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Different Water and Nitrogen Level Effects on Soil Microbial Properties of Spinach

(Kesan Perbezaan Tahap Air dan Nitrogen ke atas Sifat Tanah Mikrob Bayam)

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ABSTRACT

Understanding the interactions of plant soil environment and rhizosphere microbial changes are necessary to develop new strategies for the sustainable agriculture. A field experiment with combination of three water levels and three nitrogen rates was conducted to investigate the effect of water and nitrogen management on the changes of soil microbial properties in non-rhizosphere and rhizosphere soils of spinach. Non-Rhizosphere and rhizosphere microbial diversities were affected by water and nitrogen applications. Evenness index in the no-nitrogen treatment was more than that of 85 and 170 kg ha^{-1} nitrogen treatments in the non-rhizosphere or rhizosphere soil. Microbial biomass carbon in non-rhizosphere soil or rhizosphere soil decreased with the increase of nitrogen application, but showed the highest value in 16.5% of soil water content, followed by 12.5% and 20.5% of soil water content. Soil microbial biomass phosphorus content of 85 kg ha^{-1} nitrogen treatment in the non-rhizosphere soil or rhizosphere soil was significantly different for 0 and 170 kg ha^{-1} nitrogen treatments. Nitrification rate increased with the increase of soil water content in 0 and 170 kg ha^{-1} treatments. Our results demonstrated that water and nitrogen could impact the soil fertility and microbial activity of spinach.

Keywords: Microbial diversity; nitrogen fertilizer; rhizosphere; soil quality; water

ABSTRAK

Memahami interaksi perubahan tanah tanaman alam sekitar dan rizosfera mikrob adalah perlu untuk membangunkan strategi baru untuk sektor pertanian yang mampan. Kajian lapangan dengan kombinasi tiga paras air dan tiga kadar nitrogen dijalankan untuk mengkaji kesan pengurusan air dan nitrogen terhadap perubahan sifat mikrob tanah dalam tanih bukan rizosfera dan rizosfera bayam. Kepelbagaian mikrob bukan rizosfera dan rizosfera terjejas oleh aplikasi air dan nitrogen. Indeks kesamaan dalam rawatan tanpa nitrogen adalah lebih 85 dan 170 kg ha^{-1} daripada rawatan nitrogen dalam tanah - rizosfera atau rizosfera. Karbon biojisim mikrob dalam tanah bukan rizosfera atau rizosfera menurun dengan peningkatan aplikasi nitrogen, tetapi menunjukkan nilai tertinggi pada 16.5% daripada kandungan air dalam tanah, diikuti 12.5% dan 20.5% kandungan air dalam tanah. Kandungan tanah fosforus biojisim mikrob sebanyak 85 kg ha^{-1} rawatan nitrogen dalam tanah bukan rizosfera atau rizosfera tanah adalah berbeza bagi untuk 0 dan 170 kg ha^{-1} rawatan nitrogen. Kadar nitrifikasi meningkat dengan peningkatan rawatan kandungan air dalam tanah pada 0 dan 170 kg ha^{-1}. Keputusan kami menunjukkan bahawa air dan nitrogen boleh memberi kesan kepada kesuburan tanah dan aktiviti mikrob bayam.

Kata kunci: Air; baja nitrogen; kepelbagaian mikrob; kualiti tanah; rizosfera

INTRODUCTION

Soil microorganisms play critical roles in the cycle of soil nutrients (Adams & Frostick 2009; Doran & Zeiss 2000). Many factors such as soil properties, plant species and crop management strategy may affect the soil microbial diversity characteristics in the agricultural ecosystem. It is important to recognize the relationship between plants and microbes in the agricultural production.

Rhizosphere soil is generally defined as the zone which is the volume of soil adjacent to and affected by plant roots (Zhan & Sun 2012). Rhizosphere microorganism is directly affected by the growth of plant roots and reproduction of micro-organisms in the soil, such as bacteria, actinomycetes, fungi, algae and protozoa (Zhang et al. 2012a). Rhizosphere microorganisms are numerous and active, which constitute the rhizosphere microorganism zone (Ai et al. 2012; Tian et al. 2013). Rhizosphere microflora is mainly composed of bacteria. Beneficial rhizosphere bacteria accounted for 2-5% and harmful bacteria accounted for 8-15% in all bacteria (Hu et al. 2004). There is a close relationship between rhizosphere microorganism and plant. Microbes gather around the root system and make organic matter transform into inorganic matter to provide the effective nutrients for plants. Microbes can also secrete vitamin and growth stimulant to promote the growth of plant. Many factors such as nutrition and water can affect the rhizosphere

microorganism (Marschner et al. 2004; Meng et al. 2012; Phillips & Fahey 2008).

