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# Effect of carbendazim and pencycuron on soil bacterial community

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ABSTRACT

Soil bacterial composition is used as one of the important indicators of negative effects of the use of pesticides in agriculture. Very little is known on the effects of fungicides, particularly carbendazim and pencycuron on soil bacterial community. In this study, laboratory experiments were conducted to examine the effects of various concentrations of carbendazim and pencycuron on diversity of bacterial community in soils collected from strawberry field and incubated at different temperature conditions. The degradation rate of fungicides both in sterile and non-sterile soils were also investigated. Residues of fungicides were measured using high performance liquid chromatography (HPLC) and the change in bacterial community was examined by comparing the 16S rDNA bands on patterns by denaturing gradient gel electrophoresis (DGGE). The dissipation of carbendazim was affected by concentration applied, whereas that of pencycuron was affected by both concentration applied and incubation temperature. The microbial community could recover to its previous composition after 126 days of cultivation with the application of 10 mg/kg of carbendazim but not with the application of a high concentration, 100 mg/kg, of pencycuron. From cluster analysis, the bacterial community structure showed approximately 50% similarity throughout the experimental period, which indicated that the soil microbiota composition was not stable throughout the 120 cultivation days.



## 1. Introduction

The fungicide carbendazim [methyl benzimidazol-2-yl carbamate], a fungicide of the benzimidazole carbamate type, was introduced to control various vegetable and orchard diseases. It can also be used for eradication of disease and protection against a wide range of fungal diseases, especially caused by *Asccomycetes* spp., *Basidiomycetes*, and *Deuteromycetes* spp., in grapes, fruits and vegetables [1,2]. Moreover, carbendazim is the product of degradation of other benzimidzaole fungicides such as benomyl or thiophanate [3–5]. Carbendazim affects the environment not only by its spread but also by the degradation products of other pesticides. Previous research has shown that the application of fungicides affects the composition of the soil bacterial community [6].

The fungicide pencycuron [1-(4-chlorobenzyl)-1-cyclopentyl-3-phenylurea], a fungicide of the phenylurea type, has been used to protect crops against infection with *Rhizoctonia solani* and *Pellicularia* spp. [2]. A 23.3% suspension concentrate (SC) of pencycuron was recommended to protect rice against *R. solani* attack in Taiwan and to protect Garland chrysanthemum (*Chrysanthemum coronarium*) against Pythium disease [7]. Pencycuron residue is sometimes found in vegetables or fruit such as tomato or cantaloupe (*Cucumis melo* L.), even though the pesticide was not recommended for use in protecting these crops.

In this study, effects of carbendazim and pencycuron on the diversity of the soil microbial community using soil samples collected from a strawberry field in northern Taiwan were examined. The diversity of the microbial community in soil is an important issue in modern soil microbiology and is one of the main indicators of toxic effects of pesticides in agriculture. Here PCR amplification of 16S rDNA followed by denaturing gradient gel electrophoresis (PCR–DGGE) was used to study complex microbial populations [6,8–10]. The 16S rDNA are present in all known bacteria and their differences in sequence can reflect phylogenetic distance, and DGGE involves the electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. From the DGGE gels, DNA fragments of the same length but with different base pairs (G + C or A + T) can be separated [11].

In this paper, two fungicides with diverse dissipation patterns were compared. To comprehend the relation between pesticide usage and the bacterial community change in soil at the molecular level, not only the dissipation rates of pesticides at different temperatures and concentrations but also the PCR-amplified 16S ribosomal DNA (16S rDNA) fragments using DGGE were investigated. Because of the unevenness of pesticides during application and to magnify the change of microbial communities, 10 and 100 mg/kg carbendazim and pencycuron were used in this study, respectively.

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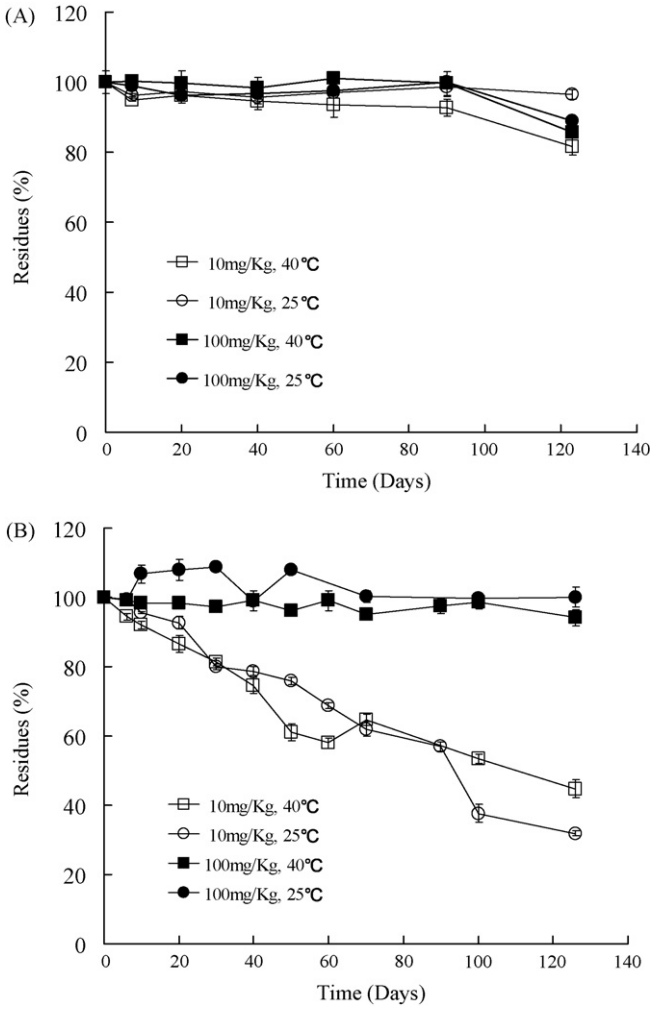


**Fig. 1.** Dissipation of 10 (open symbol) and 100 mg/kg (solid symbol) carbendazim at 25 °C (circle) and 40 °C (square) in sterile (A) and non-sterile (B) soils.

