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Soil *Pseudomonas* Community Structure and Its Antagonism Towards *Rhizoctonia solani* Under the Stress of Acetochlor

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Abstract In a microcosm experiment, the amplified ribosomal DNA restriction analysis was adopted to investigate the *Pseudomonas* community structure in soils applied with different concentrations (0, 50, 150, and 250 mg/kg) of acetochlor, and an in vitro assay was made to examine the antagonistic activity of isolated *Pseudomonas* strains acting on soil-borne pathogen *Rhizoctonia solani*. The results showed that acetochlor application changed the community structure of *Pseudomonas* in aquic brown soil. The diversity of *Pseudomonas* and the amount of isolated *Pseudomonas* strains with antagonistic activity decreased with an increasing acetochlor concentration, and the toxic effect of acetochlor reached to a steady level at 150–250 mg/kg.

Keywords Acetochlor · *Pseudomonas* · Soil microbial community · Antagonistic activity

Pseudomonas is one of the most important and well-studied bacterial species because of its wide distribution in the environment and the relatively easy cultivation. This genus includes several species of environmental interest, such as plant growth promoters (Patten and Glick 2002), plant pathogens (Samson et al. 1998; Morris et al. 2007), and xenobiotic degraders (Xu et al. 2006). In recent years, studies about *Pseudomonas* spp. were mainly focused on its biocontrol activities to soil-borne plant pathogens. The production of secondary metabolites including antibiotics and iron (Fe)-chelating siderophores is probably one of the most important mechanisms of *Pseudomonas* antifungal properties. Many effective antibiotics synthesized by *Pseudomonas* had been detected, such as 2, 4-diacetylphloroglucinol (Phl), pyoluteorin (Plt), pyrrolnitrin (Prn) and phenazine-1-carboxylic acid (PCA). *Pseudomonas* is now authorized as a key role in biological control.

Environmental factors would affect the community composition and antifungal properties of *Pseudomonas*. It has been reported that chemical pollutants, land use, agricultural systems, and plants affect the population structure of *Pseudomonas*, with specific antagonistic subpopulations which are restrained by certain ways (Bergsma-Vlamia et al. 2005; Zhang et al. 2007). Acetochlor is one of the most important herbicides which are widely used in China in order to selectively control broadleaf weeds and annual grasses growing on agricultural lands. Many studies revealed that the application of acetochlor destroyed the original composition and structure of soil bacterial and fungal communities to a certain extent (Zhang et al. 2004; Li et al. 2005) and resulted in the decline of *nif*H gene diversity (Su et al. 2007), but the information about the effects of acetochlor on soil *Pseudomonas* and its antifungal potential is less available.

The main objective of this study was to investigate the effects of acetochlor on the structure and biocontrol activities of *Pseudomonas* in soils. Culture-dependent isolates from soils under the stress of acetochlor with different concentrations were obtained and grouped by the amplified ribosomal DNA restriction analysis (ARDRA) method. Moreover, the influence of the different dosages of

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acetochlor on the prevalence of *Pseudomonas* antagonistic activity against the plant pathogen *Rhizoctonia solani* was also examined.

Materials and Methods

Surface (0–15 cm) soil samples (aquic brown soil) were collected from a fallow field in the Shenyang Experimental Station of Ecology, Chinese Academy of Sciences. Having mixed thoroughly, 2 mm-sieved, adjusted to 60% of the maximum water-holding capacity, these samples were stored at 4°C prior to treatment. According to soil-chemical analysis, the soil samples contained 1.59% of organic matter, 0.83 g/kg of total nitrogen, 0.41 g/kg of total phosphorus, and 0.93 g/kg of total potassium.

Acetochlor (90% EC, Harness, Monsanto Company, USA) was diluted to 250 mg/mL. There were four treatments (0, 50, 150, and 250 mg/kg of acetochlor) with three replicates in the experiment. Different dosages of acetochlor were mixed with 500 g soil samples and placed into pots as a microcosm system. The microcosm system was sealed with parafilm to minimize water loss, and incubated at 25°C for 5 weeks in darkness. Water was added at 1 week intervals to maintain soil moisture content, and 20 g soil samples were taken from each of the triplicated microcosms after incubation of 7, 21, and 35 days.