Water plays a key role in the microbial physiology and functions. Thus, not all soil microbiological characteristics are affected equally by nitrogen fertilizer and some farm practices like irrigation can change the effects. The microbial community's response to the soil moisture is important to indicate the nutrient cycling and soil productivity (Brockett et al. 2012). Many field experiments reported that water was the main factor affecting soil microbial structure (Chen et al. 2007). Water could greatly affect microbial community functional diversity and structural diversity (Harris 1981). However, the effects of water on the microbial community structure are still poorly understood. It is usually believed that the most influence of microorganisms in soil moisture circulation is excessive water supply or extremely poor moisture in the ecosystem.

Fertilization management in the agricultural production is necessary to maintain a healthy ecosystem for sustainable environment and agriculture (Sessitsch et al. 2001; Yevdokimov et al. 2008). Different fertilization system, soil microbial populations, quantity and activity cause different soil biological fertility (Ai et al. 2013; Zhang et al. 2008). This difference produces a significant influence on soil structure, fertility and productivity (Lupwayi et al. 2012; Zhong et al. 2010). These managements affect the soil microbial biomass carbon and nitrification rate (Fontaine & Barot 2005). The blind use of nitrogen fertilizers may deteriorate soil quality and nutrient balance. It reduces the soil microbial community diversity and the production quality of vegetables.

Vegetation influences the soil environment and then affects the soil microbial community structure and diversity. Plant roots provide the rich organic matter to the soil, and thus impact the organic matter and the number of species in the soil (Sang & Kim 2012). The organic matter in soil influences the soil microbial community structure and diversity. Vegetables need a lot of fertilizer and irrigation water (Chen et al. 2007). Different water and nitrogen applications have contributed significantly to effect the growth of plant. As frequent tilling garden soil, loose soil, frequent watering, adequacy water and nutrient, they are helpful to the growth of microbial (Agaras et al. 2012; Rani & Juwarkar 2012). Fertilization and water have great impacts on soil microbial diversity. Soil microbial quantity is numerous and microbial activity is high in the vegetable field. Therefore, it is useful to investigate the process of nitrogen transformation in vegetable field and the factors which can increase the utilization rate of nitrogen fertilizer. These managements could improve the quality of soil and vegetables.

Terminal restriction fragment length polymorphism (T-RFLP) is a semi-quantitative fingerprinting method for bacterial community analysis in environmental samples (Edel-Hermann et al. 2004; Iannelli et al. 2012). Fluorescent end-labeled PCR-amplified markers are digested with one or more restriction enzymes, resulting in the production of fluorescent labeled terminal-restriction fragments (T-RFs) of different lengths which can be separated and detected as peaks on an automated sequence analyzer (Tipayno et al. 2012). This method is used to assess microbial community structure and composition in nonrhizosphere and rhizosphere soil. However, previous researches have seldom been reported about the interactive effect of soil water and nitrogen fertilizer on soil microbial communities. Little information is known about the rhizosphere effect on the microbial community diversity and microbe biomass. In this study, we monitored the diversity of microorganism in non-rhizosphere and rhizosphere soil of spinach by PCR-RFLP method. The objectives of the research were to compare the diversity of microorganism in non-rhizosphere and rhizosphere soils from spinach communities under different irrigation water and nitrogen fertilizer applications; and to compare the microbe biomass carbon and phosphorus in non-rhizosphere and rhizosphere soils, nitrification rate.

Materials and Methods

Soil characterization

Spinach was planted on 11 December, 2011 and harvested on 15 April, 2012. Soil samples were collected at the spinach reproductive stage in late April 2012 and early May 2013 from Irrigation and Drainage Experimental Station in Qingpu District, Shanghai in China (E121°1', N31°1'). Soil properties at the initial stage of the experiment were: pH (H_2O), 5.62; organic matter, 11.88 g kg^{-1}; and total N, 1.48 g kg^{-1}, respectively. The content of inorganic nitrogen was 34.7 mg kg^{-1}. Soil particle size parameters were measured by Nano ZetaSizer laser particle analyzer (MALVERN Company). Particle size versus percentage volume of the soil samples are presented in Figure 1. The soil contained 27.05% sand, 59.86% silt, 13.10% clay and it was classified as silty loam soil.

