Soil microbial remediation to soybean field of Northeast China: Dynamic changes of fomesafen residues and phospholipid fatty acids in the black soil after application of *Shigella flexneri* FB5

Fengshan Yang¹,²,³, Cong Sun¹,²,³, Yang Lai¹,²,³, Yukun Ma¹,²,³, Haiyan Fu¹,²,³ and Chunguang Liu¹,²,³,⁴

¹Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education, Heilongjiang University, Harbin 150500, China; ²Heilongjiang Provincial Key Laboratory of Ecological Restoration and Resource Utilization for Cold Region, School of Life Sciences, Heilongjiang University, Harbin 150080, China; ³Key Laboratory of Microbiology, College of Heilongjiang Province, School of Life Sciences, Heilongjiang University, Harbin 150080, China

⁴Email: 2005013@hlju.edu.cn

Abstract. Fomesafen is widely used to control broad-leaved weeds in oil-bearing crops such as soybean and peanut. However, fomesafen has a long residual period in soil. If it is not used properly, it will cause damage to sensitive crops in different degrees. In this paper, the effects of fomesafen-degrading strains on the residue of fomesafen and the structure of soil microbial community were studied. Under field experiments, a microbial solution containing fomesafen-degrading *Shigella flexneri* FB5 was sprayed. We then detected residual fomesafen in the soil using high-performance liquid chromatography and phospholipid fatty acid content using gas chromatography-mass spectrometry. Analysis of variance suggested that the addition of fomesafen-degrading strains had a significant effect on soil microorganisms, as revealed by changes in the fungi/bacteria index, gram-negative/gram-positive bacteria ratio, stress indicators, and total phospholipid fatty acids. To sum up, our results show that the use of fomesafen-degrading strains can reduce the toxicity of fomesafen.

1. Introduction

Fomesafen is a diphenyl ether herbicide, which is mainly used to control weeds in soybean and peanut fields since 1988, when it was introduced by Zeneca Ltd. (United Kingdom). It has then been produced domestically since 1994[1]. According to the research, the use of 250-375 g/hm² of fomesafen has good efficacy, and its safety to subsequent crops is lower than the content of 250 g/hm² of effective ingredient[2]. At the time of registration, due to the small use area of fomesafen and its only use in Heilongjiang Province, the problem of residual pesticide damage is not obvious. There are few varieties of soybean herbicides to control broad-leaved weeds, and a very high dosage is recommended[3-4]. Studies have shown that when the concentration of fomesafen is more than 100 μ g/kg, it will change the antioxidant enzymes in earthworms and affect the microorganisms in the soil[5].
Soil remediation has become an important topic in China in recent years and finding ways of reducing the amount of fomesafen residue in the soil has acquired new theoretical and practical significance.

Although pesticides have greatly promoted agricultural production, their extensive use poses potential risks to soil microbial communities and ecological health[6]. Soil microorganism is not only the main engines and biomass of biogeochemical cycle in terrestrial ecosystem, they can also maintain the structure and function of an ecosystem [7-8]. Numerous studies have assessed the risk of fomesafen on soil microbes. Wu et al.[9] studied the response of fomesafen to soybean soil microbial communities in Northeast China and the results showed that the continuous use of fomesafen could stimulate the growth of bacteria and fungi. Wu et al.[3] studied the effect of fomesafen on different biological communities. Accordingly, application of 100 times the recommended dose of fomesafen (T100) had an adverse effect on soil microbial activity and microbial community. Phospholipid fatty acid (PLFA) analysis showed that the total bacterial and fungal biomass, as well as PLFA decreased with a high level of fomesafen (T100), and were accompanied by an increase in microbial stress. Therefore, it is of great theoretical and practical significance to study how to reduce the residue of fomesafen in soil and reduce its pollution to the following crops and the environment.

Microbial degradation is considered to be an important mechanism for pesticide removal and detoxification in soil[10]. Studies have shown that bacteria, fungi, actinomycetes, and algae are the main microorganisms to degrade pesticides. Among them, bacteria account for the majority because of their relatively high mutation rates and strong adaptability. The fomesafen-degrading bacterium Lysinibacillus sp. ZB-1 was isolated from contaminated agricultural soil by Liang et al.[11]. The strain could utilize fomesafen as the sole carbon source and degrade other diphenyl ethers including lactofen and fluoroglycofen. Yang et al.[12] isolated Shigella flexneri FB5, which can utilize fomesafen as the sole carbon source, from a fomesafen-contaminated soybean field in Heilongjiang Province, China. Feng et al.[13] reported that the newly isolated strain Pseudomonas zeshuii BY-1 could grow using fomesafen as the sole carbon source in pure culture. Importantly, P. zeshuii BY-1 can be potentially used for the bioremediation of fomesafen-contaminated soils. In spite of all the recent progress, however, there is known little about the detoxification of fomesafen in soil and how to eliminate any adverse effects of residual fomesafen on crop rotation. The objective of the present study was to assess the impact of a fomesafen-degrading solution on (1) the microbial community and (2) the degradation of fomesafen. To achieve the above goals, we applied the S. flexneri FB5 strain isolated from agricultural soil that had been polluted with fomesafen for a long time, and applied it in the form of a microbial degrading solution previously pioneered by us.

