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High-methionine soybean has no adverse effect on functional diversity of rhizosphere microorganisms

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ABSTRACT

A transgenic high-methionine soybean ZD91 and its non-transgenic parental soybean ZD were investigated to evaluate the potential negative impact of transgene on the microbial community in soil. The Biolog-ECO plate method was used to evaluate the functional diversity and activity of rhizosphere microbial communities at four growth stages of the soybean each year from 2012 to 2013. Results indicated that there was no difference between ZD and ZD91 in the functional diversity of microbial communities in rhizosphere soil. Besides, plant growth stage had stronger effect than cultivar. It was concluded that transgenic soybean ZD91 did not alter the functional diversity of microbial communities in rhizosphere soil.

Keywords: *Glycine max*; community level physiological profiles; community function and activity; genetically modified organisms; soil ecosystem

Genetic engineering techniques allowed the development of crops with enhanced nutrition (e.g., transgenic high-methionine soybean ZD91) (Song et al. 2013). Despite their potential to increase quality, releasing genetically modified (GM) crops into the environment may have detrimental effects on soil ecosystem (Mina et al. 2007), and GM crops may affect soil and rhizosphere microbial communities either directly or indirectly (Turrini et al. 2015). Rhizosphere microbial communities are important for plant health and to maintain ecosystem function (Berg and Smalla 2009, Yang et al. 2015). With the development of new GM crops, the safety of transgenic plants including their effects on rhizospheric microbial communities shows growing research significance (Liu et al. 2005).

Metabolic community profiles evaluated by using the Biolog MicroPlates can detect variations in metabolic diversity and physiological activity of soil microbial community (Giovannetti et al. 2005, Wu et al. 2012). In previous studies, Biolog-ECO method was used to study the impact of GM plants on soil microbial community catabolic diversity. Transient or no impact of GM plants on the soil microbial community function was found, suggest-

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ing that there is a potential for adverse transgenic plant-induced impacts on catabolic diversity of microbial communities (Lv et al. 2014, Gao et al. 2015, Sohn et al. 2015, Zhang et al. 2015). As the safety assessment of GM crops should be case by case (Liang et al. 2015), the question arises whether transgenic soybean plants with altered content of methionine will affect soil microbial community catabolic diversity. Although transgenic high-methionine soybean ZD91 was reported to have no significant impact on rhizosphere bacterial and arbuscular mycorrhizal fungal communities structures (Pu et al. 2012, Liang et al. 2014, 2015), knowledge on the effect of GM soybean ZD91 on functional diversity and activity of microbial communities in rhizosphere soil remains fairly limited so far. Therefore, the objective of this study was to investigate the effects of GM soybean ZD91 on rhizosphere microbial communities function by the Biolog-ECO plate, and to provide some information on the risk assessment of the GM soybean ZD91.

MATERIAL AND METHODS

Plant material, field design and soil sampling. Transgenic soybean cultivar (ZD91) contains the *Arabidopsis* cystathionine γ-synthase gene which was introduced artificially into the soybean cv. Zigongdongdou (ZD), and exhibits a high content of methionine in the seeds (Song et al. 2013).

Field trials were conducted during 2012–2013 (i.e., the 3rd and 4th year of the experiment) on the experimental farm of the Nanchong Academy of Agricultural Science, located in Nanchong (30°48'N, 106°04'E), Sichuan province, China. The soybean plants were grown in a randomized block design consisting of four plots (each plot 6 m × 10 m) per cultivar. Soybean was maintained in accordance with typical agronomic practices in southwest China. Sampling was carried out at four developmental stages of the plants each year from 2012 to 2013; namely seedling stage (SS); flowering stage (FS); pod-setting stage (PS); maturity-setting stage (MS); as described by Liang et al. (2015). Rhizosphere soil samples were collected in accordance with the previous method (Liang et al. 2015).

