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Intercropping of kura clover (*Trifolium ambiguum* M. Bieb) with prairie cordgrass (*Spartina pectinata* link.) enhanced soil biochemical activities and microbial community structure

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ABSTRACT

Optimizing the use of N fertilization is the major factor for establishing perennial grasses to enhance the biomass production and soil health under marginally yielding croplands. A study was conducted to investigate the influence of N fertilization and intercropping of kura clover (*Trifolium ambiguum* M. Bieb) (KC) with prairie cordgrass (PCG) (*Spartina pectinata* Link.) on soil biochemical activities and microbial community structure. The fertilization treatments included four N levels: 0, 75, 150, and 225 kg N ha^{-1}, and the grass-legume intercropping system included one grass (PCG) and one legume (KC) (PCG-KC). Data showed that soil microbial biomass C (MBC) was 90 and 55% higher with PCG-KC than the PCG-225N and PCG-75N, respectively. Enzyme activities associated with life processes of microorganisms and release of mineral nutrients from organic matter were the highest in PCG-KC, and were comparable to that observed under medium to high N fertilization. The results indicated that PCG-KC intercropping could sustainably provide soil health benefits compared to the N fertilization. The PCG-KC combination led to an increase in total PLFA (phospholipid fatty acid) and total bacterial PLFA concentration relative to the PCG-0N, PCG-75N and PCG-225N. The PCG-KC also had the highest glomalin-related soil protein content (11.9 mg g^{-1}) indicating high abundance of Arbuscular mycorrhizal fungi crucial for P nutrition. Further, N deficiency as well as high N fertilization appear to reduce the abundance of N-fixing bacteria as indicated by the presence of nifH amplicons. In summary, PCG-KC intercropping yielded comparable or more desirable soil microbial community structure and enzymatic activities compared to the N fertilization. Considering the cost and negative impact of N fertilization on the environment, intercropping KC with PCG can be desirable for improving soil health of marginally yielding croplands.

1. Introduction

Concern about the depletion of non-renewable fossil fuels has increased interest in the production of renewable and sustainable energy from plant biomass (Owusu and Asumadu-Sarkodie, 2016). The plant biomass can be used as an alternative energy source that has the advantages of reducing greenhouse gases (GHG) emissions and ensuring reliable fuel supply in the future (Hoogwijk et al., 2005). The development of renewable energy from plant biomass not only contributes to energy supply but also helps to achieve environmental and economic benefits (Demirbas et al., 2009). The use of plant biomass and animal products-the basics of the human diet-for energy production might amplify the global food crisis, especially in underdeveloped countries (Ajanovic, 2011). This concern can be alleviated by the use of bioenergy derived from different by product sources, such as wood residues, agricultural crop residues, wastes from sugar industry, and cow dung from animal husbandry (Urban and Mitchell, 2011). The major advantage of energy production based on biomass is that bioenergy is often a by-product, waste or residue product from the above sources and so does not create a competition between land for fuel and food production (Owusu and Asumadu-Sarkodie, 2016; Urban and Mitchell, 2011).

In the United States, 95% of ethanol is produced from maize (*Zea mays* L.) or sorghum (*Sorghum bicolor*) grain (Flugge et al., 2017). The increased need for maize for energy production (ethanol) has reduced its supply and created a demand in other markets including livestock feed and exports. Grass species with C_4 physiology have been widely produced as an alternative source for renewable energy (fuel)

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production because of their high biomass yield, low nutrient requirement, and ability to grow in marginal environments (Heaton et al., 2008). Many herbaceous perennial plant species have been evaluated for their energy production potential and renewable nature without competing with food, fiber, and feed production (Gonzalez-Hernandez et al., 2011).

Prairie cordgrass (PCG) (*Spartina pectinata* Link.) is a warm season rhizomatous C4-perennial crop that has significant potential for bio-fuel and feedstock production (Valentín and Karla, 2012). As interest in energy crops and feedstock production for biofuel increases, PCG is receiving more attention because it grows well on marginal lands, especially in waterlogged conditions where soils are too wet for row crop production. In low prairies, PCG is a dominant grass species where soils are not suitable for the production of maize, switchgrass, other forages, grain, and biofuel crops (Boe et al., 2013). Improved N management and optimization of N fertilization are crucial for established perennial grasses to obtain desirable biomass production and maintenance of soil health (Owens et al., 2013). Plant productivity can be greatly influenced by N fertilization but needs careful management to avoid harmful effects on the environment. Improper N fertilization leads to increased production costs and has a negative impact on the environment due to N pollution (Duran et al., 2016). Fertilization has greatly influenced the properties and processes of agroecosystems and these effects are expected to increase further in the future (Kidd et al., 2017). However, the recent increase in fertilizer N cost and inorganic fertilizers' negative impacts on GHG emissions, water contamination, and soil pollution have encouraged the use of leguminous crops as a source of N in sustainable production of plant biomass (Hirel et al., 2011; Stagnari et al., 2017). The legume crops have the ability to form a symbiotic association with N-fixing bacteria in the soil, such as *Rhizobium,* resulting in root nodule formation. Biological N fixation in root nodules converts the atmospheric N gas (N_2) into ammonia (Mus et al., 2016). Utilization of biologically fixed N is more beneficial, poses a significantly lower risk of leaching and water contamination, and is inexpensive compared to inorganic N fertilizer (Stagnari et al., 2017). The N fixed by the leguminous crops is expected to satisfy the N requirement for the grass species that may offer potential environmental benefits (Rosenblueth et al., 2018). The fixed N could be transferred to grass in various ways such as direct transfer through mycorrhizal hyphae, decomposition of dead tissues of legume crop, and root exudation of N from legume roots (Paynel et al., 2001).

