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THE IMPACT OF PERSIAN CLOVER (*Trifolium resupinatum* L.) ON SOIL HEALTH

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Abstract: Persian clover (*Trifolium resupinatum*) is an exception as it is a promising legume species due to its adaptation to environmental stress and waterlogged soil. Soil health indicator link to environment services such as nutrient management, crop practices, and biodiversity. The main comparisons in this study were conducted between annual covers (Corn and sorghum), and two grass cultivars (*Miscanthus sinensis* and *Miscanthus junceus*) that are native to East Asia, while tillage system and fertilization legume Persian clover (*Trifolium resupinatum*), were also applied in the analysis. The regression analysis revealed improved soil carbon was consistently associated with greater moisture, soil aggregate stability (WAS), and carbon mineralization under legume covers. While grasses and legume recorded sequestering more carbon, corn and corn-sorghum in rotation crops showed the worse impact on soil pH and bulk density (BD). Non-tillage practices significantly increased soil aggregate stability and soil moisture under grasses and legume. The combination of total carbon (TC) field measurements with cropping systems information has improved our understanding of how different cropping practices influence soil health improvement in full profile. The main factor for clustering treatments based on indicators was fertilization and tilling operation according to Euclidean distance that was applied to measure similarity of the groups. It can develop appropriate and cost-effective agricultural management activities, maintains or improves carbon soil accumulation to guide farmer decision making and ultimately advancing food and nutritional security.

Keywords: Soil health indicators, Perennial, Cover cropping, Total organic carbon (TOC), Soil quality

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1. Introduction

Soil health indicators play significant role especially when sustainable agriculture faced the problem of massive production to cover malnutrition and feed the rising population of the world. Concerns about environmental sustainability of annual biofuel cropping systems such as corn warrant the need for an improved understanding of soil health under different agroecosystems. Corn production requires great amount of N fertilizer, which causes environmental contamination, greenhouse gases (GHGs) emission, and raise the risk of human exposure to nitrate from contaminated drinking water as well Ward et al. (2005). The significant increase of GHGs in the atmosphere also has increased the global mean temperature by ~1°C over the last century that affects quality of agricultural crops Zhao et al. (2017). Un-balanced presence of nano-particles makes this scenario even worse, because it alters photosynthesis and induces oxidative stress by Reactive oxygen species (ROS) generation that results in DNA degradation, cell death, and Antioxidant enzymes inhibition Mirbakhsh (2023). In this situation soil can play a key role to protect health and reduce environmental contamination. Soil health indicators are responsive to different cropping system to proper sufficient food to combat with malnutrition and hunger Augustine and Lane (2014).

Integrated or coupled system-level modeling has the potential to alleviate this situation and enhance soil health. The great interest of scientists to decrease the use of fertilizer and feed the rising population simultaneously have stimulated research on perennial grasses that considered as second-generation biofuel systems as well Blanco-Canqui (2010). Evaluation of the potential benefits of biofuels over fossil fuels have greatly relied on land use conversion to perennial or cover crops (Monti et al., 2012). The conversion of cropland to biofuel cropping has been proposed to potentially enhance soil health and mitigate GHGs (Anderson-Teixeira et al., 2009). Cover cropping has also been recognized as a cost-effective management tool and beneficial practice for enhancing soil health while also providing multiple agroecosystem function such as improving water quality and use efficiency of N by reducing N leachate and preventing further environmental contamination (Tonitto et al., 2006). Cover cropping's success depends on soil conditions, climate, and management approaches (Gomez, 2017).

Cover crops are annual, biennial and perennial species grown on to protect the soil and to its fertility (Hartwig and Ammon, 2002). Persian winter legume (*Trifolium resupinatum* L.) as a cover crop has shown substantial biomass production due to a good germination and establishment beside its beneficial characteristics including weed suppression, soil aggregation and water retention improvement in Midwest. *Trifolium resupinatum* is also considered as a beneficial cover crop for minimizing nitrate leaching losses from lands under intensive grain production (Valkama et al., 2015).

