mg kg^{-1} available P, 58.5 mg kg^{-1} available K with a pH 7.10 (H_2O). 0.4% total soluble salts and had a pH in water (1:2.5 w/v) of 7.2.

Pesticides

The validamycin was produced by Qianjiang Biochemical Limited, China. This pesticide (5%) was used as commercial formulations and added to soil. The control rate of validamycin was 0 ml·kg^{-1} dry soil (treatment 1), and the treatment 2–7 were added in practice at 7.5, 15, 30, 60, 120, 240 ml·kg^{-1} dry soil respectively.

Soil treatment with pesticides

Soil was placed in cylindrical plastic pots, 2 kg in each. Then, 500 ml of sterile deionized water were added to submerge the soils to a depth of 2 cm. The soils were then incubated in the dark at 28 ± 1°C for 2 weeks for soil microorganism resumed. Pesticides were diluted with distilled water and applied to the soil as a part of the moisture required to adjust the soil to 40% of its water holding capacity (w.h.c.). These were added at rates corresponding to the treatment 1–7. Seven treatments were set up in duplicates and soil was incubated at 28°C for 5 weeks. Loss of water by evaporation was compensated everyday to avoid dryness. After 3rd, 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35^{th} day of incubation time following pesticide application soil sub-samples were taken for assaying catalase, urease, acid phophatase activities.

Assaying of soil enzymes activity

Catalase activity was measured using the titration method. Fresh soil (5 g) was added 25 ml 3% H_2O_2, the samples were placed at 0–4°C for 30 min again, before terminating the reaction with the addition of 25 ml 1 M H_2SO_4. After filtration, 4 ml 0.5 M H_2SO_4 was added to 1 ml filtrate, using 5 mM $KMnO_4$ to measure the O_2 absorbed (Xu and Zheng, 1986).

Urease activity (UA) was measured by the method of Hoffmann and Teicher (1961). 0.25 ml toluene, 0.75 ml citrate buffer (pH 6.7) and 1 ml of 10% urea substrate solution were added to the 1 g sample and the samples were incubated for 24 h at 37.8°C. The formation of ammonium was determined spectrophotometrically

at 578 nm and results were expressed as NH_3-Nmg 100 g^{-1} dry soil sample. Acid phosphatase activity was determined using p-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two millilitres 0.5 M sodium acetate buffer at pH 5.5 (Nannipieri *et al.*, 1980) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37.8°C for 90 min. The reaction was stopped by cooling at 28°C for 15 min. Then, 0.5 ml 0.5 M CaCl2 and 2 ml 0.5 M NaOH were added, and the mixture was centrifuged at 2287 g for 5 min. The p-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm. Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

Data analysis

Each treatment was performed in duplicate, the measurements of enzyme activities were evaluated by analysis of variance (ANOVA).

3 Results and discussion

All soil samples used in experiments were fetched from one place, and were sift out before mixture, so three kind of enzymes assessed had the same activities in zero day treatment between 7 different samples, and the changes of enzymatic activity in the control plot may be due to the change of temperature and the source water quality.

Catalase activity

The effect of validamycin on catalase was showed as Fig. 1 and Table 1, the catalase activity of all treatment

