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Physical and Microbiological Properties of Alfalfa-Established Soil in the Semiarid Horqin Sandy Land in Northern China

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Abstract

In a previous article, we reported that a local variety of alfalfa (*Medicago sativa* L. cv. Aohan) had high potential to be a pioneer plant for ecological restoration in the Horqin Sandy Land, China. The plantation of Aohan significantly improved the organic matter, clay, total carbon and nitrogen contents of the soils. In this study, we investigated the physical properties such as dispersion ratio, water-stable aggregates content, and the soil microbiomes, five years after alfalfa establishment in the same study site. We found no significant difference in the dispersion ratios between the soils before and after alfalfa establishment, and all the soils at the study site were erosive. Water stable aggregates mainly distributed in <1 mm fractions, which accounted for >96%, suggesting that it would take longer time for improving soil structure. However, large-size aggregates (2 - 5 mm) content was slightly higher in the alfalfa planting plots. This slight increase is presumed to have long-term importance for soil and ecosystem recovery in semi-arid areas like Horqin Sandy Land. Moreover, we also found that Actinomycetes dominated the microbial community in both bulk and rhizosphere soils, and two kinds of rhizobia, *Bradyrhizobium* and *Sinorhizobium fredii*, were identified in the rhizosphere soil.

Keywords

Dispersion Ratio, Water-Stable Aggregate, Rhizosphere, Microbiome, Rhizobia

1. Introduction

Horqin Sandy Land located in Inner Mongolia, China, was once a sparse forest meadow. However, a large-scale conversion of a natural grassland into cultivated land has occurred in this area in the past one hundred years, and it has changed into a desert due to rapid population growth, cultivated land reclamation and overgrazing [1] [2]. Now this area is recognized as an ecologically fragile region in the world [3] [4]. In 2015, we planted Aohan, a local variety of alfalfa on an abandoned farmland in the Horqin Sandy Land for the purpose of vegetation and ecosystem restoration [5] [6]. In our previous article, we reported that Aohan can be an effective pioneer plant for vegetation restoration in the Horqin Sandy Land, and that it significantly improved soil chemical properties such as organic matter content, clay content and cation exchange capacity, and decreased sand content of the soil [7].

Plantation in a semi-arid area can enhance the formation of soil aggregates, which is beneficial for mitigating wind erosion [8]. On the other hand, it is reported that land-use conversion elicits a distinct effect on the composition of soil bacterial communities [9]. Moreover, Aohan and old awn wheat mixed planting improved soil enzyme activity, microbial biomass, and enrich soil microbial diversity, which was of great significance to maintain the balance of soil ecosystem [10]. The soil physical properties have been improved 7 years after alfalfa plantation in hilly sandy areas of Shanxi Province, China [11]. Alfalfa and corn planting under both conventional and no-tillage could decrease the top soil sand contents, increase soil porosity, decrease soil bulk density and increase the water supply capacity to plant [11].

Alfalfa establishment might also impact soil aggregate distribution, dispersion ratio, microbial community, and the abundance of specific species found in the rhizobia. Therefore, the objective of our current study was to investigate the physical properties and the microbiomes of the Alfalfa-established soil in Horqin Sandy Land.

2. Materials and Methods

Study site: The study was carried out at a site in the Horqin Sandy Land of Inner Mongolia, China. *Medicago sativa* L. cv. Aohan, a local alfalfa variety in Horqin region, was established in an abandoned farmland at the Merin commune (42˚41'N to 45˚45'N, 118˚35'E to 123˚30'E), located 24 km west of Tongliao City in Inner Mongolia in 2015. The total area of the farmland was 3.3 ha, with a gentle east to west slope and an average elevation of 220 m.

Soil sampling: The experimental site was divided into sixteen plots with a size of 45 × 45 m^2 for soil sampling. Around 500 g of soil samples (0 - 15 cm deep) were collected as a composite sample from 5 points in each plot in August 2019 (**Figure 1**). After air drying, the samples were coarsely ground, passed through a 2 mm sieve, and transported to the laboratory for physicochemical analyses. Another seven samples were collected for aggregate distribution and stability analysis. Five samples taken from alfalfa plots and two taken from outside of alfalfa plots were used as controls. For this experiment, around 1500 g of soils were collected by using a plastic box with 0 - 10 cm depth without mixing and causing soil disturbance. These soils were air dried in the laboratory. In addition, a rhizosphere