Management practices can affect soil properties (Ashworth et al., 2018). The sustainability of soil resources can be maintained by promoting soil health through improved management practices (Norris et al., 2020). The choice of suitable cropping practices for increasing soil organic matter content should focus on several factors such as GHGs emissions mitigation, carbon capture improvement, and temporal yield stability (Somerville et al., 2010; Knapp and van der Heijden, 2018). For example; well-managed arable land conversion to perennial or cover cropping beside annual plots have been resulted in accumulation of SOC, due to their low nutrient requirements, extensive fibrous root systems, root exudates, and subsequent turnover of aboveground biomass (Ledo et al., 2020; Chen et al., 2022). On the other side, no-tillage reduced soil pH, but increased organic matter and Ca, Mg, and K concentrations compared to conventional tillage (Tarkalson et al., 2006). Currently maximizing stabilization and accumulation of soil C in deep horizons is a central component of successful attempts to reverse SOC loss in agricultural lands to combat the accelerated climate change and enhance soil health (Lal et al., 2013; Crow et al., 2018; Sun et al., 2021; Chen et al., 2022).

Our objective is to examine the effect of Persian winter legume, grasses, and corn with different management combination on potential of soil health indicators. We hypothesized that grass and legumes will enhance soil health indicators and crop yield compared to conventional tillage annual crop–fallow. Our experimental site has 6 treatments that have been continuously applied in a randomized complete block design with four replicates. For annual practices we have continuous corn, corn/ sorghum and sorghum/corn in rotation with conventional tillage. For cover cropping we have Persian winter clover (*Trifolium resupinatum* L) (Figure 1a), for perennial covers we have *Miscanthus sinensis* (Silver-grass) (Figure 1b) and *Miscanthus junceus* (Okavango Delta grass) (Figure 1c). Data for all soil health indicators and annualized crop yield were analyzed using regression analysis of soil components and to distinguish between 6 treatments with different cropping practices. Cluster analysis was used to split the soil health indicators to recognize the similarity of different cropping systems and cultivars.

2. Materials and Methods

2.1. Field Experiment

The experiment was conducted at a Research Farm (Islamic Azad University, Chalus Branch), located at 40° 55′ N and 53° 72′ E with an altitude of 4 m above sea level, during 2019-2020 crop year to test our alternative hypothesis. Soil samples with 0.15 m depth were collected in mid-April 2019 before tillage, planting, and fertilization. Measurements were made for intact and repacked soil cores. Mean temperature, relative humidity, and precipitation of study site are presented in Table 1.

The experimental design includes 6 treatments in completely randomized block design that have been applied since 2000. For perennial grasses there was silver-grass (*Miscanthus sinensis*) and Okavango Delta grass (*Miscanthus junceus*) with no tillage system and zero fertilizer. For cover cropping winter clover (*Trifolium resupinatum* L.) was added to corn in different plots with zero fertilizer and no tilling. The subset of three treatment chose for annual that contains corn (CC) and corn/sorghum-sorghum/corn (CS-SC) in rotation with tillage.

a b c

Figure 1. a. Persian Clover (*Trifolium resupinatum*) in a non-hairy winter annual clover with toothed margins and small pink flowers that produces olive green purple seeds. **b**. Chinese silver grass (*Miscanthus sinensis)* is used as ornamental grasses traditionally used in Japan with erect, airy, plumed seed heads that are usually produced in late summer. **c**. Okavango Delta grass (*Miscanthus junceus*) is produced by seasonal flooding and is found in permanent marshland. It is home to some of the world's most engaged species of large mammals.

Table 1. Meteorological parameters for the field sites during experiment (Mazandaran province Meteorological Office)

Months	Mean temperature (°C)		Relative humidity (%)		Precipitation (mm)	
	2019	2020	2019	2020	2019	2020
November	12.98	14.06	79.87	84.32	148.36	193.12
December	10.52	12.13	85.10	79.80	185.30	122.90
January	8.90	10.60	80.30	82.40	95.20	94.30
February	10.30	12.20	84.70	88.20	87.90	94.30
March	8.10	11.20	83.40	86.50	110.10	118.40
April	11.40	14.15	83.10	79.90	70.30	125.10
May	16.40	18.90	80.70	82.10	18.10	41.20
June	24.20	21.50	73.20	79.30	10.30	26.30

The N sources for corn treatments were urea-ammonium nitrate 38% (w/w) N (UAN) side-dressed at corn growth stage V5 at rates of 185 and 115 kg N ha^{-1} yr^{-1} for CC and CS, respectively, and liquid swine manure (80% [w/w] of N as $NH4^{+}$) injected into CC at a rate of 255 ± 24 kg N ha^{-1} yr^{-1} in either the spring (SM) or the fall (FM). Tillage operations were chisel in the fall and chisel plus disk in the spring for all cropped plots except perennials. Seeds were planted at a population density of 182 and 560 seeds ha^{-1}, respectively. Soils were tested each fall for general fertility using recommended protocols.