The experiment was arranged based on randomized block design consisting of combinations of three irrigation treatments (W) and three nitrogen levels (N). Nine

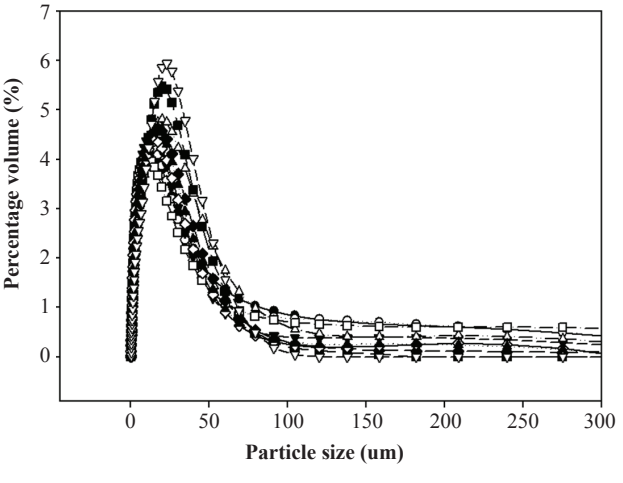

FIGURE 1. Particle size versus percentage volume of the soil samples

TABLE 1. Soil water content and nitrogen-fertilizer amounts under different treatments applied in the study

Experimental treatments	Soil water content (%)	Nitrogen fertilizer (kg (urea) ha^{-1})
W_1N_0	20.5	0
W_2N_0	16.5	0
W_3N_0	12.5	0
W_1N_1	20.5	85
W_2N_1	16.5	85
W_3N_1	12.5	85
W_1N_2	20.5	170
W_2N_2	16.5	170
W_3N_2	12.5	170

treatments in the study are shown in Table 1. The plot size was 2 m × 3.33 m. The fertilizer (urea 46% N) was applied with irrigation water at a time when spinach had five main leaves. Time Domain Reflectometry (TDR) probes were assembled to measure the soil moisture content in each treatment.

In this study, rhizosphere soil was defined as the vegetated soil in the densely rooted portion of the soil profile, adhering to the total roots after softly shaking (Zhang et al. 2012b). Non-rhizosphere soil was defined as the un-vegetated soil surrounding the root. The random sampling method was used to ensure representative sampling from the different treatments. One composite non-rhizosphere or rhizosphere soil sample was collected from each treatment. The samples were instantly transported to the laboratory in cool boxes with ice bags. Spinach roots were removed through a 200 μm sieve. A sub-sample was air dried, and then stored at room temperature for physical and chemical analysis. Another sub-sample was taken for T-RFLP analysis, freeze-dried and stored at –80°C until thawing immediately prior to DNA extraction.

DNA EXTRACTION

Total soil DNA was extracted using a Soil master™ DNA extraction kit (D5625-01 Soil DNA Kit (50), OMEGA) according to the manufacturer's instructions. The extracted DNA was diluted to 10 ng mL^{-1} prior to PCR amplification. Archaeal and bacterial 16S rRNA genes were PCR-amplified with the universal bacterial primers 8F (5'-AGA GTT TGA T (CT) (AC) TGG CTC AG-3') and 1492R (5'-GG(AGCT)(AT)AC CTT GTT ACG ACT T-3 '). Generally used fluorescent dye, 6-carboxyfluorescein (FAM), was applied to label the primers at the 5'end for T-RFLP analysis.

BIOMOLECULAR ANALYSES

A total of 0.25 mM deoxynucleoside triphosphates (2.5 mM each), 0.6 pmol/μL primer reverse, 0.6 pmol/μL primer forward, 0.03 U/μL *Taq* DNA polymerase and 2.5 μL template DNA were used in a 50 μL PCR for every sample. A Primus 96 Plus thermocycler was completed in thermocycling (MWG Biotech, Germany). The conventional PCR conditions were: initial denaturation of 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 2 min and final elongation at 72°C for 10 min.

