Effects of repeated applications of fungicide carbendazim on its persistence and microbial community in soil

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Abstract

Carbendazim, a systemic benzimidazole fungicide, is applied repeatedly to control plant diseases including soilborne diseases, over a growing season. Studies were carried out under laboratory conditions to assess the effects of repeated carbendazim applications on its persistence and microbial community in soil. The results indicate that dissipation of carbendazim in soil was accelerated with its application frequency. The degradation rate constant of carbendazim was increased significantly from 0.074 d^{-1} to 0.79 d^{-1}. The corresponding half-life was shorten markedly from 9.3 d to 0.9 d after four repeated applications. No significant inhibitory effect of carbendazim on soil microbial utilization of the carbon sources was observed after first treatment, but a slight increase in average well color development (AWCD) was shown after second, third, and fourth applications. It suggested that soil microorganisms become adapted to carbendazim after repeated application. Simpson and Shannon indexes of soil microbial community from carbendazim treated soil were also similar to those from the control soil, indicating that the richness and dominant character of soil microorganisms remain unchangeable after repeated application. However, after first, second, and third addition of carbendazim, McIntosh indexes on day 21 were significantly higher compared with the control, suggesting that balance of soil microorganisms was altered due to the enrichment of the specific carbendazim-adapting strains in soil.

Key words: carbendazim; degradation; soil microorganism; repeated application

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Introduction

Fungicides are designed to suppress the biochemical and physiological metabolism of the target phytopathogenic fungi, at the same time might have lasting effect on non-target soil-inhabiting microorganisms. A lot of research works have been conducted for assessing the dissipation of fungicides in soil and their effects on soil microorganisms (Martinez-Toledo *et al*., 1998; Smith *et al*., 2000; Sigler and Turco, 2002; Kinney *et al*., 2005; Bending *et al*., 2007). However, most of assessing tests were carried out only with a single application. In practice, a fungicide may be applied repeatedly over a growing season for disease control. Subsequent applications of same or similar pesticides could increase the degradation rate and accelerated losses (Turco and Konopka, 1990; Bischoff *et al*., 2005). There is a need to investigate residual characterization of a fungicide in soil and its possible effect on soil microbial functions for repeated application.

Carbendazim, methyl 2-benzimidazole carbamate, is a systemic benzimidazole fungicide and used to control a broad range of diseases on arable crops (e.g., cereals, oil seed rape), fruits, vegetables, ornamentals and medicinal herbs. It is also a main metabolic product of some other systemic fungicides, such as benomyl and thiophanate-methyl (Fleeker *et al*., 1974; Mongomery, 1997). The persistence of carbendazim in soil and its effect on soil microorganisms after a single application were investigated (Niewiadomska and Sawicka, 2002; Grogan and Jukes, 2003). Burrows and Edwards (2004) reported that the growth of microorganisms in lucerne planted soil was inhibited by carbendazim, and a significant inhibitory effect on soil ammonification, nitrification, and soil dehydrogenase activity was also observed. According to Shi (2007), the carbendazim is recommended to be applied as often as every 10 to 15 d. However, so far limited information is available about the persistence of carbendazim in soil following repeated field applications and the potential influence of such treatments on soil microorganisms.

The present study was designed to determine the persistence of carbendazim under laboratory conditions and to assess its potential effect on soil microbial community after repeated applications. The results will be useful for predicting the fate of subsequent carbendazim application after prior treatment, and assessing the possible effect on soil quality.

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1 Materials and methods

1.1 Chemicals and soil

Carbendazim (≥ 99.5%) was purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture, China. A commercial formulation of carbendazim (50% active ingredient, Zhenjiang Pesticide Co., Ltd., China) was used for soil treatment. The chemical reagents used in the experiments were all of analytical grade.

Surface soil (0–20 cm) of 20 g was collected from a vegetable field in the Agricultural Farm of Zhejiang University, China. The soil was freshly passed through a 2-mm sieve and then stored at room temperature for one week before use. The physiochemical properties of the soil were determined using standard methods (Liu, 1996). The soil properties were as follows: sand 21.5%; silt 71.1%; clay 7.4%; organic matter content 3.05%; water holding capacity (WHC) 39.4%; cationic exchange capacity (CEC) 10.6 cmol/kg; total nitrogen 0.14%; and pH 6.8.

