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Assessing the impact of fungicide enostroburin application on bacterial community in wheat phyllosphere

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Abstract

Fungicides have been used extensively for controlling fungal pathogens of plants. However, little is known regarding the effects that fungicides upon the indigenous bacterial communities within the plant phyllosphere. The aims of this study were to assess the impact of fungicide enostroburin upon bacterial communities in wheat phyllosphere. Culture-independent methodologies of 16S rDNA clone library and 16S rDNA directed polymerase chain reaction with denaturing gradient gel electrophoresis (PCR-DGGE) were used for monitoring the change of bacterial community. The 16S rDNA clone library and PCR-DGGE analysis both confirmed the microbial community of wheat plant phyllosphere were predominantly of the γ-Proteobacteria phyla. Results from PCR-DGGE analysis indicated a significant change in bacterial community structure within the phyllosphere following fungicide enostroburin application. Bands sequenced within control cultures were predominantly of *Pseudomonas* genus, but those bands sequenced in the treated samples were predominantly strains of *Pantoea* genus and *Pseudomonas* genus. Of interest was the appearance of two DGGE bands following fungicide treatment, one of which had sequence similarities (98%) to *Pantoea* sp. which might be a competitor of plant pathogens. This study revealed the wheat phyllosphere bacterial community composition and a shift in the bacterial community following fungicide enostroburin application.

Key words: fungicide; phyllosphere; microbial community; 16S rDNA clone library; PCR-DGGE
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Introduction

The aerial habitat part of plants is termed as the phyllosphere, which is normally colonized by a variety of bacteria, yeasts, fungi. Phyllosphere ecosystems are dynamic and ephemeral, because physical environment surrounding phyllosphere microbes change continuously with daily cycles in temperature, radiation, relative humidity, wind velocity, and leaf wetness. The phyllosphere ecology is an open system. Alien microbes can invade from the atmosphere, insects, people, biological pesticides, etc., and the microbes of the phyllosphere could also invade other ecosystems, i.e., soils, animal surface, rivers (Andrews 1992; Hirano and Upper, 2000; Lindow and Brandl, 2003). Microbial phyllosphere communities are complex and many uncultured microorganisms could be disclosed by molecular methods rather than conventional culture methods (Yang et al., 2001; Lambais et al., 2006). In our previous work, we also found many uncultured bacteria in the phyllosphere of vegetables in greenhouse by molecular methods.

Chemicals such as fertilizers and pesticides have been used in great amounts in agricultural practices across the world and have been proven to cause a disturbance in environmental microbial structure, such as soil microbial structure (Engelen et al., 1998; Yang et al., 2000) and estuarine ecosystems (Delorenzo et al., 2001). Our recent studies revealed that an increase in bacterial abundance and a shift in microbial community composition within the phyllosphere vegetable were associated with the use of pesticide in greenhouse (Zhang et al., 2008). Fungicides have become an important tool for crop protection due to their ability to control unwanted microbial pathogens. However, many fungicides are non-specific in their mode of action, resulting in changes to the composition of non-target microorganisms within the phyllosphere, including those that may have been of benefit to the plant. The effects of a particular fungicide on phyllosphere microbes are dependent upon the specific chemical properties (including any associated emulsifiers or other chemicals if the fungicide is mixed prior to application), the concentration

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at which the pesticide is used, the types of indigenous phyllosphere microorganisms present, and the environment in which the microorganisms are growing. The application of fungicides may decrease the microbial biomass due to the direct toxicity of the fungicide, or in some cases increase the microbial biomass because of the fungicide (and other associated emulsifiers or solvents) supplying a nutrient source, in addition to changes due to indirect ecological effects such as the death of microbial predators, or in some cases there may be no effect at all. As far as we know, the disturbance of fungicide in phyllosphere bacteria community in field had been seldom studied.