Kura clover (KC) (*Trifolium ambiguum* M. Bieb) is an excellent perennial leguminous crop to grow as an intercrop to reduce the fertilizer N requirement of PCG. The desirable properties of KC include the ability to withstand high moisture (flooding) and adaptability to grow well in geographical areas and marginal lands suitable for PCG cultivation (Boe et al., 2013; Kim et al., 2015). In general, grasses are more stress-tolerant than legumes; however, legumes are more productive than grasses, and the N-fixing capacity of the legumes also helps to promote the growth of the associated grass species in mixed pastures (Lauriault et al., 2008). The KC vegetatively establishes new plants by rhizomes or stolons (clone formers), which are usually better adapted to unstable environments because they expand into areas of less competition (Beuselinck et al., 1994). Several studies have documented the advantages of mixing grass species with legume crops in forages and application of N increased yield and quality (Ashworth et al., 2012; Heichel and Henjum, 1991; Zemenchik et al., 2001). However, there is limited information available regarding the impact of intercropping of legume crop and N use with grass species on soil biochemical activities and microbial community structure. Soil health indicators such as soil labile carbon (C) and N pools, microbial biomass, enzyme activities, and microbial community structure evaluate how well soil functions since soil function cannot be measured directly. Soil labile carbon (C) and N, microbial biomass C (MBC), microbial biomass N (MBN) and enzyme activities have been considered sensitive indicators of changes in SOM and biological conditions and are plays an important role in nutrient cycling (Dwivedi and Soni, 2011; Ozlu and Kumar, 2018). Studying the soil biochemical activities and distribution of microbial communities not only helps to explore soil ecological processes, but also have important implications for the management of grassland ecosystems and protection of soil microbial resources (Liu et al., 2019). Soil biochemical activities and microbial communities directly contribute to soil biogeochemical processes and play a key role in soil organic matter (SOM) decomposition, nutrient cycling, and turnover (Sekaran et al., 2018). Some of these microbes, such as N-fixing bacteria and Arbuscular mycorrhizal fungi (AMF) play a predominant role in improving plant mineral nutrition and contribute to SOM decomposition (Graham et al., 2016). Soil enzymes are the primary mediators of organic matter decomposition, release inorganic nutrients into the soil and indicate heterotrophic microbial activity (Fugen et al., 2016). The heterotrophic microbes (bacteria, fungi, and saprophytes) are responsible for the production of various enzymes in the soil which are involved in the biological oxidation of organic matter (dehydrogenase, DHA) and the release of carbohydrates (β-glucosidase), amino acids (arylamidase), orthophosphate ions from organic phosphate esters (acid and alkaline phosphatase), and sulfate-sulfur (arylsulfatase) into the soil (Acosta-Martínez et al., 2008; Margalef et al., 2017).

Thus, we hypothesized that intercropping KC with PCG can increase microbial biomass, labile organic C and N, therefore increasing the activity of enzymes involved in the major nutrient cycling viz. C, N, phosphorus (P), sulfur, and microbial communities and, in-return, improve the soil health. Therefore, the objectives of this study were to quantify the influence of different N fertilization and KC intercropping with PCG on soil biochemical activities and microbial community structure.

2. Materials and methods

2.1. Site description and sampling

The experimental site was established in 2010 at South Dakota State University Felt Research Farm (44° 22′ N; 96° 47′ W) at Brookings, South Dakota (SD), USA. This study site was characterized by a temperate continental type of climatic condition with warm and humid summers and cold winters with a mean annual air temperature of −12.2 °C in the winter and 26.7 to 32.2 °C in the summer. The mean annual precipitation was about 580 mm. Soils of the experimental field belong to moderately well-drained silty clay loam McIntosh soil (Fine-silty, mixed, superactive, frigid Aquic Calciudolls) with pH of 8.31 and electrical conductivity (EC) of 0.38 dS m^{-1}. This site was initiated to study the influence of KC intercropping and application of nitrogen (N) through urea to PCG on biomass yield (Kim et al., 2015). The experiment had randomized complete block design with four replications that had the following treatments: PCG-0N, PCG-75N, PCG-150N, PCG-225N, (0, 75, 150, and 225 N kg ha^{-1}) and PCG-KC (KC intercropping with PCG). Plants of PCG were generated from germplasm collected in SD (natural population) and the KC was an experimental line developed by AgResearch New Zealand. The PCG and KC were transplanted on 60 cm and 30 cm centers in the field in the late spring 2010. KC was inoculated with *R. leguminosarum* biovar *trifolo* strains 162C11, 162C13, and 162C14 mixture in the greenhouse. In the PCG-KC mixture treatment, KC was transplanted on 30 cm centers between and within rows for a total density of 111,111 KC plants ha^{-1}. PCG seedlings were transplanted within the KC on 60 cm centers (populations of 26,896 plants ha^{-1}). Monoculture PCG stands were also established at the same population densities of mixed stands. From 2012 to 2013, 2, 4-D (2, 4-Dichlorophenoxyacetic acid) was applied at rate of 0.6 kg a.i. per hectare to KC treatments to suppress the clover growth. Different levels of N fertilizer were applied as granular urea (46% N) to the PCG monoculture treatments once annually in May from 2011 to 2018. No other mineral fertilizers were applied in all years. The size of the individual plots was 3.0 m wide and 5.7 m long. To determine PCG yield,

after significant senescence entire plot in each treatment was harvested with a sickle-bar mower. To maintain the adequate winter ground cover and rate of regrowth after harvest to reestablish adequate ground cover, the PCG was harvested at a stubble height of about 10-cm once annually. Little or no KC was present in the biomass harvested because most KC was frozen or senesced prior to harvest. For the fresh weight, biomass samples were weighed in the field. Dry matter was determined by collecting a sample of harvested biomass and drying at 60 °C for 72 h in a forced-air oven. The yield was significantly affected by N fertilization rates and intercropping of KC in 2018, where the PCG-KC recorded higher yield than the PCG-0N treatment (data not shown).

After the harvest in November 2018, eight soil samples from random places were collected at 0–5 cm depth using hand scoop shovel from each plot and then pooled to form one composite sample. After removing visible root and plant debris, the soils were ground to pass a 2-mm sieve and thoroughly mixed. The debris free soil samples were placed in a zipper storage bag and placed on ice. These samples were kept fresh and stored in a refrigerator at 4 °C pending analysis (soil microbial biomass and enzyme activities). All the microbial parameters were analyzed within 2–3 weeks of sampling. Part of composite samples was stored at −80 °C for phospholipid fatty acid (PLFA) analysis. Soil physicochemical characterization was carried and these data included a set of soil laboratory measurements (e.g., pH, EC, nitrate-nitrogen (NO_3^-N), phosphorus (P), potassium (K), calcium (ca), and magnesium (Mg)). For analysis of pH, soil samples were air dried and ground to pass through a 2-mm sieve, and pH (1:2.5 soil/water) was determined using the procedure given by McLean (1982).

2.2. Agronomic efficiency and fertilizer urea replacement value for KC

Agronomic efficiency (AE) of N (kg kg^{-1}) was determined using the following equation: AE (kg kg^{-1}) = (yield in N fertilized plot [kg ha^{-1}] − yield in control plot [kg ha^{-1}]) / rate of fertilizer N applied (kg). The highest AE (21.1 kg kg^{-1}) was obtained from PCG-225N followed by PCG-150N (18.7 kg kg^{-1}) and lowest AE was obtained for PCG-75N (11.9 kg kg^{-1}). The AE of applied N increased with the increasing levels of N. However, the difference between the AE of PCG-150N and PCG-75 N was higher (6.37 kg kg^{-1}) compared to PCG-225N and PCG-150 N (2.33 kg ha^{-1}). The AE was increased with N application; however, PCG-150N had the greatest AE than PCG-225N and PCG-75N (data not shown).