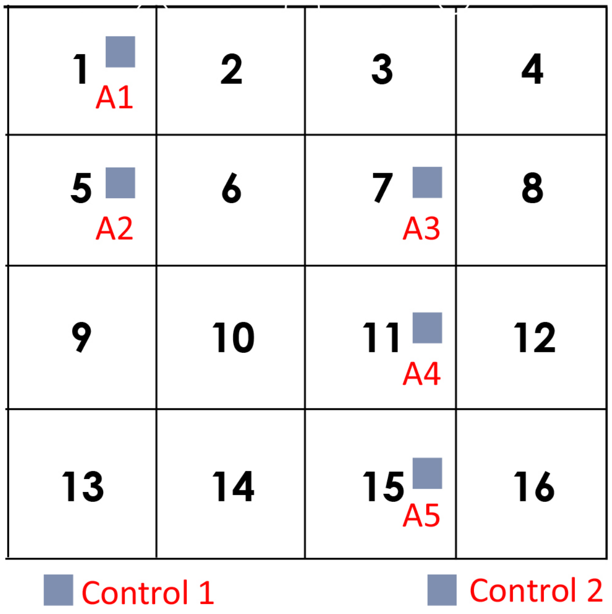

Figure 1. The plots (number) of the experimental site, and sampling points of soil aggregate analysis (square).

soil sample (0 - 30 cm) was also collected from the alfalfa planting plot 7 for analyzing microbial properties. As a control, a bulk soil within the plot 7 was collected from plant-free area in the same planting plots.

General properties: A 1:5 soil: water solution was prepared and shaken for 1 h, and the electrical conductivity (EC) was measured with an EC meter (CM-14P; TOA, Japan) and the pH was determined by glass-electrode analysis (LAQUA F-71; HORIBA, Japan). Exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) were extracted three times from each solution with 1 M ammonium acetate at pH 7.0, and the concentrations were measured with an atomic absorption spectrophotometer (AA-6800; Shimadzu, Japan). The ammonium ion absorbed in each residue was replaced by 10% sodium chloride and the cation exchange capacity (CEC) was determined using Kjeldahl distillation and titration methods. Since the studied soil was calcareous, inorganic carbon had to be removed prior to soil organic carbon analysis. Therefore, soil samples were pre-treated with 1 M hydrochloric acid (HCl) until fizzing stopped, washed with deionized water, air dried and ground again. Then, C and N contents were determined by dry combustion with a CN CORDER (JM 1000; J-Science Lab). The particle size distribution in the soil samples was determined using the sieve-pipette method (<2 μm for clay, 2 - 20 μm for silt, 20 - 200 μm for fine sand, and 200 - 2000 μm for coarse sand fractions [12].

***Aggregate distribution and stability* [13]:** Dry sieving and wet sieving methods were used to assess the size distribution of the soil water stable aggregates. For dry sieving, an air-dried soil sample (1000 g) was distributed evenly on top of a set of sieves with diameter of 15 cm (5, 2, 1, 0.5, 0.25 mm mesh width from top to bottom), and the weight of each soil fraction was determined after shaking 15 minutes at 30 rounds min^{-1}. Wet sieving involved saturating 50 g of >0.106

mm aggregates pooled with the various classes of aggregates mentioned above in a 1-litre graduated cylinder with deionized water, then transferring the saturated soil sample onto the sieve column and shaking it for 30 min at 30 rounds min^{-1}. The resulting soil fractions were transferred to aluminum cans, air dried, and weighed. For measuring water-stable aggregates, 50 g of air-dried aggregates sample was prepared according to the proportion of dry sieve aggregates, and then transferred to the top of the agglomerate analyzer set sieve with the aperture sizes of 5 mm, 2 mm, 1 mm, 0.5 mm, 0.25 mm and 0.053 mm from top to bottom. Deionized water was slowly added along the wall of the barrel until the soil sample was submerged in water, soaked and moistened for 5 minutes, and then the sample was shaken vertically at a speed of 30 times/min for 30 minutes. The particle in each fraction was dried at 105˚C, and the mass of each fraction was determined. Thus, six levels of soil water-stable aggregates of 2 - 5 mm, 1 - 2 mm, 0.5 - 1 mm, 0.25 - 0.5 mm, 0.053 - 0.25 mm, and <0.053 mm were obtained.

