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Influence of drought and dry-wet alternation on nitrogen transformation and low abundance microorganisms in tea garden soil

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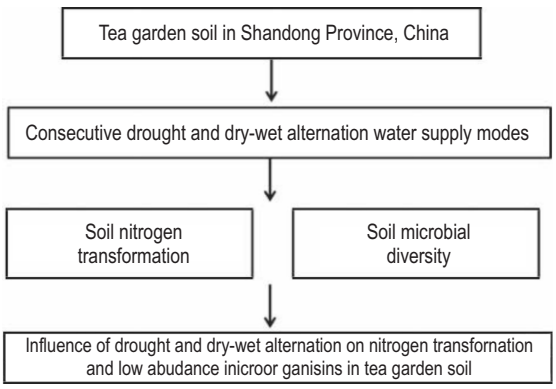
Abstract

Aim: The soil water availability seriously limits the growth and development of tea plants, and soil microorganisms are an important medium to regulate soil nutrient cycling. In this study, the effects of water supply mode on soil nitrogen nutrition and soil microbes in tea gardens were investigated.

Methodology: This experiment set up consisted two water supply modes (consecutive drought and dry-wet alternation) by using the soil microcosm incubation experiment, and four treatments were set: 20% water holding capacity for 21day (D21); 20% water holding capacity for 1-7 days and 60% water holding capacity for 8-21 days (D7W14); 20% water holding capacity for 1-14 day and 60% water holding capacity for 15-21 days (D14W7); 20% water holding capacity for 1-7 days, 60% water holding capacity for 8-14 days, 20% water holding capacity for 15-21 days (D7W7D7). Destructive sampling was carried out to determine soil NH_4^+-N, NO_3^--N, soil enzyme activities. 16S rRNA sequencing technique was used to determine the change in soil microbial diversity.



Results: The results showed that the consecutive drought reduced the content of soil NH_4^+-N to 13.97 mg kg^{-1}, and the net nitrogen mineralization was negative (-2.75 mg kg^{-1}) after 21 days of incubation. Dry-wet alternation promoted the increase in of soil net nitrogen mineralization quantity and net nitrification quantity, which rose to 3.48-26.41 mg kg^{-1} and 8.07-23.11 mg kg^{-1}, respectively. Different water supply modes had no significant impact on the structure of dominant soil microbial community, and the effect mainly focused on relative abundance, especially dry-wet alternation mode. Compared with the continuous drought treatment, the relative abundance of *Nitrospirae, Actinobacteria, Chloroflexi, Patescibacteria, Latescibacteria, Rokubacteria, Acidobacteria* were significantly different in different dry-wet alternation treatments, while the relative abundance of *Nitrospirae, Acidobacteria, Latescibacteria, Gemmatimonadetes, Patescibacteria* and *Chloroflexi* also increased or decreased significantly among different dry-wet alternation treatments. Among the physical and chemical factors of tea garden soil, NO_3- had the most significant effect on the structure of microbial community.

Interpretation: Different water supply can significantly affect the transformation of soil nitrogen and the change in soil bacterial community in tea garden, which provided a theoretical basis for tea garden to cope with adverse weather changes and maintain the stability of tea garden soil ecosystem.

Key words: *Camellia sinensis*, Nitrogen transformation, Soil microorganism, Water regime

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Introduction

Tea plant [*Camellia sinensis* (L.) O. Kuntze] is a perennial evergreen and is drought intolerant. Drought stress leads to significant structural changes in leaf organelles, resulting in higher membrane damage (Das *et al.,* 2015). Under drought stress, tea leaves turn yellow, become wilted or deformed, brown spots appear on them, and most branches die with severe damage (Chaeikar *et al.,* 2020). Cheruiyot *et al.,* (2009) reported that drought reduced tea production by up to 33% in Sri Lanka, and caused 6%-19% plant mortality and clonal variation. In addition, drought stress resulted in low catechin content in tea shoots, which affected the tea quality (Langat *et al.,* 2015). Drought is a major meteorological calamity restricting tea production. With global warming, this problem has become more intense. Soil moisture not only directly affects the physical diffusion and chemical form transformation of soil nutrients (Kaiser *et al.,* 2015), but also affects the structure and activity of soil microbial community, as well as soil processes and ecosystem functions mediated by microorganisms such as formation and transformation of soil organic matter, element biogeochemical cycles, greenhouse gas emissions (Manzoni *et al.,* 2012; Preece *et al.,* 2019).