Bacteria contribute significantly to pathogenic control, N_2 fixation, nutrient cycling, and decomposition in phyllosohere ecosystems (Ruinen, 1956; Nandi and Sen, 1981; Berg et al., 2005; Compant et al., 2005). Any effects of fungicides on microbial communities may have subsequent impacts on high trophic levels. Enostroburin is one of the new fungicides used against wheat powdery mildew. The objective of this study was to disclose the epiphytes of wheat leaves and assess the effects of enostroburin fungicide application upon the indigenous bacterial community of wheat plant cultivars in field using 16S rDNA clone library and PCR-DGGE techniques.

1 Materials and methods

1.1 Growth of wheat plants

The experiments were conducted in a native field located at the Tongzhou County near Beijing City, China. Wheat cultivars were planted in 360 m^2 plots in a field on 15 May 2006. During the growth of the plants, the rain in May was 33.1 mm and the average temperature was 19.8°C. Plants were watered and fertilized in accordance to local agricultural practices. The plants were sprayed with fungicide emulsifiable (EC, enostroburin 7.0%, *W/V*), which was diluted with water (EC:water, 1:1000, *V/V*), in accordance to the recommended dosage for wheat cultivars. Control plants were sprayed with water. Wheat leaf samples were collected randomly at day 1, 2, 3, 5, 7 after treatment. After sampling, the leaves were immediately placed into sterile stomacher bags and transported to the laboratory stored at 4°C. Leaves used for DNA extraction and subsequent 16S rDNA clone library and PCR-DGGE were stored at –20°C.

1.2 Detachment of phyllosphere microbes

Leaf samples were transferred aseptically using flame-sterilized forceps and placed in polypropylene tubes containing washing buffer (0.1 mol/L potassium phosphate buffer, pH 7.0) and sonicated (frequency 40 kHz) for 7 min in an ultrasonic cleaning bath to dislodge microorganisms from leaves. Leave debris was then removed by a slow speed centrifugation step (5 min, 500 ×*g*, 4°C) and the remaining microbial suspension centrifuged at 7,000 ×*g* for 15 min at 4°C. Microorganisms were resuspended in washing buffer and frozen at –20°C until processing (Zhang et al., 2008).

1.3 DNA extractions

The microbial pellet samples were mixed with 4.5 mL of DNA extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 100 mmol/L sodium EDTA (pH 8.0), 100 mmol/L sodium phosphate (pH 8.0), 1.5 mol/L NaCl, 1% CTAB) and 100 μL of proteinase K (10 mg/mL) in Oakridge tubes by horizontal shaking at 225 r/min for 30 min at 37°C (Zhou et al., 1996). After shaking treatment, 0.5 mL of 20% SDS was added. The samples were incubated in a 65°C water bath for 2 hr with gentle end-over-end inversions every 15–20 min. The supernatants were collected after centrifugation at 6,000 ×*g* for 10 min at room temperature and transferred into 50-mL centrifuge tubes. The microbial pellets were extracted two more times by adding 4.5 mL of the extraction buffer and 0.5 mL of 20% SDS, vortexing for 10 sec, incubating at 65°C for 10 min, and centrifuging, as described above. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroformisoamyl alcohol (24:1, *V/V*). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 hr. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 ×*g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 100 μL.

1.4 Cloning of 16S rDNA

A clone library was constructed using DNA extracted from microbial collected from the 1st day control wheat leaves. The 16S rDNA was performed with primers universal for the domain bacteria: 8-27F and 1392-1407R for the clone library. The PCR mix (50 μL) consisted of 1× PCR buffer, 2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 20 pmol of each primer, 2.5 units of TaKaRa Taq DNA polymerase and 10 ng genomic DNA. The PCR conditions were 94°C for 4 min, followed by 35 cycles of 92°C for 1 min, 50°C for 30 sec, and 72°C for 2 min followed by a final extension at 72°C for 10 min. The clone library was generated by ligating PCR products into the pGEM-T (Promega, Madison, WI, USA), which was then transformed into competent *E. coli* JM109 cells (Konstantinov et al., 2003).