1.2 Soil treatment

Considering the fact that carbendazim application rate usually is 3 or 4 times more than the recommended, the carbendazim concentration in soil was designed to be 10 mg/kg, which is 5 times of recommended dosage. A certain amount of the commercial carbendazim was well mixed with the soil and then put into 2 L flowerpots. The final soil water holding capacity was adjusted to 60%, and the pots were covered with aluminum foil. The soil without addition of carbendazim used as control and was also prepared with similar procedures. Three replicates for both the control and carbendazim treatments were performed. The soils were kept in the dark at (25 ± 1)°C. Soil water content was maintained by addition of water at 2 d intervals. The second, third, and fourth addition of carbendazim was performed on day 29, 59, and 85 after the first treatment, respectively. Soil samples were collected at 2 h, day 1, 3, 7, 14, 21, and 29 after each treatment for residual carbendazim measurement and microbial diversity analysis.

1.3 Measurement of residual carbendazim in soil

For extraction of residual carbendazim, 20 g soil was taken from the microcosms and transferred to a 250-mL Erlenmeyer flask, followed by addition of 80 mL of methanol and 10 mL of 0.2 mol/L HCl. The soil-solvent mixture was shaken for 2 h at 200 r/min on a mechanical shaker, and then through a 7-cm Buchner funnel. The filter cake was washed twice with 50 mL of methanol. The filtrates were collected in a 250-mL Erlenmeyer flask and concentrated on a rotary evaporator to about 10 mL. The concentrated extract was transferred into a separatory funnel containing 10 mL of 0.2 mol/L HCl and 50 mL of distilled water, and then washed with 50 mL of petroleum ether (60–90°C). After discharging the organic phase, pH of the left phase was adjusted to 7.0 with 1 mol/L NaOH. Subsequent to 20 mL of saturated NaCl addition, the solution was extracted three times with 50, 40, and 30 mL of methane dichloride, respectively. All extracts were then passed through a layer of anhydrous sodium sulfate, and collected in a flat-bottom flask. The sample extract was concentrated to near dryness on a rotary evaporator and dissolved in *N,N*-dimethylformamide, for HPLC analysis.

The HPLC (1200 series, Agilent Technologies, USA) was equipped with DAD set to 281 nm. A Hewlett Packard stainless steel analytical column (ZORBAX SB-C$_{18}$, 25 cm × 4.6 mm, 5 μm) was used. A Hewlett Packard stainless steel guard column packed with ZORBAX SB-C$_{18}$ (4.6 cm × 12.5 mm, 5 μm) preceded the analytical column. The mobile phase consisted of methanol and water with a ratio of 65:35 (*V/V*) and all analysises were performed at a flow rate of 0.8 mL/min. Injection of 10 μL extract sample was performed in HPLC for quantitative analysis. Five-point calibration curve was constructed from the peak area of the calibration run. The concentration of residual carbendazim in the extract was determined by comparing the peak area with that of reference standard. Under the experimental conditions, the HPLC retention time for carbendazim was about 5 min.

1.4 Analysis of soil microbial diversity

Microbial utilization of carbon substrates was assessed using Biolog Eco microplates (BIOLOG, Hayward, USA), which contained three replicate wells of 31 carbon sources and a water blank containing no carbon source. Soil microbial suspension was prepared by suspending a certain amount of fresh soil equivalent to 10 g of dry soil in 100 mL of sterile physiological saline (0.85% NaCl). The suspension was then diluted stepwise to 10^{-3} in the sterile physiological saline. A 150-μL of the diluted microbial suspension was added to each well of the Biolog Eco plate using an 8-channel repeating pipettor. The plates were incubated at 25°C in the dark for 7 d and color development in the wells was measured as absorbance at 590 nm every 24 h with a microplate reader. The average well color development (AWCD) was calculated according to the methods described by Garland (1996). The functional diversity indices expressed as Shannon-Weaver index (*H'*), Simpson index (1/*D*), McIntosh index (*U*), using following Eqs. (1)–(3).

$$H' = -\sum p_i \ln p_i \tag{1}$$

$$D = \sum \frac{n_i(n_i - 1)}{N(N - 1)} \tag{2}$$

$$U = \sqrt{\sum n_i^2} \tag{3}$$

where, p_i is the proportion of the corrected absorbance value of each well to the sum of absorbance values of all wells; n_i is the corrected absorbance value of well number i, N is the sum of absorbance values of all the wells.