The restrictions were completed separately at 37ºC for 16 h applying two enzymes: *Msp*I (0.2 U/μL final concentration) and *Hae*III (0.2 U/μL final concentration). Because the chosen *Msp*I and *Hae*III restriction endonucleases recognize and cut target sequences of 4 bp, getting average fragments of 4^4=256 bp, the 600 LIZ fragment size standard was selected. Therefore, most fragments would possibly be less than 500 bp. The 600 LIZ standard would be suitable for their detection. Although the different fragment lengths detectable using the selected primers and standards, the GeneScan™ 500 and 600 LIZ standards, along with 8F and 1492R primers, have been generally applied in many researches using analogous T-RFLP protocols. Each terminal fragment was regarded as an Operational Taxonomic Unit (OTU). Consequently, the two terms will be utilized as analogue hereafter after digesting with restriction endonucleases.

An ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) was used to perform capillary electrophoresis. GeneScan™ analysis software (Applied Biosystems, USA) was used to analyze T-RFLP profiles. Three profiles of every sample were compared and two third of visible fragments were utilized for a 'consensus' profile. Cluster analysis of a matrix was processed using the 15 'consensus' profiles. The products were sent to the Shanghai Sangon Biotech Co., Ltd. for sequencing.

DIVERSITY INDICES

The Shannon diversity index (H′) and Simpson's diversity index (1-D) were used to analyze the different treatments on the bacterial communities which were calculated from T-RFLP band data as follows:

Shannon (1949) diversity index (H')

$$H' = -\sum_{i=1}^{s} P_i \ln P_i = -\sum_{i=1}^{s} (N_i/N)\ln(N_i/N),$$

with p_i being the proportion of the i species relative to the total number of species;

Simpson's diversity index

$$D_s = 1-\sum P_i^2,$$

with p_i representing the population of the i species;
Richness (S) =the number of peaks in a sample;
Evenness (E) = $H'/\ln(S)$.
S represents the richness or total number of bands; N_i is the intensity accounted for by the ith band,

$$N = \sum N_i,$$

The Analysis of Variance (ANOVA) was used to evaluate differences (p<0.05) among different samples under the different treatments, following Bonferroni's procedure as a post-hoc test.

ANALYSIS METHOD

Soil microbial biomass carbon was measured by chloroform fumigation extraction method (Vance et al. 1987). Microbial biomass phosphorus was determined with fumigation extraction method (Wu et al. 2000). Ammonium and nitrate nitrogen in soil solution were measured using spectrophotometry.

The ratio of nitrate to the sum of nitrate and ammonium N = c(nitrate-N)/[c(ammonium-N)+c(nitrate-N)]×100%

STATISTICAL ANALYSES

All figures were drawn by SigmaPlot 10.0 software and all data were performed with SPSS 16.0 and Origin 8.0. In cases where the Tukey showed significant differences among means, the differences among treatments were compared using least significant differences (LSD) test at 5% significance level.

Results and Discussion

EFFECTS OF WATER AND NITROGEN ON SOIL MICROBIAL COMMUNITY DIVERSITY INDICES

Shannon and Simpson's diversity indices in different water and nitrogen management are presented in Table 2. There were no fertilizer effects on Shannon diversity in spinach non-rhizosphere or rhizosphere soil. Water management had a great effect on Shannon diversity and water deficit (W_3) reduced it. Drought could also reduce the soil microbial community diversity, which was consistent with the results of Kwon et al. (2013). Analysis of the data showed that application of nitrogen fertilizer had no effect on soil Simpson diversity in spinach non-rhizosphere. In rhizosphere soil, Simpson's indices of diversity showed a decrease in the treatment of N_2W_2. Soil microbial activity would reduce when the amount of chemical fertilizer was high. Fertilizer could cause the direct toxicity to microorganisms and inhibited the activity of soil microorganisms (Meng et al. 2012). This might be due to the plant root secretions, which played an important role in the change of rhizosphere microbial properties (Ai et al. 2013).

Richness index and evenness index under different water and nitrogen management are shown in Table 3. In non-rhizosphere soil, the richness index of treatment (N_0W_3, N_2W_3) decreased. The richness index in N_0W_3 treatment was the least in the rhizosphere soil. The result indicated that water deficit had negative effect on the growth of microorganism (Brockett et al. 2012). There were no changes in richness index with increasing nitrogen rate in spinach non-rhizosphere soil. Evenness was greater and it showed that few species exhibited the species dominance and species were more stable. Evenness index in N_0 treatment was more than that of N_1 and N_2 treatments in the non-rhizosphere or rhizosphere soil. More fertilization caused soil salinization, reduced species diversity, and decreased evenness index in spinach non-rhizosphere or rhizosphere soil. In the rhizosphere soil, there were no water effects on evenness index.