Water stress on soil microorganisms is mainly manifested as direct physiological stress and restricted diffusion of microbial respiratory substrates (Naylor and Coleman-Derr, 2018). Drastic changes in water potential also cause tremendous physiological pressure on some microorganisms, resulting in death and dissolvement of microorganisms, thereby reducing the total number of microorganisms in the original soil. Bacterial movement in soil environment and the process of obtaining nutrients depend on the flow of water film in the soil (Evans and Wallenstein, 2014); therefore, changes in water conditions have a greater impact on bacteria. The addition of an appropriate moisture content to arid soil has different effects on the bacterial activity. There are two main modes of bacterial growth after restoration of water conditions. Lovieno and Bååth (2008) considered that bacteria begin to grow immediately after soil rehydration, and their growth increases linearly with time, while Goransson *et al.* (2013) found that after soil rewatering, bacteria first experiences a growth retardation period, then increases exponentially to peak, and then begins to decline. Meisner *et al.* (2013) observed that the growth of bacteria in soil rewetted after four days of drought accorded with the former pattern whereas in soil rewetted after a year of drought, growth was experienced approximately after 16 hours.

Post retardation period, bacteria exponentially increased to peak and then began to decline, and when the drought duration reached a certain threshold, a transformation from the first to second mode was noted (Meisner *et al.,* 2015). This difference is affected by the soil bacterial community structure after drought stress and the total amount of effective carbon source after addition of water. When water conditions are restored after long-term drought stress in soil, the surviving bacteria first completes the physiological function of self-repair. Rainfall in Shandong tea area is relatively less and has obvious seasonality with year-to-year changes. The rainfall usually occurs in summer, indicating that moisture is the main factor restricting primary production at least during certain periods of the year. In the present study, a soil microcosm incubation experiment with two modes of water supply: consecutive drought and dry–wet alternation was conducted.

The characteristics of soil nitrogen conversion and microbial community structure were studied, so as to explore the interrelationships among nitrogen, enzyme activity, and microorganisms under different water supply modes.The results of this study can provide a reference value to further understand and predict the soil microbial processes under the change of moisture in tea gardens in Shandong and are of significance for understanding the response of tea gardens to severe weather changes, and adopting more scientific and reasonable methods to promote better development of tea industry.

Materials and Methods

Experimental design: The soil was taken from tea garden of Shandong Institute of Pomology (N-36°12'19", E-117°4'49"), and the soil was brown. The climate of Shandong Province is of warm temperate monsoon type. The annual average temperature is 11°C-14°C, and the annual average precipitation is generally 550-950mm. The annual precipitation was 60%-70% in summer, and drought occurs in winter, spring and late autumn. The basic physical and chemical properties of soil are as follows: soil texture was sandy loam soil; soil organic matter 12.2 g kg^{-1}; pH 5.3; EC 39.61μs cm^{-1}; ammonium nitrogen 20.01mg kg^{-1} and nitrate nitrogen 15.13 mg kg^{-1}. Vegetation was removed from the surface of sampling point. The top 20 cm soil was sampled randomly with a soil corer at multiple points, and a total of 30 soil points were collected. Fresh soil sample was screened through a 2-mm mesh and the roots and visible organic debris in the soil samples were removed manually. Soil water content of samples was 20% of the water holding capacity by weight after air drying in field. In total, there were 2 modes (4 different treatments) for separate destructive sampling to measure soil nitrogen mineralization, enzyme activities and microbial community structure.

One mode was consecutive drought: soil water content was set to 20%WHC, marked as D21. Another mode was dry-wet alternation: 20%WHC for 1-7d and 60%WHC for 8-21d (D7W14); 20% WHC for 1-14d and 60% WHC for 15-21d (D14W7); 20% WHC for 1-7d, 60% WHC for 8-14d, 20%WHC for 15-21d (D7W7D7). Incubation period was 21 days, and samples (3 replicates) were taken at 7 and 21 days of incubation. The samples on 7th day were marked as D7, and the samples on 21st day were marked according to different treatments. The sampling bottles were placed in incubators at 25°C, the relative humidity of 40%, and in dark. Each replicate contained 100 g of air-dried soil and was placed in 500 ml plastic bottle. Small holes were drilled on the cover of the bottles to facilitate air exchange between the inside and outside. During the

incubation period, the soil water content was adjusted based on weight with deionized water at intervals of 1-2 days.