1.5 Cloning analysis

The 278 transformants were inoculated into a 96-well microtiter plate containing 100 μL LB with glycerol and ampicillin. The 16S rDNA were amperlified with primer T7 and SP6. The PCR products were digested with RSAI and TaqI. Clones with unique restriction patterns are referred to as operational taxonomic units (OTUs). One transformant from each OTU (the clone numberof this OTU over three) was picked. The picked transformants were sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) for DNA sequencing. Sequence data obtained in this experiment were deposited in the GenBank database under accession numbers FJ515748–FJ515764.

Evaluation of the clone library was performed by rarefaction analysis using software RarefactWin Version 1.3 (Steven Holland, Stratigraphy Lab, University of Georgia (http://www.uga.edu./strata/ software/software.html)).

1.6 PCR-DGGE analysis of 16S rDNA fragments

The variable V3 region of 16S rDNA sequences from nucleotide 341 to nucleotide 534 (*E. coli* numbering) was amplified by PCR using eubacterial primers as described by Yang et al. (2001). DGGE was performed with the Dcode^{TM} universal mutation detection system (Bio-Rad, USA). PCR products (20–25 μL) were loaded onto 10% (*W/V*) acrylamide: bisacrylamide (37.5:1, *m/m*) gel containing a 40%–60% linear gradient of formamide and urea (100% denaturing solution contained 40% formamide and 7 mol/L urea). The gel was run for 7 hr at 60°C and 100 V in 1× TAE buffer. After electrophoresis, the gel was stained with ethidium bromide and photographed with a Fluor-S Multi Imager System (Bio-Rad, USA). DGGE bands of interest were excised from the gel using a sterile scalpel. The DNA was subsequently eluted overnight at 37°C in sterilized distilled water. Excised DNA was then re-amplified using the primer pair PRBA 338f and PRUN518r as described previously. The resulting PCR products were cloned into the pGEM-T (Promega, Madison, WI, USA) and then transformed into competent *E. coli* JM109 cells (Konstantinov et al., 2003) and sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. for DNA sequencing. Sequence data obtained in this experiment were deposited in the GenBank database under accession numbers FJ490423–FJ490438.

1.7 Statistics

The DGGE analysis of all samples was repeated twice. All gels were scanned at 400 dpi and analysed using the software of Quatity One (version 1.12, Bio-Rad, Hercules, USA). The cluster analysis of band patterns was performed using the method described by Li et al. (2008). The data were used to calculate the Shannon-Weaver diversity index, the evenness described by Dilly and the co-workers (Dilly et al., 2004). Firstly, a number of bands per lane were assessed using bands searching algorithm within the program. A manual check was done and the DGGE fragments constituting less than 1% of the total area of all bands were omitted. When one and the same DGGE band had a value lower than 1% of the total area of all bands in the first replicate and higher than 1% in the second one, we calculated an average value based on the two runs. Bands above 1% of the total area of all bands in two replicates of one sample were considered as dominant DGGE bands and included in the further analysis.

2 Results and discussion

2.1 16S rDNA clone library analysis

Total community DNA was extracted from the leaves before spraying fungicide samples. The 16S rDNA clone libraries were constructed and 278 clones were processed by RFLP for each library, in which at least one clone of each pattern was sequenced. Screening of these 278 clones by HhaI and HaeIII restriction enzyme analysis grouped the clones into 35 OTUs. OTUs W3, W7 and W16 were found to be the largest groups, containing 17.5%, 27.8% and 10.6% clones, respectively. In the library, *Pseudomonas* is the main group (Table 1).

Clone library was statistically evaluated by rarefaction analysis, in which the expected number of different RFLP group vs. the number of clones in the library was calculated. Rarefaction curves tended to approach the saturation plateau (Fig. 1), indicating that 278 clones used in the library screening could well cover the diversity of the 16S rDNA genes. Phylogenetic analysis of sequence data for clones representing OTUs was used to determine the phylogenetic position of each clone (Fig. 2). One third microorganisms had 16S rDNA similarities lower than 95% to database entries. Thus, this study supported the observation of previous work that more phyllosphere bacteria are uncultured (Yang et al., 2001).