Pseudomonas spp. was isolated by the procedure as Liu et al. (2006) described. After incubating the isolation plates, the colonies could be identified as *Pseudomonas* (i.e., pearl-white colored and round with entire margins), and preliminary key-tests were carried out, according to Bergey's Manual of Systematic Bacteriology. The tests included: (1) Gram and flagella staining; (2) oxidase and catalase reactions; and (3) oxidation-fermentation test of glucose. After these tests, the PCR procedure based on *Pseudomonas* specific primers PsF and PsR was carried out (Garbeva et al. 2004).

The amplified ribosomal DNA restriction analysis (ARDRA) was performed to identify the species of *Pseudomonas* isolated from different treated soils. The 16S rRNA gene was obtained from each isolate by PCR with the universal primers 27F and 1492R (Martin_laurent et al. 2001) that are targeted to universally conserved regions and permit the amplification of an $\sim$1,500 bp fragment. The PCR products were monitored by 1% agarose (w/v) gel electrophoresis at 100 V for 1 h in 0.5× Tris-acetate-EDTA buffer, and made visible by SYBR Green (TaKaRa Bio Inc., Shiga, Japan) and UV transillumination. The PCR products with the equal volume (3 μL) were digested for 12 h using restriction endonucleases *Taq* and *Hinf* under the standard conditions suggested by the manufacturer (Takara Bio Inc., Shiga, Japan), respectively. Restriction bands were separated in 10% (w/v) polyacrylamide gel, and DNA banding patterns were visualized by staining with the Gene Finder (TaKaRa Bio Inc., Shiga, Japan).

The antagonistic activity of *Pseudomonas* isolates against plant pathogenic fungi *Rhizoctonia solani* was determined by a modified method suggested by Vincent et al. (1994). The solid culture (8 mm diameter) from the growing margin of the fungal colonies was inverted and placed centrally on the potato dextrose agar (PDA) plate. Four wells were then made uniformly around the culture for inoculating *Pseudomonas*. The *Pseudomonas* for inoculation was grown in a liquid culture at 28°C for 36 h, and each 30 μL of the culture was spotted on each well. After 3 days, the diameter of inhibition zone was measured. *Pseudomonas fluorescence* Q2-87 with the ability of producing 2, 4-DAPG was served as the positive control, while *Escherichia coli* without antagonistic activity was treated as the negative control. The isolates that formed halo zones were accepted as antagonists to fungi. The scoring was performed as follows: halo ≥4 mm (+++); halo 2–4 mm (++); halo ≤2 mm (+) and no halo (−).

The Shannon diversity index (H') was used to estimate the diversity of *Pseudomonas* based on the ARDRA patterns:

$$H' = -\sum p_i \ln p_i \quad (1)$$

where $p_i = n_i / N$, n_i is the total number of the ith ARDRA type isolates, and N is the total number of the isolates in the sample. Statistical analyses to evaluate the distinction were made using the software package SPSS 13.0 for Windows. Data were considered as significant difference at $p < 0.05$.

Results and Discussion

A total of 632 *Pseudomonas* isolates were obtained from the four acetochlor treatments (0, 50, 150, and 250 mg/kg) on 7th, 21st and 35th day, and the amount of the isolates in each treatment varied from 46 to 63, respectively (Table 1).

According to the ARDRA fingerprints generated with two restriction endonucleases, the 632 isolates were divided into 20 ARDRA patters named A to T, among which, pattern A contained 118 isolates, and patterns A, C, D, J, Q, and T were mostly found and existed in all four treatments. As shown in Table 1, the patterns A, C, and D were enriched and had a high proportion in acetochlor-contaminated soils, while the patterns B, E, F, G, and P decreased or disappeared with increasing acetochlor concentrations.

