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Diversified Cover Crops and No-Till Enhanced Soil Total Nitrogen and Arbuscular Mycorrhizal Fungi Diversity: A Case Study from the Karst Area of Southwest China

Lihua Tian [1], Tao Wang [2], Song Cui [3], Yuan Li [4], Weiyang Gui [1], Feng Yang [5], Jihui Chen [1], Rui Dong [1], Xinyao Gu [1], Xuechun Zhao [1], Mingjun Zhang [6], Chao Chen [1] and Zhou Li [1,*]

[1] Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, College of Animal Science, Guizhou University, Guiyang 550025, China; TIAN7huahua@163.com (L.T.); wygui@gzu.edu.cn (W.G.); jhchen3@gzu.edu.cn (J.C.); rdong@gzu.edu.cn (R.D.); guxy@gzu.edu.cn (X.G.); xczhao@gzu.edu.cn (X.Z.); chenc@gzu.edu.cn (C.C.)
[2] Rapeseed Research Institute, Guizhou Academy of Agricultural Sciences, Guiyang 550008, China; wangtao10062023@163.com
[3] School of Agriculture, Middle Tennessee State University, Murfreesboro, TN 37132, USA; song.cui@mtsu.edu
[4] Grasslands and Sustainable Farming, Production Systems Unit, Natural Resources Institute Finland, Halolantie 31A, FI−71750 Kuopio, Finland; yuanli@lzu.edu.cn
[5] Guizhou Grassland Technology Experiment and Extension Station, Guiyang 550025, China; fengygzu@163.com
[6] Livestock and Poultry Genetic Resources Management Station of Guizhou Province, Guiyang 550025, China; mingjunzhang23@163.com
* Correspondence: zli8@gzu.edu.cn; Tel.: +86-19391112451

Abstract: The deteriorating soil health under continuous monoculture is commonly found across various cropping systems. This study evaluated the effects of different tillage practices (conventional tillage and no till) and species mixtures (legumes and grasses) on arbuscular mycorrhizal fungi (AMF) community properties, soil nutrients, and enzyme activity in a 3-year experiment. Compared with traditional tillage, the number of AMF species under no-till conditions was increased, with the *Glomus* group being dominant. Under different tillage conditions, TN (total N) and AN (available N) contents under no till were significantly higher than those under conventional tillage, while no significant differences among other nutrients were found. The activities of soil acid phosphatase (S-ACP), soil dehydrogenase (S-DHA), and soil sucrose (S-SC) under conventional tillage were significantly higher than those under no till, and the cover crop mixtures also had an exclusive advantage in yield. Soil organic matter (SOM) indicated a significant negative correlation with glomalin-related soil protein (GRSP). The increase in diversity associated with the AMF species community was strongly correlated with the increase in three enzyme activities, and AN was negatively correlated with all species. Tillage did not significantly change soil chemistry, except for AN, and the high concentration of AN led to a decrease in AMF species. The results of this study showed that no till was an effective measure for enriching soil micro-organism population. Additionally, soil AMF diversity was improved by cover crop mixtures, and microbial diversity was higher than that under monoculture cover crops. Different AMF groups responded differently to tillage and cover crop mixtures. Across all mixtures, the combination of hairy vetch (*Vicia villosa* R.) and ryegrass (*Lolium perenne* L.) performed the best.

Keywords: conservation tillage; yield; arbuscular mycorrhizal fungi; soil nutrient; cover crop

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1. Introduction

Continuous monoculture has led to enormous success in supplying food for the growing population; however, its negative impacts on soil health/quality emphasize the necessity for adopting sustainable cropping system practices in the long run. One effective way to maintain soil health is to use cover crops to enhance the functional capacity of soil

and promote soil environmental quality [1]. The incorporation of leguminous cover crops indicated positive impacts on soil health and cropping system sustainability [2]. Although some indicators did not increase significantly [3], the overall direction was positive. In recent years, planting cover crops has become a major agricultural practice to promote soil health, especially in combination with conservation tillage [4], including reduced tillage or no till. Extensive research has indicated the beneficial impacts of cover cropping on soil water storage, soil structure, and soil quality [5,6]. It can also reduce soil erosion to a certain extent [7,8]. In general, conservation tillage can increase soil microbial diversity and enrich its associated beneficial functions [9], and mulch planting and cover cropping can reduce soil erosion and suppress weeds [10].

As an important member of the microbial community in farmland soil, fungi provide important ecosystem services such as nutrient cycling, the decomposition of organic matter, and the improvement of soil structure [10]. Particularly, AMF form a symbiotic relationship with 80% of all plants on Earth [11], and their functions and benefits have been well documented, including supporting nutrient absorption, enhancing resistance to drought or root pathogens, and improving soil structure, soil aggregation, and water infiltration, as well as preventing soil erosion [12,13]. The impacts and contribution of the AMF community are affected by a variety of environmental and agricultural factors. With the increase in land use intensity, the spore density and community composition of AMF changes significantly [14]. Some AMF species can also be considered indicator species under different management styles [15]. Meanwhile, AMF communities under different tillage systems can change plant productivity, such as enhancing plant P absorption under no-till systems [16]. However, unlike other microbial groups in soil, fungi are more sensitive to physical disturbances. The differences in tolerance levels result in different structures and functions of soil fungal communities under different tillage practices. Repeated high-intensity tillage could destroy the AMF mycelial network [17], leading to irreversible impacts on soil health [18]. At the same time, no till generally reduces the physical disturbance of fungal distribution in soil and prevents the destruction of the mycelial network. Cover crops play a significant role in enhancing the mycorrhizal colonization and fungal activity of early arbuscular mycorrhizal fungi in subsequent crops [19]. In particular, the combination of cover crops with low tillage intensity can increase the abundance of AMF in soil and affect the yield of the main subsequent crops [20]. Furthermore, no till and different mixtures of cover crops could also bring greater ecosystem resilience to fungal communities and make it easier for crops to access limited resources [21]. It was found that a long-term combination of reduced disturbance and increased cover crops resulted in a more diverse and symbiotic community [22]. In general, conservation tillage practices can improve soil fertility, reduce soil erosion, and reduce the need for energy and labor. For this study, we hypothesize that the combination of two tillage methods and different mixed cover crops can provide different levels of benefits to increasing the diversity of soil AMF community, soil health, and soil enzymatic activities, ultimately leading to different impacts on cropping system sustainability.