2. Materials and Methods

2.1. Field design

Experiments were conducted in Hulan District, Heilongjiang Province, Hulan District (126°25'-127°19' N, 45°49'-46°25' E) is located in the north temperate continental monsoon climate zone of China. The average annual rainfall is about 500.4 mm and the mean temperature is 3.3℃. During both years, the soil at the experimental site was black earth.

The following soil pots were prepared, with three replicates each: a pot without fomesafen or the microbial degrading solution (CK1); a pot with fomesafen but without the microbial degrading solution (CK2); a pot without fomesafen but with the microbial degrading solution (T1); and a pot with both fomesafen and microbial degrading solution (T2). Each field measured 56 m² (0.7 × 80 m). Soil characteristics and environmental parameters were identical in all twelve fields. The herbicide was added to the soil by spraying on June 3rd in accordance with recommendations for agricultural use: 80-100 mL/667 m² at a concentration of 250 g/L. A total of 600 mL of fermented microbial culture solution was applied per ridge of soil in each experimental field. After the application of degrading bacteria, samples were taken on day 0 (May 6th), 1 (June 3rd), 7 (June 9th), 16 (June 18th), 38 (July 10th), 70 (August 11th), 101 (September 11th), and 131 (October 11th). In each field, 16 soil cores (5 cm in diameter) were collected per sample. The soil was collected, air-dried, and then samples
were passed through 40-mesh and 60-mesh sieves. The treated soil samples were stored in the refrigerator at -20℃ prior to further testing.

2.2. Fomesafen extraction and determination
8 g of treated soil samples were weighed and put into 50 mL centrifuge tubes respectively, and then 15 mL of extraction solution was added into each centrifuge tube respectively. Ultrasonic assisted extraction was performed for 5 min with ultrasonic instrument to promote dissolution. The supernatant was centrifuged at room temperature for 5 min at the speed of 5000 r/min. After centrifugation, the supernatant was drawn into a clean test tube, and the nitrogen blowing instrument was adjusted to 50℃. The supernatant was dried in a fume hood with a nitrogen blower. Then add 15 mL of extraction solution into the centrifuge tube, and repeat the above steps until 60 mL of extraction solution is added into each centrifuge tube. After all of them are dried, 2 ml of chromatographic methanol is added to the test tube respectively[14].

All analyses were performed using a Waters D600 semi-preparation/analysis mode high-performance liquid chromatography (HPLC) system equipped with a Waters C18 column (15 µm, 300 × 3.9 mm) maintained at 40℃. The mobile phases consisted of 25% methyl alcohol (A) and 75% water of formic acid (B), and were pumped at a flow rate of 0.8 mL/min.

2.3. PLFA analysis
Lipid extraction: phosphate buffer solution (concentration = 0.1 mol L⁻¹, pH = 7.0), chloroform and methanol were added to the treated soil sample in turn, with the volume ratio of 0.8:1:2. The specific amount of reagent added is generally based on the principle of adding 1 ml chloroform to 1 g treated soil, and then making appropriate adjustment according to the soil used [15]. After shaking for 2 hours in the dark place, centrifuge quickly. After centrifugation, the supernatant was transferred to a clean test tube. Phosphate buffer and chloroform were added. The supernatant was shaken violently and left overnight. The liquid is divided into two phases, and the membrane lipid is distributed in the chloroform layer (lower layer). The next step of solid-phase extraction was carried out directly with or without nitrogen.

Separation of lipids by solid phase extraction column chromatography: the lower extract obtained was put on a silica bonded solid phase extraction column (spesi) and eluted with chloroform, acetone and anhydrous methanol respectively. These reagents can split the lipids and separate neutral lipids, glycolipids and phospholipids [16-17]. Dry the part containing phospholipid (i.e. methanol part) with nitrogen.