1.5 Data analysis

The dissipation data obtained in this study were statistically analyzed with the first-order equation (Eq. (4)) using DPS Data Processing System (Tang and Feng, 2007).

ANOVA analysis was also performed with this software.

$$C = C_0 e^{-kt} \tag{4}$$

where, t is time after treatment, C is concentration of residual carbendazim in soil at time t, C_0 is concentration of carbendazim in soil at time 0.

2 Results and discussion

2.1 Method validation for the determination of carbendazim in soil

To assess the validity of the above described method for the determination of carbendazim in soil, triplicate analyses were conducted for the soil samples which carbendazim concentration was 0.1, 1.0, and 10.0 mg/kg. Extraction and analyses of canbendazim were performed as above description. The chromatograms of blank and carbendazim fortified soil samples are shown in Fig. 1. Average recoveries of carbendazim from soil at levels of 0.1, 1.0 and 10.0 mg/kg were obtained to be (83.2 ± 4.3)%, (76.9 ± 3.6)%, and (77.7 ± 1.2)%, respectively (Table 1). The result indicated that the method is satisfactory for determination of residual carbendazim in soil.

2.2 Degradation of carbendazim in soil after repeated application

Figure 2 shows the pattern of carbendazim disappearance from the test soil. Approximately 52%, 72%, 79%, and 83% of carbendazim was degraded within 3 d after first, second, third, and fourth treatments, respectively.
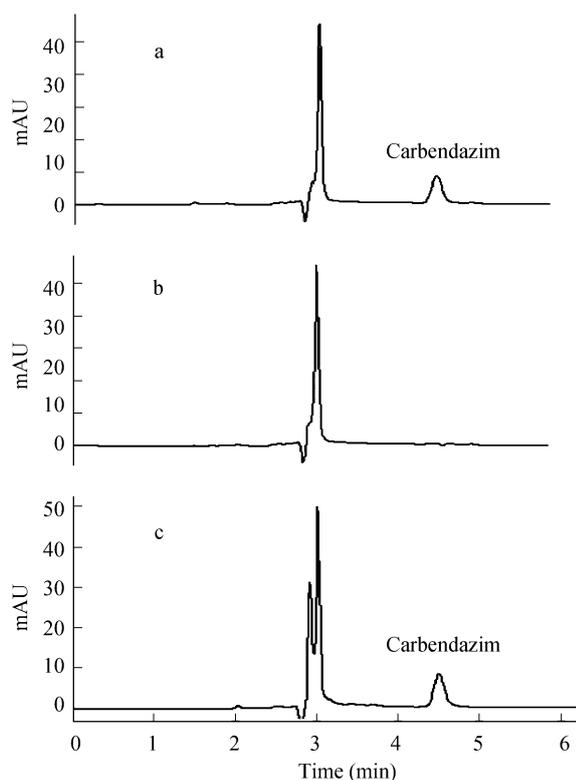
Fig. 1 Chromatograms of carbendazim standard (a), blank soil (b), and carbendazim fortified soil (c).

Table 1 Recoveries and relative standard deviation (RSD) of carbendazim spiked in the soil samples

Carbendazim content (mg/kg)	Sample weight (g)	Average recovery (%)*	RSD (%)
0.1	20.0	83.2 ± 4.3	5.1
1.0	20.0	76.9 ± 3.6	4.7
10.0	20.0	77.7 ± 1.2	1.5

* Mean ± standard deviation of three replications.