2. Materials and Methods

2.1. Experimental Sites

The experimental site was located in Sinan County, Guizhou Province (27°44′ N, 108°11′ E, altitude 400 m), with a pH of 5.8. The experimental area has abundant rainfall with a clear transition between rainy (summer) and moderately dry (winter) seasons; thus, no artificial irrigation was used. The research area features a typical subtropical monsoon wet climate, with an average annual precipitation of 1142 mm from 2019 to 2022. The average annual temperature is 17.5 °C, the dominant soil type is yellow loam with an average SOC of 18.37 g kg^{-1}, total C of 1.43 g kg^{-1}, nitrate N of 26.66 g kg^{-1}, ammonium N of 0.94 g kg^{-1}, total P of 0.66 g kg^{-1}, and available P of 1.43 g kg^{-1}.

2.2. Experimental Design

This experiment is a long-term farming experiment established in September 2019. Maize was planted in April and harvested in August, and cover crops were planted in late September and harvested in late March. It was designed as a randomized complete block setup with factorial arraignment involving two tillage practices: conventional tillage (CT) and no till (NT). For maize, the planting density was 81,000 plants ha^{-1}, and the planting variety was "Qianqing 446". Compound fertilizer (N 15%, P 15%, K 15%) at a size of 150 kg ha^{-1} was applied before seeding, and urea was applied at 73.5 and 122.25 kg ha^{-1} at the jointing stage and large trumpet stage, respectively. Weeds in the experimental field were hand removed during the growing season. Cover crops included hairy vetch (*Vicia villosa* R.), red clover (*Trifolium pratense* L.), ryegrass (*Lolium perenne* L.), and triticale (*Triticosecale* W.). Cover crop treatments included four combinations of mixed forages (M1, 2, 3, 4). The sowing rate of the four mixed cover crops was M1: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + ryegrass (15 kg ha^{-1}); M2: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + triticale (15 kg ha^{-1}); M3: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + hairy vetch (52.5 kg ha^{-1}); M4: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + red clover (52.5 kg ha^{-1}); MO: hairy vetch (60 kg·ha^{-1}); and naturally growing weeds (W). Each plot area was 32.4 m^2 (6 m × 5.4 m).

2.3. Soil Sampling

Soil sampling at sites was performed in April 2022, totaling 36 samples (12 treatments × 3 replicates). To ensure that representative soil samples were obtained, five samples were taken from within each plot, and soil layers of 0–5, 5–10, 10–20, and 20–30 cm were sampled separately from each sample point. After the four layers of soil samples were fully mixed and screened, impurities, such as stones and plastics, were removed by hand. Finally, all samples from each plot were combined into one treatment sample, and each treatment was repeated three times. Each treatment sample was sieved through 2 mm mesh and separated into two parts: one was stored in a freezer at −80 °C before microbial sequencing and the other was airdried for physicochemical analysis.

2.4. Soil Analyses

For the total N (TN), the macro-Kjeldahl digestion procedure method was used [23]. The NaOH melt–molybdenum–antimony resistance colorimetric method was used for determining the total soil P concentration (TP). The Olsen method using a solution of sodium bicarbonate was used for testing available soil P concentration (AP) [24]. The soil dehydrogenase colorimetric method, the soil acid phosphatase colorimetric method [25], and the sucrase colorimetric method [26] were used for testing key soil enzymatic activities. Soil organic carbon (SOC) was analyzed using the Walkley–Black wet combustion method [27]. GRSP extraction was determined by the colorimetric method [28], and the spore density was determined by the wet sieve decantation–sucrose centrifugation method [29].

2.5. Plant Yield

Cover crop biomass samples were collected (3 × 0.6 m^2 quadrats per subplot) in November, March, and late April, and all samples were dried to a constant weight in a standard drying oven at 60 °C. Maize biomass samples were taken at the physiological maturity stage. Both grain and stover (stalk plus leaves) were collected from the four randomly selected plants within each plot and mixed to make a composite sample. All collected plant materials were air dried until reaching a constant weight.

2.6. DNA Extraction and PCR Amplification

After each soil sample was sieved through a 2 mm screen, it was transferred into EP tubes or frozen tubes of 2 mL or larger volume. The soil content within each tube was about 0.25–0.5 g, and the overall soil sample quantity was between 1 and 2 g. Total genome DNA from the samples was extracted using the CTAB method. DNA con-

centration and purity were monitored on 1% agarose gel. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. The 18S rRNA genes of distinct regions (18SAMV4-5NF_AMDGR) were amplified using a specific primer (AMV4-5NF(AAGCTCGTAGTTGAATTTCG) and AMDGR (CCCAAC-TATCCCTATTAATCAT) with the barcode. All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 μM of forward and reverse primers, and 10 ng template DNA. The thermal cycling process consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongating at 72 °C for 30 s. The 1XTAE buffer with PCR products was placed on electrophoresis on 2% agarose gel for detection. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with the Qiagen Gel Extraction Kit (Qiagen, Germany).

2.7. Illumina NovaSeq Sequencing

Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's recommendations and index code specification. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA). At last, the library was sequenced on an Il-lumina NovaSeq platform, and 250 bp paired-end reads were generated.

