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Effects of monoculture and mixed culture of grass and legume forage species on soil microbial community structure under different levels of nitrogen fertilization

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ABSTRACT

We used PLFA analysis to monitor the soil microbial community structure in a gramineous–legume forage field under different inorganic nitrogen (N) fertilization regimes in Southwest China. The gramineous–legume forage system included one grass (*Paspalum wetsfeteini*) and one legume (*Medicago sativa*) in three planting systems: *P. wetsfeteini* monoculture, *M. sativa* monoculture, and *P. wetsfeteini* and *M. sativa* mixed culture. The fertilization treatments included three N levels: 338 (low), 450 (moderate), and 675 (high) kg N ha^{-1} yr^{-1}. The results showed that biomasses of total microbes, bacteria, fungi, and green alga were significantly greater and protozoan biomass tended to be greater under legume monoculture than that under grass monoculture; and fungal biomass was significantly greater under grass-legume mixed culture than under grass monoculture in wet season. However, principal component analysis (PCA) only revealed a tendency that the microbial PLFA composition under legume monoculture differed from that under grass monoculture in the wet season. In addition, the soil microbial community structures were not significantly different among the three planting systems in the dry season. The PCA results showed that the microbial PLFA composition under low N fertilization was apparently different from that under moderate and high N fertilization in the wet season. Particularly, the biomasses of total microbes, bacteria, and green algae were significantly greater under moderate N fertilization than under low N fertilization and the green algal biomass was significantly greater under high N fertilization than under low N fertilization in the wet season. Additionally, PCA also revealed that the microbial PLFA compositions were different under the low and moderate N fertilizations in the dry season. However, the biomass and diversity of microbial community had no significant difference among the three levels of N fertilization. The results suggest that legume cultivation increased the biomasses of soil microbial community.

1. Introduction

The amount of inorganic or industrial nitrogen fertilizers applied to agricultural ecosystems increased by 800% from 1960 to 2000 [1], and this quantity continues to increase as the demand for food increases [2], especially in developing countries [3]. Although inorganic nitrogen fertilizers have substantially increased crop yield [4], their application has adversely affected the environment by decreasing biodiversity, acidifying soils, causing water eutrophication, and increasing atmosphere nitrogen deposition [2,3,5,6].

Organic agriculture, including the application of organic rather than inorganic fertilizers, is believed to be more environmentally sound than traditional agriculture [7]. Many previous studies have indicated that organic fertilization (e.g., the application of manure or compost or the establishment of legume-based cropping systems) increases species richness and abundance, enhances food web complexity, and reduces soil carbon and nitrogen loss [8–12].

Nitrogen-fixing plant–microorganism associations increase the soil nitrogen content [13–15]. The most common nitrogen-fixing

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associations include rhizobia associated with legumes, certain non-rhizobial microorganisms associated with some crops and trees, cyanobacteria associated with rice paddies, and endophytic diazotrophs associated with sugar cane [16]. Both seed legumes (e.g., peas and beans) and forage legumes (e.g., alfalfa and clover), associated with rhizobia, transform atmospheric N_2 to reactive nitrogen (N_r) at high rates, although the average nitrogen-fixing rates are greater for forage legume than for seeds legume [14,16].

In addition to affecting plant growth, nitrogen-fixing associations between plants and microorganisms affect other soil organisms via bottom-up control. For example, the presence of legumes increased soil microbial biomass and activity in a semi-natural grassland in central Germany [17] and in a greenhouse experiment [18], the abundance of bacterivorous nematodes in grasslands in northern Sweden [19,20], the abundance of mites and omnivorous and predacious nematodes in mixed-canopy tree plantations in southern China [21], and the diversity and density of epigeic earthworms in the BIODEPTH experimental grassland in Germany [22]. Moreover, legumes increased the complexity of soil food webs, as indicated by an increase in the number of trophic links and multi-trophic interactions [10].

The soil microbial community is a key component of the nitrogen cycle and greatly affects soil fertility. The effects of inorganic nitrogen application on soil microbial communities have been extensively studied. Wallenstein et al. (2006) pointed out that the application of inorganic nitrogen increased soil microbial biomass over the short-term but suppressed it over the long-term in forest ecosystems [23]. However, not only positive but also negative and neutral effects of inorganic nitrogen application on microbial communities have been reported [24]. In contrast to the uncertainties about inorganic nitrogen fertilization, organic fertilization increased the biomass and diversity of microbial communities [7,11,25,26].

In the present study, we examined how the soil microorganisms in a gramineous–legume forage field in Southwest China are affected by different inorganic nitrogen fertilization regimes. The aim of this work was to compare the effects of a gramineous-legume forage system and inorganic nitrogen fertilization on a soil microbial community. We hypothesized that legumes would increase the biomass and diversity of soil microorganisms and higher inorganic nitrogen fertilization would suppress the biomass and diversity of soil microorganisms.