A linear regression equation was obtained to calculate the fertilizer replacement value of KC by the statistical evaluation of the dependence of the yield on fertilizer N applied (Y = 0.0215x + 7.189, where, y is yield [Mg ha^{-1}]). The mean yield of PCG-KC mixture was substituted for Y in the regression model, and then solved for X (fertilizer replacement value of KC). The fertilizer replacement value of KC obtained from this present study was 149.8 kg ha^{-1}.

2.3. Soil C and N and microbial biomass

The content of water extractable organic C and N fractions was carried out by the schematic procedure described by Ghani et al. (2003). TOC-L analyzer (Shimadzu Corporation, model-TNM-L-ROHS) was used to determine the cold-water and hot-water C (CWC and HWC) and N (CWN and HWN) fractions. Chloroform fumigation direct extraction method was used to determine the MBC and MBN in soil (Anderson and Domsch, 1978; Gregorich et al., 1990). The MBC and MBN were calculated by the difference between C and N in the fumigated and non-fumigated samples and with a correction factor of 0.45 (Beck et al., 1997). Nitrogen fertilization rates and intercropping of KC did not show any significant impact on soil organic carbon (SOC) in 2018 (data not shown). However, N fertilization rates and intercropping of KC in 2018 significantly affected TN, with PCG-KC recorded significantly higher soil TN than PCG-0N (data not shown).

2.4. Enzyme assays

The DHA (EC 1.1.1.1) enzyme activity was assayed by using 1 g of soil, and the iodonitrotetrazolium formazan (INTF) in the samples was measured by a modification of the method reported by Benefield et al. (1977) and expressed as μg INTF g^{-1} dry soil h^{-1}. Fluorescein diacetate (FDA), arylamidase (EC 3.4.11.4), and urease (EC 3.5.1.5) enzyme activities were assayed by the method of Green et al. (2006), Acosta-Martínez and Tabatabai (2000) and Kandeler and Gerber (1988), respectively. The β-Glucosidase (EC 3.2.1.21) enzyme activity was assayed by the method of Eivazi and Tabatabai (1988), using the substrate 50 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG) and expressed as μmol *p*-nitrophenol (pNP) released g^{-1} soil h^{-1}. Acid (EC 3.1.3.2) and alkaline (E.C.3.1.3.1) phosphatase and arylsulfatase (EC 3.1.6.1) enzyme activities were determined as described by Eivazi and Tabatabai (1977) and Tabatabai and Bremner (1970), respectively, and the activity was reported as μg pNP g^{-1} soil h^{-1}.

2.5. Microbial community structure

Microbial community structure was assessed by analyzing the phospholipid fatty acids (PLFA) in the soil samples. Subsamples were analyzed for PLFA at Ward Laboratories, Inc. (Lincoln, NE). Individual fatty acids have been used as signatures for various functional groups of microorganisms (Bardgett et al., 1999; Bossio et al., 1998; Grayston et al., 2001; Pankhurst et al., 2002; Yao et al., 2000). Each PLFA and the sum of all PLFAs are expressed as ng PLFA-C g^{-1} soil.

2.6. Glomalin-related soil protein (GRSP) analysis

GRSP content was determined according to the method of Wright and Upadhyaya (1998). Twenty-four milliliter of 20 mM sodium citrate (pH 7.0) was added to each tube containing 3.0 g of air-dried soil sample and mixed well. The tubes were autoclaved at 121 °C (15 psi) for 30 min. After autoclaving, the tubes were cooled and centrifuged (10,000 × *g*). Protein in solutions was assayed using the Pierce BCA protein assay kit (Thermo Scientific, IL, USA). Absorbance was measured at 562 nm in a spectrophotometer and the absorbance of the reagent and sample blank was subtracted. Protein concentration was calculated by comparing absorbance values of samples to a standard curve of 0 to 2000 μg ml^{-1} of bovine serum albumin and GRSP are expressed as mg g^{-1} of dry soil.

2.7. DNA extraction and PCR amplification of nifH

A 0.25 g aliquot of soil was used for DNA extraction using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's method. The *nifH* PCR was performed using primer sets of PolF (TGCGAYCCSAARGCBGACTC, Position 115–134) and PolR (ATSGCCATCATYTCRCCGGA, Position 457–476) (Poly et al., 2001). PCR was performed with a 30.0 μl volume containing the following: 3.0 μl taq buffer, 2.5 μl of $MgCl_2$, 0.9 μl of DNTP's, 0.9 μl of PolF and PolR primers, 0.2 μl of Taq polymerase, and 1.0 μl of template. PCR program was modified as follows: 95° for 2 min, 30 cycles of 95° for 15 s, 55° for 1 min, 72° for 30 s, and an elongation step of 72° for 5 min. To visualize the PCR products, 10 μl of the reactions were loaded onto a 50 ml, 1% agarose gel with 1 μl dye (Dahal et al., 2017). Gel ran for 45 min at 130 V and were then visualized and photographed.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was conducted to compare the effects of different treatments on soil biological parameters using the SAS Version 9.4 (SAS, 2013). Data was transformed when necessary using the Box-Cox method. Significance was determined at $\alpha = 0.05$ (McLean, 1982). Principal component analysis (PCA) was conducted by

Table 1
Effects of different treatments on soil physicochemical properties.

Treatments	pH	EC	NO_3-N	Olsen-P	Potassium	Calcium	Magnesium
		dS m^{-1}			mg kg^{-1}		
PCG-KC	8.17[a†]	0.210[a]	16.7[a]	3.67[a]	103[a]	3022[a]	1086[a]
PCG-0 N	8.34[a]	0.187[ab]	1.67[b]	4.33[a]	101[a]	3009[a]	1823[a]
PCG-75 N	8.22[a]	0.175[b]	2.67[b]	4.00[a]	94.7[a]	3059[a]	1564[a]
PCG-150 N	8.14[a]	0.188[ab]	3.67[b]	4.00[a]	128[a]	3293[a]	1089[a]
PCG-225 N	8.28[a]	0.184[ab]	5.00[b]	4.33[a]	102[a]	3187[a]	974[a]
	Analysis of Variance ($P > F$)						
Treatments	0.346	0.032	0.001	0.934	0.131	0.826	0.511

[†]Mean values within the same column followed by different small letters are significantly different at $p < 0.05$ for treatment. Note: PCG, prairie cordgrass; KC, kura clover; EC, electrical conductivity; NO_3-N, nitrate nitrogen; Olsen-P, Olsen-Phosphorus.

the PRINCOMP procedure of SAS 9.4, using the mean values of measured soil parameters (SAS, 2013). PCA is a multivariate statistical tool used to separate experimental units into subgroups based on the measured parameters by producing the loadings of these parameters (termed as eigenvector) and PC scores for each unit. The magnitude and direction of the eigenvector loadings were used to associate soil properties with the treatments (Johnson, 1998). The relationship between soil properties, biochemical activities, and microbial communities was determined by Pearson's correlation matrix using SAS version 9.4.