Dispersion ratio [14]**:** A sample of air-dried soil equivalent to 10 g of oven-dried soil was placed in a tall cylinder of approximately 1200 cubic centimeter capacity fitted with a rubber stopper. Sufficient distilled water was added to make the volume a liter. The cylinder was closed with the stopper and was shaken end over end 20 times. The suspension was then allowed to settle until the particles of a maximum diameter of 0.05 mm reached to a depth of 30 cm. Thirty ml of the suspension was pipetted at the depth. From the dry weight of the pipetted fraction, the weight of the water-dispersible silt and clay in the suspension was calculated. The dispersion ratio was calculated as the ratio of the water-dispersible silt + clay to the total silt + clay of the soils.

Soil DNA extraction and microbiome analysis [15]**:** Some properties of the bulk and rhizosphere soils are indicated in (Table 1). Soil DNA was extracted from 5.0 g soil by the Ultra Clean Microbial DNA Isolation Kit (MO Bio laboratory, Inc./QIAGEN, Carlsbad, CA, USA) according to the manufacturer's instruction. Concentration and purity of the extracted DNA were estimated by measuring the absorbance at 260 nm and the ratio of $\text{Absorbance}_{260}/\text{Absorbance}_{280}$. Microbiome analysis of the soil DNA extracts was performed by Eurofins Genomics Inc. (Tokyo, Japan), including 16S amplicon sequencing on the Illumina MiSeq sequencer for V3 - V4 regions of 16S rRNA coding region and taxonomy assignment by the QIIME tool (https://www.eurofinsgenomics.jp/jp/service/ngs/16s-rrna.aspx).

Statistical analysis: Tukey's multiple comparison test was used to identify significant differences ($p \leq 0.05$) in physicochemical soil properties between years.

Table 1. Properties of bulk and alfalfa rhizosphere soils.

Soils	Moisture (%)	pH	EC (dS $\text{m}^{-1)}$	Total C (g kg^{-1})	Total N (g kg^{-1})	Available P (mg kg^{-1})
Bulk soil	0.35	9.08	0.051	1.70	0.16	18.9
Rhizosphere soil	0.31	9.25	0.053	1.69	0.11	30.8

3. Results

Soil general physicochemical properties: We found that the pH of the soil at the site was greater than 8.6 and sand content was more than 90%, indicating the soil was both alkaline and sandy. They were classified as Chestnut soil according to Chinese soil taxonomy [16], which corresponds to Calcic-orthic Aridisol based on the United States Department of Agriculture (USDA) soil taxonomy classification [17], and Calcic Chernozem based on the World Reference Base for Soil Resources [18]. Compared to 2014, which was before alfalfa establishment, the physicochemical properties of the soils 5 years after alfalfa planting revealed some significant changes. Significantly higher moisture content, total carbon and nitrogen contents, and CEC were found in 2019 (**Table 2**). Although not significant, the coarse sand content showed a slight decrease, and the fine particles such as fine sand, silt and clay contents increased after alfalfa planting. The average values of exchangeable Ca^{2+}, Mg^{2+}, K^{+} contents, and base saturation were 1.8-, 1.4-, 2.1- and 1.5-fold higher, respectively, in 2019 compared to those in 2014. As described in the previous report, this was most likely caused by irrigation water, since the experimental site was irrigated in the early spring to promote germination [7]. However, exchangeable Na^{+} significantly decreased in 2019, which might be because the area experienced heavy rain during the sampling in 2019 and due to the easy solubility of Na in water. Similar to the results in 2015 to 2018, salinization did not appear to negatively influence the germination and growth of alfalfa in 2019.

Table 2. General physico-chemical properties of the soils before (2014) and after (2019) alfalfa establishment.