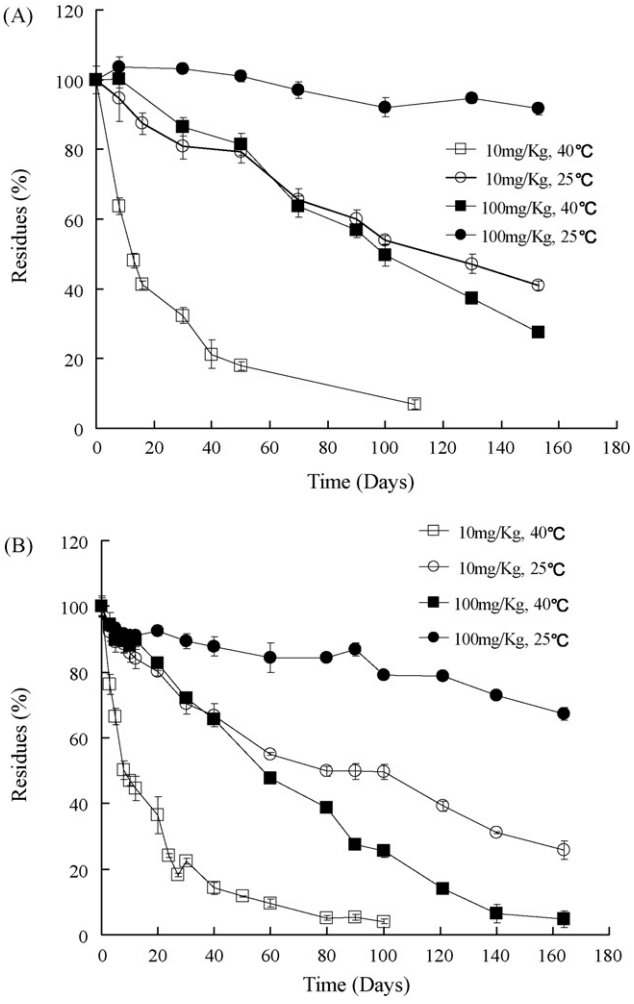


**Fig. 2.** Dissipation of 10 (open symbol) and 100 mg/kg (solid symbol) pencycuron at 25 °C (circle) and 40 °C (square) in sterile (A) and non-sterile (B) soils.

## 2. Materials and methods

### 2.1. Soil and fungicides

Soil samples were collected from a strawberry field located at Da-Hu, an important site of strawberry farming in northern Taiwan. Soil was sampled at a depth of 0–20 cm and stored at 4 °C. The soil texture was sandy loam with 0.44% organic matter content, 53.6% sand, 15.5% clay, 30.9% silt, and pH 7.47. The fungicides carbendazim and pencycuron (with purity of 99.0% and 99.5%, respectively) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

### 2.2. Sample preparation

Soil samples with their water content (8.1%) were meshed through a 2-mm sieve and treated with carbendazim or pencycuron stock solution to final concentrations of 10 and 100 mg/kg for each. Then, 6 mL of acetonitrile (solvent of the two fungicides) was added to soil samples as the control treatment. As well, 60 mL sterile water was added into each treatment to meet the field water capacity (16.7%). The two incubation temperatures were 25 °C and 40 °C to simulate room and summer-month temperatures, respectively. Soil samples underwent sterile treatments, in that prior to treatments, soil samples were sterilized (121 °C, 60 min) 3 times for 3 successive days, then the original water content was restored by adding sterile water.

### 2.3. Extraction and analysis of chemicals

To determine the residual content of carbendazim and pencycuron, 10 mL acetonitrile was added to 12 g soil samples, which were then shaken at 120 rpm for 12 h. Then, the samples were centrifuged at 3500 rpm for 10 min. The supernatants were collected and passed through 0.2-μm filters. The filtrate was analyzed by use of high performance liquid chromatography (HP 1100 Series, Agilent, Santa Clara, CA, USA), equipped with a C-18 reverse phase column (Purospher STAR RP-18, E. Merck). The mobile phase involved use of acetonitrile and water (80:20; v/v), with a flow rate of 1.0 mL/min. Carbendazim and pencycuron were detected with use of a UV–vis detector at 206 and 200 nm, and the retention times were 2.20 and 5.30 min, respectively [3,12–14]. There were two standard curves for each fungicide, concentrations of 1, 2.5, 5, 7.5, 10 and 10, 25, 50, 75, 100 mg $L^{-1}$. Each of them was conducted in triplicates with $R^2$ above 0.99. The recovery of carbendazim and pencycuron was 103.7% and 101.7%, respectively.

### 2.4. DNA extraction

Total genomic DNA was extracted by use the of an UltraClean™ Soil DNA Isolation kit (MO BIO Laboratories, West Carlsbad, CA, USA). Extracted DNA was stored at −20 °C.