The kinetic data derived from first-order function are shown in Table 2. It is remarkable that dissipation of carbendazim was increased with its application frequency. The degradation rate constant of carbendazim in soil increased significantly from 0.074 to 0.7972 d^{-1}, and the corresponding half-live reduced markedly from 9.3 to 0.9 d after four successive applications.

The increase of degradation rate of carbendazim was probably resulted from the enhanced catabolism of the target chemical by soil microorganisms after repeated applications. The result is in agreement with that the degradation of *N,N*'-dibutylurea was accelerated after its repeated applications (Bischoff *et al.*, 2005), and that iprodione degradation rate is influenced by the number of application times (Joan and Maria, 1998). Similar results also have been reported by other researchers (Spain and Van-Veld, 1983; Kearney and Kellog, 1985; Samuel and Pillai, 1991; Bromilon *et al.*, 1996; Mitchell and Cain, 1996; Walker *et al.*, 1996; Arthur *et al.*, 1997; Mohapatra and Awasthi, 1997; Bharati *et al.*, 1998). One possible explanation for the increased pesticide degradation is that: a specific microbial strain or community, which might causes the pesticide degradation, was enhanced by repeatedly applying the pesticide. The other one is that the overall microbial community in soil was stimulated by addition of pesticide as nutrients (Bischoff *et al.*, 2005). Carbendazim contains 23% N and 56% C. Correspondingly, the addition of carbendazim at a rate of 10 mg/kg to soil would yield 5.6 mg/kg C and 2.3 mg/kg N. Compared to total amounts of C (14500 mg/kg) and N (1400 mg/kg) of the tested soil, the addition of carbendazim as nutrients could be negligible. Therefore, the potential ability of a microbial community to break carbendazim is induced and increased after the repeated applications, and resulting in an enhancement in the degradation.

### 2.3 Effect of repeated carbendazim applications on soil microbial diversity

Observations on soil microbial diversity in the carbendazim treated soil samples using Biolog Eco plates were carried out over the 7-d incubation period (Fig. 3). No significant inhibition on the AWCD was observed after first application. It is likely that carbendazim does not have inhibitory effect on soil microbes in the tested soil. Sousa *et al.* (2004) reported that carbendazim has significant inhibitory effect on substrate induced respiration (SIR) of soils sampled in Bangor (UK) and Coimbra (Portugal), while no effect on SIR in Flörsheim (Germany) or Amsterdam (Netherlands) soils for any sampling time. Such inconsistent results suggest that the effect of carbendazim

Table 2 Kinetic data for dissipation of carbendazim in soil after repeated application

Application frequency	C_0 (mg/kg)	k (d^{-1})	R^2	$t_{1/2}$ (d)
1	8.709 ± 0.1627 a	0.074 ± 0.0014 a	0.8243 ± 0.0163	9.3 ± 0.3 a
2	10.7247 ± 0.1067 b	0.3625 ± 0.0104 b	0.8809 ± 0.0038	1.9 ± 0.0.1 b
3	9.822 ± 0.0359 b	0.6494 ± 0.0158 c	0.9431 ± 0.0030	1.1 ± 0.04 c
4	9.5097 ± 0.2210 c	0.07972 ± 0.0713 d	0.9150 ± 0.0051	0.9 ± 0.1 c