Soil inorganic nitrogen and enzyme activity: The concentration of ammonium nitrogen and nitrate nitrogen in the supernatants extracted by KCl solution (50 ml of 2 mol l^{-1}, 10 g soil) were determined by continuous flow analyser (Kou *et al.,* 2018). Urease activity was determined by phenol blue colorimetry (Lu, 2000). 1 ml toluene was added to 2 g soil. After 15 min, 10 ml urea solution (100 g l^{-1}) and 20 ml citrate buffer (pH = 6.7) were mixed into the solution which then were incubated for 3 hrs at 37°C. The accumulated NH_4^+-N (mg NH_4^+-N 100g^{-1} dry soil $3h^{-1}$) was measured colorimetrically at 690 nm. Soil denitrifying enzyme activity (DEA) was determined by nitrate residual method (Guan *et al.,* 1987). 1 ml glucose solution (10g l^{-1}) and 10 ml potassium nitrate solution (10g l^{-1}) were added to 1.0 g soil samples and incubated at 37°C for 48 hrs to determine NO_3^--N concentration. After 48 hrs of incubation with NO_3^--N as substrate, the residual amount of NO_3^--N (NO_3^--N g^{-1} dry soil 48 hr^{-1}) was measured. According to Guan *et al.* (1987), $NaNO_2$ was used as substrate to determine the activity of nitrite reductase.

Extraction of total DNA: Soil total DNA extraction was performed using "PowerSoil DNA" Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA), following the manufacturer's protocol. Quality of the extracted DNAs was examined on a 1.0% agarose gel. The concentrations and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA was stored at -20°C.

Soil bacterial community composition and diversity determination: The V4 variable region of the 16S rRNA gene was amplified by high fidelity PCR. 16S rRNA sequencing was conducted on the Illumina MiSeq Benchtop Sequencer (Illumina, USA). Sequencing primers were Primer F: GTGCCAGCMGCCGCGG/ Primer R: GGACTACHVGGGT WTCTAAT. The original offline data were controlled and filtered to obtain high quality sequencing data using the TrimGalore, FLASH2, Mothur and Usearch software. The non-repetitive sequence was extracted, and then the singleton sequence was removed by Uparse software. Sequences with more than 97% similarity were clustered into the same OTU. The denovo mode was used to remove the chimera sequence. The resulting OTU representative sequence was used for the analysis.

Statistical analyses: Statistical analysis was conducted using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and least significant difference (LSD) test were used to compare the averages of two treatments. The significance level was set at P□= 0.05. Spearman analysis was performed on soil factors and soil microorganisms. Origin 8.0 was used to make figures. STAMP was used to analyze differences in microbial communities at the phylum level. Redundancy analysis (RDA) was used to explore the relationship between soil bacteria with soil environmental factors at the 97% OTU level using Canoco 5.0. The NMDS was performed using R version 3.4.3.

Results and Discussion

The effects of different treatments on soil nitrogen varied significantly. The content of NH_4^+-N in D21 (13.97 mg kg^{-1}) decreased by 12.22% after 21 days compared with that after 7 days (15.68 mg kg^{-1}); the content of NH_4^+-N increased by 8.34% and 73.91% in D7W14 (16.99 mg kg^{-1}) and D14W7 (27.27 mg kg^{-1}), respectively, but decreased by 1.61% in D7W7D7 (15.43 mg kg^{-1}). After incubation, the order of NH_4^+-N content under different treatments was D14W7 > D7W14 > D7W7D7 > D21, and the differences were significant ($p< 0.05$) (Fig. 1a). No significant difference was observed in the soil NO_3^--N content between the consecutive drought treatment (D21,18.40 mg kg^{-1}) and seven days of cultivation (18.47 mg kg^{-1}). The contents of NO_3^--N in three treatments increased significantly ($p< 0.05$), and those in D14W7, D7W14, and D7W7D7 were 1.07, 0.85, and 0.25 times that of D7, respectively. After cultivation, the soil NO_3^--N content of each treatment was in the order: D7W14>D14W7>D7W7D7>D21, and the difference between the treatments reached a significant level ($p<0.05$) (Fig. 1b). Differences in soil net nitrogen mineralization and nitrification under different water supply modes were found to be significant. After 21 days of incubation, the order of net nitrogen mineralization was D14W7 > D7W14 > D7W7D7 > D21 (Fig.1c), which indicated that the dry–wet alternation mode increased the soil net nitrogen mineralization. Significant positive correlation was observed between soil moisture and net nitrogen mineralization within a certain range (Guntiñas *et al.,* 2012).