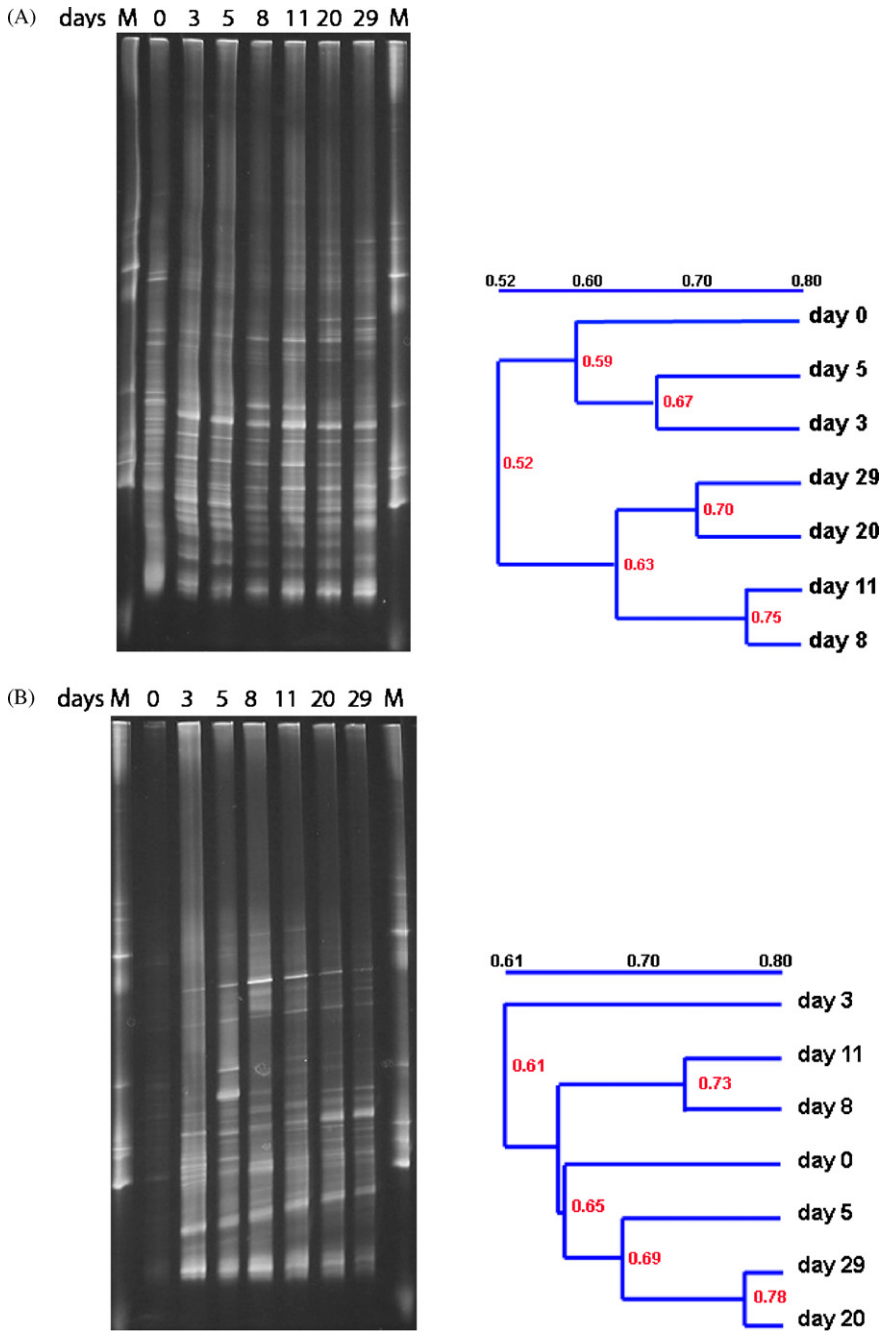


**Fig. 3.** PCR–DGGE analysis of 16S rDNA sequence fragments obtained from pesticide-free soil incubated at 25 °C (A) and 40 °C (B) and cluster analysis of bacterial community structures by UPGMA. Lane M: marker.

### 2.5. PCR–DGGE

PCR was performed with ABgene DNA polymerase and a buffer kit obtained from Applied Biosystems (Forster city, CA, USA). The PCR mixture (50 μL) contained 0.2 mM of each dNTP, 3.0 mM $MgCl_2$, 1× buffer solution, 1 unit of DNA polymerase, 0.4 μM of each primer, and 1 μL of template DNA. Primers 968F and 1401R were used for the amplification of bacterial 16S rDNA [15]. A GC-clamp was attached to the 5′ end of 968F [11]. The GeneAMP® PCR system 9700 (Applied Biosystems, Forster city, CA, USA) was used for PCR amplification. The 16S rDNA was amplified at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were confirmed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining. The reproduction of the PCR–DGGE results has previously been confirmed by performing PCR–DGGE with three replicate DNA samples from the same treatments. DGGE analysis involved use of a D-Code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). Samples of 20 μL of PCR products were loaded onto an 8% (w/v) polyacrylamide gel that contained 40–60% denaturing gradient of formamide and urea. Electrophoresis was run at 60 °C in 1× TAE for 12 h at a constant voltage of 75 V. After electrophoresis, the polyacrylamide gel was stained with SYBR Green I nucleic acid gel stain and visualized on a UV transilluminator. The gel was photographed with use of a CCD camera.