The numbers of the ARDRA patterns decreased with an increase in acetochlor concentrations (Table 1). CK had

Table 1 Comparison of special ARDRA patterns enriched or disappeared with increasing acetochlor concentrations

ARDRA genotype	Percentage of isolates on 7th day (%)				Percentage of isolates on 21st day (%)				Percentage of isolates on 35th day (%)			
	CK[a]	50	150	250	CK	50	150	250	CK	50	150	250
A[b]	**7.9**	**8.8**	**14.5**	**20.0**	**5.7**	**12.5**	**22.4**	**24.1**	**8.5**	**31.9**	**45.7**	**31.4**
B[c]	*7.9*	*3.5*	*0*	*0*	*11.3*	*0*	*0*	*0*	*8.5*	*0*	*0*	*0*
C	**3.2**	**5.3**	**9.1**	**14.0**	**5.7**	**12.5**	**18.4**	**16.7**	**1.7**	**17.0**	**15.2**	**21.6**
D	**4.8**	**7.0**	**12.7**	**20.0**	**3.8**	**6.3**	**14.3**	**13.0**	**3.4**	**12.8**	**13.0**	**17.6**
E	*11.1*	*5.3*	*0*	*0*	*7.5*	*4.2*	*0*	*0*	*8.5*	*6.4*	*0*	*0*
F	*4.8*	*0*	*0*	*0*	*7.5*	*0*	*0*	*0*	*5.1*	*0*	*0*	*0*
G	7.9	7.0	0	0	7.5	0	0	0	5.1	2.1	0	0
J	6.3	8.8	12.7	6.0	1.9	6.3	10.2	14.8	8.5	6.4	4.3	3.9
P	3.2	0	0	0	1.9	0	0	0	1.7	0	0	0
Q	1.6	12.3	21.8	18.0	3.8	6.3	2.0	5.6	5.1	2.1	4.3	9.8
T	4.8	5.3	1.8	2.0	1.9	10.4	2.0	3.7	6.8	2.1	4.3	3.9
Total genotypes	19	16	13	10	18	14	12	10	19	13	9	11
Total isolates	63	57	55	50	53	48	49	54	59	47	46	51
Shannon index	2.89	2.65	2.08	2.04	2.87	2.55	2.16	2.09	2.81	2.13	1.70	1.92

[a] Concentration of acetochlor (mg/kg). CK = 0

[b] ARDRA patterns in boldface were enriched ones

[c] ARDRA patterns in italics were disappeared antagonistic ones

almost all ARDRA patterns (a total of 19, 18, and 19 patterns on the 7th, 21st, and 35th day, respectively), and the isolates in the patterns were evenly distributed, with a proportion of 1.6%–11.3%. The numbers of ARDRA patterns in other treatments were significantly less. The Shannon *H* values (Table 1) also showed that CK had the highest biodiversity, and the biodiversity decreased with the increase of acetochlor concentrations from 0 to 150 mg/kg. However, similar numbers of ARDRA patterns and similar Shannon *H* values were found from the soil samples under stress of acetochlor with 150 and 250 mg/kg, and no significant differences of Shannon H' values were observed in soils treated with the same concentration of acetochlor among different incubation time.

The ARDRA profile is a useful biomarker to analyze the community structure of soil bacteria (Prosser 2002), and has been adopted to examine the effects of pesticides and other chemicals on soil bacterial diversity and composition (Viti and Giovannetti 2005; Braun et al. 2006; Wang et al. 2008). In this work, the *Pseudomonas* population in soils applied with different concentrations of acetochlor had significantly different ARDRA profiles. The higher the applied acetochlor concentration, the lesser number the ARDRA patterns presented, and the lower the *Pseudomonas* diversity. Similar results about the abundance of bacteria were obtained in a previous study by Zhang et al. (2004). They observed using the denaturing gradient gel electrophoresis (DGGE) analysis method that the diversity of soil bacterial population decreased, and the population structure changed with a decrease in the concentration of acetochlor. Foley et al. (2008) also found that the diversity of freshwater bacterial community generally decreased under exposure to acetochlor, as the average number of DGGE bands in most treatments was significantly less than that in the control.