Soil samplings occurred within the week of corn planting in early July (11 July 2019 and 3 July 2020) and again at corn growth stage R1 in late September (25 September 2019 or 27 September 2020).

2.2. Soil Sampling and Analysis

A slice of soil (4 m wide by 1.5 cm thick by 15 cm depth) was removed from three sides of the hole using a soil knife. Soils from three sides of six holes were placed in a plastic bag to prepare a composite sample for analysis and the bag was put in a cooler packed with ice for transportation to the laboratory. A subsample of 400 g soil from each plot was sealed in a plastic bag, put in a cooler packed with ice, and shipped to soil testing laboratories for analysis of biological properties. The remaining soil was shipped to other laboratories where soils were air-dried, ground, and sieved to 2 mm prior to the analysis of physical and chemical properties.

Measurements were made for intact and repacked soil cores. For total C and N concentration (g Kg^{-1}) in the soil the samples sieved through 2 mm sieve then 0.5 gram sub-sample from each core was combusted (CN analyzer) to quantify C:N ratio. The soil C data was square root transformed prior to the analysis to make the data more normally distributed. The accurate measurement of bulk density that is directly influenced by organic matter (OM) content is critical for converting SOC on a weight basis to content per unit volume. Cores were oven dried to get a dry mass for measuring bulk density. Bulk density was calculated by dividing the oven-dried soil samples with the volume of soil core Grossman and Reinsch (2001). Samples were taken from the center of each depth interval to represent depth increment.

Aggregation is a complex process of the interactions between roots and mycorrhizal fungi in relation to plant community composition which is improved by cover cropping/fresh root exudation and its influences on SOC sequestration Lal (2015). Samples for measuring wet aggregate stability (WAS) of the soil in this part of the project were taken by using hydraulic probe 5.3 cm in diameter from four mentioned depths. Samples were push through 8 mm sieve while still is moist and then dried and sieved to remove 2 mm fraction. From each sample two 25 g subsample were derived for analyzing using wet aggregate size distribution method. Aggregate stability was determined using the sprinkle infiltrometer as the amount of 0.25–2.00 mm aggregates remaining in a 0.25 mm sieve after a 5 min simulated hard rainfall (Schindelbeck et al., 2016). The average mean weight diameter was calculated for each depth.

Soil pH and electrical conductivity were determined using 1:2 soil/water ratio with a pH meter. Soil P, Ca, N, K concentrations were determined by extracting the soil with Mehlich-3 solution and quantification by inductive coupled plasma-atomic emission spectroscopy (ICP-AES) (Sikora and Moore, 2014).

2.3. Data Analysis

Data for all soil health indicators and different treatments were analyzed using R version 4.0. The regression analysis of the soil health components at particular time of soil sampling provides an estimate of relative influence of each soil health indicators. Hierarchical clustering with Ward's algorithm was performed to assess common soil health indicators among different treatments that was analyzed by correlation matrix to delineate the relationships between treatments. This type of clustering shows which treatments tend to have similar variations in concentrations.

3. Results and Discussion

The regression and correlation patterns for soil health indicators in 2019 showed high positive correlation of soil moisture with total Carbon (TC), Carbon mineralization, Wet aggregate stability (WAS) under winter clover and silver-grass. WAS significantly influenced by carbon and nitrogen mineralization under winter clover (Figure 2). Since, soil aggregates are formed through physical and biochemical activities treatment and tillage effect had strong and direct impact on it. The positive correlation also recorded between total N (TN) and TC under treatments with winter clover and silver-grass. WAS recorded high relation to carbon content of the soil and C-mineralization in grasses and legume (Table 2). The results indicates that physical protection of SOC can be achieve through positive interaction between SOC and soil structure such as WAS and moisture. On the other site the positive interaction of TN and TC reveals the main mechanism of soil organic matter (SOM) protection with TN and TC (Zhang et al., 2020). Accumulation of greater SOC under perennials and no-tillage system effectively promotes soil aggregate, which can store SOC for long-term and increase moisture to protect roots (Aziz et al., 2013; Hernandez et al., 2019). These finding is so important, especially regarding soil moisture that has a direct impact on other health factors and associated for agro-meteorological variables at different time-frequency domains Jamshidi et al. (2021).