Microbial community level physiological profiles. Rhizosphere microbial community fingerprints were performed using the Biolog ECO microplate (Biolog, Hayward, USA) for soil community. 5 g portions of soil were shaken in 45 mL of 0.85% sterile saline solution for 30 min, and the 10^{-3} dilution was used to inoculate. A 150 µL aliquot was placed into ECO microplate well, and microplates were incubated at 25°C in the dark. The colour development was recorded at 590 (colour development plus turbidity) and 750 nm (turbidity only) every 24 h until 168 h. The final values were the absorbance at 590 nm minus absorbance at 750 nm after correction for readings in the control well.

Microbial response in each microplate that expressed average well colour development (AWCD) was determined as before (Garland and Mills 1991). Plate readings at 72 h were used to calculate substrate richness (S); Shannon's diversity index (H); and substrate evenness (E); where S is the number of utilized substrates, $H = -\Sigma (P_i \times \ln P_i)$ and P_i is the ratio of activities on each substrate to the sum of activities on all substrates, and $E = H/\ln S$ (Xie et al. 2009, Zhao et al. 2010). Principal component analysis (PCA) was implemented by using data normalized by dividing the absorbance value of each well at 72 h by the AWCD (Wang et al. 2007).

Statistical analysis. One-way analysis of variance and Duncan pair-wise comparisons ($P < 0.01$) were used to determine if there is a reliable difference between soybean cultivars by employing the SPSS 17.0 software (SPSS Inc., Chicago, USA). ANOVA comparisons of the diversity indices between years, growth stages and cultivars were made by the SPSS 17.0.

RESULTS AND DISCUSSION

The AWCD value reflects the ability of soil microbial communities to utilize different carbon sources and a higher AWCD corresponds to a greater metabolic activity (Lv et al. 2014). There was an increasing trend in the AWCD values, showing that all of the microbial samples can continue to use the various carbon sources (Figure 1). To compare the catabolic diversity among different cultivars, substrate richness, Shannon's diversity index, and substrate evenness in the incubation time of 72 h were calculated. The microbial diversity of ZD and ZD91 were not statistically different throughout the growth cycle (Table 1). Significant differences in Shannon's diversity index and substrate evenness were observed among growth stages, however,

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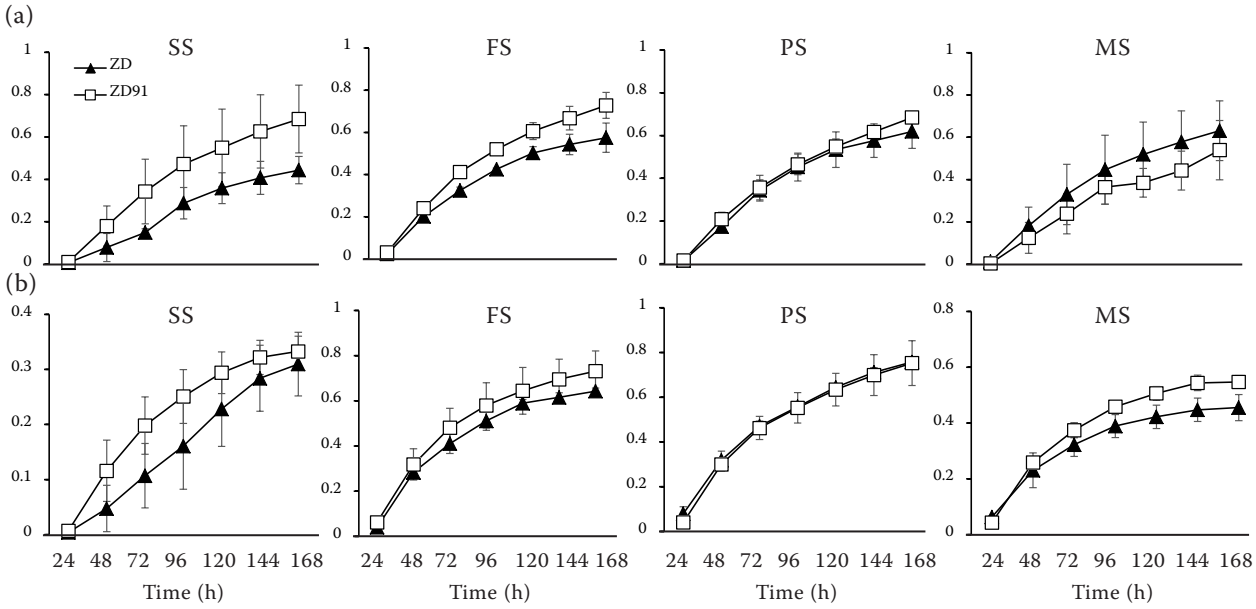