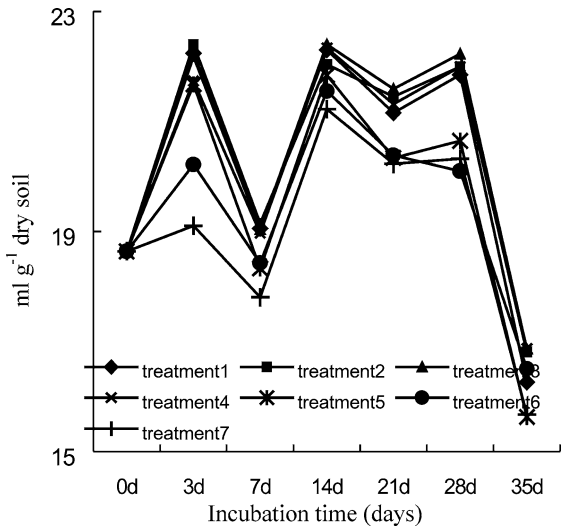

Fig. 1 The effects of validamycin on the activity of soil catalyse

fluctuated during the whole experiment, the fluctuation extent of 7 treatments were different but had the same current. After 3th day treatment, the activity of catalase began to change, the activity of treatment 7 (the concentration of validamycin was 240 ml·kg^{-1} dry soil) was significant lower than that of the control (Table 1), drop down to about 86%, while catalase activity of other treatments had no significant different with that in the control pot. During 7–28th day treatment, the catalase activities of 5, 6, 7 treatments (the concentrations of validamycin were 60, 120, 240 ml·kg^{-1} dry soil, respectively) were also inhibited by validamycin in different degrees, the degree of the inhibition had the dose-effect relationship with the concentration of validamycin, the higher of treated concentration of validamycin, the stronger inhibition of

Table 1 The influence of validamycin to the activity of soil catalase

	0d	3d	7d	14d	21d	28d	35d
Treatment 1	18.64 ± 0.01	22.24 ± 0.05 a	19.05 ± 0.04 a	22.30 ± 0.11 a	21.15 ± 0.19 abc	21.85 ± 0.17 ab	16.26 ± 0.31 a
Treatment 2	18.64 ± 0.01	22.40 ± 0.11 a	19.14 ± 0.15 a	22.03 ± 0.16 ab	21.45 ± 0.23 a	21.99 ± 0.03 ab	16.81 ± 0.12 a
Treatment 3	18.64 ± 0.01	22.18 ± 0.11 a	19.03 ± 0.13 a	22.40 ± 0.04 a	21.59 ± 0.04 a	22.23 ± 0.04 a	16.86 ± 0.15 a
Treatment 4	18.64 ± 0.01	21.73 ± 0.08 a	18.97 ± 0.13 ab	22.33 ± 0.09 a	21.31 ± 0.24 ab	22.00 ± 0.25 ab	16.87 ± 0.46 a
Treatment 5	18.64 ± 0.01	21.67 ± 0.31 a	18.33 ± 0.21 cd	21.84 ± 0.18 ab	20.34 ± 0.43 c	20.65 ± 0.81 bc	15.63 ± 0.68 a
Treatment 6	18.64 ± 0.01	20.21 ± 0.15 ab	18.43 ± 0.21 bc	21.55 ± 0.10 bc	20.38 ± 0.25 bc	20.10 ± 0.04 c	16.5 ± 0.27 a
Treatment 7	18.64 ± 0.01	19.11 ± 1.56 b	17.80 ± 0.12 d	21.22 ± 0.00 c	20.23 ± 0.04 c	20.33 ± 0.42 c	15.67 ± 1.51 a

Means with the same letter are not significantly different at $P = 0.05$.

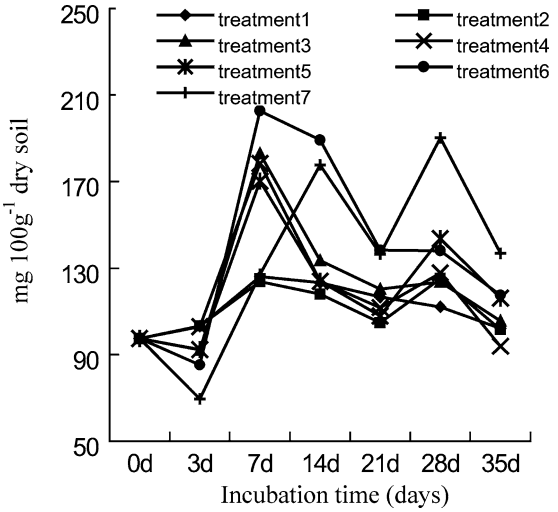

Fig. 2 The effect of validamycin on the activity of soil urease

catalyse suffered. The activity was close to that of the control after 35th day later.

