

Full Length Research Paper

Phyllosphere bacterial communities associated with the degradation of acetamiprid in *Phaseolus vulgaris*

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Much effort has been focused on microbes that inhabit soil and water and their degradation abilities to chemical pollutants. Few studies were performed on interactions between pollutants and phyllosphere microbes (phyllomicrobes) in plant leaves, although, plant leaves exert critical effects on the quality of agricultural product. In this study, acetamiprid degradation by phyllomicrobes and its ecological effects on phyllobacteria were investigated by *in situ* and *ex situ* methods. *In situ* degradation results showed that the half-lives of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid on disinfected and natural leafage were 4.99 and 3.24 d, respectively, but the half-lives of 30 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid displayed no significant difference on the two kinds of leafage. *Ex situ* cultivable microbes from phyllosphere of *Phaseolus vulgaris* completely degraded 11 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid within 42 days in plant leaves medium (PLM), but not in chemical medium. However, only 30% acetamiprid was decreased within 56 days when acetamiprid concentration increased up to 30 $\mu\text{g}\cdot\text{ml}^{-1}$ in PLM. Bacterial population investigation and 16S rRNA gene PCR-DGGE (denaturing gradient gel electrophoresis) fingerprints analysis indicated that, low concentration of acetamiprid showed minor effects on phyllobacterial community or population, but phyllobacterial community structure was significantly changed by acetamiprid at the concentration higher than 30 $\mu\text{g}\cdot\text{ml}^{-1}$. In conclusion, phyllomicrobes of *P. vulgaris* were able to degrade acetamiprid at the proper concentration, however, phyllomicrobes biodegradation capability was inhibited by high concentration of the pesticide and phyllomicrobes' community was also destroyed at the same conditions.

Key words: Microbial community structure, phyllosphere, acetamiprid degradation, 16S rDNA, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Foliage is always inhabited by a large number of microbes including epibiosis and endogenesis. Phyllosphere microflora is mainly composed of bacteria, filamentous fungi, yeasts and a small bit of algae, protozoa and nematodes. Bacteria are the most abundant inhabitants within phyllosphere and also the greatest contributors to phyllosphere ecosystem (Lindow and Brandl, 2003; Richard et al., 2003; Wilson et al., 1999). Phyllosphere environment is generally considered as a rather hostile

habitat for microorganisms due to the tremendous physical fluctuation during day and light, direct UV radiation and oligotrophy. Interestingly, bacteria are often detected in population averaging from 10^6 to 10^7 cells every centimeter (up to 10^8 cells·g⁻¹) in the hostile habitat (Lindow and Brandl, 2003; Yang et al., 2001). It is reasonable to believe that, such organism possess special ecological relationships with the hosts. Inconsequential (transient epiphytic saprophytes) and substantial (commensals, mutualistic symbionts, endophytes or pathogens) could be sum-up between the 3810 relationships of plant-microbe and microbe-microbe in phyllosphere ecosystem (John and Harris, 2000).

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Leaf-associated microbes play important roles in their hosts, such as nitrogen fixation, pathogen prevention, growth stimulation and residual pesticide decomposition. Researches about microbial ecology and disease protection of phyllosphere microbes have been so abundant, but interactions between phyllobacteria and pesticides were rarely investigated (Lindow and Brandl, 2003; Wilson et al., 1999).

At present, two kinds of bioremediation methods are applied. One is to isolate degradative microbes from environmental samples (or construct multifunctional degradative bacteria by genetic technologies). The other is to stimulate degradation ability of indigenous microbes by creating appropriate conditions for contaminated areas (Zwillich, 2000; Debarati et al., 2005). Although, efficient degradative microbial strains for acetamiprid were very hard to get, the pesticide has been reported as a degradable chemical for environmental microbes (Dai et al., 2010a, b). Thus, bioremediation technology of indigenous microbes is a useful method for acetamiprid remediation (Abramovitch et al., 2002). In order to explore more efficient bioremediation methods for acetamiprid, this study was designed to: (1) test phyllobacteria's degradation ability to acetamiprid; (2) to detect acetamiprid ecological effects on phyllobacterial community; (3) to determine the relationships between acetamiprid degradation and phyllobacteria fluctuation.

MATERIALS AND METHODS

Phaseolus vulgaris (a kind of common bean), sown in culture dishes (10 cm in diameter and 10 cm height) at 30°C, 15000 Lx light intensity (12 h) with black land soil for three weeks were chosen as plant materials. The black land soil was collected from Liaoning province, northeast of China with soil organic carbon (SOC) of about 18.3 g·kg⁻¹, total nitrogen 1.9 g·kg⁻¹ and pH 6.5. Tryptone-yeast-beef extracts medium (TYB medium, contained 0.3% yeast extract, 0.2% beef extract, 0.6% tryptone and 0.3% NaCl) was selected as chemical medium for bacteria cultivation and population investigation. Acetamiprid was provided by Nippon Soda Co., Ltd. Japan.