After 21 days of cultivation, the accumulation of net nitrogen mineralization was negative in the consecutive drought mode (Fig. 1c), which might be attributed to depletion of soil nutrients during the entire cultivation period that promoted the death of microorganisms and decreased the ability of organic nitrogen to transform into NH_4^+-N. Simultaneously, under the combined action of ammonia volatilization and nitrification, the NH_4^+-N content was consumed rapidly and the accumulation of net nitrogen mineralization was reduced (Marcos *et al.,* 2016; Francisco *et al.,* 2011). In dry–wet alternation mode, the soil net mineralization showed a "pulsing" change trend, but the overall trend was downward, and the soil NH_4^+-N content did not effectively accumulate (Zheng *et al.,* 2012).

Compared with the D14W7 treatment, D7W14 treatment maintained a longer time after rehydration, quickly activated soil microbial activity (Hamer *et al.,* 2007), accelerated soil NH_4^+-N consumption, and resulted in lower net nitrogen mineralization (20.08 mg kg^{-1}) (Fig.1c). Soil net nitrification increased under different water supply modes; however, the soil net nitrification in the dry–wet alternation mode was significantly higher than that in the continuous drought mode. In dry–wet alternation, the soil net nitrification was highest in D7W14 (23.11 mg kg^{-1}), followed by D14W7 (19.16 mg kg^{-1}) and D7W7D7 (8.07 mg kg^{-1}) (Fig. 1d). The increase in soil moisture led to an increase in the net nitrogen mineralization rate, particularly in the increased soil NO_3^--N content (Klein *et al.,* 1996; Marcos *et al.,* 2016). In this study, the