Soil parameters	2014 (n = 15)	2019 (n = 16)
Moisture content (%)	0.80 ± 0.25 a	4.63 ± 1.12 b
pH	8.64 ± 0.49 a	8.67 ± 0.17 a
Electric conductivity (mS m^{-1})	5.34 ± 1.51 a	8.26 ± 1.70 b
Total carbon (g kg^{-1})	2.68 ± 1.20 a	5.11 ± 1.47 b
Total nitrogen (g kg^{-1})	0.26 ± 0.06 a	0.42 ± 0.10 b
Coarse sand (%)	67.2 ± 11.2 a	63.0 ± 5.9 a
Fine sand (%)	27.2 ± 10.5 a	31.4 ± 5.5 a
Silt (%)	1.3 ± 0.7 a	1.42 ± 0.54 a
Clay (%)	3.7 ± 1.4 a	4.15 ± 1.45 a
CEC (cmolc kg^{-1})	2.32 ± 0.44 a	3.05 ± 0.54 b
Exchangeable Ca^{2+} (cmol$_c$ kg^{-1})	7.33 ± 4.66 a	13.3 ± 8.51 b
Exchangeable Mg^{2+} (cmol$_c$ kg^{-1})	1.80 ± 1.59 a	2.46 ± 1.78 a
Exchangeable K^{+} (cmol$_c$ kg^{-1})	0.22 ± 0.06 a	0.46 ± 0.15 b
Exchangeable Na^{+} (cmol$_c$ kg^{-1})	0.08 ± 0.01 a	0.05 ± 0.02 b
Base saturation (%)	381.5 ± 194.1 a	588.4 ± 254.3 a

Average value with standard error. (Data for plot 10 of 2014 is not available). Different alphabets indicate significance levels according to Tukey multiple comparison test ($p < 0.05$).

Dispersion ratio: The dispersion ratio of the soils before and 5 years after alfalfa establishment ranged between 25.2 - 39.4% and 20.1 - 52.0%, respectively (**Figure 2**). Although the dispersion ratio of plots 2 - 4 showed some decrease in 2019, no significant difference was apparent in the average values between 2014 and 2019.

Aggregate distribution and stability: The soil water stable aggregates (WSA) were mainly distributed in <1 mm fractions, which accounted for 96.64% - 99.99% of WSA. Only 0.07% - 3.36% of WSA occurred in >1 mm fractions (**Figure 3**). Although the percentages of >1 mm WSA was extremely low, the difference clearly appeared in 2 - 5 mm fraction. Compared to control samples where no 2 - 5 mm WSA were observed, the alfalfa planting plots contained 0.07% to 2.08% of 2 - 5 mm WSA.

Rhizosphere microbiome: A total of 118 and 102 species of microorganisms were identified in the bulk and alfalfa rhizosphere soils in alfalfa plot, respectively. As shown in **Figure 4**, seven species of the top 10 were the same in both soils, but in the rhizosphere, the density of *Conexibacter* sp., *Solirubrobacter* and *Streptomyces* was reduced while that of *Pelobacter* sp. AOP6, unclassified Bacteria

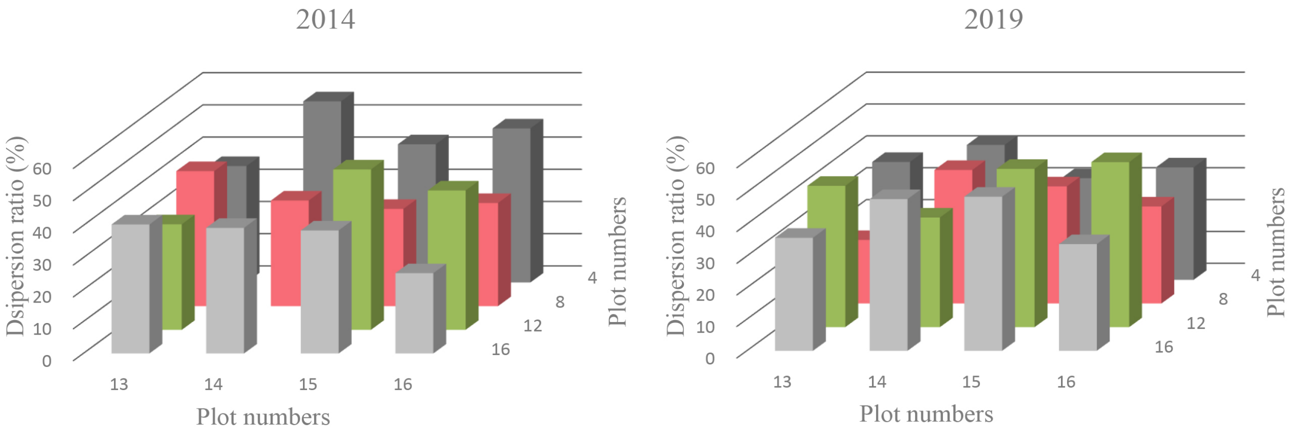

Figure 2. Dispersion percentage of the soils before and five years after alfalfa establishment.