Acetamiprid degradation on phyllosphere of *P. vulgaris* (in situ)

Leaves surface was sterilized by UV irradiation and ethanol as described by Wilson et al. (1999). Acetamiprid solution was prepared by technical product (acetamiprid content ≥ 97%) with distilled water at different concentrations. Different concentrations (0, 10, 30 and 50 µg·ml⁻¹) of acetamiprid solution were sprayed onto the sterilized materials, respectively, with a misting bottle, until the applied solution was run off from leaves (50 ml acetamiprid solution was applied onto every tested culture dishes). Unsterilized natural materials were set as control. Samples were collected for acetamiprid residue analysis after being incubated at the same conditions as mentioned earlier for 2 h and 1, 3, 7, 14 and 21 days, respectively. Considering the residue of acetamiprid on plant leaves was declined quickly for the first two cultivation days and became relatively stable at the third day, the samples incubated for 3 days were selected to analyze phyllobacterial community. The acetamiprid degradation dynamics on the two kinds of leaves were

represented using the regression equation $C_t = C_0 e^{-Kt}$ and half-lives were calculated by $T_{0.5} = 1/K \ln 2$ (t , incubation time; C_t , acetamiprid residue; C_0 , original deposition; K , degradation constant; $T_{0.5}$, half-life).

Preparation of plant leaves medium

120 g well-cut and well-mixed fresh leaves of *P. vulgaris* were homogenized for 15 min by a glass homogenizer after 1000 ml sterilized water was added, the homogenized solution was the prepared plant leaves medium (PLM). Another 1000 ml homogenized solution was sterilized thoroughly by C₀₆₀ gamma radiation to construct sterilized PLM.

Acetamiprid degradation and microbial community dynamics in PLM 'microcosms'

Different concentrations (0, 10, 30 and 50 µg·ml⁻¹) of acetamiprid was added into 250 ml PLM to construct four tested 'microcosms'. Sterilized PLM and distilled water that mixed with the same concentrations of acetamiprid were set as control. Appropriate samples were withdrawn from the constructed microcosms after incubated at 30°C (150 r/min) for 2 h and 7, 14, 28, 42 and 56 days to analyze the acetamiprid residue and cultivable bacterial population. At the time point of the 14th day, the bacterial communities were analyzed. The population dynamics of bacteria were determined using plating dilutions with TYB agar and the bacterial communities were analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

Phyllobacterial community structure in TYB microcosms

Different concentration (0, 10, 30 and 50 µg·ml⁻¹) of acetamiprid was added into 250 ml TYB broth and each broth was further mixed with 0.5 ml PLM to inoculate phyllobacteria. The TYB microcosms were incubated at the same condition as PLM microcosms. Sample collection, acetamiprid residue determination and microbial community investigation were performed as described in PLM microcosms.

In order to make PCR-DGGE data more informative, the PLM homogenates prepared for PLM microcosms and TYB microcosms were obtained from the same homogenized solution. The same origin of homogenized solution insured the identical microbial community for these treatments at the time of zero ($T = 0$).

Determination of acetamiprid

Acetamiprid residue of plant leaves (or PLM) was determined as described by Obana et al. (2003). In detail, 5 ml liquid sample (or 5 g leaves' homogenate) was mixed with 25 ml methanol extracting for 30 min in airtight flask and was filtered under decompression conditions. Filtrate was extracted by 50 ml petroleum ether and 100 ml 5% NaCl for three times. Aqueous extracts were collected and followed by further extraction with 80 ml dichloromethane (CH₂Cl₂) for three times. The dried CH₂Cl₂ extracts were dissolved with 10 ml petroleum ether/ethyl acetate (3:7 v/v) and then was loaded onto silica gel column which had been equilibrated by washing buffer (petroleum ether/ethyl acetate, 3:7 v/v). The extracted acetamiprid in silica gel column was firstly purified with 10 ml washing buffer, then was collected with 10 ml elution buffer (petroleum ether/ethyl acetate, 1:9 v/v). The elution was dried and re-dissolved with 1 ml CH₃OH/H₂O (3:2, v/v) for chromatogram analysis. Analysis was performed by high performance liquid chromatograph (HPLC; Agilent 1100) which was connected to a C₁₈ reverse phase column

Table 1. Recovery efficiency and regression equation determined by different concentrations of acetamiprid for the analytical method evaluation.

Append concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Detected concentration	Recovery efficiency (%)	Regression equation
10	10.52 \pm 0.57	105	
30	27.90 \pm 1.70	93	$y=12.496x+0.0239$
50	44.54 \pm 0.23	89	$R^2=0.999$
60	54.71 \pm 2.21	91	

Regression equation was determined by HPLC using different concentrations of standard acetamiprid; y, acetamiprid concentration; x, the peak area of HPLC; R^2 , correlation coefficient of the regression equation.

(250 \times 4.6 mm) and a UV detector with absorbance at 239 nm. The flow rate was 0.8 ml \cdot min $^{-1}$ with CH₃OH/H₂O (3:2, v/v). Recovery efficiency of the stated method was evaluated at the concentrations of 10, 30, 50 and 60 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid that appended in clean plant leaves.