2.2 DGGE and diversity analysis

In this study, the change of bacterial community after spraying enostroburin fungicide was elucidated by the

Table 1 16S DNA clone library sequence homologies

OTU	Closest identity	Accession number	Identity (%)	Percentage (%)
W1	*Pseudomonas putida*	AY958233	99	1.2
W2	Bacterium m5	DQ453814	96	1.2
W3	*Pseudomonas* sp.	AM410625	99	17.5
W4	*Pantoea agglomerans*	EU047555	99	1.2
W5	Candidatus *Hamiltonella defensa*	AF293621	94	1.6
W6	Uncultured bacterium	DQ256313	94	4.3
W7	Antarctic seawater bacterium	DQ064630	99	27.8
W8	Candidatus *Regiella insecticola*	AY296734	94	0.8
W9	Antarctic seawater bacterium	DQ064630	93	0.8
W10	*Pseudomonas* sp.	AY247063	99	1.2
W11	*Buchnera* sp.	AB033776	95	2.4
W12	*Pseudomonas* sp.	EU158318	99	0.8
W13	Uncultured bacterium	EF179845	95	1.2
W14	*Pseudomonas* sp.	AY247063	99	2.0
W15	*Buchnera* sp.	AB033776	98	1.6
W16	*Pseudomonas* sp.	EU781733	97	10.6
W17	*Erwinia* sp.	Z96086	93	0.8
Total				77.0

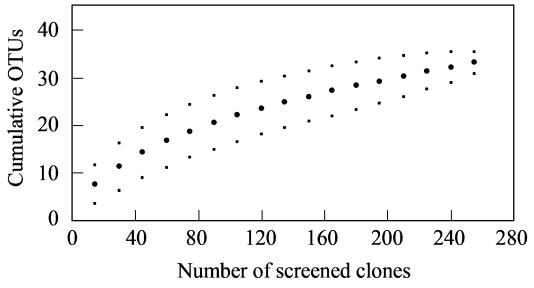

Fig. 1 Rarefaction analysis of the 16S rDNA clones using software Rarefactwin Version 1.3. Dashed lines represent 99% confidence intervals.