2.8. Bioinformatics Analysis

The analysis was conducted by following the "Atacama soil microbiome tutorial" of Qiime2docs along with customized program scripts (https://docs.qiime2.org/2022.2/ 2.8 (accessed on 12 May 2024)). Briefly, raw data FASTQ files were imported into the format that could be operated by the QIIME2 system using the QIIME tools import program. Demultiplexed sequences from each sample were quality filtered and trimmed, de-noised, and merged, and then the chimeric sequences were identified and removed using the QIIME2 dada2 plugin to obtain the feature table of amplicon sequence variant (ASV) [30]. The QIIME2 feature-classifier plugin was then used to align ASV sequences to a pretrained GREENGENES 13_8 99% database (trimmed to the AMV4-5NF_AMDGR region bound by the 338F/806R primer pair) to generate the taxonomy table [31].

2.9. Statistical Analysis

Data on soil nutrients, soil microbiological properties, and plant yields were analyzed using IBM SPSS 27.0 software. We used univariate analysis of variance (ANOVA, $p < 0.05$) based on a randomized complete block design with repeated measures to test the treatment effect (before performing ANOVA, all data were tested for normality, and ANOVA was performed after conforming to a normal distribution). Duncan's Multiple Range Test was used for mean separation. Principal component analysis (PCA) was used for analyzing the AMF community composition in the soils with the different management practices, and PCA analysis was implemented with the "factoextra" package in R. The AMF component species and soil-related properties data were analyzed using the R programming language, and the "ggplot2" package was used to show the relationship between dominant species and soil properties. Diversity metrics were calculated using the core-diversity plugin within QIIME2. Feature level alpha diversity indices, such as observed OTUS, Chao1 richness estimator, the Shannon diversity index, and Faith's phylogenetic diversity index, were calculated to estimate the microbial diversity within an individual sample. Redundancy analysis (RDA) was performed to reveal the association of microbial communities in relation to environmental factors based on relative abundances of microbial species at different taxa levels using the R package "vegan" (Use R version 4.1.3).

3. Results

3.1. Soil Nutrient Status

After three years of experiments, compared with conventional tillage, TN and AN under no-till conditions were significantly higher than those under conservation treatment (0–30 cm) (Figure S1B,D). Under different cover cropping treatments, TN, TP, and AN showed no significant differences (Figure S2B–D). Under all combinations, SOM and TN of NTW were significantly higher than those of other combinations, but AP of NTW was significantly lower than those of other combinations. SOM content is the highest in NTW, about 1.25 times the lowest, and the lowest is in NTM2, followed by 19.95 g kg^{-1} in CTM1 (Table 1). Among all the mixed sowing combinations, the SOM content of the M1 combination was the highest, and the SOM content of the M2 combination was the lowest (Figure S2A). SOM content in no-till treatment was higher than that in conventional tillage treatment, but it was not significant (Figure S1A). The highest content of TN in NTW was more than 1.2 times the lowest content of CTM3 (Table 1). There was no significant difference in the content of TN among different forage mixture combinations, among which the content of the M1 combination was the highest and the content of the M3 combination was the lowest (Figure S2B). No-till treatment was significantly higher than conventional tillage treatment ($p < 0.01$) (Figure S1B). The highest content of TP was in CTM4, and the lowest content was found in CTM3 (Table 1). There was no significant difference between different forage mixture treatments and different tillage treatments (Figure S2C). The content of TP in no till was higher than in conventional tillage. The highest content of AN in NTM4 was about 1.37 times the lowest content of CTW (Table 1). There was no significant difference between different forage mixture combinations, but no-till treatment was significantly higher than conventional tillage treatment ($p < 0.001$) (Figure S1D). The content of AP in CTM2 was 1.53 times the lowest content of NTW. NTM2 had a high pH value, NTM1 had the lowest pH value, and the pH values of all treatments were between 5.43 and 6.25 (Table 1). The pH values of different mixed sowing treatments were greatly different, and the pH value of the M2 and M4 combination was significantly higher than W (Figure S2F), and the value of pH tillage treatment was higher than no-till treatment (Figure S1F).

Table 1. Soil nutrient variables for the various systems in the study site.

Treatment	SOM (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	pH
NTW	20.32a	1.31a	0.63ab	115.20b	20.38e	5.63cde
NTM1	19.80abc	1.22bcd	0.64abc	115.71b	26.44bc	5.43e
NTM2	16.24f	1.23bc	0.63bc	112.18bc	28.23abc	6.25a
NTM3	19.59abcd	1.28ab	0.62cd	112.69bc	26.62bc	5.86bcd
NTM4	18.36bcde	1.23bc	0.66ab	125.26a	30.91ab	6.17ab
NTMO	19.36abcd	1.22bcd	0.59de	112.69bc	24.79cd	5.58de
CTW	17.85def	1.18cd	0.61bc	91.06f	24.35cde	5.99cde
CTM1	19.95ab	1.26ab	0.62abc	103.63d	28.63abc	6.01ab
CTM2	17.37ef	1.28ab	0.63bc	97.60def	31.35a	6.05ab
CTM3	17.91cdef	1.09e	0.57e	100.11de	21.22de	5.90bc
CTM4	18.36bcde	1.16d	0.67a	104.64cd	27.87abc	6.10ab
CTMO	16.75ef	1.16d	0.63bc	95.08ef	27.60abc	5.54de

Note: SOM: soil organic matter, TN: total N content, TP: total P content, AN: available N, AP: available P and soil pH. NT: no tillage, CT: conventional tillage, W: weeds, MO: monoculture, M1: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + ryegrass (15 kg ha^{-1}), M2: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + triticale (15 kg ha^{-1}), M3: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + hairy vetch (52.5 kg ha^{-1}), M4: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + red clover (52.5 kg ha^{-1}). The different lowercase letters are significantly different among cultivation times at the 0.05 probability level according to Duncan's test.