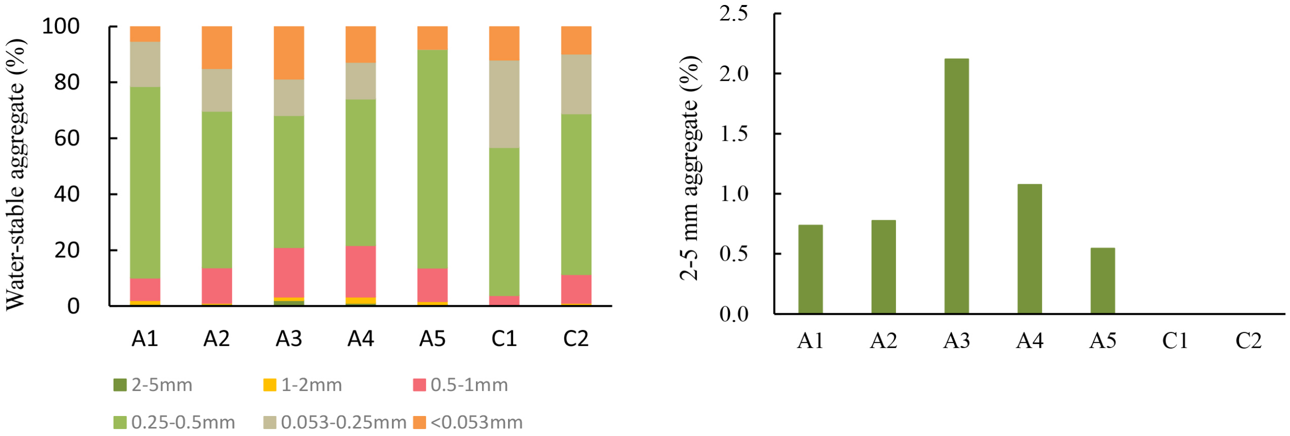

Figure 3. Water-stable aggregate distribution (left) and 2 - 5 mm aggregate (right) of the soils (A1 - A5 show the soils 5 years after alfalfa establish; C1 and C 2 are control sample collected from outside of alfalfa plots).

strains and *Rhodococcus* increased. More than 40% of the top 10 species were actinomycetes in both bulk and rhizosphere soils, although some of the species were different. Moreover, 8 species in the bulk soil were gram-positive, while 6 species in the rhizosphere soil were gram-negative. Two kinds of rhizobia, *Sinorhizobium fredii* and *Bradyrhizobiaceae*, were identified in the rhizosphere soil, and nodule-like structures were observed on the alfalfa root, although their sizes were smaller than those formed by typical rhizobia (**Figure 5**).

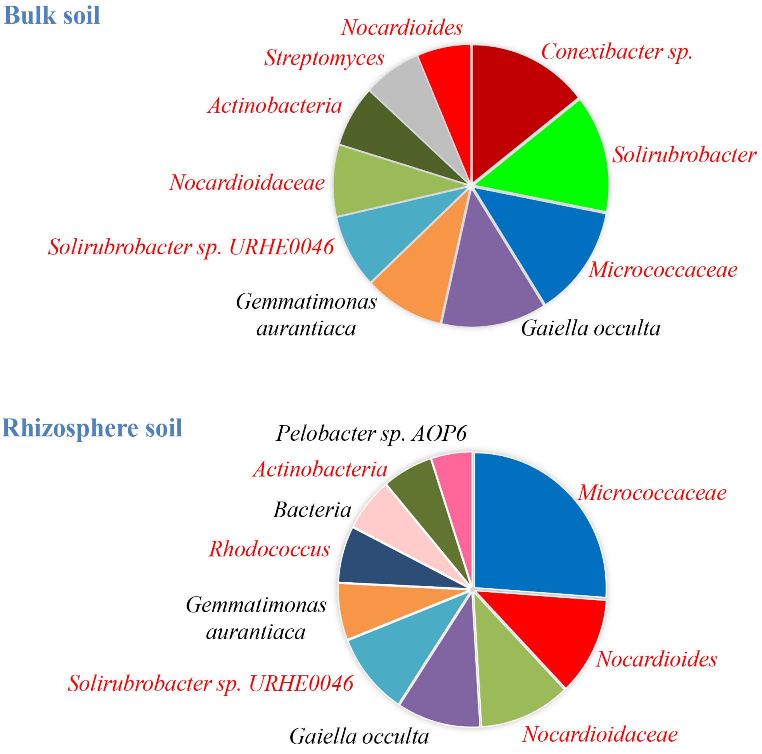

Figure 4. Top 10 species of microbial community in bulk and alfalfa rhizosphere soils (Red letter indicates gram-positive bacteria, and blue letter indicates gran-negative bacteria).