THE EFFECTS OF DIFFERENT WATER AND NITROGEN TREATMENTS ON MICROBE BIOMASS CARBON

The effects of water and nitrogen fertilizer on soil microbial biomass carbon were different (Figure 2). The microbial biomass carbon was significantly influenced by the water treatment. When the same amount of nitrogen fertilizer was applied, changes of microbial biomass carbon were as $W_2>W_1>W_3$ in spinach non-rhizosphere soil. The microbial biomass carbon was significantly different between W_2 and W_3 in N_1 treatment ($p<0.05$). There was an obvious difference among W_3, W_1 and W_2 water in N_0 treatment ($p<0.05$). The microbial biomass carbon could show the status of soil available nutrients and biological activity, and largely reflect the soil microorganism quantity. Microbial biomass carbon was an important index to evaluate the soil microorganism

TABLE 2. Water and nitrogen effects on Shannon diversity (H') and Simpson's diversity (1-D) indices of bacterial populations in the non-rhizosphere soils and rhizosphere soils based on T-RFLP analysis, respectively.

Nitrogen application	Irrigation method	Non-rhizosphere soil		Rhizosphere soil	
		Shannon diversity (H′)	Simpson's diversity (1-D)	Shannon diversity (H')	Simpson's diversity (1-D)
N_0	W_1	2.03a	0.92a	2.13a	0.92a
	W_2	1.95a	0.82a	2.22a	0.87a
	W_3	1.07b	0.88a	1.75b	0.95a
N_1	W_1	2.32a	0.93a	2.84a	0.89a
	W_2	2.72a	0.77b	2.27b	0.93a
	W_3	1.91b	0.88a	2.74a	0.93a
N_2	W_1	2.44a	0.91a	1.97b	0.94a
	W_2	2.38a	0.90a	2.78a	0.65b
	W_3	1.70b	0.85a	2.74a	0.93a

The results were given as mean (n=3). Within each treatment, values accompanied by different letters differ significantly at $p<0.05$. N: nitrogen treatment; W: water treatment

TABLE 3. Water and nitrogen effects on richness and evenness indices of bacterial populations in the N_0

Nitrogen application	Irrigation method	Non-rhizosphere soil		Rhizosphere soil	
		Richness index	Evenness index	Richness index	Evenness index
N_0	W_1	2.44a	0.84a	2.09a	0.86b
	W_2	1.75b	0.91a	2.22a	0.81b
	W_3	1.07c	0.88a	1.84a	0.95a
N_1	W_1	2.32a	0.89a	1.97b	0.87a
	W_2	2.38a	0.78a	2.74a	0.77a
	W_3	1.7b	0.85a	2.78a	0.80a
N_2	W_1	2.44ab	0.65ab	2.84a	0.55a
	W_2	2.72a	0.72a	2.27a	0.67a
	W_3	1.91b	0.58b	2.74a	0.76a

Richness (S) = the number of peaks in a sample; evenness (E) = $H'/\ln(S)$. Values given were the means of three replicates. Values with a column followed by different letters were significantly different at $p<0.05$. N: nitrogen treatment; W: water treatment