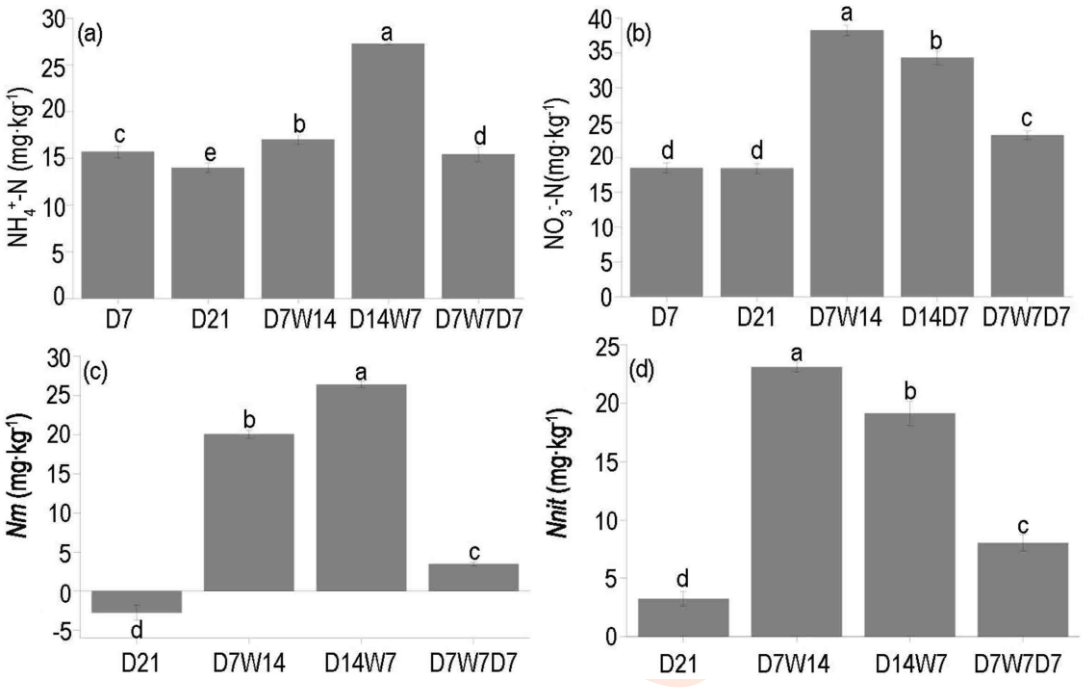

Fig. 1: Contents of ammonium (a), nitrate (b), Nm: Net nitrogen mineralization quantity (c), Nnit: Net nitration quantity (d) in soil under different water treatments. Values are mean of three replicates ± 3 S.D. Bars with different letters indicate significant differences at $P< 0.05$ based on One-way ANOVA.

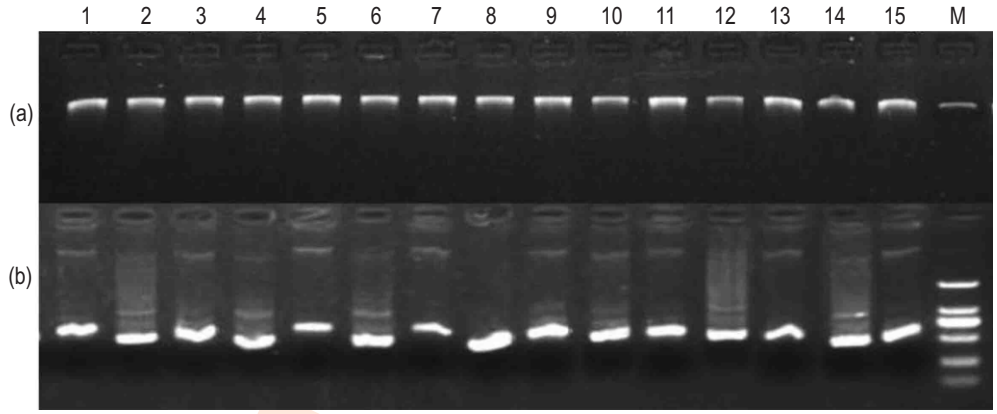

Fig. 2: Agarose gel electrophoresis of total soil DNA extracted (a) and DNA PCR (b).V4 variable regionsequencing primers were Primer F: GTGCCAGCMGCCGCGG/ Primer R: GGACTACHVGGGTWTCTAAT. 1-15samples (3 replicates):D7-1, D7-2, D7-3, D21-1, D21-2, D21-3, D7W14-1, D7W14-2, D7W14-3, D14W7-1, D14W7-2, D14W7-3, D7W7D7-1, D7W7D7-2, D7W7D7-3. M, Marker.

net nitrification content of soil after soil rehydration (8.07-23.11 mg kg^{-1}) was higher than that after consecutive drought (3.27 mg kg^{-1}) (Fig. 1d). Because the maintenance of a high soil water content is beneficial to the mineralization and nitrification of organic matter, increasing the conversion of NH_4^+-N to NO_3^--N and net nitrification (Ma *et al.,* 2013; Wang *et al.,* 2018). The dry–wet alternation also promoted the transformation of NH_4^+-N to NO_3^--N. The net nitrification rapidly increased after rewatering, which was more conducive for accumulation of NO_3^--N. In dry–wet alternation, the higher soil water content and the longer the rehydration time, the higher the NO_3^--N content in soil (Fig. 1b). The total DNA was about 23 kb, with single band, no dispersion and good integrity (Fig. 2a). The A260/280 and A260/230 of total DNA extracted from soil were higher than 1.8 and 2.0,