Correlation patterns for 2020 soil health indicators showed correlation of soil moisture with C-mineralization, wet aggregate, N-mineralization, TC (Figure 2). It was pretty the same as 2019 and did not have a change (Table 2). Retaining crop residues in perennials and cover crops reduce exposure of the soil to external environmental factors such as heat and increase moisture, which has direct positive impact on TC Zheng et al. (2018). The negative correlation between TC and bulk density was recorded in 2020 as well (Figure 2). Increased SOC content under perennial or/and cover cropping with no-tillage system can subsequently improve soil physical properties such as bulk density and WAS Maiga et al. (2019). Carbon mineralization showed correlation to TC, TN, N-mineralization, and wet aggregate in all treatments. High correlation was recorded between C-mineralization and N-mineralization and also TN and TC specially under legume (Table 2). Glomalin that is produced from cover crops root might be one of the main reasons in adhesion and stabilization of soil under cover crops treatments Liu et al. (2020).

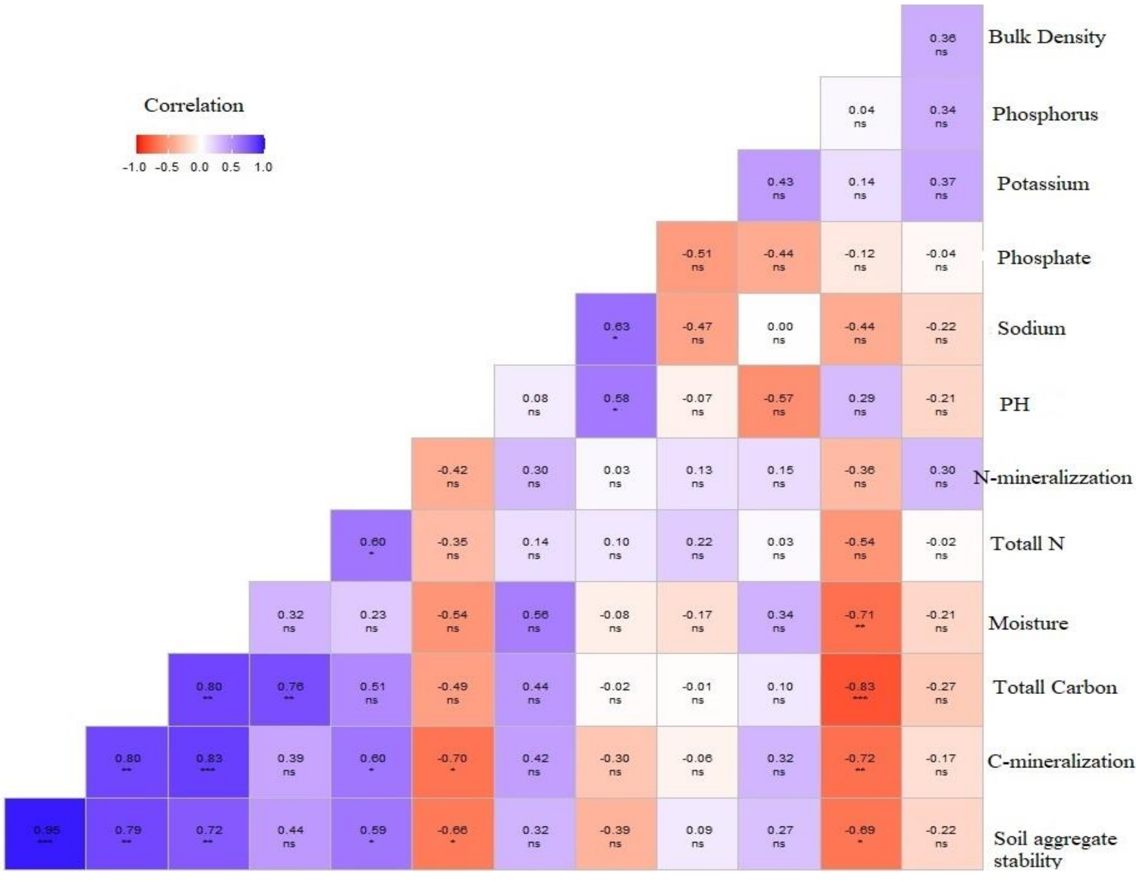

Figure 2. Correlation patterns between soil health components for 2019 and 2020 in average. The results recorded strong relation between soil aggregate stability and mineralization that increase total carbon as well in both year, and strong negative relation between Bulk density with total N and total C is shown.