3. Results

3.1. Soil physicochemical properties

Soil physicochemical properties of all treatments were summarized in Table 1. Compared with N fertilized treatments, soil NO_3^--N content was significantly increased under PCG-KC intercropping treatment. The PCG-KC treatment had a significantly higher EC compared to PCG-75N treatment. No significant differences were found in soil pH, available P, K, Ca, and Mg content between intercropping and N fertilizer treatments.

3.2. Soil C and N fractions, and microbial biomass

Data for CWC, CWN, HWN, MBN, and HWC and MBC under different treatments are shown in Fig. 1. The effect of the application of different N fertilizer levels and intercropping of KC on CWC was significant. The CWC content varied from 15.2 to 21.1 μg C g $soil^{-1}$. Compared to PCG-75N and PCG-0N fertilization, the PCG-KC combination significantly increased the CWC content. However, no significant differences were observed between treatments for CWN, HWC, and HWN. The PCG-KC treatment increased the MBC by 90 and 55% compared with the PCG-225N and PCG-75N, respectively. The PCG-KC treatment significantly increased soil MBN concentration more than all other treatments except PCG-0N treatment.

3.3. Soil enzymatic activities

Data for soil enzyme activities of the different treatments are presented in Table 2. DHA enzyme activity was increased under PCG-KC treatment compared to PCG-0N, PCG-150N, and PCG-225N treatments. The arylamidase activity was higher with PCG-KC and PCG-150N treatments compared to PCG-225N treatment. The PCG-KC significantly increased the urease enzyme activity by 81 and 41% compared with the PCG-0N and PCG-75N, respectively. The N application gradually increased the urease activity from PCG-0N to PCG-225N. The PCG-KC and PCG-150N treatments resulted in significantly higher β-glucosidase enzyme activity than in the PCG-225N treatment ($p < 0.05$). Acid phosphatase enzyme activity under treatment PCG-KC was 2.5, 2.1, 1.8, and 1.6 times higher than PCG-0N, PCG-225N, PCG-75N, and PCG-150N treatments, respectively. Compared with PCG-0N and PCG-75N, alkaline phosphatase enzyme activity increased by 37 and 32% under PCG-150N, respectively. The treatment PCG-KC increased the arylsulfatase enzyme activity by 75, 52, and 44% compared with the PCG-0N, PCG-75N, and PCG-225N treatments, respectively.

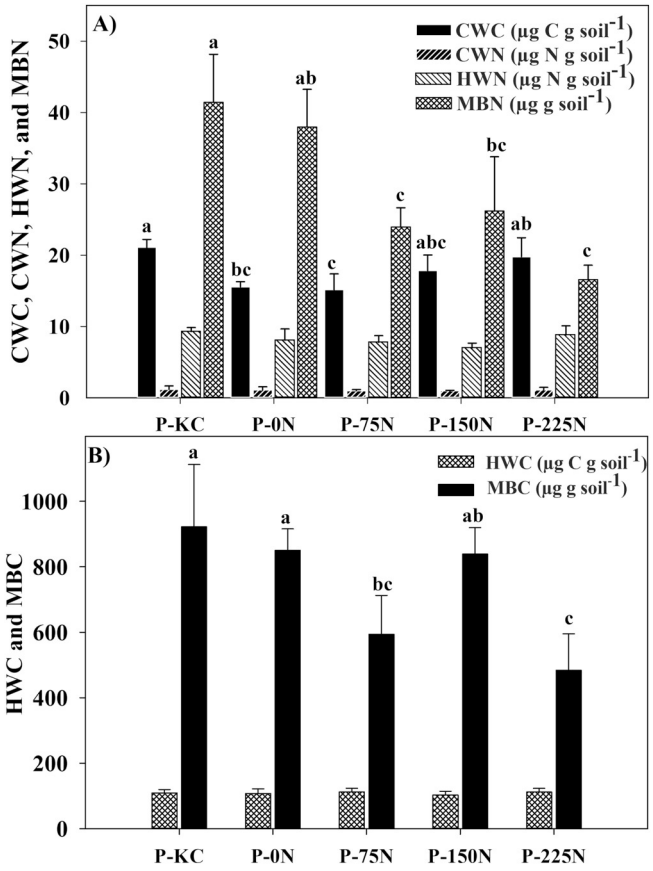

Fig. 1. (A) Cold water soluble organic carbon (CWC) and nitrogen (CWN), hot water soluble organic N (HWN), and microbial biomass N (MBN) and (B) hot water soluble organic C (HWC), and microbial biomass C (MBC) concentrations influenced by various treatments. Note: P-prairie cordgrass; KC: kura clover; N: nitrogen rates (0, 75, 150, and 225 kg ha^{-1}). Small letters represent the significant differences between treatments at $p < 0.05$.

3.4. Soil microbial community structure

We performed PLFA analysis to investigate the effects of KC and N fertilization on microbial community structure. We observed treatment-induced changes in some key microbial communities among the treatments (Tables 3a and 3b). Introducing KC with PCG increased the

Table 2
Response of soil enzyme activities to different nitrogen rates and kura clover (KC) intercropping in prairie cordgrass (PCG).

Treatments	DHA	FDA	Arylamidase	Urease	β-glucosidase	Acid P	Alkaline P	Arylsulfatase
	μg INTF g^{-1} dry soil h^{-1}	mg fluorescein kg^{-1} dry soil ha^{-1}	μg β-naphthylamine g^{-1} dry soil ha^{-1}	μg N-NH_4^+ g^{-1} soil h^{-1}	μmol pNP kg^{-1} soil h^{-1}	μg pNP g^{-1} soil h^{-1}		
PCG-KC	2.09^{a†}	0.152	18.6^{a}	2.39^{a}	14.5^{a}	249^{a}	714^{ab}	600^{a}
PCG-0 N	1.42^{b}	0.178	15.5^{ab}	1.32^{c}	8.09^{b}	101^{b}	583^{b}	343^{c}
PCG-75 N	1.56^{ab}	0.162	14.3^{ab}	1.69^{bc}	10.5^{ab}	139^{b}	605^{b}	396^{c}
PCG-150 N	1.44^{b}	0.133	18.2^{a}	2.02^{ab}	15.9^{a}	154^{b}	801^{a}	541^{ab}
PCG-225 N	1.48^{b}	0.143	11.1^{b}	2.49^{a}	7.13^{b}	121^{b}	706^{ab}	417^{bc}
	Analysis of Variance ($P > F$)							
Treatments	0.02	0.07	0.01	0.001	0.001	< 0.0001	0.02	0.0004

†Mean values within the same column followed by different small letters are significantly different at $p < 0.05$ for treatment. Note: DHA, Dehydrogenase; FDA, Fluorescein Diacetate; Acid P, Acid Phosphatase; Alkaline P, Alkaline Phosphatase.