Alkaline methanol hydrolysis and saponification (methylation) of phospholipids: phospholipids are easily soluble in toluene: select the prepared methanol mixture and methanol solution with the concentration of 0.2 mol L⁻¹ sodium hydroxide, water bath at 37℃ for 15 min, cooling to room temperature, and then extract with hexane: chloroform mixture, acetic acid with concentration of 1 mol/L and distilled water [18], and the upper organic phase is phospholipid fatty acid methyl ester (FAME).

3. Results

3.1. Determination of fomesafen residue in the soil
The presence of fomesafen in the soil could be described by a linear relationship according to the following linear regression equation: \( y = 14966x - 77848 \), \( R^2 = 0.999 \). As shown in Figure 1, prior to its application, the fomesafen residue was below 0.1 mg/kg (May 6th). Fomesafen was applied to the field on June 3rd, at which point its residue reached a peak of 4.3 mg/kg. Compared to CK1, the concentration of fomesafen in CK2 dropped significantly on day 7, while T1 maintained a more nuanced trend. Compared to CK2, the concentration of fomesafen in T2 dropped to a significantly lower level on days 7 and 101, but exhibited only a slow decline between days 16, 38, and 70. These
findings indicated that addition of fomesafen-degrading *S. flexneri* FB5 could rehabilitate herbicide-contaminated soil.

Figure 1. Degradation curve of fomesafen in the presence of a solution containing *S. flexneri* FB5. CK1: without fomesafen or the microbial degrading solution; CK2: with fomesafen but without the microbial degrading solution; T1: without fomesafen but with the microbial degrading solution; T2: with fomesafen and with the microbial degrading solution.

As shown in Figure 2, the following exponential regression equations were obtained for CK2: \( y = 3.233e^{-0.011x} \), \( R^2 = 0.8461 \); and T2: \( y = 2.1133e^{-0.015x} \), \( R^2 = 0.7993 \). The half-life of fomesafen in the soil was calculated to be 63.01 days for CK2 and 46.21 days for T2. These results showed that *S. flexneri* FB5 could degrade fomesafen and reduce its half-life in the soil. Thus, addition of a fomesafen-degrading microorganism played an important role in soil remediation.

Figure 2. Dissolution curve of fomesafen. CK2: with fomesafen but without the microbial degrading solution; T2: with fomesafen and with the microbial degrading solution.

3.2. Effect of fomesafen-degrading *S. flexneri* FB5 on soil microorganisms

Consistent with the CK1 control, the fungi/bacteria index, GN/GP ratio, and the stress indicator increased in T1 on days 6, 30, and 120. Consistent with the CK2 control, these same values decreased in T2. In contrast, total fatty acid content was lower in T1 but higher in T2, except on days 16 and 101.

As shown in Figure 3A, the fungi/bacteria index of all four treatments was about 0.45 in the non-application stage or in a normal environment. Compared to CK1, the fungi/bacteria index was higher in CK2 on days 1, 7, 38, 70, 101, and 131; analysis of similarities revealed that the effect was significant on days 1, 7, 38, 70, and 101 (\( p<0.05 \)), and extremely significant on day 60 (\( p<0.01 \)). The fungi/bacteria index was higher in T1 than CK1 on days 7, 38, and 131; exhibiting extremely significant difference on days 1, 7, 16, 38, and 131 (\( p<0.01 \)). Compared to CK2, the fungi/bacteria index of T2 was lower on days 1, 7, 16, 38, 70, 101, and 131; with an extremely significant difference on day 16 (\( p<0.01 \)). Finally, the fungi/bacteria index of T2 was lower than that of T1 on days 1, 7, 16, 38, 70, and 131; with an extremely significant difference on days 1, 7, and 101 (\( p<0.01 \)).
As shown in Figure 3B, addition of fomesafen-degrading *S. flexneri* FB5 to the soil changed the microbial GN/GP ratio. The GN/GP ratio of the four treatments was about 1.60 in the non-application stage. The ratio was higher in CK2 than in CK1, although the difference was not significant. Similarly, the ratio was higher, albeit not significantly, in T1 than in CK1 on days 7, 38, and 131. The GN/GP ratio was lower in T2 than in CK2, but the difference was significant only on day 1 (p<0.05). Finally, the ratio was lower in T2 than in T1 on days 7, 38, 70, and 131, but significantly so only on day 1 (p<0.05).

As shown in Figure 3C, addition of fomesafen-degrading *S. flexneri* FB5 to the soil altered microbial stress indicator levels. The stress indicator was at its lowest in the non-application stage. The stress indicator was higher in CK2 than in CK1, with an extremely significant difference on days 16, 70, 101, and 131 (p<0.01). It was higher also in T1 compared to CK1, with an extremely significant difference on day 131 (p<0.01). The stress indicator was lower in T2 than in CK2, and significantly so on days 1 and 70 (p<0.05), or with respect to T1, with a significant effect on days 1, 7, 70, 101, and 131 (p<0.05).