Urease activity

Urease catalyses the hydrolysis of urea to CO_2 and NH_3, which is of particular interest because urea is an important N fertiliser. Urease is released from living and disintegrated microbial cells, and in the soil it can exist as an extracellular enzyme absorbed on clay particles or encapsulated in humic complexes (Nannipieri, 1994; Megharaj *et al.*, 1999; Marcote *et al.*, 2001). The effect of validamycin on urease was showed as Fig. 2 and Table 2: the activity of urease treated by validamycin rose at first and then dropped down slowly as showed in Fig. 2. The fluctuation extent of high dose treatment was different with that of the control. After 3rd day later, the concentration of 240 ml·kg^{-1} dry soil began to inhibit the activity of urease significantly. According to AVONE analysis, the activity was only 67.3% of the control's (Table 2). Following the extend of treatment time, the soil urease was stimulated and the activities of urease in other treatments (like treatment 3, 4, 5, 6) were higher than the control's after 7th day treatment, but the stimulative effect could only be maintain for a short time (about one week) in low dosage concentration of validamycin, while validamycin treatment with high dosage (like treatment 6) could stimulate the activity of urease until 21th day or

Table 2 The influence of validamycin to the activity of soil urease

	0d	3d	7d	14d	21d	28d	35d
Treatment 1	97.51 ± 1.53	103.04 ± 0.33 a	126.10 ± 2.28 bc	123.22 ± 1.14 b	116.53 ± 4.08 ab	112.04 ± 8.48 b	102.12 ± 3.26 bc
Treatment 2	97.51 ± 1.53	103.16 ± 7.01 a	123.68 ± 0.82 c	117.92 ± 7.01 b	104.77 ± 4.40 b	125.07 ± 5.06 b	101.66 ± 0.98 bc
Treatment 3	97.51 ± 1.53	92.21 ± 9.13 a	182.95 ± 1.14 a	133.48 ± 7.5 b	120.22 ± 8.32 ab	123.57 ± 19.90 b	105.58 ± 0.33 bc
Treatment 4	97.51 ± 1.53	102.81 ± 6.52 a	178.34 ± 10.27 a	123.91 ± 12.23 b	111.69 ± 10.27 ab	127.83 ± 2.12 b	93.82 ± 0.00 c
Treatment 5	97.51 ± 1.53	92.32 ± 2.45 a	170.15 ± 4.57 a	123.8 ± 6.85 b	107.89 ± 4.89 b	143.63 ± 17.28 ab	116.07 ± 5.38 b
Treatment 6	97.51 ± 1.53	85.17 ± 4.73 ab	202.78 ± 14.84 a	189.17 ± 10.60 a	138.33 ± 9.13 a	137.98 ± 20.38 ab	117.57 ± 8.81 b
Treatment 7	97.51 ± 1.53	69.38 ± 2.83 b	127.03 ± 30.66 bc	177.76 ± 18.91 a	136.48 ± 5.5 a	190.21 ± 20.22 a	136.83 ± 2.82 a

Means with the same letter are not significantly different at $P = 0.05$.

even longer (like treatment 7, which stimulated urease during the whole experiment process).

Acid phosphatase

In soil, phosphatases, extracellularly secreted by plants and microorganisms, play a key role in the phosphorus cycle, allowing the formation of inorganic phosphorus, the only phosphate-form taken up by plants and microorganisms (Marcote *et al.*, 2001). These enzymes are usually not free in solution but associated with soil constituents. Indeed, they have been localized within structured soil particles, by means of experimental probes utilizing microscopic observations of soil sections (Ladd *et al.*, 1996).

The effect of validamycin on acid phosphatase was shown as Fig. 3 and Table 3. Acid phosphatase activity in soil treated with the seven rates showed different responses (Table 3). Validamycin had not significantly changed the enzyme activity during the most incubation periods, only validamycin with high concentration (treatment 6 and 7) could stimulate the activity of acid phosphatase. Promotion was recorded only once by the two rates of treatment in one week (7–14th day) after soil treatment.