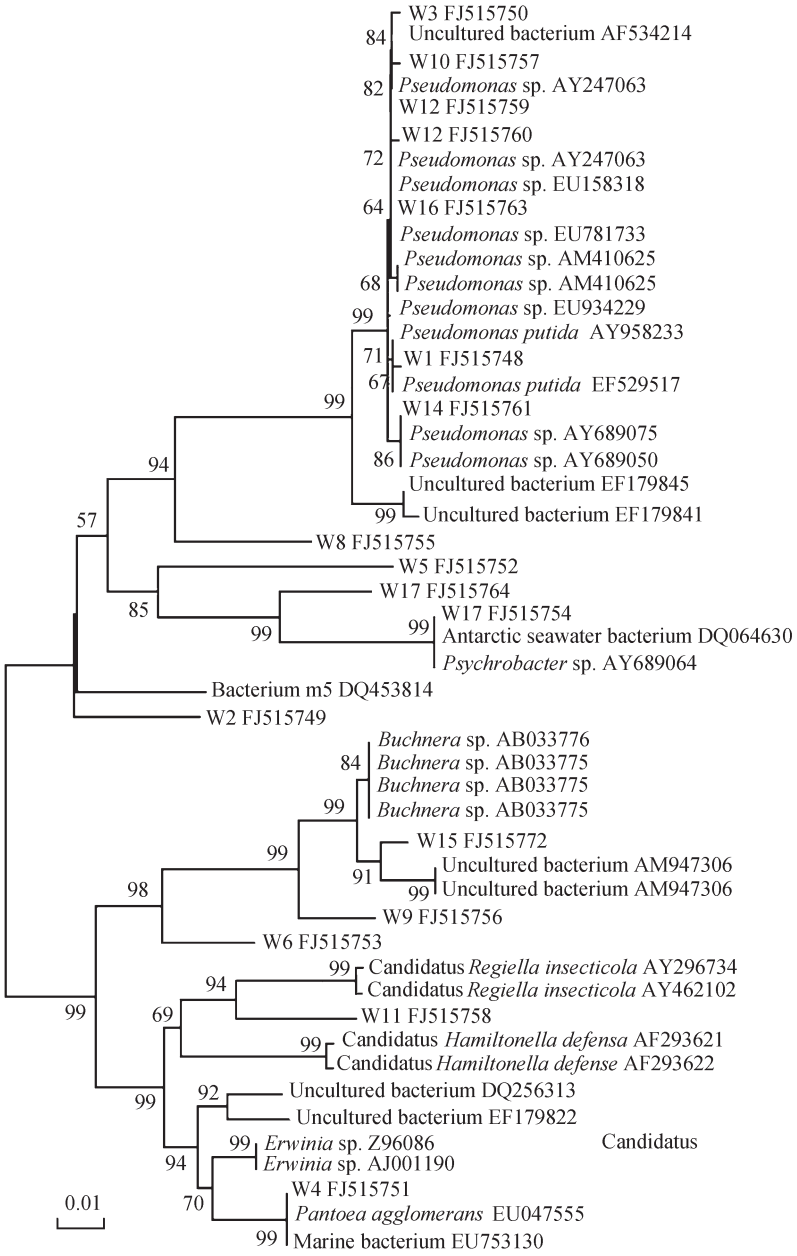

Fig. 2 Neighbor-joining analysis showing the phylogenic relationship of clone library 16S rDNA sequences to other related organisms. Clone identities are described in Table 1.

DGGE phylotype. As shown in Fig. 3, for control samples, 13 bands were sequenced, in which 2 strains of the genus *Buchnera* (WD1, WD10), 4 strains of the genus *Pseudomanas* (WD2, WD4, WD14 and WD16) and 2 strains of the genus *Pantoea* (WD6 and WD13) were identified. A total of 9 bands were identified from the enostroburin fungicide treated samples, which consisted of 3 strains of the genus *Pseudomanas* (WD4, WD14 and WD16), 2 strains of the *Pantoea* (WD6 and WD13), one strain of genus *Buchnera* (WD7), and one strain of genus *Orientia* (WD15).

DGGE band WD5 was detected in all profiles in control, while bands WD10, WD13 and WD15 were found to be unique in the treated samples. Meanwhile, several bands like WD14 and WD16 were detected in all samples, indicating that the bacterial groups represented by these bands possibly played an important role in phyllosphere ecology. A similar difference of the bacterial community structure between the treated samples and the control samples are shown in Fig. 4.

In the treated phyllosphere, the Shannon-Weaver diversity index, evenness, and equitability showed that microbial diversity of the treated samples are higher than the control samples from the first day to the fifth day (Table 3).

Band WD13, which was closest to *Pantoea* sp., emerged in all the treated samples, and became a dominant bacterium during the investigation period. *Pantoea* sp. could accumulate glycine-betaine, ectoine and amino acids in the bacterial cells, which enable the *Pantoea* sp. to resist environmental stress (Canamas et al., 2007). It is reported that *Pantoea* sp. can effectively mobilize cellular to