2. Materials and methods

2.1. Site description and experimental design

This study was carried out at the Huanjiang Observation and Research Station for Karst Ecosystems (107°51′–108°43′E, 24°44′–25°33′N), Chinese Academy of Sciences (CAS), Guangxi Province, China. The climate is subtropical monsoon with a distinct wet season (from April to September) and dry season (from October to March). The mean annual temperature and precipitation are 18.5 °C and 1389 mm. The soil is brown calcareous soil developed from a dolostone base. According to the Chinese Soil Taxonomy system, it belongs to primitive soils [27,28].

The experimental site was formerly used as an experimental grassland that was abandoned in 2009. In January 2013, the land was plowed to a depth of 30 cm with an excavating machine, and large roots and stones were removed with a rake (2-cm tine spacing). In early March 2013, we established an experiment with treatments representing a complete factorial combination of three levels of nitrogen fertilization and three planting systems (a 3 × 3 factorial design) with three replicates. Therefore, a total of 9 treatments and 27 experimental plots (5 m × 4 m per plot) were established. The three levels of nitrogen fertilization were 338 (N1), 450 (N2), and 675 (N3) kg N ha^{-1} yr^{-1}; urea (containing 45% N) was chosen as the nitrogen fertilizer. One gramineous species (*Paspalum wetsfeteini*) and one legume species (*Medicago sativa*) were selected to form the following three planting systems: *P. wetsfeteini* monoculture (G), *M. sativa* monoculture (L), and *P. wetsfeteini* and *M. sativa* mixed culture (G + L).

Seeds of *P. wetsfeteini* and *M. sativa* were purchased from a local farmer and were directly planted in rows of the designated plots in early April 2013. The sowing rates for *P. wetsfeteini* and *M. sativa* were 4 g and 1g m^{-2}, respectively. At the end of April 2013, the seeds of *P. wetsfeteini* and/or *M. sativa* were re-sown in plots where seeds had germinated poorly. The nitrogen fertilizer was applied to all of the nitrogen fertilization plots at the end of April, July, and November 2013. Before seeds were sown, all of the fertilized plots were also treated with phosphorus and potassium fertilizers at 150 kg ha^{-1} yr^{-1}, respectively. Each month, germinating weeds were manually removed from all plots; herbicide was not used to avoid potential effects on soil organisms.

2.2. Soil sampling and analysis

Soil was sampled in July 2013 and January 2014. Soil cores (2.5 cm in diameter) were taken at 0–10 cm depth from 10 random locations within each plot. The 10 cores from each plot were combined to form one composite sample. The surface litter was carefully removed before soil cores were collected. Samples were transported to the laboratory in insulated boxes. The soil was passed through a 2-mm sieve to remove the roots. Then, each fresh soil sample was divided into two subsamples. One subsample was immediately used to determine the soil water content and the other was kept in a refrigerator at −20 °C for no more than two weeks before processed to determine microbial community structure.

Soil water content (g of water per 100 g dry soil) was measured by oven-drying the soil for 48 h at 105 °C. Phospholipid fatty acids (PLFAs) were extracted from 8 g of fresh soil and were analyzed as described [29]. Concentrations of each PLFA were calculated relative to methyl nonadecanoate (19:0) internal standard concentrations. Bacterial biomass was considered to be represented by 10 PLFAs (i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0, cy17:0, and cy19:0); fungal biomass was considered to be represented by the PLFA 18:2ω6,9; and actinomycete biomass was considered to be represented by three PLFAs (10 Me 16:0, 10 Me 17:0, and 10 Me 18:0) [30–32]. Microbial biomass was considered to be represented by the 10 bacterial PLFAs, the one fungal PLFA, and PLFA 16:0. In addition, protozoan biomass was considered to be represented by two PLFAs (20:0 and 20:4ω6,9,12,15), and green algal biomass was considered to be represented by the PLFA 18:1ω9 [33–35]. Other PLFAs such as 16:1ω5, 18:1ω7, and 18:3ω6,9,12 were also used to analyze the soil microbial community structure.

2.3. Data analysis

Soil microbial community composition was analyzed by transforming the PLFA data to their principal components (PCA) and analyzing these using ANOVAs [24,36]. The main and interaction effects of nitrogen fertilization and planting system on dependent variables describing the soil microbial community were examined by two-way ANOVAs. One-way ANOVAs were used to evaluate statistical differences among the 9 treatments of each microbial variable. Data were transformed (natural log, square root, or rank) to meet assumptions of normality and homogeneity of variance. Statistical significance was determined at $p < 0.05$. ANOVAs and