### 2.6. Statistical analysis

The DGGE profiles were analyzed by an unweighted pair-group method with arithmetic averages (UPGMA), and the similarity was

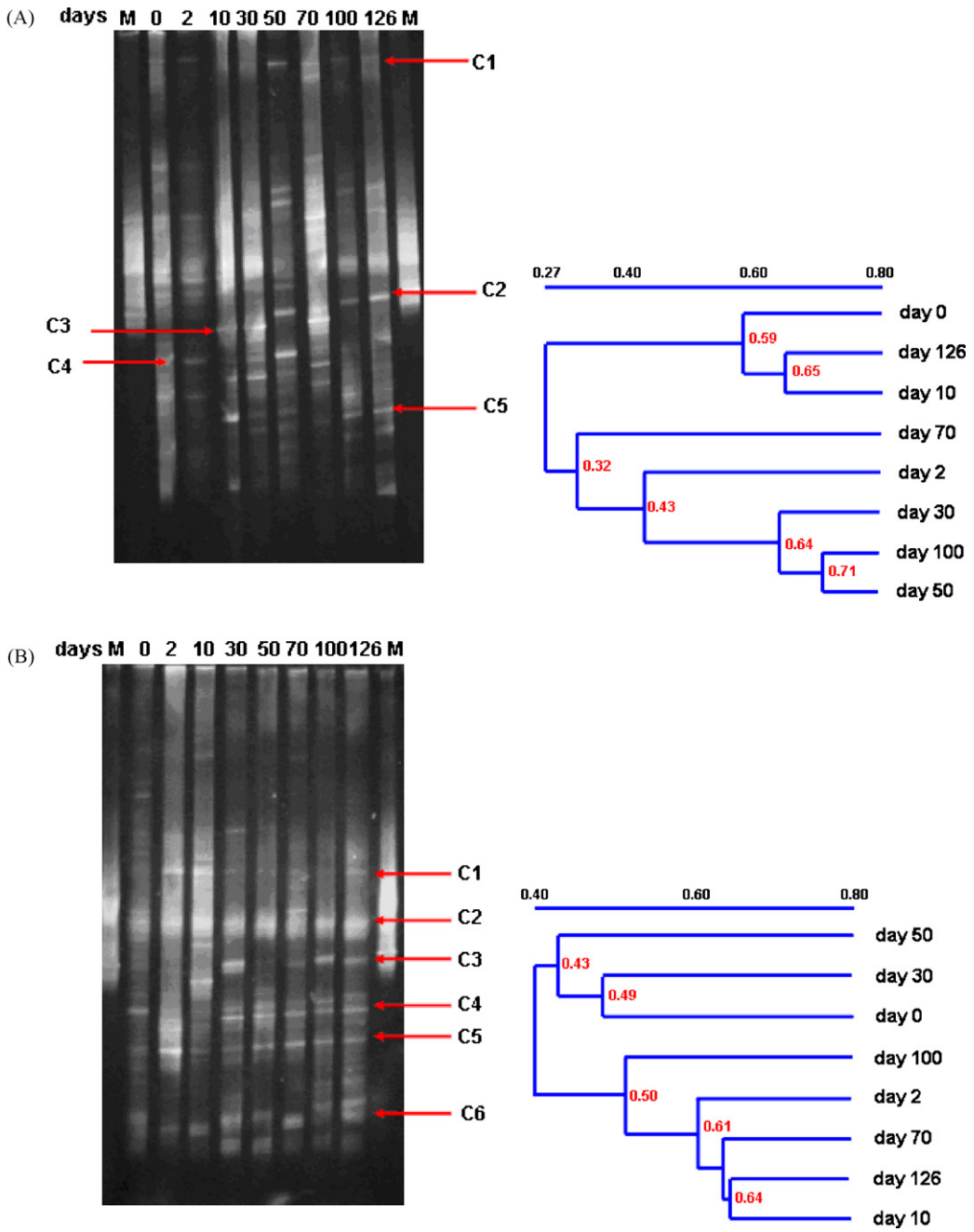


**Fig. 4.** PCR–DGGE analysis of 16S rDNA sequence fragments obtained from soil incubated with 10 mg/kg of carbendazim at 25 °C (A) and 40 °C (B) and cluster analysis of bacterial community structures by UPGMA. Lane M: marker.

calculated by the coefficient of DICE with Quantity One® software (Bio-Rad, USA). The UPGMA method was used to examine the change in the bacterial community under different temperatures and incubation days.

## 3. Results

### 3.1. Dissipation of carbendazim in sterile and non-sterile soils

Fig. 1A shows the residual amounts of carbendazim during 90 days of incubation at 25 °C in sterile soil amended with 10 and 100 mg/kg of carbendazim to be 98.6% and 99.9%, respectively, and those incubated at 40 °C to be 92.7% and 99.7%, respectively. Thus, carbendazim was barely degraded in sterile soils even with changed incubation conditions. Fig. 1B shows the residual amounts of fungicide after 126 days of incubation at 25 °C in non-sterile soil amended with 10 and 100 mg/kg carbendazim to be 31.8% and 100%, respectively, and those in 40 °C to be 44.8% and 94.2%, respectively. Carbendazim dissipated faster at lower concentrations. From the DGGE patterns in Figs. 4 and 5, soil incubated with 10 mg/kg carbendazim had a more complex bacterial community structure than soil incubated with 100 mg/kg; therefore, a high concentration of carbendazim may be toxic to soil microbes in altering their composition, size of populations, or deterring the activities of microbial communities in soils. The effect of incubation temperatures was small, which demonstrates that soil microbes may utilize carbendazim under both room temperature and summer-month temperatures.