Genomic DNA preparation

Microbial cells collection from leaf samples was carried out following the method as described by Yang et al. (2001). Genomic DNAs which isolated from collected microbes or TYB medium were prepared using the method described by Marmur (1961). The microbial genomic DNA from PLM was extracted using the methods as described by Zhou et al. (1996) with moderate modification. In brief, 5 g pretreated sample was suspended in 5 volumes TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 0.01 g \cdot ml $^{-1}$ PVP, pH 8.0) and incubated at 37°C for 30 min. Pellets were collected by centrifuged at 13,000 g for 2 min at 4°C and resuspended in 400 μ l extraction buffer (100 mM Tris, 100 mM EDTA, 200 mM NaCl, 1% PVP, 2% CTAB, pH 8.0) by blazing vortexing for 5 min. Reversed tubes acutely for 5 min after 400 μ l 2% SDS was added. The mixture was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was transferred to a fresh tube and was extracted with the same volume of chloroform-isoamyl alcohol (24:1, v/v). After extraction, the tube was centrifuged for 15 min at 13,000 g at 4°C. The supernatant was transferred to another fresh tube and genomic DNA was precipitated with 0.6 volume of isopropanol at -20°C for 2 h.

PCR-DGGE

PCR primers and GC clamp used by Yang et al. (2001) were selected to amplify the 338-f to 518-rRNA region which contains one variable loop of the 16S rRNA gene. PCR amplification was performed in a volume of 50 μ l containing 25 pmol of each primer, 25 mM dNTPs, 1.5 mM Mg²⁺, 1 \times PCR buffer and 2.5 units of Taq polymerase (Takara, Dalian). The amplification procedure was as follows: 1 cycle of denature at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally, was extended at 72°C for 10 min.

DGGE analysis of 16S rRNA gene was performed using the procedure of Muyzer et al. (1993). It was performed with Dcode universal mutation detection system (Bio-Rad, USA). 15 μ l of the PCR products were loaded onto an 8% (wt/v) polyacrylamide gel (acrylamide/bis acrylamide, 37.5:1) with a denaturing gradient ranging from 30 to 60%. The 100% denaturant contained 7 mol \cdot L $^{-1}$ urea and 40% formamide (Blackwood et al., 2007; Sei et al., 2004). The electrophoresis was conducted at 55°C, first for 10 min at 20 V and subsequently, for 14 h at 100 V in 0.5 \times TAE buffer (Sambrook and Russell, 2002). After electrophoresis, the gels were

stained with silver nitrate according to the procedure as described by Sanguinetti et al. (1994).

The gel images were transformed into digital data using quantity one from Bio-Rad system. The Shannon-weaver index of diversity (H') and Simpson index of dominance (D) were calculated from the results of DGGE profiles to quantify the diversity of microbial community (Shannon and weaver, 1963; Simpson, 1949); Dice's coefficient of similarity (S_D) which represents band similarities between pairs of samples were calculated as described by Sei et al. (2004).

RESULTS

Evaluation of the analytical method for acetamiprid determination

The recovery efficiency of acetamiprid in plant leaves (or PLM medium) are arranging from 89 to 105%. The evaluation results displayed that the analytical method applied in this study well satisfied the requirement of the pesticide analysis standard (Table 1; satisfy scopes are from 80 to 120%). The LOD (limit detection of the apparatus) and LOQ (limit of quantification of the analysis method) of the method were 1.0 \times 10 $^{-10}$ g and 0.002 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively.

Processes of acetamiprid degradation on plant leaves (*in situ*)

The residue of acetamiprid decreased rapidly at all treatments in the early stage of incubation and the degradation speed came into slow phase (the second phase) about three days later. The dynamics of acetamiprid degradation represented by the regression equation $C=C_0\cdot e^{-kt}$ and the half-lives calculated from the equation $T_{0.5}=1/k\cdot\ln 2$ were displayed in Table 2.

The half-lives of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid on unsterilized and sterilized leafage are 3.24 d and 4.99 d, respectively, but 30 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid showed similar half-lives on the two kinds of leafage (3.1 to 3.5 d) (Table 2).

After 21 days incubation, the residue of unsterilized sample with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid treatment was almost

Table 2. Residual regression equations and half-lives of acetamiprid on two kinds of plant leaves at the *in situ* conditions.

Treatment ($\mu\text{g}\cdot\text{ml}^{-1}$)	Original deposition (C_0 , $\mu\text{g}\cdot\text{ml}^{-1}$)	Regression equation ($C=C_0\cdot e^{-kt}$)	Correlation coefficient (R^2)	Half-life ($T_{0.5}=1/k\cdot\ln 2$, day)
Unsterilized leaves				
10	8.60±0.11	$C=9.605\cdot e^{-0.2225t}$	0.939	3.24±0.25*
30	11.37±0.76	$C=9.731\cdot e^{-0.1075t}$	0.982	3.08±0.29
50	18.73±0.31	$C=16.010\cdot e^{-0.093t}$	0.957	3.20±0.25
Sterilized leaves				
10	8.65±0.12	$C=6.9442\cdot e^{-0.139t}$	0.957	4.99±0.50*
30	12.22±0.48	$C=11.308\cdot e^{-0.1001t}$	0.974	3.23±0.31
50	17.98±0.44	$C=15.842\cdot e^{-0.0809t}$	0.986	3.46±0.48

Original deposition (C_0), regression equation and half-life ($T_{0.5}$) were calculated from the mean values of three subsamples, the standard deviations were given for $T_{0.5}$ and C_0 . C_0 is the value of actual original deposition determined in the present tests and C_0 is the original deposition calculated by regression equation. Because the correlation coefficient of every regression equation lower than 1 ($R^2 < 1$), the values of C_0 are not as the same as C_0 . *Significant difference.