Table 3a
Response of Total, total bacterial, actinomycetes, gram-negative (-ve) bacterial, gram-positive (+ve) bacterial, total fungi, and Arbuscular mycorrhizal fungi (AMF) biomass PLFAs to different nitrogen rates and kura clover (KC) intercropping in prairie cordgrass (PCG).

Treatments	Total	Total Bacterial	Actinomycetes	Gram (-ve)	Gram (+ve)	Total Fungi	AMF
	ng PLFA-C g^{-1} soil						
PCG-KC	12748^{a†}	6641^{a}	1018^{a}	3524^{a}	3117^{a}	2119^{a}	910^{a}
PCG-0 N	10004^{b}	5106^{b}	904^{ab}	2773^{ab}	2333^{b}	1809^{ab}	764^{ab}
PCG-75 N	9574^{b}	4992^{b}	843^{b}	2718^{ab}	2274^{b}	1707^{ab}	683^{b}
PCG-150 N	10480^{ab}	5417^{ab}	909^{ab}	2817^{ab}	2600^{ab}	1880^{ab}	860^{ab}
PCG-225 N	9593^{b}	5073^{b}	924^{ab}	2557^{b}	2516^{ab}	1579^{b}	740^{ab}
	Analysis of Variance ($P > F$)						
Treatments	0.01	0.02	0.01	0.03	0.01	0.02	0.02

†Mean values within the same column followed by different small letters are significantly different at $p < 0.05$ for treatment

overall soil microbial community compared to the N fertilization. PCG-KC treatment significantly increased the mean abundance of total PLFA, and total bacterial PLFA compared to PCG-0N and PCG-225N. The mean abundance of actinomycetes was increased by 21% under PCG-KC treatment compared with PCG-75N. Compared to PCG-225N, PCG-KC treatment significantly increased the gram-negative bacterial concentration. A decrease in the gram-negative bacterial composition was observed with an increase in N fertilization. The concentration of gram-positive bacteria was 1.33 and 1.37 times higher than the PCG-0N and PCG-75N, respectively. Data showed that the treatment PCG-KC increased the total fungi PLFA concentration significantly compared to the PCG-225N treatments. The concentration of AMF was increased by 33% with PCG-KC treatment compared with the PCG-75N. Similarly, PCG-KC treatment increased the saprophytes biomass 44%, compared with PCG-225N treatment. With regard to protozoa biomass, PCG-0N treatments significantly increased their concentration by 32% compared with the PCG-225N treatment. The undifferentiated PLFA biomass concentration of PCG-KC was 42, 37, 33, and 28% higher as compared to that of PCG-75N, PCG-225N, PCG-0N, and PCG-150N, respectively. However, predator:prey ratio was significantly higher with PCG-0N than the PCG-225N. PCG-225N treatment significantly increased the gram-negative: gram-positive bacterial ratio compared to PCG-75N and PCG-0N treatments.

3.5. GRSP content

The content of GRSP was in the range of 2.90 to 11.9 mg g^{-1} (Fig. 2). GRPS content increased in the order of PCG-0N < PCG-75N < PCG-225N < PCG-150N < PCG-KC. Among the treatments, PCG-KC significantly increased GRSP content (11.9 mg g^{-1}) compared to all other treatments. In our study, the GRPS content in soil increased first (0 to 150 kg N) and decreased with increasing N level (at 225 kg N) and reached the peak level 9.27 mg g^{-1} at 150 kg N.

Table 3b
Response of saprophyte, protozoa, undifferentiated biomass PLFAs, fungi-bacteria, predator:prey, and gram-negative: gram-positive bacteria ratio to different nitrogen rates and kura clover (KC) intercropping in prairie cordgrass (PCG).

Treatments	Saprophyte	Protozoa	Undifferentiated	Fungi: Bacteria Ratio	Predator:Prey Ratio	Gram (+ve): Gram (-ve) Ratio
	ng PLFA-C g^{-1} soil					
PCG-KC	1209^{a†}	221^{ab}	3768^{a}	0.32^{a}	0.034^{b}	0.89^{ab}
PCG-0 N	1045^{ab}	250^{a}	2839^{b}	0.36^{a}	0.050^{a}	0.85^{b}
PCG-75 N	1024^{ab}	217^{ab}	2658^{b}	0.34^{a}	0.044^{ab}	0.84^{b}
PCG-150 N	1020^{ab}	231^{ab}	2952^{b}	0.35^{a}	0.043^{ab}	0.92a^{b}
PCG-225 N	839^{b}	190^{b}	2750^{b}	0.31^{a}	0.037^{b}	0.99^{a}
	Analysis of Variance ($P > F$)					
Treatments	0.02	0.07	0.003	0.10	0.01	0.002

†Mean values within the same column followed by different small letters are significantly different at $p < 0.05$ for treatment.

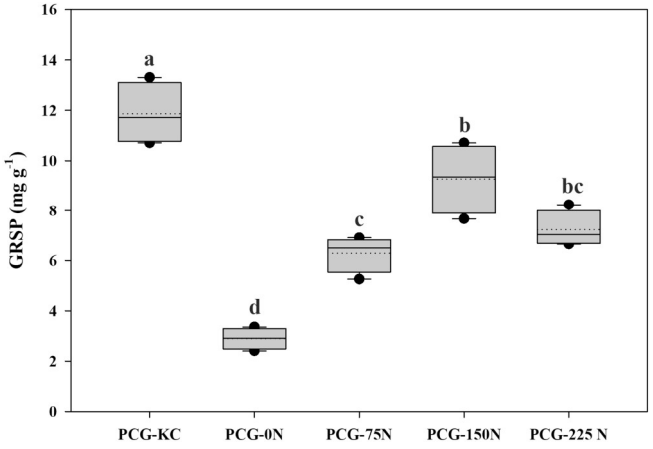

Fig. 2. Glomalin related soil protein (GRSP) content influenced by various treatments. Note: PCG-prairie cordgrass; KC: kura clover; N: nitrogen rates (0, 75, 150, and 225 kg ha^{-1}). Small letters represent the significant differences among treatments at $p < 0.05$.