3.2. Analysis of the AMF Community Composition in the Soils with the Different Management Practices

In this field, a total of five AMF were identified at the genus level (Table 2). The number of taxa in no till was higher than in conventional tillage (Figure S3). The genera observed were *Archaeospora*, *Acaulospora*, *Diversispora*, *Glomus*, and *Paraglomus*. At the

absolute level, *Acaulospora* accounted for 23.39% in tillage treatment, which was higher than that in no-till treatment. *Glomus*, as the highest genus in no-till treatment, accounted for 70.39%, followed by *Paraglomus*, accounting for 6.73% (Figure S4). At the relative level, the relative *Glomus* quantity under no till was higher than that under tillage treatment, and the relative *Glomus* quantity under monoculture was significantly higher than that under forage mixture combination (Figure 1). The relative *Paraglomus* quantity under no-till conditions was higher than that under tillage treatment (Table S1), and the relative *Paraglomus* quantity under forage mixture combination was higher than that in monoculture and weed plots, and the M1 combination had the highest relative *Paraglomus* quantity (Table S2). The relative quantity of *Acaulospora* under conventional tillage conditions was higher than that under no-till treatment. *Archaeospora*, as a relatively small genus, can be called a rare genus. There were significant differences between *Archaeospora* and no-till treatment. Under no-till treatment, *Archaeospora* did not appear in some plots (Table S1). In addition, NTM4, CTMO, and CTM1 indicated the largest number of rare genera (Figure 1).

Table 2. Taxonomic information of the arbuscular mycorrhizal fungi (AMF) identified in the study site.

Phylum	Class	Order	Family	Genus	Species
Glomeromycota	Glomeromycetes	Archaeosporales	Archaeosporaceae	*Archaeospora*	Archaeospora VTX00005
					Archaeospora VTX00245
			Acaulosporaceae	*Acaulospora*	Acaulospora VTX00030
					Acaulospora VTX00037
		Diversisporales	Diversisporaceae	*Diversispora*	Diversispora VTX00062
					Diversispora VTX00054
		Glomerales	Glomeraceae	*Glomus*	Glomus VTX00150
					Glomus VTX00280
					Glomus VTX00114
					Glomus VTX00143
					Glomus VTX00125
					Glomus VTX00222
					Glomus VTX00113
					Glomus VTX00310
					Glomus VTX00309
					Glomus VTX00195
					Glomus VTX00092
					Glomus VTX00248
					Glomus VTX00056
					Glomus VTX00278
					Glomus VTX00057
					Glomus VTX00193
					Glomus VTX00279
					Glomus VTX00307
					Glomus VTX00333
					Glomus VTX00063
		Paraglomerales	Paraglomeraceae	*Paraglomus*	Paraglomus VTX00337

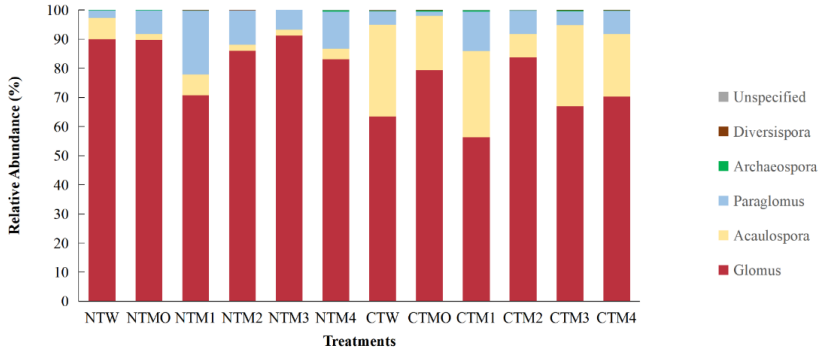

Figure 1. Relative abundance of the genus of arbuscular mycorrhizal fungi (AMF) in the different treatments. NT: no tillage, CT: conventional tillage, W: weeds, MO: monoculture, M1: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + ryegrass (15 kg ha^{-1}), M2: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + triticale (15 kg ha^{-1}), M3: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + hairy vetch (52.5 kg ha^{-1}), M4: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + red clover (52.5 kg ha^{-1}).

According to the Shannon diversity index, the AMF diversity under no-till treatment was higher than conventional tillage treatment, and NTM4 had a significantly higher diversity index than other treatments (Table S3), indicating that AMF community diversity under M4 was the highest (Table S4). However, the Shannon diversity index was the lowest under CTMO treatment (Table S3). Overall, there was a higher diversity under no till than conventional tillage, and a higher diversity under forage mixture treatment and weed plots than monoculture treatment (Table S4). It can be seen that the diversity of NTM4 and NTW was significantly higher than other treatments.

3.3. Soil Microbiological Properties

All treatments resulted in significant changes in different soil enzymes, and the contents of three enzymes in tillage treatment were significantly higher than those in no-till treatment ($p < 0.001$) (Figure S5). The activity of S-ACP in CTM4 treatment was the highest, and the content was 2.27 times that of the lowest content, followed by CTM3, and NTM1 had the lowest S-ACP activity (Table 3). There were no significant differences in the content of all mixture combinations, but M4 had the highest content and the M3 combination had the lowest (Figure S6A). The highest content of S-DHA was CTMO, which was 1.67 times the lowest content of NTM3 (Table 3), and there was no significant difference across all the mixed combinations (Figure S8B). For S-SC, the highest CTMO content was 1.99 times the lowest NTM3 content (Table 3), and there was no significant difference between different mixture treatments (Figure S6C). Spore density also showed a great difference among different treatments. The spore density of NTMO was 6.19 times that of CTW with the lowest spore density (Table 3). Although there was no significant difference among different mixture treatments, M1 was higher than other mixture combinations, and weed treatment was the lowest (Figure S8D). The spore density under no-till treatment was significantly higher than that under tillage treatment ($p < 0.001$) (Figure S5D). GRSP had the highest content of CTM3, followed by NTM2 (Table 3), and the M2 combination of different mixture treatments was significantly higher than that of weed plots (Figure S6E,F). There was no significant difference in EGRSP among all treatments (Table 3).