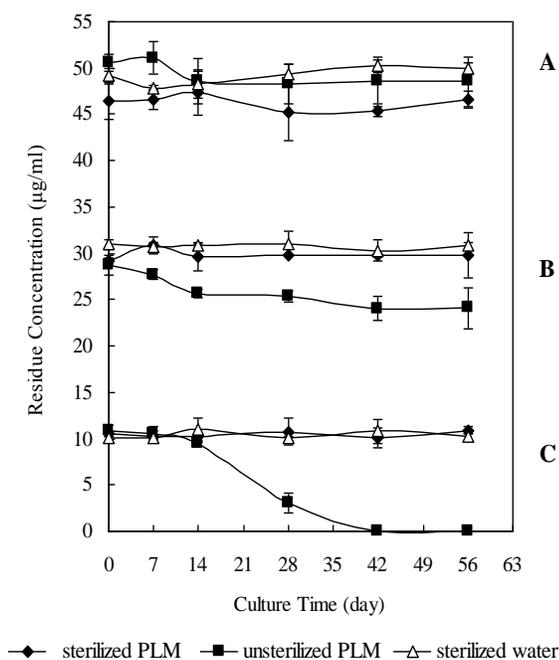


Figure 1. Processes of acetamiprid degradation in the culture mediums and sterilized water (*ex situ* degradation processes). Treatment of acetamiprid concentrations: A, 10 $\mu\text{g}\cdot\text{ml}^{-1}$; B, 30 $\mu\text{g}\cdot\text{ml}^{-1}$; C, 50 $\mu\text{g}\cdot\text{ml}^{-1}$.

cleared up ($C_{21}=0.051 \mu\text{g}\cdot\text{ml}^{-1}$), but there were still about 1.10 and 2.60 $\mu\text{g}\cdot\text{ml}^{-1}$ residues at high concentration of treatments (30 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$). The residual concentration of sterilized samples was 0.30 $\mu\text{g}\cdot\text{ml}^{-1}$ (10 $\mu\text{g}\cdot\text{ml}^{-1}$ treatment), 1.60 $\mu\text{g}\cdot\text{ml}^{-1}$ (30 $\mu\text{g}\cdot\text{ml}^{-1}$ treatment) and 2.91 $\mu\text{g}\cdot\text{ml}^{-1}$ (50 $\mu\text{g}\cdot\text{ml}^{-1}$ treatment), respectively. *In situ* degradation results on sterilized and unsterilized leafage at different concentrations implied that, phyllosphere of *P. vulgaris* showed significant degradative abilities to the new kind of pesticide at the properly applied

concentration.

Processes of acetamiprid degradation in *ex situ* culture microcosms

At the treatment concentration of 11 $\mu\text{g}\cdot\text{ml}^{-1}$, acetamiprid was completely degraded by phyllosphere within 42 days in PLM (Figure 1c), however, only 30% acetamiprid was decreased within 42 days when acetamiprid concentration in PLM increased up to 30 $\mu\text{g}\cdot\text{ml}^{-1}$ (even the incubation time extended to 56 days, the degradation ratio was still approximately 30%) (Figure 1b). If the applied concentration of acetamiprid in PLM was higher than 50 $\mu\text{g}\cdot\text{ml}^{-1}$, the residual concentration displayed almost no significant changes within the whole culture time, that means phyllosphere did not displayed any degradative effects to the pesticide. According to the degradation results in PLM, phyllosphere's degradative capability was inhibited obviously by high concentration of acetamiprid (Figure 1). Different from unsterilized PLM microcosms, acetamiprid was not degraded in sterilized PLM microcosms or sterilized water at every tested concentration (Figure 1). These results suggested that the composition of kidney bean's leafage (include the enzymes of the plant leaves) did not displayed any degradation ability to the pesticide and the pesticide was quite stable in sterilized water at tested pH and temperature. TYB microcosms and TYB medium (not inoculated phyllosphere) also did not displayed obvious degradative abilities to acetamiprid at each applied concentration (data not shown). TYB microcosms' degradation results further proved that, the degradative microbes of phyllosphere could not be cultured by the chemical medium and the PLM was a kind of suitable culture medium for this group of microbial cultivation test. The PLM microcosm's degradation results were consistent with that of *in situ* conditions, phyllosphere