3.6. PCA

PCA was used to identify the variation of soil biochemical properties and microbial communities. The first two PC scores explained 90% variability among the treatments based on the measured soil biochemical properties and microbial communities and other PCs are explained below 10% variability. PC1 is highly correlated with total PLFA, total bacterial PLFA, AMF PLFA, β-glucosidase, and arylsulfatase. Explanation of variability in this axis can thus be related to the variability of available substrate and other soil environmental conditions. PC2 correlates fairly positively with urease enzyme activity and CWC; and negatively correlates with MBC, MBN, and arylamidase enzyme activity. Overall, the PCA showed that the PCG-KC and PCG-150N treatments have a significant influence on CWC, MBC, MBN, arylamidase, urease, β-glucosidase, arylsulfatase, total PLFA, total bacterial PLFA, AMF, and GRSP content (Fig. 3). PCA reveals that the biochemical nature of soil and they were different in N fertilizer and KC intercropping treatments. The majority of the soil biochemical properties and microbial community structure exhibited good association with PCG-KC and PCG-150N, showing greater abundance along PC1. The PCA signifies the importance of the optimum dose of N fertilization and intercropping of KC with PCG on soil microbial properties.

3.7. Estimation of rhizobial abundance

We used a well-established *nifH* PCR assay to evaluate rhizobia abundance (Fig. 4). As expected, a clear presence of rhizobia was detected in PCG-KC treatment soils. The presence of a legume in this treatment is expected to enrich the soil with rhizobia. Among the different N treatments, a clear reduction in *nifH* amplicon intensity was observed with increasing amounts of N applied (Fig. 4). At 225 kg N ha^{-1}, *nifH* was below detectable limits suggesting very low rhizobia abundance.

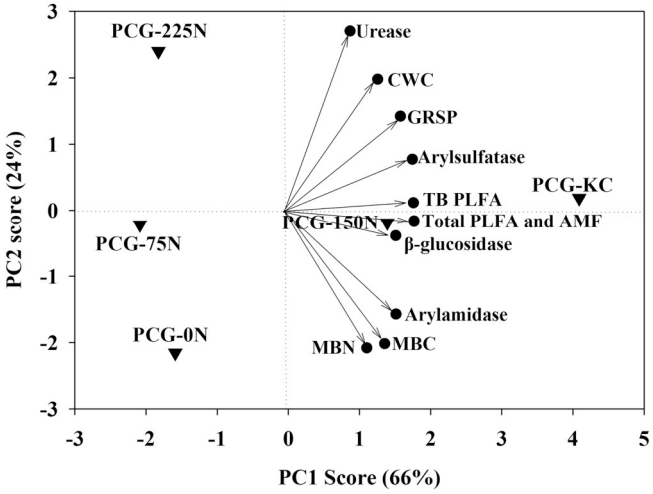

Fig. 3. Principal component (PC) analysis scores (1 and 2) for soil biological parameters as determined between different treatments. The eigenvectors (of PC1 and PC2) of the soil biological characteristics (circle) are also superimposed with the PC scores biplot at a similar scale reflecting their association. The eigenvectors were multiplied by five to obtain a clear and superimposed figure. Note: PCG-prairie cordgrass; KC: kura clover; N: nitrogen rates (0, 75, 150, and 225 kg ha^{-1}); PLFA: phospholipid fatty acid; AMF: Arbuscular mycorrhizal fungi; CWC: cold-water soluble organic carbon; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen; GRSP: glomalin related soil protein.

4. Discussion

Intercropping is an environmentally friendly method that plays an important role in maintaining soil biodiversity and stability, improving nutrient content, and controlling pests and disease occurrence (Dai et al., 2019). Previous papers showed that intercropping improved soil physicochemical properties (Bedoussac and Justes, 2010; Liu et al., 2014). We found that intercropping of KC with PCG did not significantly influence the soil physicochemical properties such as soil pH, available P, K, Ca, and Mg content. However, soil NO_3^--N increased under intercropping treatment, which may be caused by the biological atmospheric N fixation by the KC plants (Kermah et al., 2018).

In this present study, significant increase in labile C (CWC), MBC, and MBN were observed under PCG-KC, suggesting that intercropping KC with PCG had beneficial effects on soil microbial activity, probably due to the supply of readily available C substrates, through root exudates and SOM, and availability of mineral N through N-fixation (Yang et al., 2012). The differences in microbial biomass in the PCG-KC relative to other N fertilizer treatments may be attributed to higher levels of organic compounds. The SOM decomposition mostly depends on the quantity and quality of organic matter added. The fibrous rooting system under PCG and dense rhizomatous root system in KC might have supplied a significant proportion of the C through the process mediated in the rhizosphere. Perennial plants offer significantly greater C inputs than annual crops due to their prolonged photosynthetic activity and greater root biomass (Marshall et al., 2016). Elgharably and Marschner (2011) also observed an increase in soil microbial biomass with