All values are expressed as means ± SD of triplicate samples.
Means followed by the same letter within a column were not significantly different ($P < 0.05$).
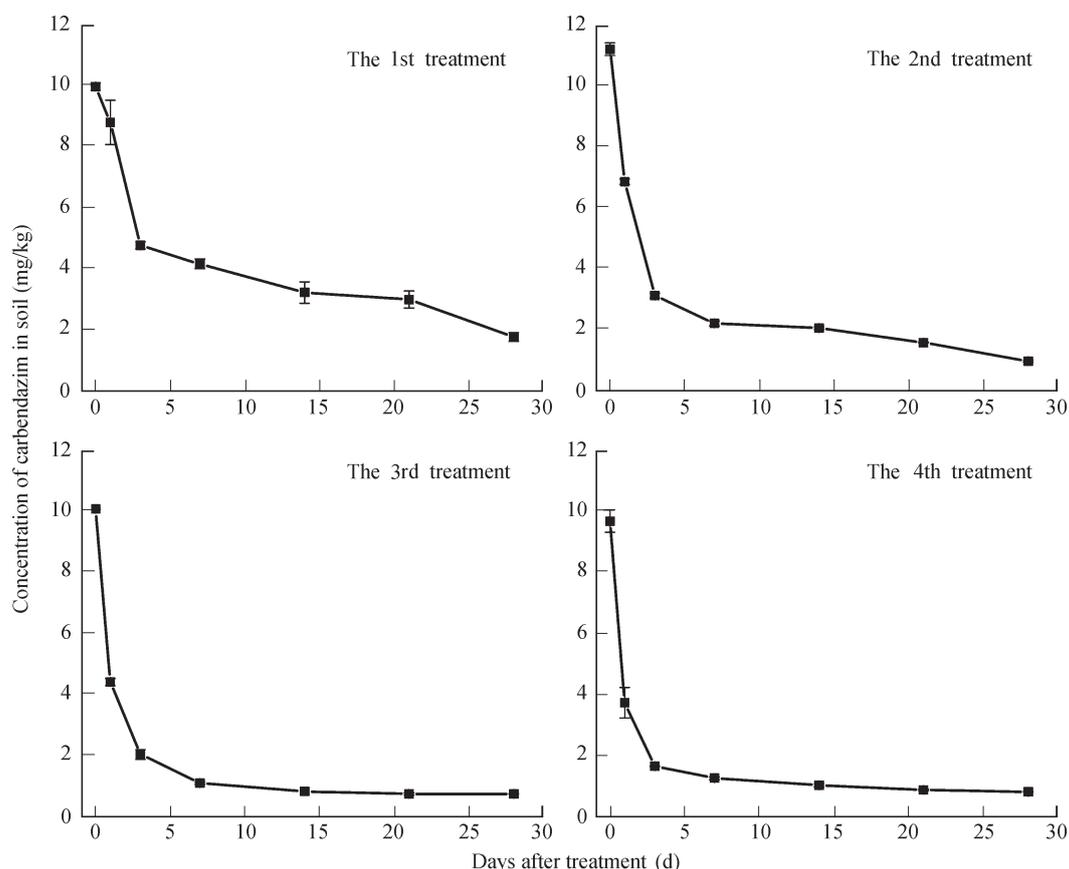
Fig. 2 Dissipation of carbendazim in soil after repeated applications.

on soil microbial community probably depend on soil types.

With repeated applications of the fungicide, soil microorganisms might become adapted to it. A slight increase in AWCD was observed on day 21, day 7 and 21, day 3 and 7 after second, third, and fourth additions of carbendazim, respectively (Fig. 3). In agreement with the changes in AWCD, Simpson and Shannon indexes from carbendazim applied soil were similar to those from the control soil (Table 3). The result indicates that the richness and dominant soil microorganisms in the tested soil were not altered by the repeated applications of carbendazim. However, McIntosh index on day 21, after first to third repeated additions of carbendazim, were much higher than those in the controls, indicating that the balance between the various types of microorganisms might be altered by the enrichment of the specific carbendazim adapted strains in the soil.

Fungicides, designed to control pathogenic diseases,

could have an inhibitory effect on some types of soil microorganisms. However, the effect of a fungicide on soil microorganisms is not always negative. Both enhancement and inhibition of a variety of dominant microorganisms were observed following a single chlorothalonil application and a short incubation period (Sigler and Turco, 2002). In the present study, no significant inhibition of repeated carbendazim application on Simpson and Shannon indexes was observed. Contrarily, McIntosh indexes on day 21 after first, second, and third applications increased dramatically. This might be resulted from adaptation of microbes to carbendazim under the selective pressure caused by carbendazim residues in soil. The adaptation of microbes to carbendazim leads to the accelerated degradation observed after repeated applications, especially 3rd and 4th applications.