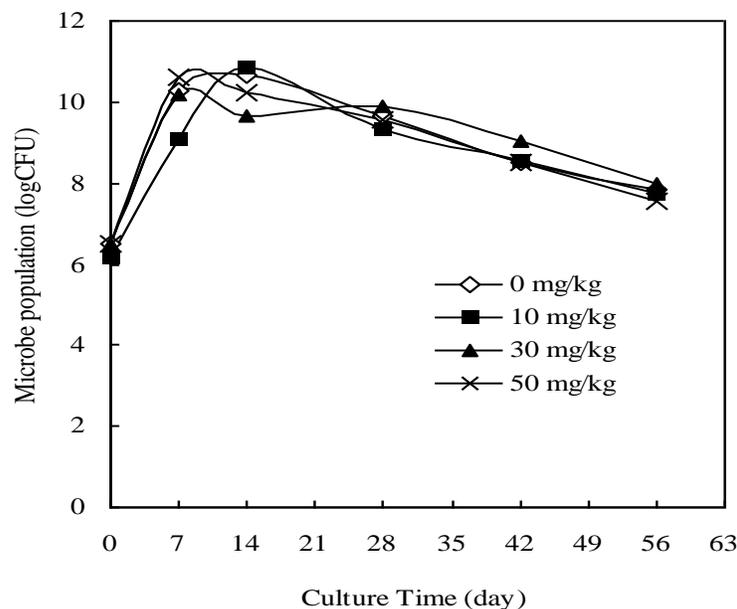


Figure 2. Bacterial population dynamics in PLM microcosms at different concentrations of acetaminophen.

showed significant capability to low concentration of acetaminophen than that of high-loading conditions.

Population dynamics of phyllobacteria under acetaminophen stress

The population of phyllobacteria in PLM microcosms increased rapidly at all applied concentrations of acetaminophen within the first two weeks. Investigation result of population dynamics showed that, all treated concentrations of acetaminophen displayed limited effect on bacterial population that cultured in PLM. The bacterial population at the time point of 14th day was about 10^5 times larger than that of *in situ* conditions. After 14 days, phyllobacterial population in PLM microcosms decreased drastically at all concentrations treatment, the negative results may be caused by nutrition shortage, metabolized products accumulation or other negative factors (Figure 2). The incubation time of 14th day was the largest bacterial population time point in PLM microcosms for every concentration treatment. The bacterial population investigation result showed that, phyllobacteria of this time point was the most abundant period during the whole cultural time and the samples collected at this point were selected for phyllobacterial community analysis.

Qualitative analysis of the microbial community

As displayed in Figure 3, the DGGE band patterns at conditions *in situ*, PLM microcosms and TYB microcosms

were all changed drastically at the high concentrations of acetaminophen. However, the band patterns remained quite stable at low concentration of acetaminophen treatment compared with their controls (Figure 3). The band pattern of sterilized leafage was completely distinct from those of the natural leaves or natural leaves treated with different concentrations of pesticide (Figure 3a).

For the *in situ* conditions (Figure 3a), bands S1 and S2 were the primary members for the sterilized leafage and the two groups of phyllobacteria should be the sterilize reagents resistant bacteria species. Natural leaves treated with $10 \mu\text{g}\cdot\text{ml}^{-1}$ acetaminophen showed highly similarity band pattern to the natural leaves, but the L1 and L2 were the new appeared bands that were not existed in natural leaves and these two bands also appeared in high concentrations treatment of acetaminophen. Interestingly, bands L1 and L2 are the groups of bacterial communities that can be significantly promoted by high concentrations of acetaminophen. Bands M1 and M2 are the particular communities for the treatments of $30 \mu\text{g}\cdot\text{ml}^{-1}$ acetaminophen of *in situ* conditions and bacterial community of M2 were strongly promoted by this concentration level of acetaminophen. If the acetaminophen concentration increased to $50 \mu\text{g}\cdot\text{ml}^{-1}$, M2 will be restrained and another new band H1 was appeared.

For the PLM microcosms (Figure 3b), bands P1 and P2 were the most sensitive bacterial communities to acetaminophen, the two bands disappear when PLM microcosms was treated with acetaminophen even under the concentration of $10 \mu\text{g}\cdot\text{ml}^{-1}$. Bands A1, A2 and A3 were the typical bacterial groups that can be stimulated by low concentration of acetaminophen and inhibited by high concen-