Table 2 DGGE band sequence homologies

DGGE band	Closest identity	Accession number	Identity (%)
WD1	*Buchnera aphidicola*	M63249	99
WD2	*Pseudomonas* sp.	EU935094	95
WD3	Restionaceae environmental sample	EF024965	98
WD4	Uncultured *Pseudomonas* sp.	EU169683	98
WD5	Uncultured bacterium	EU153089	98
WD6	*Pantoea* sp.	FJ235635	98
WD7	Uncultured bacterium	EF521199	95
WD8	*Pantoea* sp.	FJ235635	100
WD9	*Buchnera* sp.	AB033776	98
WD10	*Buchnera* sp.	AB033776	99
WD11	Uncultured bacterium	FJ235654	99
WD12	*Pseudomonas* sp.	EU446220	99
WD13	*Pantoea agglomerans*	AF130948	98
WD14	Uncultured bacterium	FJ235654	97
WD15	*Orientia tsutsugamushi*	M63248	97
WD16	*Pseudomonas argentinensis*	EU723826	98

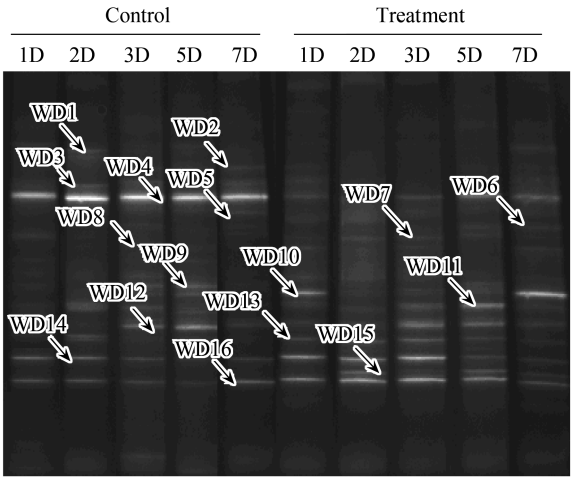

Fig. 3 Composite 16S rRNA gene directed PCR-DGGE: banding profiles of epiphyte bacteria. Lanes: 1D, 2D, 3D, 5D, 7D mean the day in the trial.

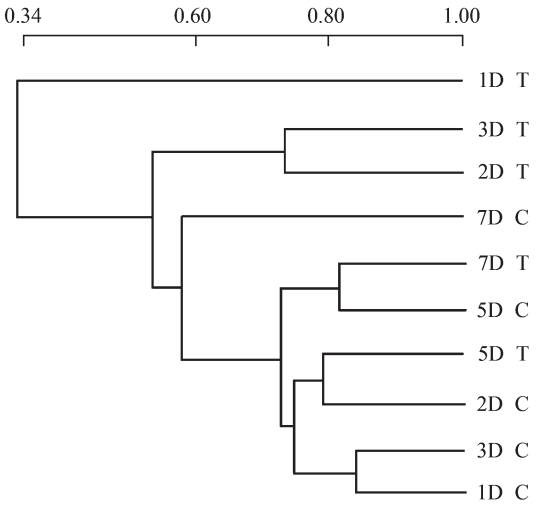

Fig. 4 UPGMA dendrogram computed from Dice similarity matrix among DDGE profiles of amplified 16S rDNA gene. Scale bar indicates Dice similarity values. C: control, T: treatment.

defense responses upon pathogen attack, such as wheat rust

Table 3 Diversity analysis of the control and enostroburin treated samples

Material	Day	No. of DNA bands	Shannon-Weaver diversity index	Even-ness	Equita-bility
Control	1	12	1.86	1.86	0.714
Control	2	13	2.06	1.85	0.763
Control	3	14	2.28	1.99	0.844
Control	5	14	2.04	1.78	0.755
Control	7	14	1.95	1.70	0.722
Treatment	1	20	2.39	1.91	0.921
Treatment	2	15	2.44	2.03	0.902
Treatment	3	16	2.45	2.04	0.907
Treatment	5	16	2.46	2.07	0.910
Treatment	7	16	2.30	1.91	0.850