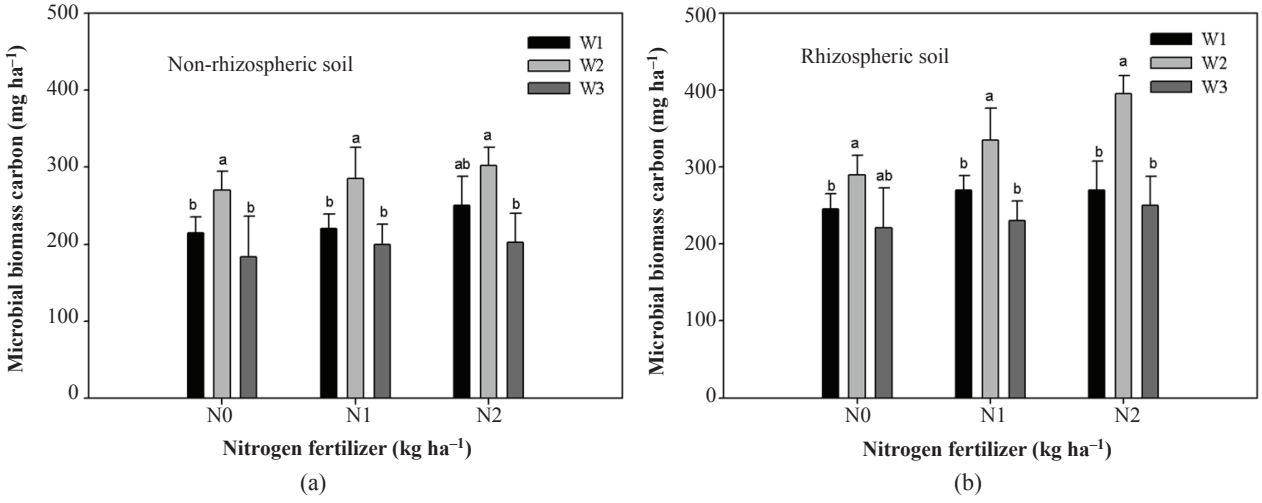

FIGURE 2. The effects of different water and nitrogen treatments on microbe biomass carbon in spinach non-rhizosphere and rhizosphere soil. Within each treatment, values accompanied by different letters differ significantly at $p<0.05$. N: nitrogen treatment; W: water treatment

quantity and activity, and soil fertility (Lupwayi et al. 2012).

Under the same nitrogen treatment, microbial biomass carbon could be ranked as order: $W_2>W_1>W_3$ in spinach rhizosphere soil (Figure 2b). It indicated excessive or limited water could reduce soil microbial biomass carbon and microbial activity in the rhizosphere soil. However, the microbial biomass carbon increased with the increase of nitrogen fertilizer at the same water treatment. These results revealed that more nitrogen could affect the microbial activity, and thus influenced the microbial biomass carbon. It was similar to the result of Li et al. (2013). The rhizosphere soil microbial biomass carbon content was more than non-rhizosphere soil.

THE EFFECTS OF DIFFERENT WATER AND NITROGEN TREATMENTS ON MICROBE BIOMASS PHOSPHORUS

Fertilization could significantly increase the soil microbial biomass phosphorus. Microbial biomass phosphorus content also showed significant differences in the rhizosphere or non-rhizosphere (Figure 3). It was alike to the trends of microbial biomass carbon. Microbial biomass phosphorus in the rhizosphere soil was more than the biomass phosphorus in the non-rhizosphere soil. The soil microbial biomass phosphorus content of the N_1 treatment was significantly different in non-rhizosphere or rhizosphere soil. The fertilizer application provided the nourishment for the microorganism activity and this could promote the microbial propagation (Chan et al. 2012). Part organic phosphorus and mineralized inorganic phosphorus were transformed to microbial biomass phosphorus through the microorganism. The soil microbial biomass phosphorus increased in the process.

THE EFFECTS OF DIFFERENT WATER AND NITROGEN TREATMENTS ON NITRIFICATION RATE

The soil nitrification was greatly affected by water and fertilization in non-rhizosphere or rhizosphere soil (Figure 4). Nitrification rate increased with the increase of soil moisture content in N_0 and N_1 treatments, and they