Table 3. Biological variables for different systems in the study site.

Treatment	S-ACP (U g^{-1})	S-DHA (U g^{-1})	S-SC (U g^{-1})	Spore Density (g $soil^{-1}$)	GRSP (mg g^{-1})	EGRSP (mg g^{-1})
NTW	82.25d	300.10f	2.73g	8.52f	101.80d	1.94a
NTM1	60.51e	295.87f	3.13f	25.58b	106.20bc	1.90a
NTM2	78.23d	324.89e	3.32e	15.47c	108.90ab	1.89a
NTM3	34.56f	245.83h	2.37h	15.77c	105.26bcd	1.99a
NTM4	76.33d	274.23g	2.61g	24.03b	105.92bcd	1.94a
NTMO	40.92f	253.46h	2.65g	29.92a	104.27cd	1.95a
CTW	106.58c	383.68c	3.86cd	4.83f	105.45bcd	1.94a
CTM1	125.73b	396.94b	4.33b	11.40cde	106.18bc	1.96a
CTM2	113.85c	378.93e	4.02c	12.68cde	107.72abc	1.94a
CTM3	133.14ab	343.71d	4.27b	14.33cd	110.37a	1.97a
CTM4	137.69a	387.35c	3.72d	11.22cde	107.20abc	1.96a
CTMO	126.57b	411.51a	4.73a	10.23de	105.74bcd	1.97a

Note: S-ACP: soil acid phosphatase, S-DHA: soil dehydrogenase, S-SC: soil saccharase, GRSP: glomalin-related soil protein, EGRSP: easy extract glomalin-related soil protein. NT: no tillage, CT: conventional tillage, W: weeds, MO: monoculture, M1: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + ryegrass (15 kg ha^{-1}), M2: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + triticale (15 kg ha^{-1}), M3: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + hairy vetch (52.5 kg ha^{-1}), M4: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + red clover (52.5 kg ha^{-1}). The different lowercase letters are significantly different among cultivation times at the 0.05 probability level according to Duncan's test.

3.4. Plant Yield

There was a significant difference in plant yields from different cover crop mixtures. CTM1 had the greatest cover crop yield, followed by NTM3, CTM2, and CTM3 (Figure S7).

There was a significant difference in the content of cover crop yield among different forage mixture combinations, among which the content of the M3 combination was the highest and the content of the M4 combination was the lowest. In general (Figure S8), the yield of cover crops and maize under conventional tillage treatment was greater (Figures S9 and S10). For maize production, tillage treatment generally resulted in greater maize yield (Figure S10A), especially CTM1 and CTM2 treatments, with their yields significantly greater than other treatments. NTW treatment had the lowest maize yield, which was 11.59 t ha^{-1} (Figure 2). The mixture combinations had a greater maize yield and biomass than W (Figure S10C,D), and the tillage treatment had a greater maize yield and biomass than the no-till treatment (Figure S10A,B).

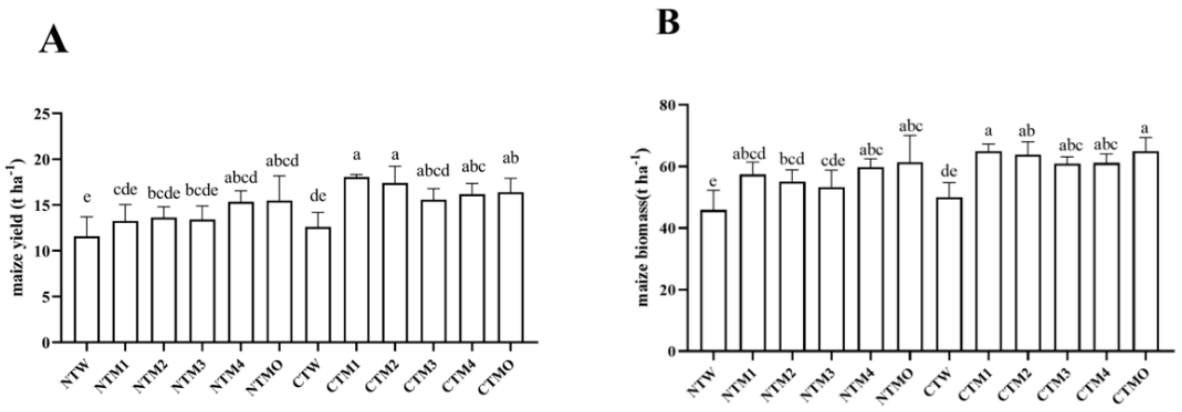

Figure 2. Maize yield (**A**) and biomass (**B**) for different systems in the study site. CT: conventional tillage, W: weeds, MO: monoculture, M1: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + ryegrass (15 kg ha^{-1}), M2: hairy vetch (37.5 kg ha^{-1}) + red clover (22.5 kg ha^{-1}) + triticale (15 kg ha^{-1}), M3: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + hairy vetch (52.5 kg ha^{-1}), M4: triticale (7.5 kg ha^{-1}) + ryegrass (7.5 kg ha^{-1}) + red clover (52.5 kg ha^{-1}). The different lowercase letters are significantly different among cultivation times at the 0.05 probability level according to Duncan's test.

3.5. The Relationship between the AMF and the Soil Properties

RDA analysis of all nutrient properties, enzyme activity indexes, and species at the detected genus level showed that *Acaulospora*, as a representative species in conventional tillage, was positively correlated with S-ACP, S-SC, and S-DHA in soil, among which S-SC had the highest correlation. AN, spore density, maize yield, SOM, TP, TN, and TP negatively correlated with the AP, with AN yielding the highest correlation. Soil S-ACP, S-SC, and S-DHA negatively correlated with *Glomus*, which was the most representative in no-till treatment. GRSP and the rare genus *Archaeospora* were positively correlated (Figure 3). After PCA analysis of nutrient indexes and microbial-related property indexes, the first two principal components accounted for 51.52% of the total variance of soil properties, in which PC1 retained 35.43% of the variance and PC2 retained 16.09% of the variance. The correlation between each attribute and its respective principal component is shown in Table S5. Among those, S-SC (0.44), S-DHA (0.42), S-ACP (0.42), AN (−0.39), and spore density (−0.31) were the most relevant indicators of PC1, while AP (0.61) and TP (0.57) were the most relevant indicators of PC2. (Table S5).