severity caused by *Puccinia* (Ortmann and Moerschbacher, 2006). There are other reports that the *Pantoea* sp. is the most ubiquitous bacterial participants of phyllosphere communities (Legard et al., 1994; Wilson and Lindow, 1994) and the populations could have a fluctuation for some reasons, such as biological competitions (Wilson et al., 1999). In our experiment, *Pantoea* sp. was proved to be a phyllosphere bacterium, but only had a small population (1.2%) when the wheat had not been sprayed enostroburin fungicide according to the 16S rDNA clone library. Therefore, it can not be detected by DGGE. On the treated wheat leaf surface, however, *Pantoea* sp. was found to be a dominant bacterium. *Pantoea* sp. could have the ability to synthesize indole-3-acetic acid (IAA) (Brandl and Lindow, 1998; Manulis et al., 1998). IAA could promote cell wall loosening at a very low concentration, and exogenously applied auxin stimulates the release of saccharides from the plant cell wall (Lindow and Leveau, 2002). This may be the reason that the phyllosphere had a great diversity from day 2 to day 5 in the fungicide treated phyllosphere.

Band WD15 was emerged in the treated samples and shows a sequence similarity very close to *Orientia tsutsugamushi* (97%), which is a causative organism for scrub typhus (Watt and Parola, 2003). Although we can not identify the band WD15, we should pay close attention to the potential ecology risk.

There are also other possible reasons which lead to an increase in microbial diversity. Annapurna and Rao (1982) reported the effect of foliar application of captan, dithane, carbaryl and atrataf on the quantity of total carbohydrates, total amino acids and total nitrogen leached from corn leaves and on the phylloplane counts of fungi, bacteria and actinomycetes. Leaf extracts were analyzed for amino acids, carbohydrates and nitrogen in association with microbial counts. Generally, more microbial species were found in the treated samples than the control samples. A significant change in Gram-negative bacteria in all treatments was recorded. Less total amino acids and total nitrogen were measured in all treated leaf extracts, which were rich in carbohydrates as compared with the control samples. Another reason is that the fungi could be restrained after spaying enostroburin fungicide and some bacteria could use the carbon source, nitrogen source occupied by fungi before.