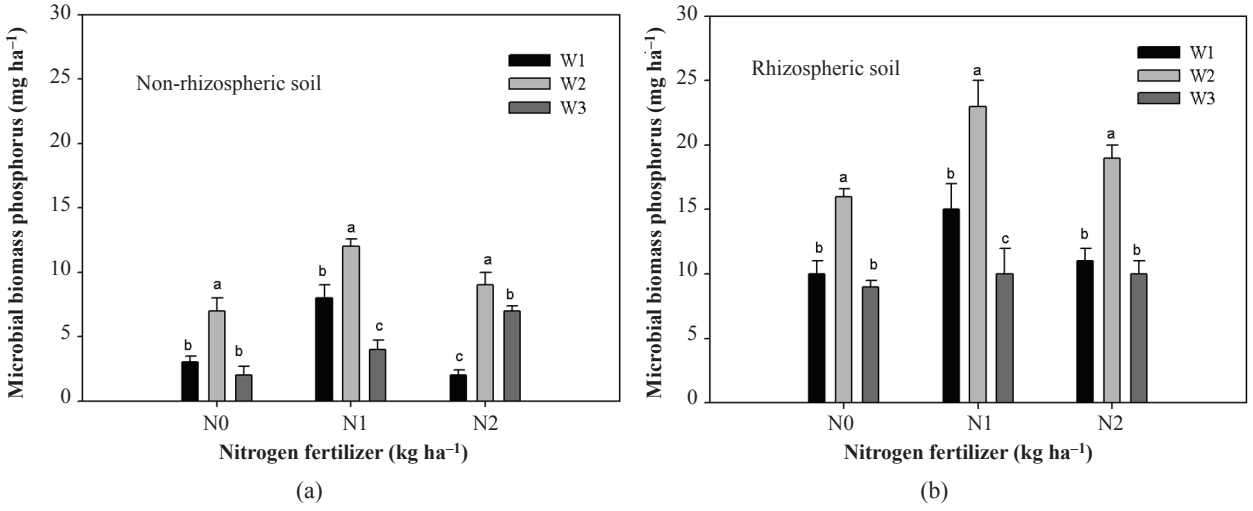

FIGURE 3. The effects of different water and nitrogen treatments on microbe biomass phosphorus in spinach non-rhizosphere and rhizosphere soil. Within each treatment, values accompanied by different letters differ significantly at $p<0.05$. N: nitrogen treatment; W: water treatment

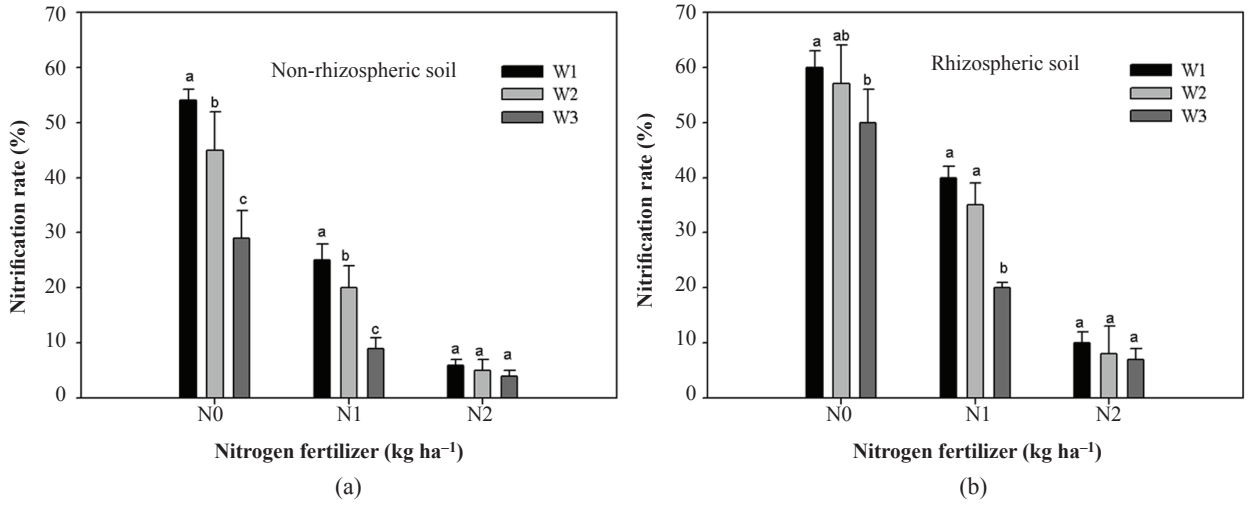

FIGURE 4. The effects of different water and nitrogen treatments on nitrification rate in spinach non-rhizosphere and rhizosphere soil. Nitrification rate means the conversion rate of ammonium to nitrate. Within each treatment, values accompanied by different letters differ significantly at $p<0.05$. N: nitrogen treatment; W: water treatment

ranked in the following order: $W_1>W_2>W_3$. There was a significant difference among W_1, W_2 and W_3 ($p<0.05$). This was possibly due to the reduced soil nitrification with decreased soil moisture content. The soil nitrification microbial activity reduced when the soil moisture content was too low (Cheng et al. 2012; Zaman et al. 1999). Under higher nitrogen fertilizer applications, the nitrification rate was usually low, and there were no significant differences between the different water treatments.

This study showed a clear shift in the root-associated microbial community structure of spinach which grew in the soil treated by different water and nitrogen treatments. Water and nitrogen fertilization had important effects on the microbial community, microbe biomass carbon and phosphorus, and nitrification rate. However, the response in the rhizosphere soil to water and nitrogen treatments was significantly different from that in the non-rhizosphere soil. Application of water and nitrogen fertilizers could impact the soil fertility and microbial activity. In conclusion, microbial community parameters were most closely associated with soil water and nitrogen fertilizer.

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