The similarity of ARDRA patterns between soil samples under the stress of acetochlor at the same concentration indicated that acetochlor had a persistent effect on the *Pseudomonas* population until the 35th day of incubation, which was consistent with the results by Luo et al. (2004) and Li et al. (2005). They found that the toxic effect of acetochlor on soil bacterial and fungal communities lasted for at least 40 days. Therefore, the potential ecotoxicological risk of acetochlor affecting the structural and functional characteristics of soil bacterial community could not be ignored.

Approximately 35.4% (224 of 632) of all isolates showed antagonistic capacity to *R. solani* (Fig. 1). The highest isolation frequency was found in CK soils, with 57.7% at the 7th day, 54.7% at the 21st day, and 50.8% at the 35th day of incubation. Most (91.9%) of the isolates belonged to ARDRA patterns B, E, F, I, K, and T were antagonistic, and one-third of the isolates belonged to the patterns A, D, H, P, Q, R, and S were effective to suppress *R. solani*. No antagonist was found in the other seven patterns. The frequency of the antagonists in patterns B, E and F decreased with an increase in the concentration of acetochlor, and disappeared under the stress of acetochlor at high dosage. Most isolates with the antagonistic activity (+++) were found in CK soils.

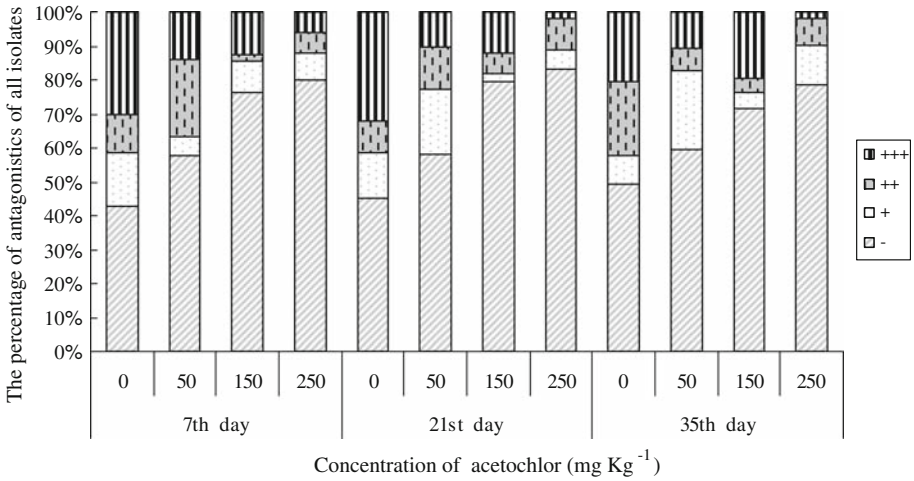

Fig. 1 The frequency of the isolates with different antagonistic ability in treated soils

The antifungal activity of *Pseudomonas* isolates supported the general view that acetochlor strongly affects the *Pseudomonas* population which is active towards to plant pathogen *R. solani* in soils. With an increasing acetochlor concentration, the antagonistic potential of soil *Pseudomonas* decreased. Interestedly, the ARDRA patterns B, E, and F with most of antagonists disappeared under the stress of acetochlor, especially at the middle and high dosage (150 and 250 mg/kg), and patterns A and D with only a few of the antagonists enriched with an increasing acetochlor concentration. No isolates with antifungal activity was found in pattern C, which was also enriched with an increasing acetochlor concentration. Hence, acetochlor decreased the antifungal activity of soil *Pseudomonas* by diminishing some subpopulations which were active in biocontrol, along with the enrichment of some other subpopulations that were non-antagonistic. This may be explained by the fact that some *Pseudomonas* spp. could degrade and utilize acetochlor as carbon sources (Ye 2003; Xu et al. 2006). Foley et al. (2008) also found that the stress of acetochlor increased the number of carbon sources utilized by the microbial community in freshwater.

In conclusion, the application of acetochlor changed the community structure of *Pseudomonas*, decreased the diversity, and reduced the relative amount of *Pseudomonas* strains with antagonistic activity towards *Rhizoctonia solani*. As *Pseudomonas* is one of the most important populations in soil suppression to plant diseases, acetochlor is likely to decrease soil fungistasis. The further work is still exigent.