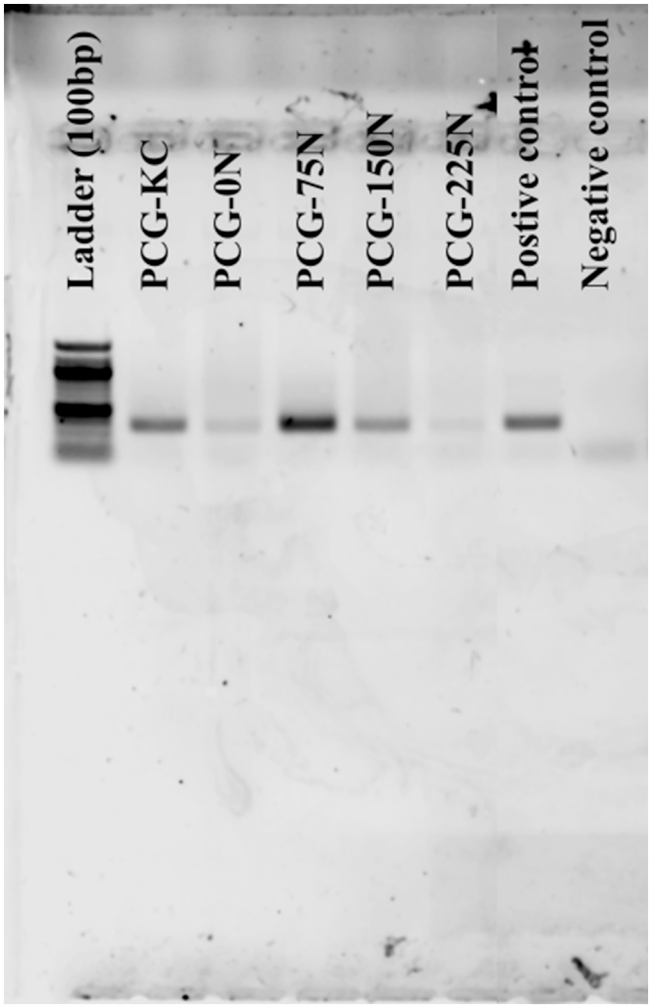

Fig. 4. Electrophoretic pattern of the PCR products obtained using primers for the nifH gene under different treatments. Note: Positive control is USDA 110 (*bradyrhizobium diazoefficiens*), PCG-prairie cordgrass; KC: kura clover; N: nitrogen rates (0, 75, 150, and 225 kg ha^{-1}).

increasing residue addition. It has been a widely accepted fact that the addition of SOM markedly increases the labile C and microbial biomass directly or indirectly (Gong et al., 2009), which is consistent with our present findings.

The content of MBC and MBN in PCG-KC was 90 and 45% higher than PCG-225N, respectively. Nutrients through mineral fertilizer may meet the mineral nutrition demand of the microbes, but not that of C, which is a major component of microbial cells and substrates (Mohammadi et al., 2012). The increased level of N might have reduced the amount of root exudates released to the soil, which contributes significantly to promoting soil microorganisms (Golchin et al., 1994), and consequently resulted in the reduction of MBC and MBN under the high N fertilization rate. Our results were in agreement with previous findings in the grasslands in the USA, Europe and China that increased level of N fertilization, and decreased MBC and MBN due to the reduced root exudation (Huang et al., 2008; Sekaran et al., 2018; Yang et al., 2012).

There were more profound effects of intercropping of KC with PCG on the production of enzyme activities compared to the N fertilization. The increased heterotrophic microbes (bacteria, fungi, and saprophytes) are responsible for the production of various enzymes in the soil. In general, leguminous crops contain a low C:N ratio and easily available compounds, such as carbohydrates and amino acids, which can stimulate the microbial activity and increase their population (Dinesh et al., 2004; Piotrowska-Długosz and Wilczewski, 2014). Increased DHA enzyme activity under PCG-KC mixture might be linked to more substrate availability for microbes in the soil, which in turn helped enzyme production and release into the soil. This is consistent with Herencia (2015), who found that soils with higher levels of crop residues increased DHA activity more than the low-residue soils. According to Marschner et al. (2003), enzymes excreted by microorganisms are required to degrade the SOM to obtain nutrients. Increased β-glucosidase and urease activity with N fertilization and KC incorporation with PCG suggest that decomposition of organic C and hydrolysis of urea are limited by N addition, whereas external N addition through urea and N-fixation (*Rhizobium* sp.) through KC overcame these limitations and stimulated the microbes to utilize organic C and hydrolyze urea. Cellulose represents the significant proportion of plant residues returned to the soil in the grassland, and the production of β-glucosidase enzyme is therefore very important for hydrolysis of cellobiose to glucose. Higher activity of the β-glucosidase enzyme could be related to higher cellulose-rich substrates pool available for decomposition (Sinsabaugh and Shah, 2011). Mergel et al. (1998) showed that application of N stimulated the production of C containing compounds in soils, compounds that originate from plant-root decomposition and root exudates secretion. We also explain this positive influence of N fertilization on β-glucosidase and urease enzyme activity by the fact that the activity of C and N acquiring enzymes tends to respond to changes in substrate availability (Allison and Vitousek, 2005).

Increased activity of acid phosphatase enzymes under PCG-KC compared to all other N fertilization treatments could be responsible for the hydrolysis of organically bound phosphate ions and release the available P to the soil environment (Mohammadi et al., 2012). Arbuscular Mycorrhizal fungi (AMF) in soil mainly involved in the production of phosphatase enzymes (Hallama et al., 2019). PCG-KC combination was most successful in increasing soil AMF abundance and they also might have enhanced the production of higher phosphatase enzyme activity. Zhang et al. (2011) also observed that phosphatase activities were higher with AMF-colonized plants than non-colonized plants. In the present study, intercropping KC with PCG enhanced the soil enzymatic activities, which implies that the PCG-KC mixture is well suited to increase the microbial activity and soil enzymatic production.

We performed PLFA profiling of soil microbial community structure as an indicator of soil metabolic health. We observed significantly higher microbial biomass structure (PLFA) in PCG-KC samples compared to the N fertilization (Tables 3a and 3b), suggesting that the PCG-KC mixture is more supportive for microbial community structure, likely resulting from higher levels of labile soil C and enzyme activities. PCG-KC mixture significantly increased total, total bacterial, actinomycetes, Gram-negative and Gram-positive bacterial, total fungi, AMF, saprophytes, and undifferentiated PLFAs. The increase in soil microbial community biomass observed in the PCG-KC treatment may be because of the addition of easily biodegradable compounds in the organic material and higher availability of labile C, which stimulate the activity of soil microbes (Franco-Andreu et al., 2017). The plant residue of the legume crop is N-enriched and readily decomposed (Zhao et al., 2014). In general, legume crops benefit the bacterial-mediated decomposition more than the fungi-mediated decomposition due to more accessibility of N enriched plant litter to bacteria than fungi (Viketoft et al., 2009). However, both fungi and bacterial biomass were increased with PCG-KC mixture and the ratio of fungi to bacteria was not significantly affected by planting system and N fertilization in the present study. Therefore, the PCG-KC mixture may not only affect the bacterial-mediated decomposition but also fungi-mediated decomposition. The PCG-KC mixture induced enhancements in both fungi and bacteria might be due to the accessibility of N-riched plant residues. Another possible reason might be that the N-enriched plant residue is more accessible to bacteria and increases the bacteria-mediated decomposition pathway activity, which may accelerate the fungi-mediated decomposition pathway through supplying resource input of recalcitrant

substrates such as cellulose, hemicellulose, lignin, and tannins (Zhao et al., 2015). Chai et al. (2005) reported that legume mixed culture significantly increased the soil microbial biomass and diversity.