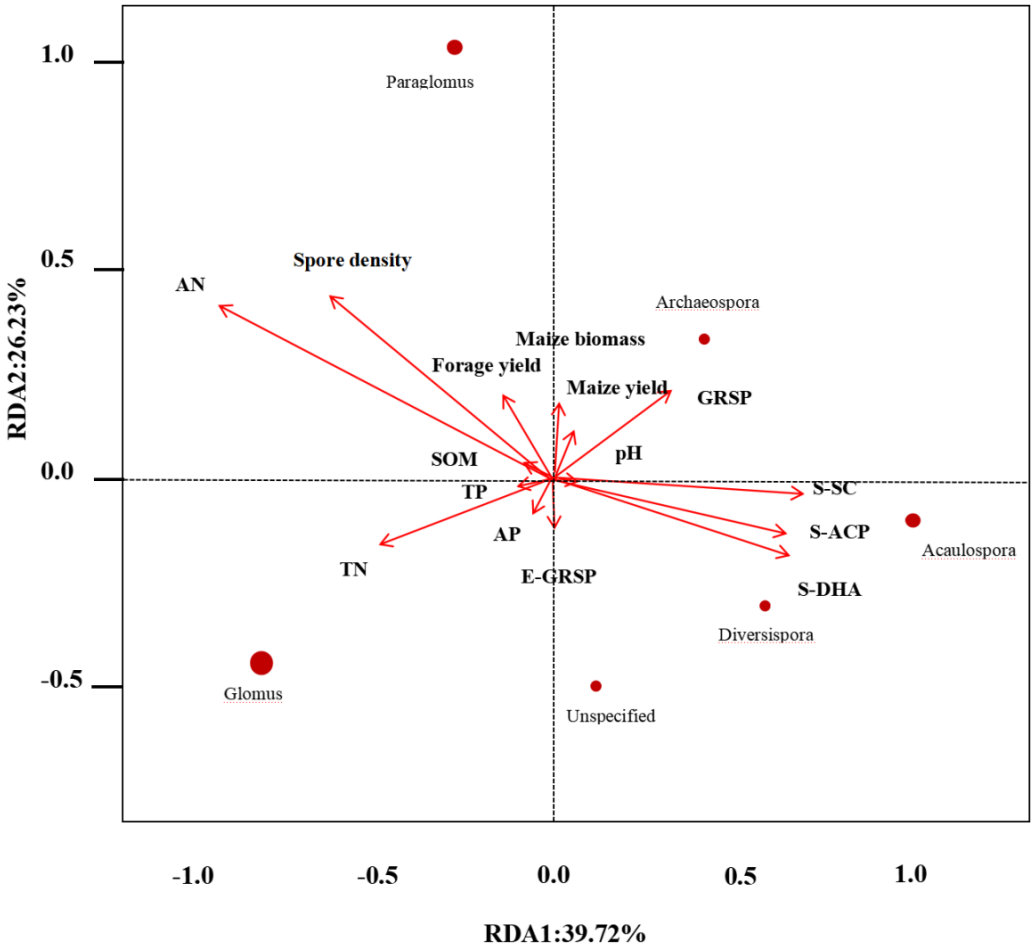

Figure 3. Arbuscular mycorrhizal fungal community response and potential drivers, redundancy analysis (RDA) of the chemical factors and genus level of the arbuscular mycorrhizal fungi (AMF) community. Abbreviation: S-ACP (soil acid phosphatase), S-DHA (soil dehydrogenase), S-SC (soil saccharase), SOM (soil organic matter content), AN (available N), AP (available P), TN (total N), TP (total P), GRSP (glomalin-related soil protein), E-GRSP (easy extract glomalin-related soil protein).

4. Discussion

4.1. Effects of Tillage Methods and Different Forage Mixture Combinations on Soil Nutrients

The effects of different tillage treatments on soil properties were different, driving microbial community structure changes in the soil environment. AMF play a role in nutrient regulation between plant roots and soil nutrients. AMF extraneous mycelia and GRSP form stable soil aggregates increase soil carbon content and promote nutrient storage. Soil SOM and TP showed no significant difference between the two tillage methods (Figure S1), but these indexes were generally higher under no-till treatment than under tillage treatment, and TN and AN under no-till treatment were significantly higher than under tillage treatment (Figure S1). A reduction in tillage intensity is usually coupled with increased SOM and nutrient content reserve, which is consistent with the classical model of carbon decomposition and loss function mediated by fungal humus [32]. The increase in extrarical mycelium and the expansion of the mycelium network can more effectively promote N and P uptake, which will help enhance C assimilation. Similarly, increased organic matter and nutrient content in no-till systems can lead to more AMF diversity. The content of AN in no-till soil was significantly higher than that in tillage treatment (Figure S1), while the content of AN in a tillage weed field was the lowest, and there was a significant negative correlation between AN and *Acaulospora*, indicating that *Acaulospora* was the most dominant group under tillage treatments (Figure 1). Additionally, Lu et al. [33] found that N addition had little impact on the abundance of *Acaulospora*,

indicating that *Acaulospora* might possess a better tillage tolerance level than other genus groups. This contradicts the finding reported by [34], stressing that *Acaulospora* is very vulnerable to soil disturbance. We attribute this finding to the cover crop factor and its interaction with different species selection in this study. Our experiments showed that after three years of trial setup, no till showed higher soil quality, especially chemical properties, compared to conventional tillage, particularly reflected in soil N and P content. Schmidt et al. [21] found that total N was higher under no till, but NO_3^- concentrations were higher in conventional tillage plots. Mhlanga et al. [35] found that soil chemistry of mulch systems under no till varied between systems in sandy regions but generally led to increases in soil organic carbon and total N, with conventional tillage treatments having higher available P content. This contradicts our research findings and is probably due to the effects of introducing different cover crop species.