Carbendazim is usually applied to control a broad range of diseases on arable crops as well as soilborne diseases. Practically, it is recommended to be applied repeatedly
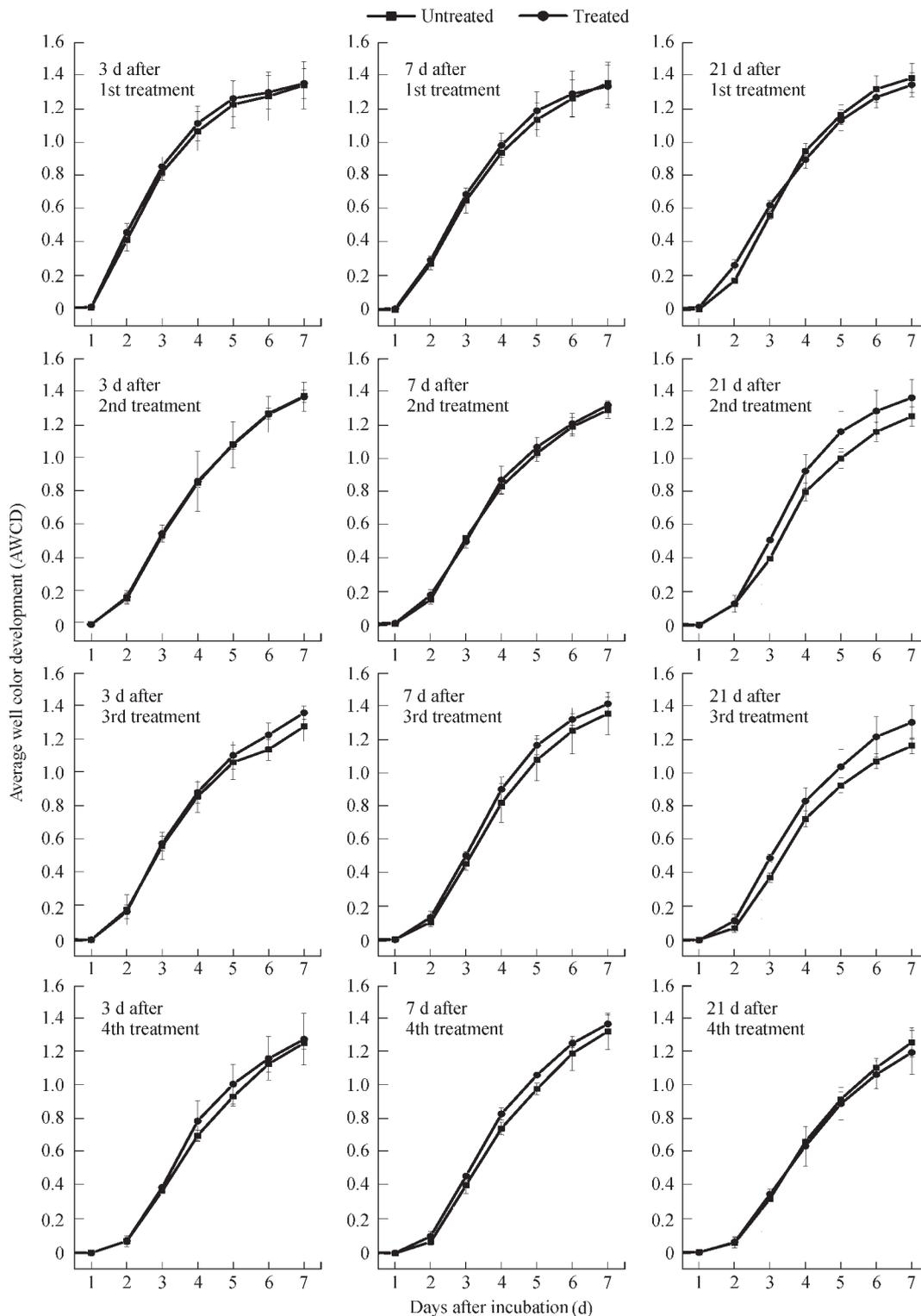
Fig. 3   Average well color development changes over the incubation period of the soil repeatedly treated with carbendazim.

over a growing season if necessary. For instance, carbendazim is applied repeatedly every 10 d for the control of grain smut and leaf blight in the production of herb Semen Coicis. The results demonstrated that the prior treatment with carbendazim to soil can substantially reduce the persistence of subsequently applied fungicide. The half-life of carbendazim reduced from 9.3 d to 0.9 d. As a result, the time period for soilborne pathogen exposed to carbendazim must be shortened, which might lead to a poor performance of carbendazim for the control of soilborne diseases.