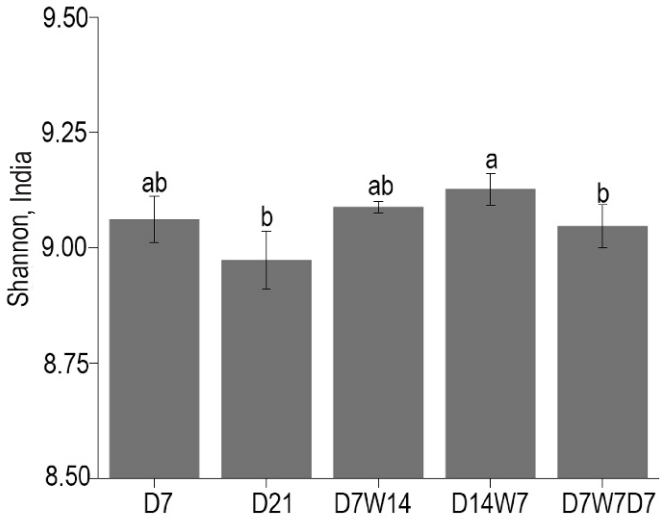

Fig. 3: *Shannon Index* of bacterial community structure in different soils. Values are mean of three replicates±S.D. Bars with different letters indicate significant differences at $p < 0.05$.

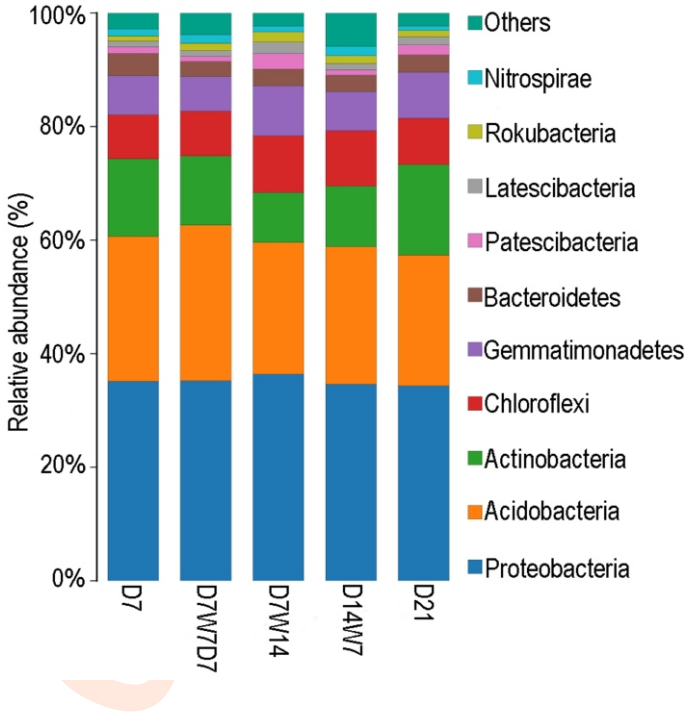

Fig. 4: Structure composition of bacterial community with different water treatments at phylum level. The relative abundance of phylum > 1% is shown, and those <1% are classified as others.

respectively, and the concentration was above 50 ng μl^{-1}. The V4 variable region of 16S rRNA gene was amplified by high fidelity PCR. The target band amplified by PCR were bright and single (Fig. 2b). The above results showed that the extraction quality of soil total DNA were good and could meet the requirements of sequencing. The drastic fluctuation in soil moisture not only had a direct impact on the current microbial community structure but also continuously affected the subsequent soil processes and ecosystem services (Meisner *et al.,* 2015). Shannon Index analysis revealed that as compared with soil microbial diversity