4.2. Effects of Tillage Methods and Different Forage Mixture Combinations on Soil AMF

Long-term no-tillage soil systems can better increase AMF biomass, improve soil quality, and increase soil carbon sequestration and soil organic matter content [36]. Soil disturbance by conventional tillage has a particularly adverse effect on AMF diversity and the stability of cropping systems [37]. A large number of studies have found that conventional tillage reduces the total fungal biomass in agricultural soils [38]. For example, in a 15-year long-term field trial, reduced tillage significantly increased the abundance of different micro-organisms [39]. No till and reduced tillage have a positive impact on the abundance and diversity of microbial flora [40], and no-till soil has a greater AMF diversity index [15]. Additionally, for some plots with crop residues, AMF spore abundance and root colonization are enhanced under no-till conditions. In our three-year short-term experiment, different tillage treatments also brought some changes to AMF abundance (Figure S4). Overall, we found that the best way to protect native AMF and improve crop yield is to minimize tillage and incorporate crop residues. Furthermore, we found that Glomus remains the most abundant genus group compared to all other AMF groups, and it appeared to be more sensitive to the negative impacts caused by tillage (Figure S3). This observation agrees partially with the results reported by [41] but provides more information on cover cropping integrated no-till practices on a broader selection of AMF groups. Likewise, *Paraglomus* also indicated rapid responses towards the negative impacts caused by tillage, as indicated in a previous study [42]. The relative abundance of *Acaulospora* was higher than that of no-till treatment. Additionally, with the increase in tillage intensity, the relative abundance of *Acaulospora* also gradually increased (Figure 3). We consistently observed this across all cover crop mixtures. As one of the important genera of AMF, *Acaulospora* is widely distributed in various terrestrial ecosystems and is the dominant genus in most environments [43]. Thus, with the increase in tillage, the relative abundance of *Acaulospora* should decrease, as indicated in a previous study [34]. However, information related to the impact of soil disturbance intensity on *Acaulospora* is extremely limited, and we concluded that there might be more confounding factors driving its community structure change across different environmental conditions. Clearly, the impacts of tillage and cover cropping on the proportion of different AMF groups appear complex. Future research is needed in this knowledge area.

In general, compared with conventional tillage treatment, AMF biomass was lower under no-till conditions (Figure S3), but AMF microbial species diversity was increased (Table S3). This agrees well with many previous studies. For example, in a grassland ecosystem study, fungal diversity declined by 19% under standard tillage conditions compared to no till [44]. In another crop rotation study, species diversity in no-till soils was 4% higher than in standard tillage plots [45]. In a data synthesis study, 654 research projects were collectively investigated, indicating that soil available P was the main driver of AMF diversity and that both soil available P and pH are the dominating factors for determining AMF abundance [46]. Judging from the limited differences in soil P and pH status across all treatments in this study (Table 1, Figures S1 and S2), we argue that the impacts from both

soil disturbance and cover cropping practices on AMF diversity and abundance should outweigh basic soil physiochemical and geospatial factors.

4.3. Effects of Tillage Methods and Different Forage Mixture Combinations on Soil Enzymes and Their Microbial Properties

A recent study found that tillage had a significant negative effect on AMF diversity and that the destruction of mycelium by tillage was also strongly associated with reduced AMF abundance [47]. Generally speaking, tillage-induced soil disturbance can lead to microbial drying, mechanical killing, limited matrix availability, and disrupted access to food resources, which are detrimental to microbial growth and activity [48,49]. Conservation tillage practices can generally enhance soil enzyme activities, particularly when combined with straw mulching [50,51]. The results from this study, on the other hand, indicated that the activities of soil acid phosphatase, dehydrogenase, and sucrase in soil under conventional tillage were significantly higher than those under no-till treatment (Figure S5). This phenomenon could be explained by the increased bulk density of no-till treatments, which could negatively affect major soil enzyme activities, and the optimum bulk density could be cropping system/site specific [52]. The activities of available P and acid phosphatase in the no-till area are higher than those in the tillage area, which is similar to the results from a previous study [53]. This may be due to the fact that the soil is more acidic under no-till conditions, which promotes acid phosphatases in organic soils [54]. As expected, this response is the opposite of alkaline phosphatase towards tillage [53].

The change in the AMF community is mainly driven by vegetation change. In this study, mixed sowing crops increased the biomass of fungi and AMF compared with monoculture crops. This pronounced advantage of mixed cover cropping vs. monoculture could be caused by the fact that no Brassicaceae species were introduced in our treatment, as antifungal compounds released from Brassicaceae species could reduce AMF spore germination and proliferation [55]. Therefore, the enzyme responses observed in this study were primarily caused by enhanced plant biomass production, increased symbiotic development between roots and microbes, and the coupling effect of the proliferation of plant growth-promoting micro-organisms, including AMF. Specifically speaking, different mixed sowing combinations of legume and grass bring root tissues with different C:N ratios and biochemical properties to the underground. Over time, the continuous metabolism of root growth can release different types and quantities of enzymes, leading to an alteration in the soil microbial community and population [56]. As indicated in this study, both mixed sowing combinations have greater enzyme activity, and different enzymes have different sensitivities to different mixed sowing combinations. For example, the M4 combination has higher S-ACP enzyme activity, the M2 combination has higher S -DHA activity, and the M1 combination has higher S-SC activity (Figure S6A–C). It was also found that cover cropping generally has a stronger impact on the AMF community structure than cash crops [57]. Thus, we argue that AMF responses towards other cash crops, such as rice or soybean, should be similar to maize.