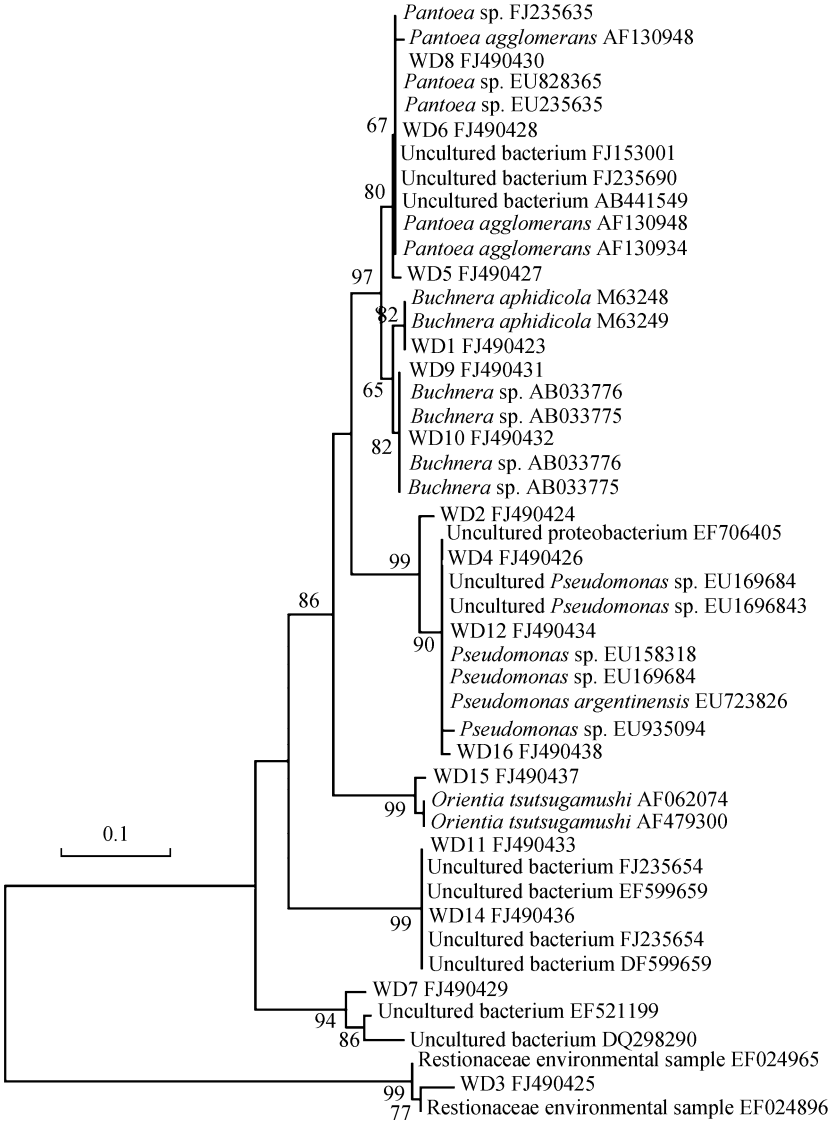

Fig. 5 Neighbor-joining analysis showing the phylogenic relationship of 16S rDNA sequences from excised DGGE bands to other related organisms. Band identities are described in Table 2.

Pseudomonas sp. (WD4, WD14 and WD16) were the favorite bacteria and grew well on the control wheat leaves according DGGE through this experiment, which was similar to the clone library analysis. Schreiber et al. (2005) reported that the inoculation of *Pseudomonas* sp. increased water permeability of *Hedera* and *Prunus* cuticles, which in turn increase the availability of water and dissolved compounds in the phyllopshere. Consequently, living conditions in the habitat phyllosphere are improved. It can be concluded that the ability to change leaf surface properties will improve epiphytic fitness of pseudomonads (Schreiber et al., 2005). Legard et al. (1994) reported 15 bacteria were frequently recovered and identified on wheat leaves in the glasshouse and field, but only *Pseudomonas aureofaciens* kept similar frequency in all experiments. Egamberdiyeva and Hoflich (2003) also found that the number of *Pseudomonas fluorescens* PsIA12 increased significantly on the root and shoot of winter wheat. Whereas, the main OTUs W7 in the 16S rDNA clone library was not detected by DGGE analyses. The reason might be the PCR bias caused by the different PCR conditions and primers (Li et al., 2009).

In this study, we found that enostroburin fungicide application resulted in the changes of bacterial community structure and diversity in wheat phyllosphere. The shifts of bacterial communities could be driven by different environmental factors, such as crop types, soil physico-chemical properties and other environmental disturbances (e.g., temperature, nutrition). For example, some studies showed explicit shifts in fertilizer treatment (Ge et al., 2008). However, significant shifts of phyllosphere microbial communities following certain chemical applications were seldom studied.

2.3 Phylogeny of bacteria in phyllosphere

Their phylogenetic relationship to other closely related species is shown in the Neighbour-joining tree in Figs. 2 and 5. Results of this study showed that all the sequences belonged to strains of γ-Proteobacteria. It is different to the results from other plants growing in greenhouse,

such as the microbial community of the pepper plant phyllosphere were composed of Firmicutes, Bacteroidetes, and γ-Proteobacteria phylum (Zhang et al., 2009).

In conclusion, this study for the first time examined the changes of the microbial community within the phyllosphere of wheat plant cultivars following the application of enostroburin fungicide. Using two culture-independent techniques, 16S rDNA clone library and PCR-DGGE, results here suggested that the application of enostroburin fungicide not only caused a significant change in bacterial community composition, but also resulted in an overall increase in bacterial diversity within the phyllosphere. Future studies are recommended to investigate the influence of different fungicides upon the composition and structure of phyllosphere microbial communities for different plants. In turn, further understanding will be made regarding how the microbial communities may actually result in the degradation of pesticides.

Acknowledgments

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