As shown in Figure 3D, addition of fomesafen-degrading *S. flexneri* FB5 to the soil altered microbial total fatty acid content. The concentration of total fatty acids in soil microorganisms was at its lowest level in the non-application stage. Total fatty acid concentration began to rise on June 3rd. Total PLFA was lower, albeit not significantly, in CK2 than in CK1; similarly, total PLFA was lower in T1 than in CK1, with an extremely significant difference on day 7 (p<0.01). Total PLFA was higher, albeit not significantly, in T2 than in CK2. Finally, total PLFA was higher in T2 compared to T1, with an extremely significant difference on day 7 (p<0.01).

**Figure 3.** Effect of fomesafen-degrading *S. flexneri* FB5 on soil microorganisms. CK1: without fomesafen or the microbial degrading solution; CK2: with fomesafen but without the microbial degrading solution; T1: without fomesafen but with the microbial degrading solution; T2: with fomesafen and with the microbial degrading solution. Means marked with different letters indicate significant differences (p<0.05) among treatments for the designated dates. Extremely significant differences are indicated by *p<0.01.

### 4. Discussion

#### 4.1. Determination of fomesafen residue in the soil

Following its application, the concentration of fomesafen first decreased rapidly and then slowly, reflecting earlier results by Wu *et al.* [9]. The initial rapid drop in pesticide concentration was due to
microorganisms using such compounds as a carbon source for growth. The slow decline was due to the strong adsorption of pesticides by clay and organic substances, which resulted in lower bioavailability and slower degradation [19-20]. The initial decrease and subsequent increase in fomesafen concentration may be affected by a number of factors, such as seasonality, day and night changes in temperature, soil humidity, salt-alkaline differences, sunlight exposure, and air flow. Moreover, the existence of other organisms in the soil will affect the microbial degradation of fomesafen to varying degrees. Tian et al.[21] showed that degradation was higher in black soil than in loess. The reason may be that black soil contains more organic matter, which affects the growth of microorganisms, and thus promotes degradation.

4.2. Effect of fomesafen-degrading S. flexneri FB5 on soil microorganisms

Our results indicate that addition of fomesafen can inhibit the growth of bacteria, leading to a higher fungi/bacteria index. Therefore, fomesafen has a significant effect on soil microbial community structure. Fomesafen-degrading microbes reduced the amount of fomesafen residue, and thus lowered the fungi/bacteria index, the GN/GP ratio, and stress indicator levels, while increasing total PLFA. Hence, addition of fomesafen-degrading S. flexneri FB5 had a significant effect on soil microorganisms.

Differences in carbon-chain structure, length, saturation, and hydroxyl groups of specific fatty acids in various soil microbial populations, allow the study of the effects of herbicide application on the composition of biological populations and communities. So far, the effects of glyphosate, acetochlor, 2,4-D, and atrazine on microbial communities have been studied by GC-MS. However, little has been known about the effect of herbicide-degrading strains on soil microbial communities and their activities. Similarly, the use of PLFAs as a biological indicator to study the response of microbial communities to degrading bacteria has not been tried before.

Therefore, here we used GC-MS, together with the fungi/bacteria index, GN/GP ratio, stress indicator, and total fatty acid content, to determine the effect of degrading strains on soil microorganisms. We report that PLFAs can be used to study changes in soil microbial communities after the application of herbicide-degrading bacteria. The ensuing information can provide an important theoretical basis for the study of ecological and environmental safety of biodegradable bacteria.

5. Conclusions

The present study shows that application of a microbial degrading solution can lower the residual concentration of fomesafen and its half-life in the soil, while resulting also in a clear reduction of fungi, gram-negative bacteria, total PLFA, and stress indicators. This change impacted significantly on microbial community structure. In conclusion, fomesafen-degrading strains play an important and promising role in soil remediation.

Acknowledgement

This study was supported by Heilongjiang Natural Science Project (C2018051), Heilongjiang University Special Project of the Basic Scientific Research Service Fee of the University in Heilongjiang Province (HDRCCX-201614) and University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Proince,(UNPYSCT-2017119).