3 Conclusions

The investigation indicates that multiple and successive applications of carbendazim to soil accelerated its degra-
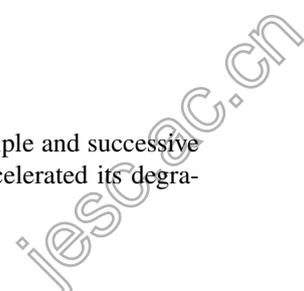
Table 3 Effect of repeated carbendazim applications on diversity of soil microbial community

Application frequency	Days after treatment	Simpson index		Shannon index		McIntosh index	
		0 mg/kg	10 mg/kg	0 mg/kg	10 mg/kg	0 mg/kg	10 mg/kg
1	3	20.47 ± 0.2 a	22.34 ± 1.0 a	3.15 ± 0.05 a	3.19 ± 0.08 a	5.41 ± 0.3 a	5.78 ± 0.1 a
	7	18.89 ± 1.1 a	19.26 ± 1.2 a	3.06 ± 0.07 a	3.09 ± 0.05 a	4.96 ± 0.2 a	4.92 ± 0.2 a
	21	16.33 ± 0.3 a	16.44 ± 0.9 a	2.95 ± 0.01 a	2.95 ± 0.05 a	4.34 ± 0.2 a	4.78 ± 0.1 b
2	3	15.95 ± 1.3 a	15.55 ± 1.2 a	2.93 ± 0.09 a	2.96 ± 0.11 a	4.17 ± 0.1 a	4.32 ± 0.3 a
	7	15.96 ± 0.9 a	15.39 ± 1.0 a	2.93 ± 0.05 a	2.95 ± 0.07 a	4.07 ± 0.2 a	4.21 ± 0.5 a
	21	13.05 ± 1.0 a	15.13 ± 0.5 b	2.80 ± 0.07 a	2.90 ± 0.03 a	3.42 ± 0.1 a	4.06 ± 0.1 b
3	3	15.98 ± 1.8 a	15.76 ± 0.5 a	2.92 ± 0.09 a	2.92 ± 0.02 a	4.36 ± 0.4 a	4.53 ± 0.4 a
	7	13.94 ± 0.9 a	15.39 ± 0.8 a	2.86 ± 0.07 a	2.95 ± 0.04 a	3.85 ± 0.3 a	4.02 ± 0.2 a
	21	13.21 ± 0.2a	15.85 ± 0.8 b	2.77 ± 0.04 a	2.96 ± 0.05 b	3.21 ± 0.2 a	3.84 ± 0.2 b
4	3	11.33 ± 0.34 a	13.11 ± 1.64 a	2.92 ± 0.09 a	2.92 ± 0.02 a	3.43 ± 0.1a	3.37 ± 0.2 a
	7	13.05 ± 2.55 a	14.20 ± 0.99 a	2.74 ± 0.13 a	2.82 ± 0.07 a	3.47 ± 0.2 a	3.87 ± 0.2 a
	21	11.03 ± 0.94 a	11.47 ± 0.65 a	2.69 ± 0.10 a	2.72 ± 0.04 a	3.04 ± 0.3 a	3.34 ± 0.2 a

Data are expressed as mean ± SD.
Data in the same row followed by the different letter are significant in difference ($P < 0.05$).

dation in soil and the half-life was shortened significantly from 9.3 d to 0.9 d, which shortened its exposure time to the soilborne diseases as well. Such treatment had no inhibitory effect on soil microbial community with respective to the richness of soil microorganisms, but altered the balance of soil microorganisms by the enrichment of specific strains adapted to carbendazim.

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