Figure 5. Photo of nodules bearing with alfalfa root in Horqin Sandy Land.

4. Discussion

Similar to the results obtained in our previous work in 2018 [7], the results from 2019 showed that alfalfa establishment improved the soil moisture, organic matter content, soil particle distribution and nutrient retention capacity. The significant increase in soil moisture reflects the abundant rainfall of 2019 (428.9 mm compared to the average annual precipitation of 385 mm). The above ground biomass return from alfalfa in autumn might have contributed to the increase in soil organic matter and CEC.

Soils with a dispersion ratio of <15% are classified as nonerosive, and those with a dispersion ratio >15% are classified as erosive [14]. Although the dispersion ratio decreased in some plots, no significant difference was observed in the average values between the soils before and after alfalfa plantation, and all the soils at the study site are erosive. Our results revealed that five years of alfalfa establishment is still too short to reduce soil dispersion ratio, and a longer-term plant coverage will be necessary for reducing soil erodibility.

Compared to the soils before alfalfa plantation, clay content increased about 0.5% in 2019, five years after alfalfa cultivation. As with clay, the large-scale aggregate content (2 - 5 mm) was also a little higher in the alfalfa planting plot in 2019. The influence of these slight increases may be negligible in humid regions but are presumed to be very significant for soil and ecosystem restoration in semi-arid areas such as the Horqin Sandy Land.

Actinomycetes are well known for their ability to decompose lignocellulose components, which makes them important in decomposition processes [19]. High percentage of actinomycetes was observed in microbial community from both the bulk and rhizosphere soils, which might be important in the decomposition of organic matter derived from alfalfa. Kramer and Gleixner [20] proposed that gram-negative bacteria use more plant-derived C sources that are relatively labile, while Gram-positive bacteria use C sources derived from soil organic matter that are more recalcitrant. Although more gram-negative bacteria were found in the rhizosphere soil compared to bulk soil, gram-positive bacteria dominated in both the soils. It is probably because the levels of moisture and nutrients in the rhizosphere is also extremely low in the study site (**Table 2**). Generally, *Bradyrhizobium* dominates in weakly acidic soil, and *Sinorhizobium fredii* dominates under dry alkaline condition. However, both rhizobia were identified in the soils of this study. Unfortunately, we could not identify rhizobia in nodules, because the nodule-like structures were too small to harvest enough amount of sample [21]. However, the occurrence of rhizobia and nodule-like structures indicated that the leguminous plant alfalfa can form nodules in the alkaline sandy soils of a semi-arid area, and possibly fix nitrogen in them [22]. *Sinorhizobium fredii and Bradyrhizobiaceae* may play an important role for nitrogen supply to alfalfa and soil in the Horqin Sandy Land.

5. Conclusion

Five years after alfalfa was established in the Horqin Sandy Land of Inner Mongolia, China, there was a slight increase in soil clay content and large size WSA, but no obvious improvement in soil dispersiveness. Our results indicate that it would take longer periods of time to improve the soil structure. On the other hand, two types of rhizobia were identified in the alfalfa rhizosphere soil, and nodules were also found on the roots of alfalfa. From these results, we speculate that the local variety of alfalfa, Aohan, can coexist with rhizobia and fix nitrogen in the Horqin Sandy Land. As a future outlook, it is necessary to examine changes in soil physical properties over longer spans. There is also room for improvement in cultivation methods in order to maximize the sand-fixing effect of alfalfa. One idea is to stagger the sowing times between rows so that alfalfa roots are always present in a part of the land.

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Author Contributions

Yumei Kang and Jun Kawamoto designed and managed the study, Tatsuya Kawazawa, Taisei Kono and Yong Zhang conducted the analysis with support from Yumei Kang and Jun Kawamoto, Yumei Kang drafted the manuscript, which was edited by Jun Kawamoto and Yong Zhang.

Additional Information

Requests for materials should be addressed to Yumei Kang.

Competing Interests

The authors declare no competing financial interests.

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