References

[1] Guo J F, Zhu, G.N., Shi, J., Sun, J.J. 2003 Adsorption, desorption and mobility of fomesafen in Chinese soils Water Air Soil Pollut 148 77-85
[2] Wei-Dong L I . Control Effect of Fomesafen 250 g/L SL on Broadleaf Weeds in Soybean Field[J]. Journal of Anhui Agricultural ences, 2011
[3] Wu, X. H., Xu, J., Dong, F. S., Liu, X. G., Zheng, Y. Q. 2014 Responses of soil microbial community to different concentration of fomesafen J. Hazard. Mater 273 155-164
[4] Cobucci, T., Prates, H. T., Falcão, C. L. M., Rezende, M. M. V. 1998. Effect of imazamox, fomesafen, and acifluorfen soil residue on rotational crops. Weed Sci. 46 258-263

[5] Rauch, B. J., Bellinder, R. R., Brainard, D. C., Lane, M., Thies, J. E. 2007. Dissipation of fomesafen in New York state soils and potential to cause carryover injury to sweet corn. Weed Technol. 21 206-212

[6] Álvarez-Martín, A., Hilton, S. L., Bending, G. D., Rodríguez-Cruz, M. S., Sánchez-Martín, M. J. 2016. Changes in activity and structure of the soil microbial community after application of azoxy streobin or pirimicarb and an organic amendment to an agricultural soil. Appl. Soil Ecol. 106 47-57

[7] Van Der Heijden, M. G., Bardgett, R. D., Van Straalen, N. M. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. 11 296-310

[8] Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., Owens, S., Gilbert, J. A., Wall, D. H., Caporaso, J. G. 2012. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. Proc. Natl. Acad. Sci. USA 109 21390-21395

[9] Wu, X. H., Zhang, Y., Du, P. Q., Xu, J., Dong, F. S., Liu, X. G., Zheng, Y. Q. 2018. Impact of fomesafen on the soil microbial communities in soybean fields in Northeastern China. Ecotoxicol. Environ. Safety 148 169-176

[10] Gao, Y., Chen, S. H., Hu, M. Y., Hu, Q. B., Luo, J. J., Li, Y. N. 2012. Purification and characterization of a novel chlorpyrifos hydrolase from Cladosporium cladosporioides Hu-01. PLoS ONE 7 e38137

[11] Liang, B., Lu, P., Li, H. H., Li, R., Li, S. P., Huang, X. 2009. Biodegradation of fomesafen by strain Lysinibacillus sp. ZB-1 isolated from soil. Chemosphere 77 1614-1619

[12] Yang, F. S., Liu, L., Liu, C. G. 2011. Screening, characterization, and application of Shigella flexneri FB5 in fomesafen-contaminated soil. Procedia Eng. 18 277-284

[13] Feng, Z. Z., Li, Q. F., Zhang, J., Zhang, J., Huang, X., Lu, P., Li, S. P. 2012. Microbial degradation of fomesafen by a newly isolated strain Pseudomonas zeshuii BY-1 and the biochemical degradation pathway. J. Agric. Food Chem. 60 7104-7110

[14] Zhang, Y., Li, X. G., Xu, J., Dong, F. S., Liu, X. G., Zheng, Y. Q. 2012. Determination of fomesafen residues in soybean and soil using QuECHERS and UPLC-MS/MS. Environ. Chem. 31 1399-1404

[15] Bossio, D. A., Scow, K., Gunapala, N., Graham, K. J. 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microb. Ecol. 36 1-12

[16] Zhang, C. P., Xu, J., Liu, X., Dong, F., Kong, Z., Sheng, Y., Zheng, Y. 2010. Impact of imazethapyr on the microbial community structure in agricultural soils. Chemosphere 81 800-806

[17] Papadopoulou, E. S., Karpouzas, D. G., Menkissoglou-Spiroudi, U. 2011. Extraction parameters significantly influence the quantity and the profile of PLFAs extracted from soils. Microb. Ecol. 62 704-714

[18] Chaudhary, Doongar R. Dick, Richard P. Linking Microbial Community Dynamics Associated with Rhizosphere Carbon Flow in a Biofuel Crop (\r, Jatropha curcas\r, L.)[J]. Communications in Soil Science & Plant Analysis, 47(9)1193-1206

[19] Lee, S., Gan, J., Kim, J. S., Kabashima, J. N., Crowley, D. E. 2004. Microbial transformation of pyrethroid insecticides in aqueous and sediment phases. Environ. Toxicol. Chem. 23 1-6

[20] Muñoz-Leoz, B., Ruiz-Romera, E., Antigüedad, I., Garbisu, C. 2011. Tebuconazole application decreases soil microbial biomass and activity. Soil Biol. Biochem. 43 2176-2183

[21] Tian, S., Wang, X. P. 2015. Localization and properties of fomesafen-degrading Enzyme. J. Anhui Agri. Sci. 43 111-113+135