Soil enzymes could behave differently when exposure to one kind of pollutant (Margesin *et al.*, 2000). Pesticides are used for increasing crop production, but increased usage caused hazardous effects to the environment and human health (Dimitrios *et al.*, 2000). The major problem with the recommended use of pesticides

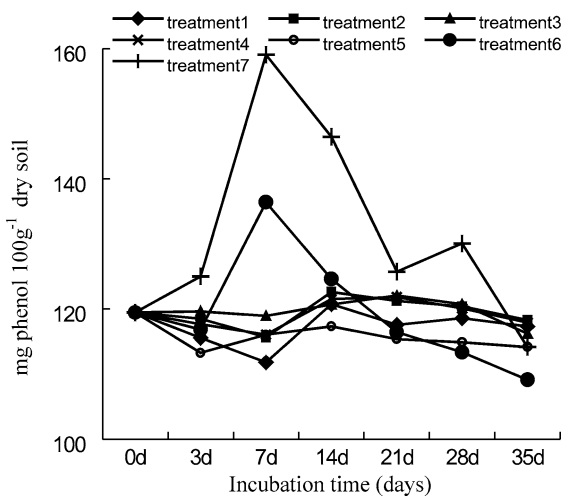

Fig. 3 The effect of validamycin on the activity of soil acid phosphatase

Table 3 The influence of validamycin to the activity of soil acid phosphatase

	0d	3d	7d	14d	21d	28d	35d
Treatment 1	119.47 ± 1.03	115.56 ± 2.54 a	111.81 ± 0.69 c	120.77 ± 0.46 b	117.60 ± 1.73 a	118.57 ± 1.27 ab	117.35 ± 2.54 a
Treatment 2	119.47 ± 1.03	118.49 ± 1.61 a	115.64 ± 2.19 c	122.65 ± 0.58 b	121.26 ± 0.69 a	120.45 ± 0.00 ab	118.33 ± 1.61 a
Treatment 3	119.47 ± 1.03	119.63 ± 2.07 a	118.98 ± 3.69 bc	120.69 ± 0.58 b	122.08 ± 0.46 a	120.77 ± 0.23 ab	116.21 ± 1.61 a
Treatment 4	119.47 ± 1.03	117.68 ± 2.07 a	116.05 ± 4.38 c	121.51 ± 4.03 b	121.83 ± 4.96 a	120.12 ± 2.54 ab	117.92 ± 3.34 a
Treatment 5	119.47 ± 1.03	113.28 ± 5.07 a	116.05 ± 2.31 c	117.35 ± 4.84 b	115.40 ± 3.00 a	114.91 ± 1.61 b	114.17 ± 2.65 a
Treatment 6	119.47 ± 1.03	116.86 ± 16.60 a	136.42 ± 11.53 b	124.6 ± 1.04 b	116.45 ± 0.12 a	113.36 ± 0.58 b	109.12 ± 0.35 a
Treatment 7	119.47 ± 1.03	125.01 ± 18.21 a	159.08 ± 1.61 a	146.45 ± 1.50 a	125.75 ± 5.65 a	130.07 ± 7.61 a	114.17 ± 4.5 a

Means with the same letter are not significantly different at $P = 0.05$.

is that so little actually reaches the target pests. Pesticides is applied and worldwide about 3 billion kg is applied each year, estimates are that less than 0.01% of the pesticides that are applied reach the target pests (Pimentel and Levitan, 1986). This, of course, means that 99.9% of the pesticide that is applied pollutes the environment. In addition, pesticides contaminate human food and water resources. For example, about 35% of the food that is purchased by consumers has measurable levels of pesticide residues, with 1% to 3% having residues that are above the accepted tolerance level (Pimentel, 2002). Methods for controlling pests and diseases using chemical pesticides may be highly effective, but at the same time may be damaging to the environment. Arising concerns on the use of chemical pesticides have recently led to the development and commercialization of biological pesticides. In contrast to synthetic chemicals, biological pesticide is biological in origin (i.e., viruses, bacteria, pheromones, natural plant compounds). And now, a number of biological pesticides have been already put into use to control various types of insects responsible for the destruction of forests and agricultural crops.