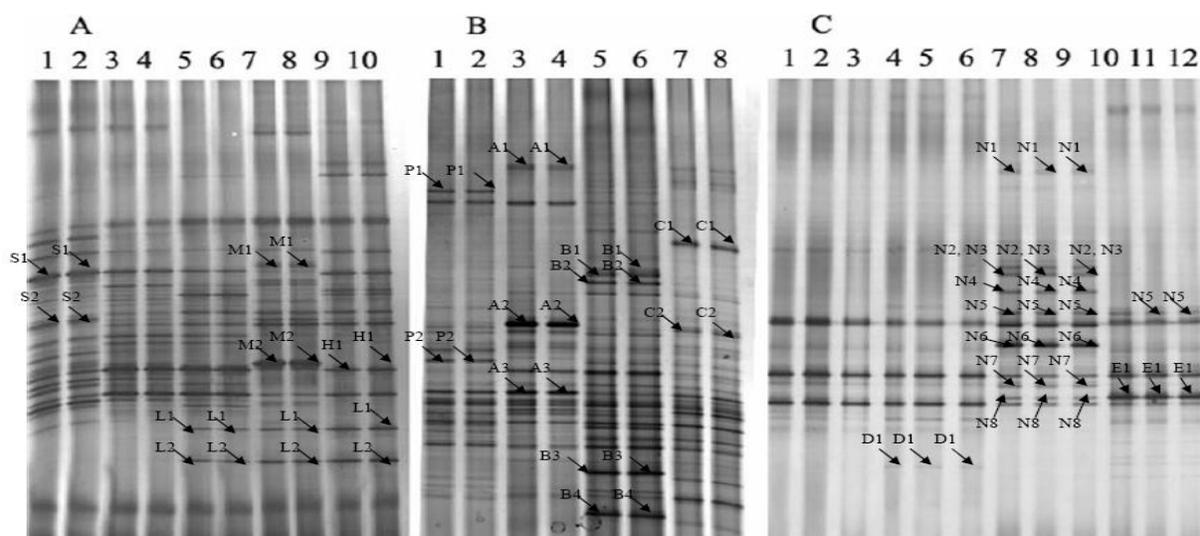


Figure 3. 16S rRNA gene PCR-DGGE profiles for phyllobacterial community structure at different cultural conditions. A, *In situ* conditions: Lanes 1 to 2, sterilized leafage; lanes 3 to 4, unsterilized leafage; lanes 5 to 6, unsterilized leafage with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 7 to 8, unsterilized leafage with 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 9 to 10, unsterilized leafage with 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; B, PLM microcosms: Lanes 1 to 2, PLM without acetamidrid; lanes 3 to 4, PLM with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 5 to 6, PLM with 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 7 to 8, PLM with 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid. C, TYB microcosms: Lanes 1 to 3, TYB without acetamidrid; lanes 4 to 6, TYB with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 7 to 9, TYB with 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid; lanes 10 to 12, TYB with 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid.

concentrations of the pesticide, especially the group A2 and A3 which were the most abundant groups for the 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid treatment, but almost disappeared under the conditions of high concentration treatments. Many special bacterial groups could be promoted by 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid but inhibited by 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid in PLM microcosms, such as bands B1, B2, B3 and B4. Bands C1 and C2 were the new appeared bacterial groups under the conditions of 50 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid, the two bands were the acetamidrid resistant bacteria groups in PLM microcosms test.

For the TYB microcosms (Figure 3c), low concentration treatment also showed similar band pattern as control (TYB microcosm without acetamidrid treated), except the faint band D1. TYB microcosms showed the same reactions as the *in situ* conditions and PLM microcosms, that many bacterial groups (bands N1 to N8) could be promoted by 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid but completely inhibited by the pesticide at the concentration of 50 $\mu\text{g}\cdot\text{ml}^{-1}$. Band E1 was significantly stimulated by high concentration of acetamidrid and the bacterial groups of E1 and N5 were the resistant communities in the TYB microcosms test.

In all tested conditions (*in situ*, PLM and TYB), the number of bands at 30 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid treatment were more abundant than that of other applied concentrations. The PLM microcosms (Figure 3b) showed similar band numbers to the *in situ* conditions (Figure 3a) and they were all much more abundant than that of chemical microcosms (Figure 3c).

Cluster analysis constructed from PCR-DGGE profiles

displayed that in chemical and PLM microcosms, the sample treated with high concentration of acetamidrid (30 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$) formed the farther distant branch from its corresponding control than those treated with 10 $\mu\text{g}\cdot\text{ml}^{-1}$ acetamidrid (Figure 4b, c). Under *in situ* conditions (Figure 4a), the microbial community of sterilized leaves represented a deep-separated lineage from the natural leaves and natural leaves treated with different concentrations of acetamidrid. The stated result indicated that, the community structure of phyllobacteria was destroyed heavily by UV and alcohol sterilization. Cluster analysis results of *in situ* and *ex situ* tests indicated that, low concentration of acetamidrid showed minor effects on phyllobacteria community, but the bacterial communities were significantly changed by the pesticide at the concentration higher than 30 $\mu\text{g}\cdot\text{ml}^{-1}$.

Quantitative analysis of the microbial community

The Shannon-Weaver indexes of diversity (H') of the PLM microcosms and *in situ* conditions showed similar values, which were all higher than that of chemical microcosms (TYB). The Simpson indexes of dominance (D) were significantly lower in PLM microcosms and *in situ* conditions than that of chemical microcosms (Figure 5). High H' and low D values indicated high diversity in a microbial community (Shannon and weaver, 1963; Simpson 1949). The results indicated that, the diversities of PLM microcosms and *in situ* conditions were significantly higher than that of chemical microcosms.