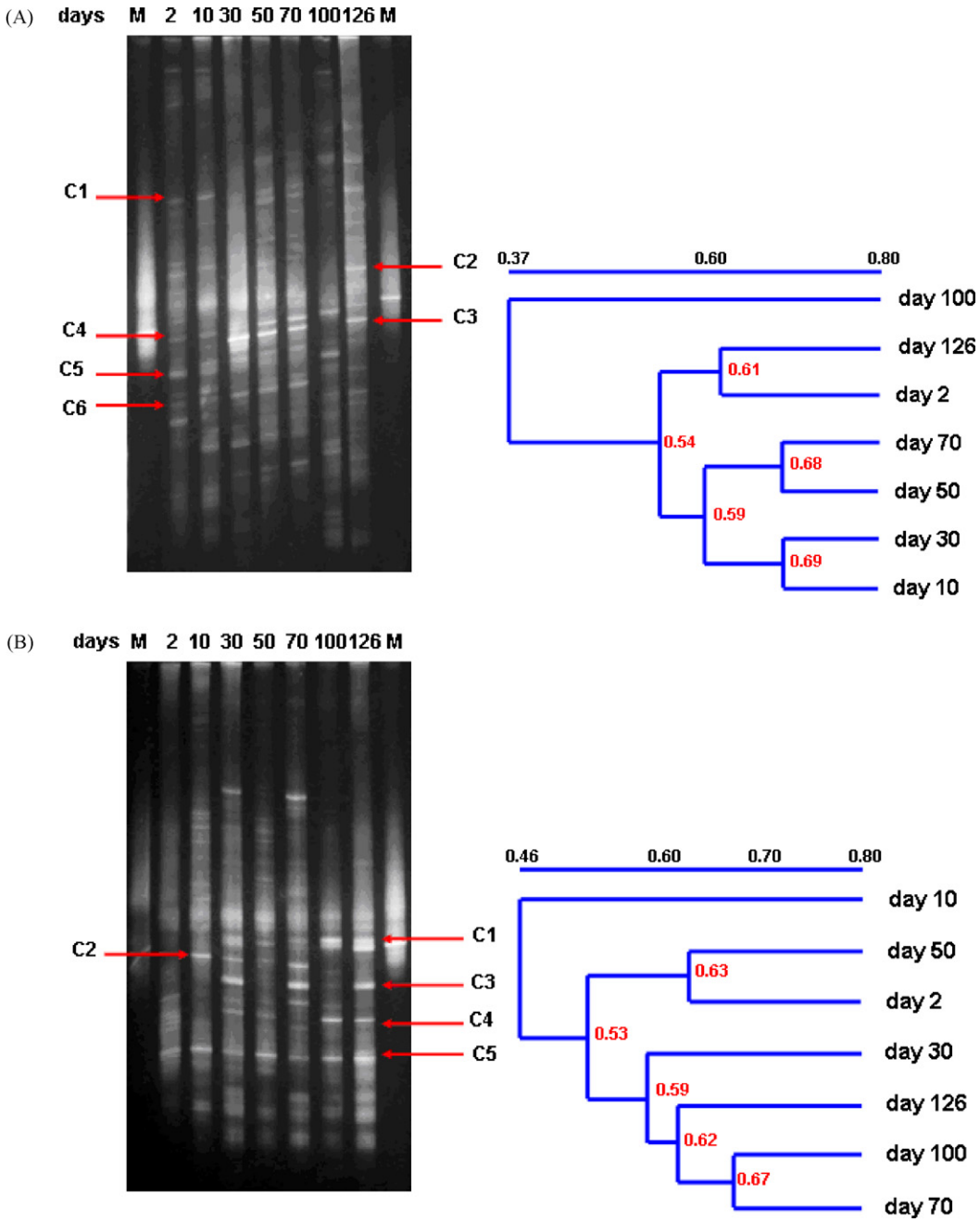


**Fig. 5.** PCR–DGGE analysis of 16S rDNA sequence fragments obtained from soil incubated with 100 mg/kg of carbendazim at 25 °C (A) and 40 °C (B) and cluster analysis of bacterial community structures by UPGMA. Lane M: marker.

### 3.2. Dissipation of pencycuron in sterile and non-sterile soils

Fig. 2A shows the residual amounts of pencycuron during 100 days of incubation at 25 °C in sterile soil amended with 10 and 100 mg/kg pencycuron to be 54% and 92.1%, respectively, and those at 40 °C to be 10% and 49.7%, respectively. Fig. 2B shows the residual amounts of fungicide during 100 days of incubation at 25 °C in non-sterile soil amended with 10 and 100 mg/kg pencycuron to be 49.7% and 86.9%, respectively, and those at 40 °C to be 4% and 25.5%. Therefore, biological and non-biological treatments did not differ for this fungicide. The change in incubation temperature affected dissipation rate, which could be attributed to the acceleration of both biological and chemical reaction rates.Changes in soil bacterial community without pesticide amendment.

The similarity among bacterial communities in pesticide-free soils was 52% and 61% under 25 °C and 40 °C, respectively, within 0–29 days (Fig. 3), which suggests that the diversity did not change abruptly in this cultivation system.