Figure 1. Average well colour development (590–750 nm) of substrate utilization patterns in Biolog ECO microplate ($P < 0.01$) in (a) 2012 and (b) 2013. SS – seedling stage; FS – flowering stage; PS – pod-setting stage; MS – maturity-setting stage

there was no significant difference in substrate richness among growth stages (Table 2). These results indicated that the soil microbial diversity was the most strongly influenced by growth stage.

The analysis of substrate groups can provide much more information than that obtained by analysing the individual substrates alone (Zak et al. 1994). The 31 carbon substrates in the ECO microplate can be subdivided into 6 categories (i.e., carbohydrates, carboxylic acids, amino acids, polymers, phenolic compounds and amines) (Choi and Dobbs 1999). According to Figure 2, there were no distinct differences in the substrate utilization patterns of the microbial communities in ZD or ZD91. Besides, it was found that carbohydrates were primarily expended in soil.

PCA can be used to analyse the differences in the patterns of carbon source utilization (Donegan et al. 1995). When cultivar was examined as an explanatory variable, there were no significant differences in the carbon substrate utilization profiles of ZD or ZD91 within the same year (Figure 3a). However, the carbon substrate utilization profiles were clearly distinct among the different growth stages (Figure 3b). The PCA revealed that growth stage was a stronger explanatory factor for differences in community function, while cultivar was not a major factor. These results demonstrated that

Table 1. Substrate richness (S), Shannon's diversity index (H) and substrate evenness (E) of microbial community carbon resources catabolism in the Biolog ECO microplate incubated for 72 h (means ± standard errors) ($P < 0.01$)

	Diversity indices	Seedling stage		Flowering stage		Pod-setting stage		Maturity-setting stage	
		ZD	ZD91	ZD	ZD91	ZD	ZD91	ZD	ZD91
2012	S	26.33 ± 1.53^A	29.00 ± 2.00^A	28.67 ± 0.58^A	29.33 ± 0.58^A	29.33 ± 1.53^A	28.67 ± 0.58^A	28.67 ± 1.15^A	28.33 ± 1.53^A
	H	2.69 ± 0.29^A	2.91 ± 0.15^A	2.99 ± 0.03^A	3.04 ± 0.06^A	2.94 ± 0.13^A	2.95 ± 0.01^A	2.92 ± 0.08^A	2.84 ± 0.14^A
	E	0.83 ± 0.09^A	0.87 ± 0.06^A	0.89 ± 0.01^A	0.90 ± 0.01^A	0.87 ± 0.05^A	0.88 ± 0.01^A	0.87 ± 0.02^A	0.85 ± 0.02^A
2013	S	28.33 ± 2.08^A	28.33 ± 2.08^A	28.00 ± 2.65^A	29.00 ± 0.00^A	30.00 ± 1.00^A	30.00 ± 0.00^A	29.67 ± 1.15^A	28.33 ± 2.08^A
	H	2.34 ± 0.42^A	2.38 ± 0.21^A	3.00 ± 0.06^A	3.08 ± 0.10^A	2.99 ± 0.06^A	3.19 ± 0.26^A	2.78 ± 0.08^A	2.94 ± 0.10^A
	E	0.70 ± 0.14^A	0.71 ± 0.07^A	0.90 ± 0.03^A	0.91 ± 0.03^A	0.88 ± 0.01^A	0.94 ± 0.08^A	0.82 ± 0.03^A	0.88 ± 0.04^A

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Table 2. ANOVA comparisons of three diversity indices from Biolog ECO microplate between year, growth stage and cultivar (*$P < 0.01$)

Diversity indices	Effect	*F*-value	*P*-value
Substrate richness	year	0.91	0.34
	growth stage	2.12	0.11
	cultivar	0.33	0.57
Shannon's diversity index	year	0.93	0.34
	growth stage	12.71	0.00*
	cultivar	1.18	0.28
Substrate evenness	year	1.36	0.25
	growth stage	9.36	0.00*
	cultivar	0.87	0.36

the ZD and ZD91 soil communities had similar metabolic characteristics in the corresponding stages. Previous studies also showed that transgenic plants had no significant adverse effect on the metabolic activity of the microbial community (Shen et al. 2006, Lv et al. 2014).