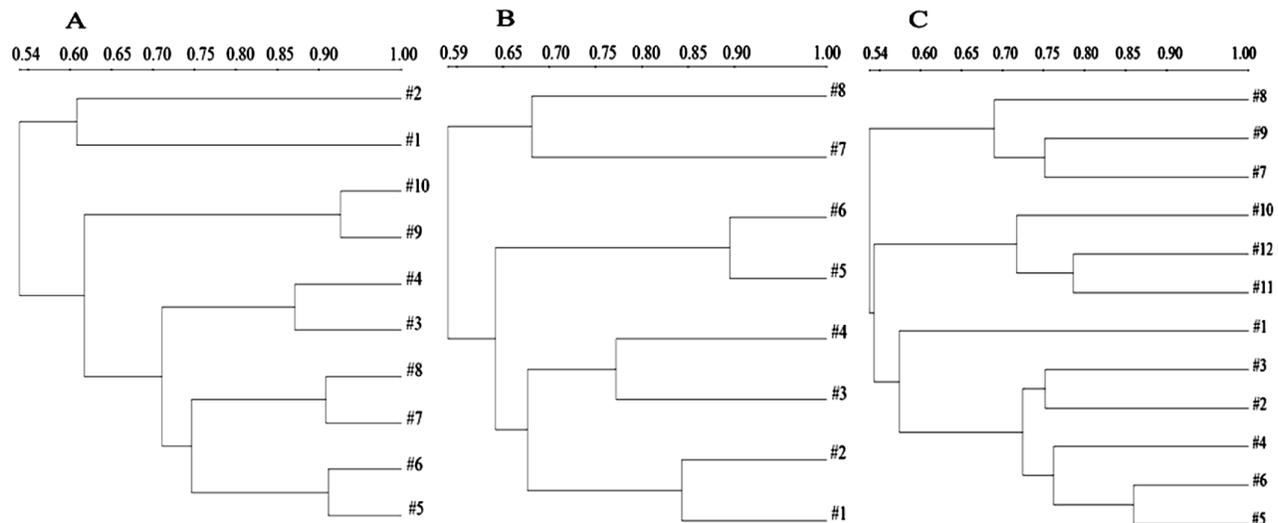


Figure 4. Phyllobacterial dendrogram constructed from PCR-DGGE banding profiles by cluster analysis for community similarities at different cultural conditions. A, In situ conditions: #1 to 2, sterilized leafage sample; #3 to 4, unsterilized leafage sample; #5 to 6, unsterilized leafage with $10 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #7 to 8, unsterilized leafage with $30 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #9 to 10, unsterilized leafage with $50 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; B, PLM microcosms: #1 to 2, PLM without acetamiprid; #3 to 4, PLM with $10 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #5 to 6, PLM with $30 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #7 to 8, PLM with $50 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; C, TYB microcosms: #1 to 3, TYB without acetamiprid; #4 to 6, TYB with $10 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #7 to 9, TYB with $30 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid; #10 to 12, TYB with $50 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid. Note: The numbers listed are corresponding to the lanes' number the figure.

Diversity results of PLM microcosms also suggested that, the composition of kidney bean's leafage was more suitable for phyllobacterial nutrition requirements than that of chemical mediums. The PLM is one of the best choices for phyllobacteria cultivation tests.

Diversities of PLM microcosms and *in situ* conditions remained relatively stable at different concentrations of acetamiprid treatments compare with chemical microcosms. In chemical microcosms, phyllobacterial diversity fluctuated drastically according to different concentrations of acetamiprid applied. The similar disciplinary in all culture conditions is that, the phyllobacterial diversity could be stimulated by acetamiprid at appropriate concentration, but inhibited at the high concentration.

In summary, the new kind of pesticide showed minor negative effects on phyllobacterial community of *P. vulgaris* at the properly applied concentration (not higher than $10 \mu\text{g}\cdot\text{ml}^{-1}$ in this study) and the phyllobacteria also displayed significant degradation abilities to acetamiprid. However, the phyllobacteria's community structure could be destroyed heavily by high concentration of the pesticide, and the phyllobacteria's degradation capability was also inhibited at the same conditions.

DISCUSSION

After pesticides come into environment and contact with water, soils, plants or other substance, they can be cleaned up gradually by processes such as volatilization, photolysis, absorption, eluviations and biotic or abiotic

decomposition (Petit et al., 1995). On the other hand, residual pesticides played complicated effects on environmental organisms in turn (Julien and Reeledr, 1987). Interactions between acetamiprid degradation and phyllobacterial community dynamics were determined in this study. The fact that acetamiprid residue decreased rapidly at all concentration treatments in the early stage of *in situ* conditions was mainly attributed to the results of eluviations and volatilization (the melting point of acetamiprid is 98.9°C and the vapor pressure 1×10^{-8} mm Hg), in this stage, other factors such as biotic or abiotic degradation may only displayed limited contributions. Biotic and abiotic degradation ought to play more important role after residual chemicals were steadily absorbed to the plant or other substance.

Once plant leaves were sterilized by ultraviolet, ethanol or H_2O_2 , phyllobacteria were killed or restrained during the phyllobacteria recover period, the biotic degradation abilities would be reduced or even lost (Wilson et al., 1999). In this study, phyllobacterial community structure was seriously destroyed by ultraviolet and ethanol disposal, in the mean while, the pesticide degradation results showed that, the half-life of $10 \mu\text{g}\cdot\text{ml}^{-1}$ acetamiprid on sterilized leafage were significantly longer than that of unsterilized leafage (unsterilized leafage was 3.24 days compared with 4.99 days on sterilized leaves). By comparing acetamiprid half-lives on sterilized and unsterilized leafage at different concentrations, we concluded that, phyllobacteria of kidney bean's leafage showed the degradative abilities to new kind of neonicoti-