### 3.3. Soil bacterial community with carbendazim treatment

The effect of 10 mg/kg carbendazim treatment on soil bacterial communities under 25 °C and 40 °C is shown in Fig. 4A and B. As seen in Fig. 4A, the bands C1, C4 and C5 were present throughout the cultivation period, which suggests the high tolerance for carbendazim. The C3 community appeared after 10 days but disappeared after 70 days of cultivation, which suggests a low tolerance of carbendazim metabolites. C2, which appeared after 50 days, may have displaced the less-tolerant communities and became one of the

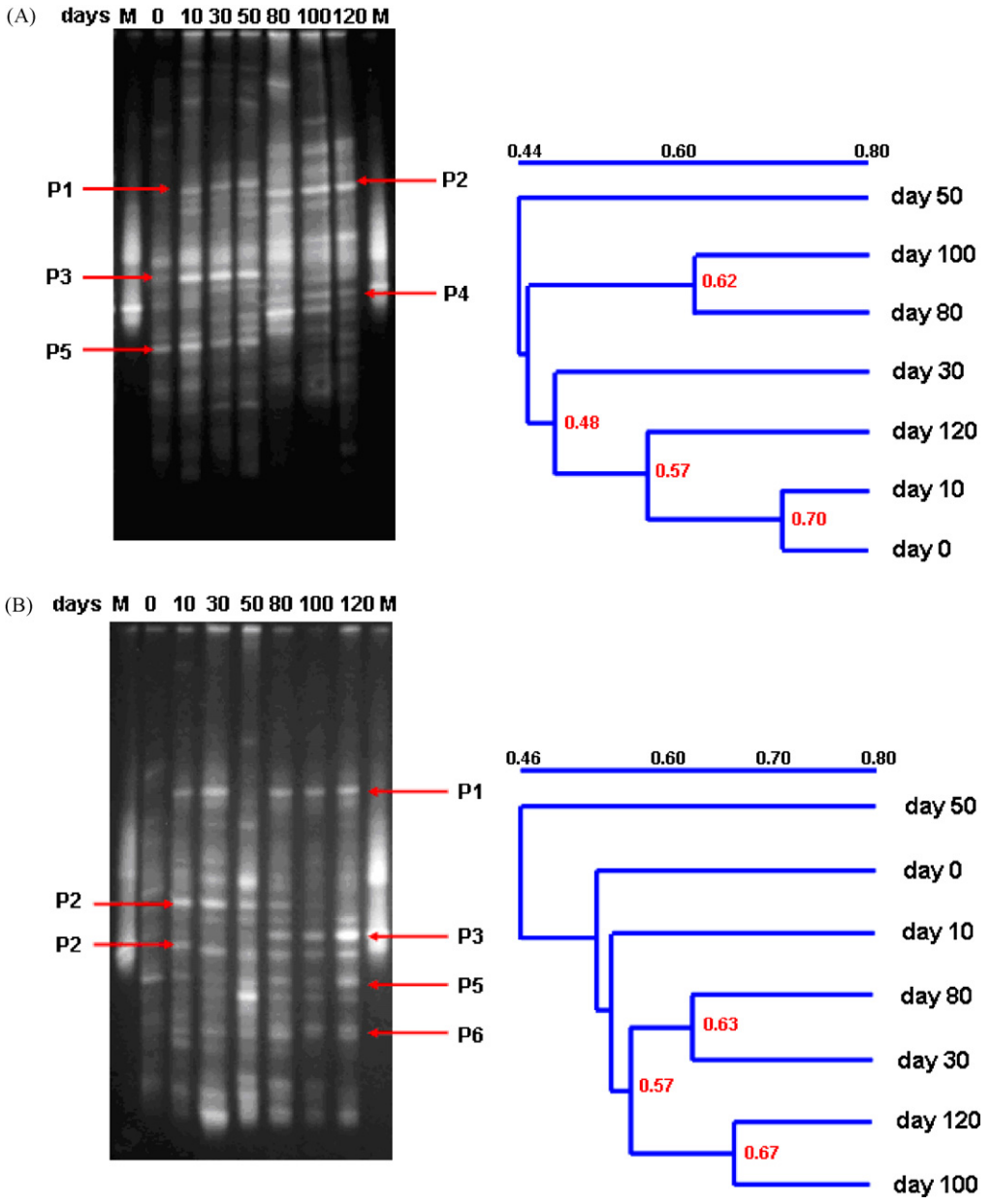


**Fig. 6.** PCR–DGGE analysis of 16S rDNA sequence fragments obtained from soil incubated with 10 mg/kg of pencycuron at 25 °C (A) and 40 °C (B) and cluster analysis of bacterial community structures by UPGMA. Lane M: marker.

dominant species among all communities. The similarity between the control and all the carbendazim treatments was 27%, which indicates the violent disturbance in soil microbial communities with the addition of carbendazim. The divergence remained at 41% after 126 days, which resembled the diversity level right after carbendazim application, and reflected the recovery of the microbial communities.

From Fig. 4B, the bands C1, C2, C5 and C6 were present throughout the cultivation period; bands C3 and C4 appeared after 30 days of cultivation. The 30- and 50-day bacterial composition differed remarkably from that of other cultivation periods. This finding was in accordance with data showing changes in 50-day dissipation rate.