According to the previous studies, natural factors rather than GM plants played a major role in the number of rhizosphere microbes. For example, it was reported that in the rhizosphere communities, there were similarities between transgenic potato and its non-transgenic counterpart, whereas environmental factors had a greater influence on rhizosphere microbes (Heuer et al. 2002). Lv et al. (2014) also revealed that different growth stages had stronger influence than ecotypes (*GFP*-transformed tobacco and wild tobacco) on rhizobacterial community structure. In addition, plant developmental stage was found to have a greater effect than plant species on bacterial community structure and resource utilization profiles (Houlden et al. 2008). In this study, no significant differences in the carbon substrate utilization profiles of ZD and ZD91 were found. However, the catabolic profiles at different growth stages were distinct, which was consistent with previous studies.

In conclusion, it was found that the transgenic soybean ZD91 had no significant effect on soil microbial catabolic diversity. The functional diversity of microbial communities was markedly affected by natural variations in the environment related to soybean growth stage. Further detailed analyses using culture-independent approaches, such as pyrosequencing, are needed to study the potential

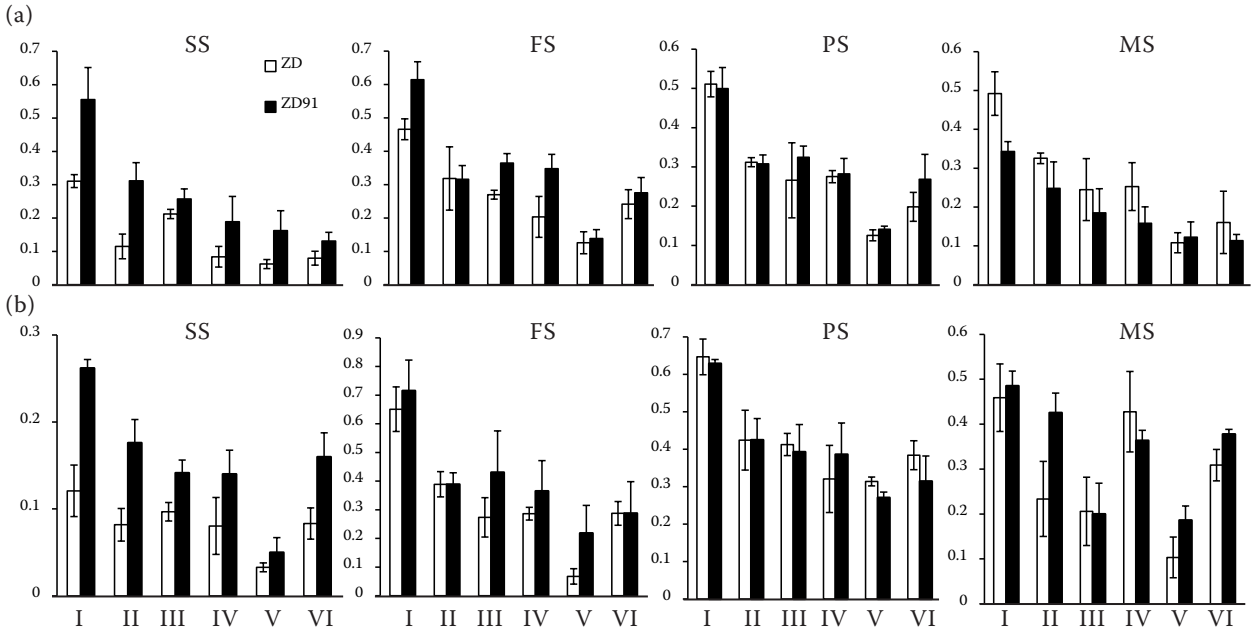

Figure 2. Group-wise average well colour development on carbon sources (six groups) at 72 h incubation time in Biolog ECO microplate by the microbes from ZD and ZD91 ($P < 0.01$) in (a) 2012 and (b) 2013. SS – seedling stage; FS – flowering stage; PS – pod-setting stage; MS – maturity-setting stage. I – carbohydrates; II – carboxylic acids; III – amino acids; IV – polymers; V – phenolic compounds; VI – amines

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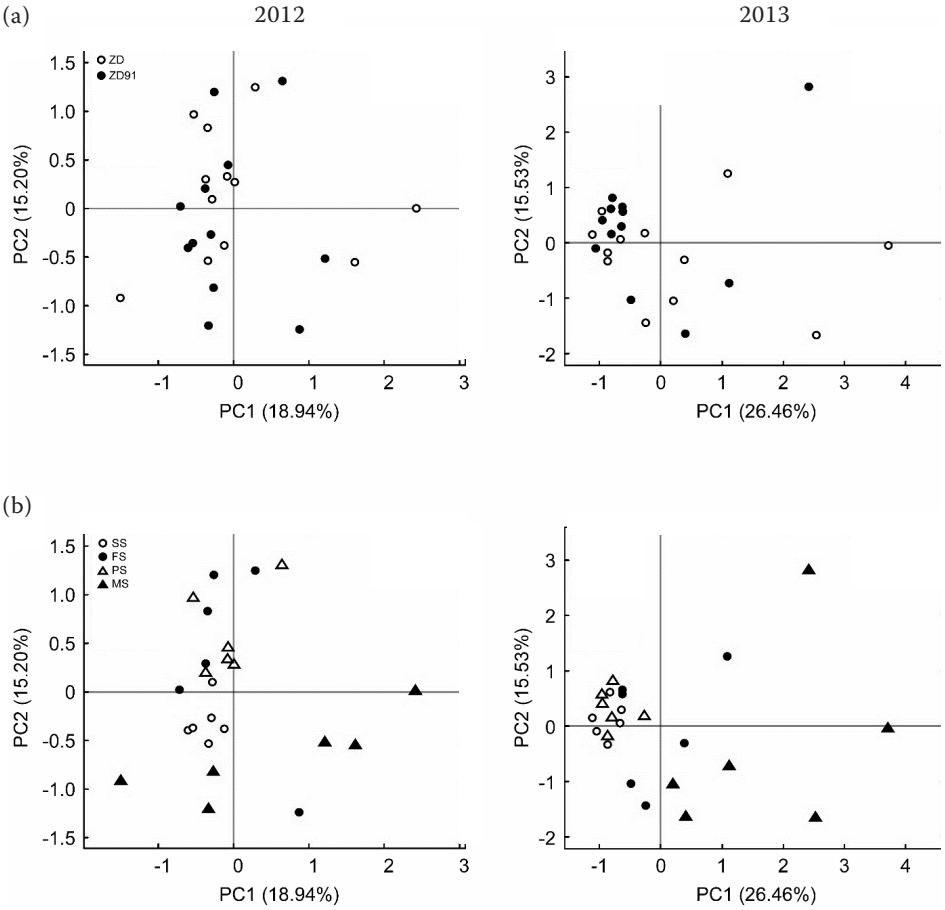

Figure 3. Principal component analysis of substrate utilization by microbial communities within the rhizosphere samples of ZD and ZD91. The eigenvalues displayed on the diagram axes refer to the percentage variation of the respective axis. (a) carbon substrate utilizing profiles between cultivars; (b) carbon substrate utilizing profiles between growth stages. SS – seedling stage; FS – flowering stage; PS – pod-setting stage; MS – maturity-setting stage

effects of transgenic soybean on the soil microbial community structure. This study addresses the biosafety issues aroused by the cultivation of GM crops, and it could provide useful parameters for assessing the effects of transgenic soybean.

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