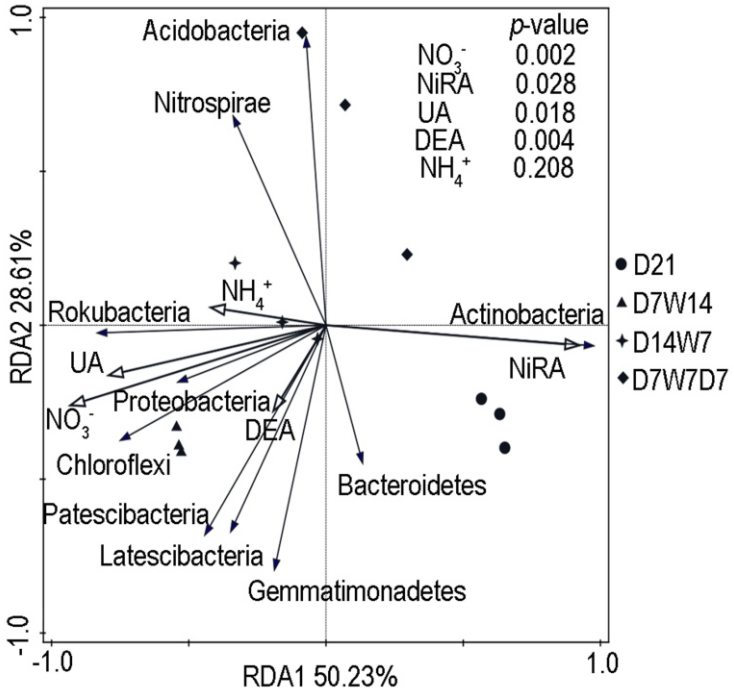

Fig. 5: Redundancy analysis (RDA) of soil bacteria (phylum lever) with soil environmental factors, *viz*. NH_4^+, NO_3^-, UA, DEA, and NiRA.

cultured for 7 days, the soil microbial diversity of D21 and D7W7D7 treatments decreased, and that of D14W7 treatment increased, while no difference was found between D7W14 treatment and soil microbial diversity incubated for 7 days. At 21 days, the soil microbial diversity index of D14W7 treatment was highest and that of D21 and D7W7D7 treatments was lowest (Fig. 3). The results revealed that soil microbial diversity decreased by consecutive drought, due to the elimination of drought-tolerant microorganisms under excessive water stress (Fahimipour and Gross, 2020), resulting in the decrease in soil microbial diversity. Soil microbial diversity increased after rewatering, however, on extending the rewetting time, soil microbial diversity decreased (Shannon index of D14W7 treatment was greater than that of D7W14 treatment). Likewise, an increase in drying and wetting frequency significantly reduced soil microbial diversity.

Previous studies have reported that after drought stress, the soil forms a vacant niche in the microbial food web (Fahimipour and Gross, 2020). Opportunistic microorganisms with strong drought tolerance and the response to soil water change accelerate their growth and occupy the niche in the microbial food web after rewatering, resulting in changes in the microbial community composition and soil function (Fukami, 2015; Lynch and Neufeld, 2015). With the extension of rewatering time, the "eutrophic" microbial community accelerates growth, occupies a large part of niches, and becomes the dominant population, which results in decrease in microbial diversity (Kakumanu *et al.,* 2013). The phyla of soil microorganisms with relative abundance > 1% were selected for the analysis. The distribution and relative abundance of dominant phyla were found to vary under different modes. *Proteobacteria, Actinobacteria, Acidobacteria* and *Chloroflexi* were the dominant bacteria in the soil under different water supply modes, accounting for approximately 80% of the total abundance of soil microorganisms (Fig.4). In previous studies, *Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi* and *Bacteroides* have been found to be dominant bacteria in all soil samples (Cheng *et al.,* 2018; Gao *et al.,* 2019). They belong a group of bacteria that can adapt to drought stress well, can survive in poor soil, and are also a major component of dry soil microbial population in the world (Neilson *et al.,* 2012).

The relative abundance of dominant phyla (*Proteobacteria* and *Acidobacteria*) did not change significantly because *Proteobacteria* were more suitable for proliferation in a nutrient-rich environment. However, the soil nutrient resources of the tea garden selected in this experiment were relatively rich, and the short-term drought would not reduce the availability of soil resources (Johnston-Monje *et al.,* 2016), although it had little effect on *Proteobacteria*. Previous studies have found that *Acidobacteria* was also highly sensitive to drought, and its abundance was low in arid soil (Barnard *et al.,* 2013). However, in this study, the abundance of *Acidobacteria* did not change significantly, because the soil of tea garden is acidic, and more suitable for the proliferation of *Acidobacteria* or *Acidobacteria* synthesizes many extracellular polymers to encapsulate the

entire microbial cells in case of decreased soil water content to reduce the damage caused by drought (Kielak *et al.,* 2016). Previous studies have found that 69–74% of the revived microbial communities in response to water changes belong to previously undetectable, low-abundance, rare species (Aanderud *et al.,* 2015; Lynch and Neufeld, 2015). Aanderud *et al.* (2015) studied grassland and farmland ecosystems and found that the soil would usher in the outbreak of low-abundance microbial populations after drought stress, such as α -*Proteobacteria* in grassland ecosystems and *Gemmatimonas* in farmland ecosystems, and the abundance of OTUs increased rapidly after humidification post drought, becoming the dominant population in the microbial community. The abundance of dominant bacteria was also significantly different after 21 days of culture. At the phylum level, the relative abundance of 4,6 and 3 microbial populations changed significantly under dry–wet alternation (D14W7, D7W14 and D7W7D7) and continuous drought treatment (D21).