The effect of treatment with 100 mg/kg carbendazim on soil bacterial communities under 25 °C and 40 °C are shown in Fig. 5A and B. As seen in Fig. 5A, the bands C4 and C5 were consistently present throughout the cultivation period, which suggests their higher tolerance towards carbendazim and the possibility of its being used as a carbon source. C3 and C2 appeared after 30 and 100 days, respectively, whereas C1 and C6 disappeared after 70 and 100 days, respectively, which revealed the community shift in soils after the addition of carbendazim. Except for 100 days' cultivation, all treatments produced greater than 54% similarity, which indi-

**Table 1**
Dissipation rate constants, half-life values, and determination coefficients by first-order kinetics on soils treated with carbendazim or pencycuron.

| Fungicide | Conc. (mg/kg) | Temp. (°C) | $K$ | $R^2$ | $t_{1/2}$ |
|---|---|---|---|---|---|
| Carbendazim | 10 | 25 | 0.0066 | 0.9817 | 105 |
| | | 40 | 0.0067 | 0.9102 | 103 |
| | 100 | 25 | –[a] | – | – |
| | | 40 | – | – | – |
| Pencycuron | 10 | 25 | 0.0072 | 0.9610 | 96 |
| | | 40 | 0.0306 | 0.9486 | 23 |
| | 100 | 25 | 0.0019 | 0.9469 | 365[b] |
| | | 40 | 0.0134 | 0.9834 | 52 |

[a] No significant dissipation observed.
[b] Half-life exceeds the cultivation period.

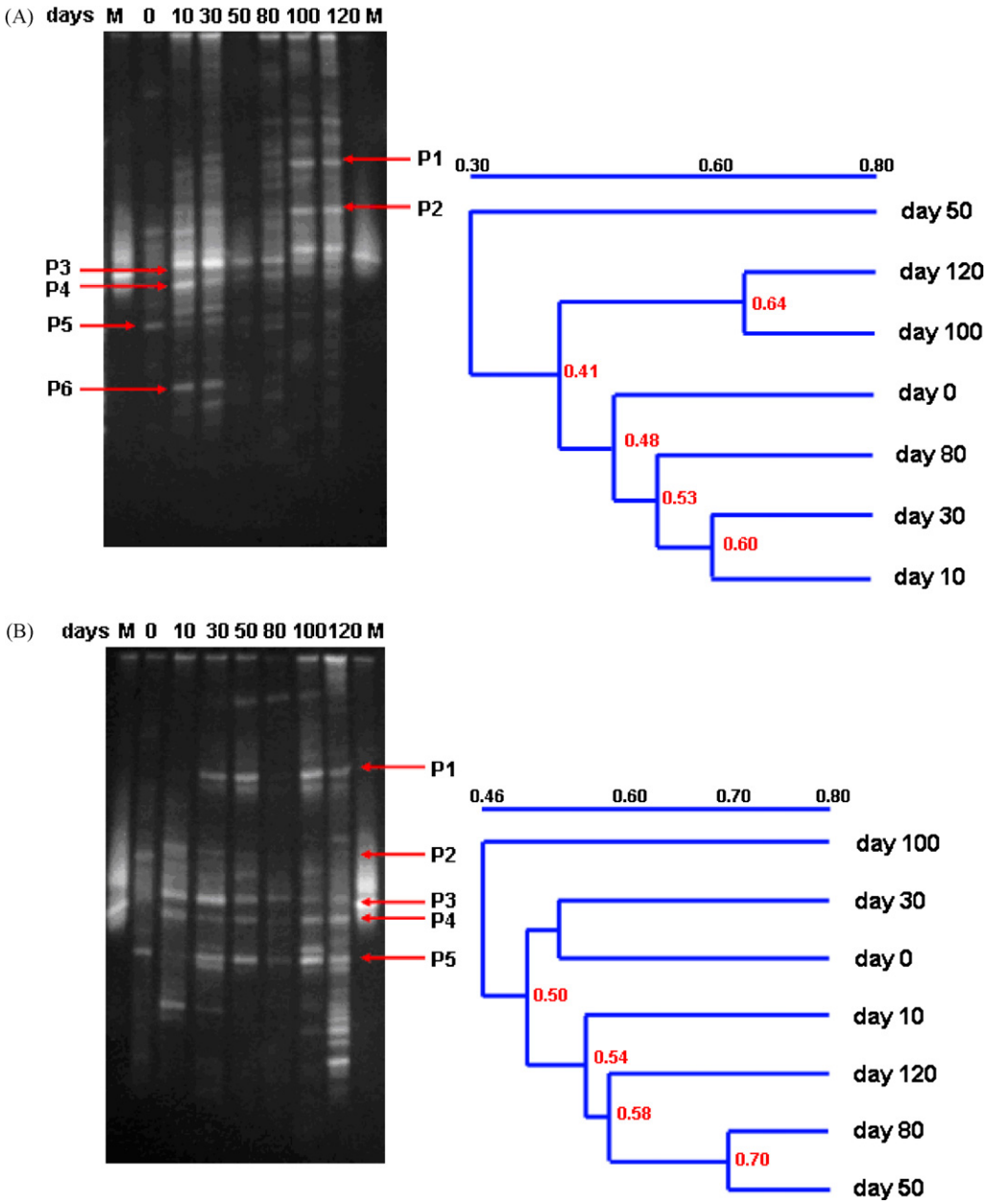


**Fig. 7.** PCR–DGGE analysis of 16S rDNA sequence fragments obtained from soil incubated with 100 mg/kg of pencycuron at 25 °C (A) and 40 °C (B) and cluster analysis of bacterial community structures by UPGMA. Lane M: marker.

cated a minor community shift and was in accordance with the low dissipation rate under treatment with a high concentration of carbendazim.