A misconception, held by some proponents of biological pesticides, is that because they are 'natural' they are inherently safe. That may be true in some particular cases, biological pesticides, however, may have the potential to risk human beings as well. Naturally produced molecules also can be extremely toxic. Nicotine or *Clostridium botulinum* toxins are, respectively, of similar or greater toxicity than the most acutely toxic synthetic pesticides. Organisms such as *Plasmodium falciparum*, *Escherichia coli* 0157 or *Listeria spp*. have produced significant numbers of deaths or cases of disease in humans. There is therefore a need to assess the potential of biological pesticides to adversely affect ecosystem (Dewhurst, 2001). Validamycin is one of biological pesticides, and when it is sprayed into soil, it can affect soil ecosystem. In our research, the activities of catalyse, urease, acid phosphatase were changed significantly in soil samples which treated with validamycin, and the fluctuation extents were related with the kinds of soil enzymes, the treatment concentration of validamycin and treated time. For example, validamycin could inhibit catalyse and urease in initial stages, but the catalyse activity could resume to normal level, while the activity of urease could be stimulated and became higher than that of the control during treatment process; validamycin had no significant inhibitory effect on the acid phosphatase activity during initial stages, on the contrary, it stimulated the enzyme activity one week later. All of the experiment results also showed that the influent time of validamycin to soil enzyme was very short, only about one month (to catalyse and acid phosphatase), while validamycin could maintain a litter longer time to urease stimulation than experiment designed time (35th day).

As other reports, the phenomenon of soil enzymes stimulated or inhibited was coexistent, the fluctuation extent was also different among different enzyme sorts. Researchers reported that the enzyme-complexes mostly investigated are those attained by adsorption or interaction of enzyme molecules with pure clays, humus materials or humus-like compounds (Theng, 1979; Boyd and Mortland, 1990; Ladd and Butler, 1975; Rao *et al.*, 2000). Validamycin or other pesticides may combine with active site of soil enzyme or effect on substrate accessibility to the active site and caused chemical or conformational changes of enzymatic structure (McLaren and Packer, 1970; Nannipieri and Gianfreda, 1998). But these effect models were difficult to explain the effect of soil treated with validamycin on urease activity fluctuated between inhibition and promotion. So, the mechanism of how validamycin changed soil enzymes could not only validamycin influence enzyme active site, but also influence other soil physical and chemical, especially biological factors. We concluded that it was very possible that validamycin in soil disturbed diversity, biomass, and activity of soil microorganisms (or lethality, inhibit and stimulation), and changed the excretion of soil microbes, which can affect soil enzymes activity, accordingly. The results that the higher concentration of validamycin, the stronger catalyse inhibited and the earlier coming of the inhibition, also proved that one or some microorganism species were died or inhibited under high dosage of validamycin, and treatment time extended, the activity could resume slowly.

From these results, we speculated there was two kind of explanations: 1) validamycin may be easy to be degraded in soil environment, for the change of soil enzymatic activities maintained for a short time, but no matter degraded completely or not, validamycin or its degraded products were somewhat toxic on microorganisms biomass and soil enzymes activity; 2) validamycin may be toxic to some microorganism species, so the activities of catalyse and urease were inhibited, but the high activities of urease and phosphatase

showed that it was far more likely that validamycin (or its degraded product) was acting as a kind of organic carbon source (to some microorganism species) which increased microbial biomass and in turn increased the activities of urease and acid phosphatase, when this kind of carbon resource exhausted, soil microorganism would resume to normal level.

To illuminate the relationship between the fluctuation of soil enzyme and the succession of microorganism species, and which kind of soil microorganism can utilize or be inhibited by validamycin (or its degraded product), we will use molecular biology techniques including temperature or denatured gradient gel electrophoresis (TGGE or DGGE) to study the composition and structure of the whole microbial community in the soil.

4 Conclusion

Our results indicate that validamycin significantly affect enzymatic activities in soil in short time, which could be stimulated or inhibited. The present findings mean that the biological pesticides are only relative safe pesticides which could also cause environmental risk and the field to evaluate the environmental safety of other biological pesticides should be attracted attention.

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