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References

Bergsma-Vlamia M, Prinsa ME, Raaijmakers JM (2005) Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. FEMS Microbiol Ecol 52:59–69. doi:10.1016/j.femsec.2004.10.007

Braun B, Böckelmann U, Grohmann E, Szewzyk U (2006) Polyphasic characterization of the bacterial community in an urban soil profile with in situ and culture-dependent methods. Appl Soil Ecol 31:267–279. doi:10.1016/j.apsoil.2005.05.003

Foley ME, Sigler V, Gruden CL (2008) A multiphasic characterization of the impact of the herbicide acetochlor on freshwater bacterial communities. ISME J 2:56–66. doi:10.1038/ismej.2007.99

Garbeva P, van Veen JA, van Elsas JD (2004) Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiol Ecol 47:51–64. doi:10.1016/S0168-6496(03)00234-4

Li XY, Zhang HW, Zhou QX, Su ZC, Zhang CG (2005) Effects of acetochlor and methamidophos on fungal communities in black soils. Pedosphere 15:646–652

Liu HM, Dong DX, Peng HS, Zhang XH, Xu YQ (2006) Genetic diversity of phenazine- and pyoluteorin-producing pseudomonads isolated from green pepper rhizosphere. Arch Microbiol 185:91–98. doi:10.1007/s00203-005-0072-6

Luo HF, Qi HY, Zhang HX (2004) The impact of acetochlor the bacterial diversity in soil. Acta Microbiol Sin 44:519–522

Martin_laurent F, Philippot L, Hallet S, Chaussod R, Germon JV, Soulas G, Catroux G (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl Environ Microbiol 67:2354–2359

Morris CE, Kinkel LL, Xiao K, Prior P, Sands DC (2007) Surprising niche for the plant pathogen *Pseudomonas syringae*. Infect Genet Evol 7:84–92

Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. Appl Environ Microbiol 68:3795–3801

Prosser JI (2002) Molecular and functional diversity in soil micro-organisms. Plant Soil 244:9–17

Samson R, Shafik H, Benjamo A, Gardan L (1998) Description of the bacterium causing blight of leek as *Pseudomonas syringae* pv. porri (pv. nov.). Phytopathology 88:844–850

Su ZC, Zhang HW, Li XY, Zhang Q, Zhang CG (2007) Toxic effects of acetochlor, methamidophos and their combination on nifH gene in soil. J Environ Sci - China 19:864–873

Vincent MN, Harrison LA, Brackin JM, Kovacevich PA, Mukerji P, Weller DM, Pierson EA (1994) Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Appl Environ Microbiol 57(10):2928–2934

Viti C, Giovannetti L (2005) Characterization of cultivable heterotrophic bacterial communities in Cr-polluted and unpolluted soils using Biolog and ARDRA approaches. Appl Soil Ecol 28:101–112

Wang MC, Liu YH, Wang Q, Gong M, Hua XM, Pang YJ, Hu SJ, Yang YH (2008) Impacts of methamidophos on the biochemical, catabolic, and genetic characteristics of soil microbial communities. Soil Biol Biochem 40:778–788

Xu J, Qiu XH, Dai JY, Cao H, Yang M, Zhang J, Xu MQ (2006) Isolation and characterization of a *Pseudomonas oleovorans* degrading the chloroacetamide herbicide acetochlor. Biodegradation 17:219–225

Ye C (2003) Environmental behavior of the agrochemicals acetochlor in soil. Bull Environ Contam Toxicol 71:919–923

Zhang HW, Zhou QX, Zhang QR, Zhang CG (2004) Toxic-effects of acetochlor, methamidophos and their combination on bacterial amount and population richness at molecular levels in agricultural black soils. Environ Sci 25:143–148

Zhang Q, Zhang HW, Su ZC, Li XY, Zhang CG (2007) Effects of long-term petroleum and heavy metals pollution on the diversity and community structure of *Pseudomonas* populations in agricultural soils. Chin J Appl Ecol 18:1327–1332