The relative abundance of *Nitrospirae* significantly improved by dry–wet alternation treatment. The relative abundance of *Actinobacteria* significantly reduced ed by 79.43% and 48.99% ($p< 0.05$) and that of *Chloroflexi* increased by 22.07% and 18.84% ($p< 0.05$) in the D14W7 and D7W14 treatments, respectively. The relative abundance of *Patescibacteria* significantly reduced with the D14W7 and D7W7D7 treatments. The relative abundance of *Rokubacteria, Latescibacteria* and *Proteobacteria* increased significantly in the D7W14 treatment and that of *Acidobacteria* significantly increased only in the D7W7D7 treatment; no significant change was observed among other treatments. The results revealed that the effects of dry–wet alternation treatment on microorganisms were also different. Compared with the D7W14 treatment, the D14W7 treatment significantly increased the relative abundance of *Nitrospirae* and *Acidobacteria* by 59.17% and 4.50%, respectively ($p< 0.05$).

The decrease in *Latescibacteria*, *Gemmatimonadetes*, and *Patescibacteria* was 81.50%, 26.17%, and 187.64%, respectively ($p< 0.05$). The relative abundance of *Latescibacteria, Gemmatimonadetes, Patescibacteria*, and *Chloroflexi* in the D7W14 treatment was 90.91%, 44.23%, 212.59%, and 26.39% higher than that in the D7W7D7 treatment, ($p< 0.05$) and that of *Nitrospirae* and *Acidobacteria* decreased by 46.09% and 18.22%, respectively ($p< 0.05$). Compared with the D7W7D7 treatment, the D14W7 treatment significantly improved the relative abundance of only *Chloroflexi.* These results demonstrated that the effects of different water supply modes on soil dominant microbial flora mainly focused on the relative abundance, and the microbial community structure did not change.

The effect of consecutive drought on soil microbial population in tea garden was weak, however, the abundance of soil microbial population changed greatly after rehydration, which was mainly reflected in the effect on rare microbial groups with low abundance. To summarize, the soil moisture change frequency was small, and the long-term maintenance of sufficient water could more effectively regulate the beneficial microbial groups in the soil. Drought indirectly affects the bacterial community of soil by changing the soil nutrients. This impact is greater than the direct impact of drought on the soil bacterial community (Bastida *et al.,* 2019; Naylor and Coleman-Derr, 2018). Therefore, the relative abundance of dominant flora is often significantly affected by soil factors (Johnston-Monje *et al.,* 2016). Redundancy analysis (RDA) was used to find out that NO_3-, NiRA, UA, and DEA play a crucial role in the regulation of bacterial community ($p< 0.05$), and the contribution rate of NO_3- was highest, reaching 52% whereas NH_4^+ did not reach a significant level in the regulation of bacterial community ($p = 0.208$). It means that NO_3- played a leading role in the influence of soil microorganisms in this experiment.

The NO_3^--N correlated positively with Proteobacteria, Chloroflexi, and Rokubacteria and negatively with Actinobacteria, which might be that the increase of soil nitrogen content is conducive to the accumulation of soil microbial population and microbial biomass (Nishiyama *et al.,* 2001; Ralte *et al.,* 2005); UA correlated positively with *Chloroflexi* and *Nitrospirae* and negatively with *Actinobacteria*; no significant correlation was observed between DEA and flora, and NiRA correlated positively with *Actinobacteria* and negatively with *Chloroflexi* and *Rokubacteria*. Based on the above research, compared with consecutive drought, the dry-wet alternation promoted the increase of soil net nitrogen mineralization and net nitrification. Consecutive drought had a weak effect on soil microorganisms. The dry-wet alternation can adapt to the change of soil moisture by affecting the flora with low relative abundance in the dominant soil microbial flora.

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Add-on Information

Authors' contribution: Z. Zhao: Participated in experimental design, carried out the experiment, wrote manuscript; **X. Han:** Designed proposal, guide experiments, revised manuscript; **S. Yu:** Carried out the experiment; **S. Yang:** Designed proposal, analyzed data.

Research content: The research content is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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