In the present study, plant species had a stronger influence on the soil microbial community structure, emphasizing the importance of plant species and their rhizodeposition on soil microbes. It is shown that different plant species can influence the microbial community and its composition due to variations in the quality and quantity of root exudates (Söderberg et al., 2002). Bardgett et al. (1998) and Wardle et al. (1999) have reported that activity, composition, and abundance of soil decomposer communities may markedly vary with specific functional plant groups such as legumes or different plant species. Leguminous crops positively affect the microbial community due to high plant residue quality, that is, plant residues with lower C/N ratio (Dinesh et al., 2004; Piotrowska-Długosz and Wilczewski, 2014). Many experiments on monoculture versus mixed plant species have demonstrated that plant mixtures can improve soil nutrient cycling and soil fertility and produce higher plant biomass (Forrester et al., 2004; Montagnini, 2000). The increase in plant biomass in mixed cropping may have a strong influence on soil microbial communities. Microbial community composition for PCG-KC mixture differed from that of PCG-0N and PCG-225N, which could be due to lower root C/N ratio of the legumes and greater root exudation rates of legume crop. This increase in soil microbial community biomass associated with legume cropping is consistent with previous studies using legumes in legume-grass intercropping (Piotrowska-Długosz and Wilczewski, 2014; Zhao et al., 2015). Meimei et al. (2008) describe significant differences in soil microbial community biomass with the legume crop compared to monocrop grass species. Jesus et al. (2010) have also observed a differentiated bacterial community according to the species present. This suggested that PCG in combination with KC (PCG-KC) and N fertilization released a greater amount of assimilated N and C into the soil, through N fixation and either as root exudates or dead root cells and supported the growth of soil microorganisms. The abundance of AMF in PCG is not surprising as several of the prairie grasses are known to be mycorrhizal fungi network dependent (Koziol and Bever, 2017; Sekaran et al., 2018). The greater microbial biomass in soil with PCG-KC mixture at no N fertilizer suggested that a plant mixture of PCG-KC would be able to maintain more favorable soil environmental conditions for soil microbes than the application of N fertilizer to PCG.

In this work, GRSP concentration was significantly higher in PCG-KC treatment than all other N fertilization treatments. Such different results may be attributed to the long-term incorporation of PCG-KC combination influencing AMF populations and the addition of N through N fixation, and as a consequence increased GRSP content in the soil. AMF is responsible for the production glomalin through its hyphal and spore walls (Driver et al., 2005) and the activity of fungal community can be considered related to the quantity of GRSP content in soil (Bedini et al., 2007). The variation in GRSP content in soils due to the N addition mainly attributed to the changes in soil microbial activities and plant growth (Xie et al., 2015). Application of 150 kg N to PCG might have increased soil microbial activities and plant growth and inhibited at 225 kg N. Sun et al. (2018) found that the highest GRSP contents were not obtained at the highest amount of N fertilizer. A high level of N fertilization can quickly satisfy the N limitation in the soil and increase the microbial activities at the time of application. At a later stage, high-level N application may cause N saturation in the soil and inhibit the activities of microorganisms that decrease the production of GRSP content (Yu et al., 2013). However, the GRSP content under high N level was higher than that under low N level. Our results indicated that intercropping of KC with PCG could increase the GRSP contents, but a high level of N addition inhibited the GRSP contents in the soil.

Rhizobial abundance in the soil is an indication of soil health and ability to provide biological nitrogen fixation. Rhizobia bacteria can survive in soils as saprophytes, in association with roots of non-legumes, or in symbiotic association with legumes in root nodules (Zahran, 1999). The presence of rhizobia can be evaluated by polymerase chain reaction (PCR) amplification of *nifH* gene fragment from soil DNA preparations (Gaby and Buckley, 2012). This gene encodes an essential component of nitrogenase, the enzyme responsible for N fixation, and shows high degree of conservation in DNA sequence. In agreement with previous observations (Depret et al., 2004; Yan et al., 2014), this indicated that excessive amounts of N reduced the abundance of rhizobia in soil. Interestingly, *nifH* was not detectable by PCR in the no-exogenous-N treatment either (Fig. 4). This treatment also had the lowest levels of GRSP content suggesting both rhizobia and AMF were poorly abundant under N deficiency. This is likely due to the lack of well-established host plants for these symbionts in N deficient conditions. The no-exogenous-N treatment had total available C that was similar to or higher than other N treatments. This was reflected in the total microbial abundance which was also similar in other N treatments. Therefore, reduced symbiont abundance in no-exogenous-N treatment appears to be a specific effect of soil N deficiency on rhizobia ales fungi.

The PCA summarized the study and placed N fertilization treatments and KC treatment on the opposite side of the quadrants except for PCG-150N treatment. Application of PCG-150N and intercropping of KC with PCG had a higher degree of similarity followed by the application of PCG-0N and PCG-75N treatments. These results exactly demonstrated that the intercropping of KC with PCG has more influences on soil microbial biomass, enzyme activities, and microbial community structure than N fertilizer treatments except PCG-150N. Intercropping of KC with PCG and application of 150 kg N to PCG might have created relatively stable environment resulting in more microbial activity, which in turn enhanced the labile C, microbial biomass, soil enzymatic activities, microbial community structure, and GRSP content that are considered good indicators of soil health. Our results show that biochemical and microbial community structure respond positively to long-term N addition (especially at PCG-150N) and intercropping of KC to PCG. PCG-KC was able to yield comparable or better soil health parameters as 150 kg N fertilizer application. This positive response may be explained by the fact that increased accumulation of SOM, biological N fixation, and root exudate secretion may provide a variety of C and N substrates which support the microbial activity and enzyme production in soil (Franco-Andreu et al., 2017).

5. Conclusions

The current study examined the response of soil biochemical properties and microbial community structure to PCG-KC mixture and N fertilization to PCG. PCG-KC mixtures appear to be good candidates for biofuel feedstock production and improving soil biological conditions for better plant growth. Intercropping KC with PCG showed good potential for producing higher soil enzymatic activities and GRSP production than that of PCG monocultures with different N fertilization. This implies that the PCG-KC mixture created a more favorable environment for the growth of plant roots and soil microorganisms that secrete enzymes into the soil. Thus, long-term maintenance of PCG-KC mixture can supply the N requirement through N fixation and improve soil biochemical activities and microbial community structure which are sensitive indicators of soil health. The N-fixing bacteria (rhizobia) were increased under PCG-KC and N fertilization (particularly, 75N and 150N) than PCG-0N and PCG-225N. Overall, N application also had a greater impact on soil labile C, biochemical activities, and soil microbial community structure, especially at 150N. However, when considering the potential negative impact and input cost of mineral N fertilizer, PCG-KC mixtures are desirable over N fertilizer application. More studies are required to optimize the PCG-KC mixtures for the improvement of soil biological health. Also, further studies should investigate the influence of PCG and KC mixtures on soil microbial composition at the gene level to explore the specific activities of microorganisms.

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Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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