GRSP, as a thermally stable, viscous, and hydrophobic protein produced on the AMF mycelial wall, is a refractory protein that resides in soil for many years. It can resist microbial attack, help stabilize long-term carbon sequestrations and aggregates, and become more abundant with increasing clay and soil organic matter [58]. Our study found that GRSP content is more sensitive towards cover cropping mixtures than tillage methods (Figures S5 and S6). Additionally, GRSP and SOM showed a negative correlation, which may be due to the low viscosity of the soil in the experimental site and the existence of large soil aggregates in the soil, preventing the generation of GRSP, as well as the fast SOM turnover and mineralization rate in our environment [59]. GRSP was positively correlated with S-ACP and S-SC, and there was a very significant correlation between soil enzymes and GRSP, which is consistent with the findings reported by a previous study [60].

4.4. Effects of Tillage Methods and Different Forage Mixture Combinations on Plant Yield

Both cover crops and cash crops had greater crop yields under tillage conditions than no-till treatment (Figure S9). We attribute this finding to the effectiveness of tillage practice in adjusting soil moisture content and reducing bulk density based on our soil conditions [61]. It has also been shown that maize yield is more sensitive to tillage changes than legume crop yield, which is consistent with our findings. Legume–grass mixtures typically outperform grass or legume monocultures due to the symbiotically fixed N credit and rapid responses from companion grasses [62,63]. In our study, the higher yield of monoculture legumes under no-till treatment than those under grass mixtures might be caused by the superior adaptability of hairy vetch and red clover to lower soil pH conditions and limited productivity contributed by triticale and ryegrasses. Additionally, in our study, no till resulted in greater AMF diversity, which can greatly favor the biomass yield of N-fixing crops (e.g., legumes) than non-N-fixing crops (e.g., grasses and forbs). According to a recent meta-analysis study [64], long-term soil health and nutrient enhancement should be more beneficial for increasing yield and resource use efficiency in wheat production. Likewise, under tillage treatment, M1 cover crops had the greatest yield (Figure S8), which may be due to the high proportion of legumes in this mixed sowing combination and the slightly more active AMF community. We argue that after a longer duration of treatment implementation, the effect of AMF in the soil will become more pronounced, ultimately resulting in a more significant crop productivity increase than the short-term findings [64]. This study explored the effects of short-term conservation tillage and covered cropping practices on the diversity and structure of soil clumps mycorrhizal fungi. We expected that a longer period of implementation could result in more pronounced treatment effect yields and nutrients of cover crops on subsequent maize and will be continuously observed in the next few years, which may lead to more interesting agronomic and AMF findings.

4.5. Correlation between Soil Enzyme Activity and AMF

Generally speaking, under low P concentrations and neutral pH conditions, AMF diversity should correlate well with soil enzyme activity and plant growth responses [65]. However, our study indicated that this response could be AMF genus specific. For example, *Glomus*, *Acaulospora*, and *Archaeospora* indicated a much greater correlation, with all three major soil enzymes tested in this study compared to *Paraglomus* and *Diversispora*. The significant correlation between *Acaulospora* and S-ACP was previously mentioned by Nopphakat et al. [66], indicating that *Acaulospora* was the major soil AMF group in charge of the secretion of soil phosphatase. However, information relating to its strong correlation with S-SC and S-DHA is nearly unavailable. This emphasizes the importance of conducting future studies investigating the impact of *Acaulospora* in affecting hydrogen-consuming micro-organisms in the soil. S-SC and *Glomus* were significantly negatively correlated ($p < 0.01$), which was not documented in previous studies; thus, their mechanisms should be important in future research directions.

5. Conclusions

Under conventional tillage and no-till treatment, soil fungal communities showed significant differences in response to tillage and forage cover crop mixture combinations. No till significantly increased soil TN and alkali-hydrolyzed N contents and changed the species diversity of the community. The correlation between species composition, chemical properties, and soil enzymes indicated that *Glomus*, as the dominant genus of no-till treatment, was negatively correlated with S-DHA. Soil S-ACP, S-SC, and S-DHA are positively correlated with *Acaulospora*, which is the predominant AMF group under greater tillage intensity. Soil enzymes are closely correlated with soil AMF species diversity/abundance. Tillage treatment had a greater effect on soil fungal community and enzyme activity than cover cropping. Compared with no tillage, tillage treatment was more beneficial to increase corn kernel and maize plant yield. In general, despite limited cash crop responses, no-till and diversified forage cover crop mixtures promote a more diverse and nutrient-rich soil

AMF fungal community. Interestingly, increased tillage intensity could favor the abundance level of certain AMF genus groups.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture14071103/s1, Figure S1: Soil nutrient variables for the different mixture combinations in the study site; Figure S2. Soil nutrient variables for the different mixture combinations in the study site; Figure S3. The number of taxa in different tillage treatments; Figure S4. Absolute abundance of the genus of arbuscular mycorrhizal fungi (AMF) in the different treatments; Figure S5. Biological variables for different systems in the study site; Figure S6. Biological variables for different systems in the study site; Figure S7. Cover crop mixture yields in different treatments in the study site; Figure S8. Cover crop mixture yields for the different mixture combinations in the study site; Figure S9. Cover crop mixture yields for the different tillage types in the study site; Figure S10. Maize yield and maize biomass for different systems in the study site; Table S1. The relative quantity proportion of the genus under different tillage conditions; Table S2. The relative quantity proportion of the genus under different cover crop mixtures; Table S3. Shannon diversity, Simpson index, Chao1 index, and faith in different treatments; Table S4. Shannon diversity, Simpson index, Chao1 index, and faith in different treatments; Table S5. Correlation between attributes and each principal component (PC1 and PC2).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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