From Fig. 5B, the band C5 was present throughout the cultivation period; the communities C1, C3 and C4 appeared after 30 days, whereas C2 disappeared after 70 days' cultivation. All treatments produced greater than 46% similarity. This result fits well with that of Fig. 5A, in which the high genetic similarity can be ascribed to the low dissipation rate with high concentration of carbendazim amendment.

### 3.4. Soil bacterial community affected by pencycuron

The effect of treatment with 10 mg/kg pencycuron applied to soil bacterial communities under 25 °C and 40 °C is shown in Fig. 6A and B. As seen in Fig. 6A, the band P3 was present throughout 120 days of cultivation, which suggests it as one of the pencycuron-tolerant species. The communities P2 and P4 appeared after 80 and 100 days, whereas the P1 and P5 communities disappeared after 50 days. The similarities decreased from day 30 to day 100 but returned to the steady state after 120 days' cultivation (57% similarity). Thus, the soil microbial communities tended to recover after more than 50% of pencycuron was dissipated.

As shown in Fig. 6B, the bands P4, P5 and P6 were present throughout 0–120 days of cultivation. P1 and P3 appeared after 10 and 80 days; P2 disappeared after 80 days. Similarity of the microbial community remained at a steady state of approximately 50% as compared with controls, which indicates that amendment with pencycuron at 40 °C caused irreversible changes in soil microbial communities.

The effect of treatment with 100 mg/kg pencycuron applied to soil bacterial communities under 25 °C and 40 °C are shown in Fig. 7A and B. As seen in Fig. 7A, P4 and P5 were present throughout 120 days' cultivation. P1 and P2 appeared after 10 days; P3 and P6

disappeared after 80 and 30 days, respectively. The diversity of the microbial community was 59% after 120 days' cultivation, which indicates a greater change (in comparison with treatment at lower concentration) in soil microbiota with pencycuron treatment.

As shown in Fig. 7B, P3, P4 and P5 were present throughout the 120 days' cultivation. P1 appeared after 30 days, whereas P2 disappeared after 50 days. Changes in the soil microbial community was not associated with the duration of cultivation. However, throughout the experimental period, the similarity was approximately 50%, which indicates a greater change in soil microbiota (in comparison with treatment at lower concentrations) with pencycuron treatment.

## 4. Discussion

We investigated the dissipation and effects of the fungicides carbendazim and pencycuron in a strawberry field in Taiwan. Table 1 displays the results calculated from the dissipation data of carbendazim and pencycuron. The dissipation of carbendazim may be attributed to biologically relative mechanisms and was influenced by increased concentration and not incubation temperature. Thus, carbendazim at a high concentration, of 100 mg/kg, may be harmful to soil microbes. Previous research of $^{14}$C-labelled 2-aminobenzimidazole, the primary degradation product of carbendazim, in soil showed that the residual amount increased exponentially from 1 °C to 22 °C, peaked at 22 °C, remained almost constant up to 35 °C, and then became almost zero at 40 °C [16]. However, in this study, the dissipation of carbendazim was fastest under 40 °C (+13%) with 10 mg/kg carbendazim, which indicates that 2-aminobenzimidazole may not be the only intermediate. Alternative intermediates (benzimidazole and an unknown metabolite) after carbendazim amendment have been proposed [17].

The dissipation of pencycuron was due to both non-biologically and biologically relative mechanisms and was influenced by both temperature and concentration. The half-life of 100 mg/kg pencycuron was more than 3.8 times longer than that of 10 mg/kg pencycuron at 25 °C, but at 40 °C, it was only 2.3 times longer. This finding indicated that increased temperature enhances the dissipation of pencycuron. According to our dissipation data, the enhancement was attributed to the acceleration of chemical reaction rates. The result was in contrast to that of Pal et al. [18], who found the dissipation of pencycuron greater in non-sterile than sterile soil. The rate of degradation of pesticides in soils may be largely governed by a complex interaction of biotic and abiotic components [12,18,19]; thus the change in environmental factors such as soil properties, water content, and organic matter content can influence the dissipation rate of chemicals.

In this study, the effects of biological and non-biological degraded fungicides on change in microflora diversity were investigated. On adding 10 mg/kg carbendazim, the change in soil microbial composition was greater than 40% at both 25 °C and 40 °C cultivation temperatures after 126 days. Comparatively less change was observed with a high concentration of carbendazim applied because rigorous growth conditions may only allow for a small portion of soil microbial communities. Although previous reports have demonstrated that carbendazim has some effect on the composition of soil microflora, the effects were not long lasting and therefore, carbendazim had low toxic effects on microbial activities [20]. This study monitored changes in the entire microbial community at field application rate.

With the application of pencycuron, the microbial composition was approximately 50% similar throughout most of the experimental period, which showed that under our experimental conditions, the dissipation of pencycuron largely depended on the chemical reaction. Moreover, soil microbiota were unstable in composition throughout the 120 days of cultivation.

## 5. Conclusions

The degradation of carbendazim and pencycuron in a strawberry field is influenced by different environmental factors, and the composition of micro-organisms changed with the application of pencycuron but not carbendazim after 120 days of incubation.

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