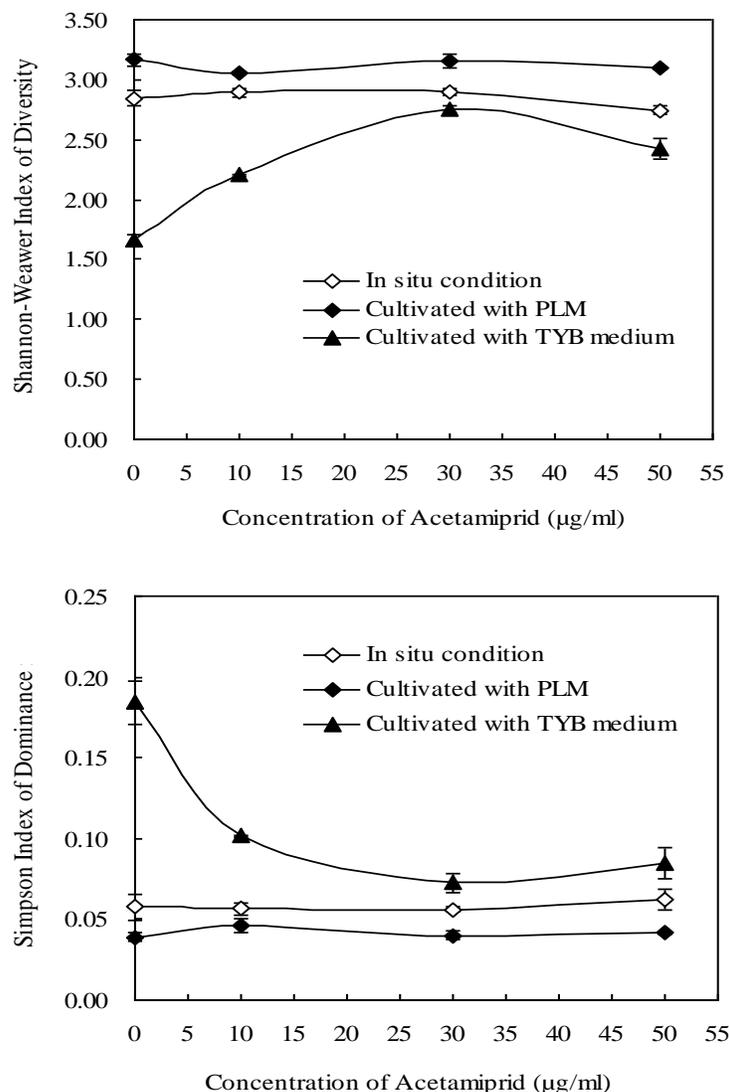


Figure 5. Biodiversity analysis of phyllobacteria at different cultural conditions (*in situ* and *ex situ* conditions).

showed the degradative abilities to new kind of neonicoti- 3816 noids pesticide at proper applied concentration. Degradation process combine with PCR-DGGE analysis further displayed that, phyllobacterial community structure was heavily destroyed by high concentration of acetamiprid and phyllobacteria degradation ability was restrained at the same time. The previous acetamiprid biodegradation results showed that, the pesticide could be biotransformed by *Stenotrophomonas maltophilia*, *Rhodotorula mucilaginosa*, *Rhizopus* sp. and *Penicillium* sp., but the biotransformation efficiency of these soil microbes were not very high (Dai et al., 2010a, b). The half-life of acetamiprid in sucrose mineral salt medium with the *R. mucilaginosa* IM-2 were about 3.7 days and there was still 8.8% acetamiprid residue detected after 20 days incubation (Dai et al., 2010b). Soil microbes degradation researches described earlier displayed

similar results as in the plant leaves investigation of *in situ* conditions, that is, the pesticide could be biotransformed by microbes from soil and plant leaves at the proper concentration, but the transformation ability were not good enough (this study was the first report for the acetamiprid degradation by microbes of phyllosphere).

In order to investigate the relationships between phyllobacterial community dynamics and acetamiprid degradation further, two kinds of *ex situ* culture methods were adopted and compared. According to results of uncultured scientific reports, only a small bit of environmental microorganisms could be cultivated using single chemical mediums, the closer medium composition is similar to original environment, the larger proportion of the environmental microbes could be isolated (Ward et al., 1990; Ghezzi and Steck, 1999). In this study, PLM and TYB mediums were selected and compared for phyllo-

microbial cultivation tests. Obviously, bacterial diversity values in PLM microcosms were notably higher than that of TYB chemical microcosms. PLM was proved to be one of a proximal medium for phyllobacterial nutrition requirements. Perhaps the results of this study were not exhaustive enough, the microbial cultures in PLM represented the phyllobacterial community and functions of phyllosphere better than other mediums do. Degradation result of PLM microcosms further proved that, acetamiprid is a degradable chemical for phyllo-microbes of *P. vulgaris* at proper applied concentration. However, the degradation ability was inhibited gradually with increasing of acetamiprid concentration and the degradation ability was lost completely when the treated concentration is higher than $50 \mu\text{g}\cdot\text{ml}^{-1}$ (Figure 1a). Although, the culture-dependent results displayed that all tested concentrations of acetamiprid showed no significant negative effects on phyllobacterial population, phyllobacterial dominant communities were changed obviously, when PLM microcosms is treated with high concentrations of acetamiprid. Our work confirmed that, the new kind of pesticide could be degraded by phyllosphere microbes at proper concentration and the results could provide useful instructions for the pesticide applications.

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