Moisture had greater impact on TC in grasses than corn, which increased over time and its greatest impact was recorded in 2020 that was about ten-fold compares to annual (Table 2). The impact of TN on TC was greater in perennial than annual, which increased over the time. However, the impact of TC on TN regarding (perennial and legume: annual) was not constant and the highest record was shown in 2019. (Table 2). Wet aggregate recorded higher impact on TC in perennial than annual, which increased over time and in 2020 and was greater in perennial than annual. However, regarding the impact of wet aggregate on TN comparing perennial to annual;

although perennial recorded greater than annual but it decreased to equal ratio over time (Table 2). The impact of respiration on TC was greater in perennial compares to annuals, which increased over time and the highest record was shown in 2020. However, carbon mineralization showed pretty constant impact on TN in both perennial and annual (Table 2). The impact of Nitrogen mineralization on TN was greater than on TC, but in both ratios, perennial recorded higher impact on TN and/or TC than annual. Although perennial showed the potential for storing C in ground and reducing atmospheric CO_2 concentration, management practices such as reduced- or no-tillage, residue retention may reduce SOC losses and increase soil health. Plant residue management is also so important in retaining soil moisture and increase WAS. Our results agree with the idea of using winter legume and grasses for restoration of degraded agriculture lands, either on their own such as our grasses or in combination with annual such as our Persian clover that was dressed with corn.

Hierarchical clustering with Ward's minimum variance algorithm creates a hierarchical decomposition of the given set of data objects forming a dendrogram – a tree which splits the database recursively into smaller subsets. Euclidean distance was applied to measure similarity of the groups. The dendrogram shows two main big cluster around 20,000 Euclidean distances. Fertilizer application had significant impact on clustering our treatments. No fertilizer was added to legume and two type of grasses that are clustered separately from other treatments that were under fertilization. Moreover, all perennial grasses and legume are grouped in this cluster and are separated from annual. However, silver and delta perennial grasses recorded the highest similarity with minimum Euclidean distances, then legume joined them. Tillage effect was significant in grouping treatments because half of treatments in each group were under no-tillage and the rest of them were with conventional tillage (corn), (corn and sorghum in rotation), and (sorghum and corn in rotation) (Figure 3).

Table 2. Pearson correlation among comprehensive assessment of soil health indicators for all 6 treatments among 209 and 2020. Correlation patterns for legume clover showed high correlation of soil moisture with TC, C-mineralization, wet aggregate. Positive correlation recorded between total N and total C. Carbon mineralization showed correlation to total C and wet aggregate. High correlation was recorded between wet-aggregate and C-mineralization and TC under silver-grass. High correlation was recorded between C-mineralization and N-mineralization and also TN and TC in Persian legume. Factors that showed correlation to TC and TN are; Wet aggregate, N-mineralization and bulk density (with negative correlation)

Indicators	Moisture	PH	Bulk density	Aggregate	Nmineralization	P	K	P-	Na	TN	TC	Cmineralization
Moisture	1	-0.03278522	-0.665762172	0.772172028	0.199550022	-0.41995	0.548526	0.349383	-0.18667	0.188424764	0.904864	0.749973426
PH	-0.032785216	1	0.337759339	0.017979778	0.430068968	0.38453	0.004185	0.515873	-0.06595	-0.197605264	-0.28602	0.008516348
Bulk density	-0.665762172	0.33775934	1	-0.687174436	-0.388247529	0.410672	-0.45417	-0.00097	-0.05399	-0.398160841	-0.86577	-0.692139627
Aggregate	0.772172028	0.01797978	-0.687174436	1	0.568231388	-0.01269	0.284375	0.377384	-0.42219	0.396793951	0.797971	0.907894344
Nmineralization	0.199550022	0.43006897	-0.388247529	0.568231388	1	0.310394	0.36138	0.202081	0.034686	0.136273095	0.231185	0.617660213
Phosphorus	-0.419946781	0.3845304	0.410672252	-0.012690605	0.310393568	1	-0.53205	0.313879	-0.35585	0.131682043	-0.39645	-0.094194469
Potassium	0.548526284	0.00418546	-0.45417183	0.28437489	0.36137954	-0.53205	1	0.025232	0.58453	-0.067479694	0.431537	0.356391514
Phosphate	0.349382822	0.51587298	-0.000968516	0.377383827	0.202081312	0.313879	0.025232	1	-0.38589	-0.046972493	0.123176	0.323750712
Sodium	-0.186665491	-0.0659472	-0.053989705	-0.422187574	0.034685981	-0.35585	0.58453	-0.38589	1	0.124212903	-0.13811	-0.418843826
Total N	0.188424764	-0.19760526	-0.398160841	0.396793951	0.136273095	0.131682	-0.06748	-0.04697	0.124213	1	0.434136	0.084956843
Total C	0.904864244	-0.28602188	-0.865773618	0.797970977	0.231185015	-0.39645	0.431537	0.123176	-0.13811	0.434135841	1	0.739562375
Cmineralization	0.749973426	0.00851635	-0.692139627	0.907894344	0.617660213	-0.09419	0.356392	0.323751	-0.41884	0.084956843	0.739562	1

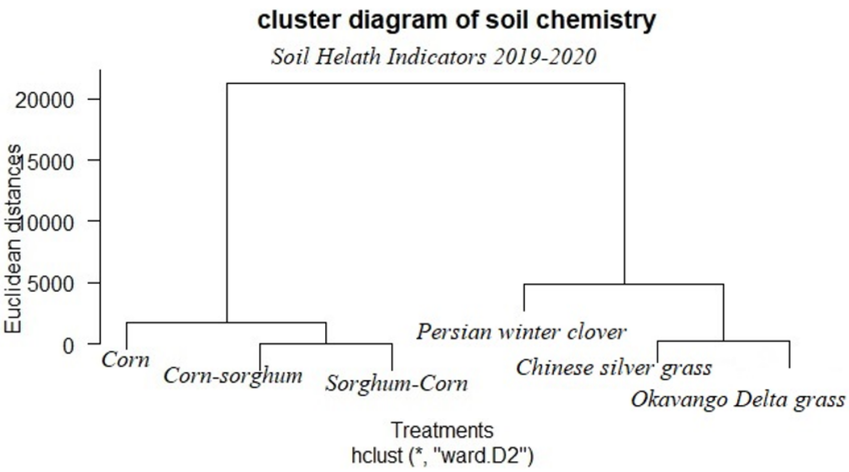

Figure3. According to the dendrogram first legume and Miscanthus (Silver grass and Okavango), then Corn and Sorghum in rotation are the first and second two lines that merged. Rather than different cropping system there are two other factors that impact the separation in this dendrogram, which are fertilization and tilling system that should be considered in improving agriculture systems. According the range of similarity corn and sorghum in rotation and Silver-grass and Delta-grass recorded the highest rate.

The results recorded the most similarity between corn and sorghum in rotation and Silver with Delta grasses. The results agree with the theory that when perennial and legumes are deployed on the landscape it is generally assumed that soil health increase in comparison to annual. However, there exists a critical need to synthesize and evaluate data on greenhouse gas emissions and SOC sequestration at meaningful temporal and spatial scales following land use changes to second-generation biofuel cropping systems as well. Thus, *Miscanthus,* and winter clover have potential to improve Soil health contents while supplying feedstock for biofuel production.

4. Conclusion

Our results demonstrate that routine soil test should include analysis of total carbon/ nitrogen for the long-term sustainability under different cropping practices and agricultural system. The most promising soil health indicator that related to different cropping practices were organic Carbon, total carbon, followed by total Nitrogen, bulk density, moisture. Our study of different indicators of soil health that included physical and chemical properties under different cropping systems experiments showed that no-fertilization in combination to perennial and covers caused the most promising distinction between treatments. Cover cropping such as Persian winter clover side dress in corn had similarity to perennial grasses (*Miscanthus sinensis* and *Miscanthus junceus*) regarding improving soil health as well. Our results indicates that perennial covers and legume cultivated with corn increase soil health and quality, where organic carbon, total carbon and nitrogen has highest contribution to these cropping practices in the most top soil layers. Grasses and legumes have therefore the potential to be a climate change mitigator, by resulting a negative C balance system or at least storing some C in the ground and thus reducing atmospheric CO_2 concentration. C stored in ground during perennial and cover cropping may be lower than C and other GHGs emitted during combustion, which resulting in net positive emission from plantation and potentially contributing to food security and local economic. The full potential of these biofuel cropping systems to increase soil health can be best realized with proper N fertilization, particularly, when the land use conversion involves grasslands.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	M.M.	S.S.S.S.	Z.Z.
C	40	30	30
D	70	20	10
S	50	50	
DCP	30	40	30
DAI	40	30	30
L	60	20	20
W	50	50	
CR	70	20	10
SR	50	50	
PM	40	40	20
FA	40	40	20

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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