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Seasonality of Arbuscular Mycorrhizal Fungal Diversity and Glomalin in Sodic Soils of Grasslands Under Contrasting Grazing Intensities

Ileana García [1,*], Karla Cáceres-Mago [2] and Alejandra Gabriela Becerra [2,*]

[1] Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Ángel Gallardo 470, Buenos Aires C1405DJR, Argentina
[2] Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET), Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, Córdoba X5016GCN, Argentina; kcaceresmago@imbiv.unc.edu.ar
* Correspondence: igarcia@macn.gov.ar (I.G.); abecerra@imbiv.unc.edu.ar (A.G.B.)

Abstract

Arbuscular mycorrhizal fungi (AMF) taxa, glomalin protein, and hyphal density are potential indicators of soil functionality of temperate grasslands in marginal environments subject to grazing over the years. This study evaluated how the AMF community composition, glomalin protein, and hyphal density vary in response to grazing intensity (low or high) and seasonality (spring and autumn) in sodic soils of Argentinian temperate grasslands. The AMF community was dominated by Glomeraceae species. *Funneliformis geosporus* and *Glomus brohultii* were the most abundant in both seasons and all grasslands. No AMF species were associated with a particular grazing intensity. However, *Entrophospora etunicata*, *Glomus fuegianum*, *Septoglomus constrictum*, and *Acaulospora* sp. occurred only in spring, and no species were exclusive to autumn. Hyphal density was highest in grasslands with low grazing intensity and can be considered an indicator of soil functionality. Glomalin protein was the highest in spring in all grasslands. The lower grazing intensity in grasslands with poor livestock control showed no changes in AMF diversity. The AMF community showed high adaptation to soil conditions, indicating high resilience. We concluded that longer periods of controlled grazing management are needed to improve soil conditions and, consequently, change the AMF species composition.

Keywords: arbuscular mycorrhizal community; glomalin protein; grazing; sodic soils; seasonality; temperate grasslands

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1. Introduction

At a global level, the sustainability of temperate grasslands is threatened by the poorly controlled use of the soil in these environments [1,2]. In general, these grasslands are used for raising livestock due to their forage supply. In Argentina, the scenario is similar, and temperate grasslands are considered marginal environments, with nutrient-deficient soils, salinity and sodicity problems, and heavy soil with scarce drainage [3–5].

In general, poorly controlled grazing or overgrazing reduces plant species diversity, vegetation cover, and forage quality due to the presence of agronomically undesirable species [6,7]. Another negative effect of overgrazing is the increase in the percentage of bare soil, which consequently decreases soil fertility and water retention, increasing soil compaction [1]. The combination of these factors leads to the deterioration of the grassland,

also affecting soil microbial communities [5]. In recent years, there has been increasing concern about the degradation of grasslands and the negative effects on forage production and the environment. This has led to a holistic rethinking of livestock management systems towards a more environmentally friendly approach, associated with increased resting periods and reduced stocking rates [6]. In long-term experiments, the decrease in grazing pressure has been shown to improve forage quality and soil physical and chemical properties compared with high-intensity grazing grasslands at a similar annual stocking rate [8,9]. However, the question remains as to how long it takes for significant changes in soil properties and plant and microbial communities to occur after grazing intensity is reduced. This question has no single answer, and the answer depends on each particular system. We consider that Argentinian temperate grasslands are a good scenario to study the effects of reduced grazing intensity on soil properties and microbial communities in different marginal environments.

Regarding soil microbial communities, arbuscular mycorrhizal fungi (AMF) are a widely distributed fungal group capable of establishing a symbiotic relationship with 85% of terrestrial plants [10]. AMF are a key soil community that plays a vital role in maintaining the productivity and stability of grasslands [11]. In this sense, AMF confer benefits at the individual plant and ecosystem levels [10,12]. Individual plant benefits include improved nutrition and water balance and tolerance to grazing and environmental stresses [13–15]. At the ecosystem level, it is interesting to study the AMF species that can be indicators of environmental deterioration or restoration following a change in grazing practices. In this sense, the effects of grazing on the AMF community are controversial because the AMF response to grazing is context-dependent, may be influenced by soil properties [16,17], and is directly related to the intensity and extent of grazing over time [9,18]. Thus, knowledge of the composition of the AMF community in response to grazing is of great importance to understand the maintenance of AMF diversity and community stability over time and its relationship with soil properties, especially in marginal ecosystems, such as Argentinian temperate grasslands. To the best of our knowledge, this aspect has been little studied.

At the ecosystem level, a relevant aspect to highlight is that the AMF hyphal network established in the soil is able to connect different plants and promote nutrient transport between species [10,12]. In addition, the hyphae and spores produce and store glomalin, a glycoprotein that is characteristic of this group of fungi. When the hyphae and spores die, glomalin is deposited in the soil, where it accumulates up to 5% of soil carbon and acts as a soil structuring agent [19]. Glomalin is isolated from the soil as a fraction known as total glomalin-related soil proteins (GRSPs). However, a fraction of total glomalin-related soil proteins (T-GRSPs), known as easily extractable glomalin-related soil proteins (EE-GRSPs), is more frequently measured due to its simplicity [20]. It has been reported that EE-GRSPs are sensitive to soil changes caused by their use and management [21,22]. Therefore, it has been suggested that GRSPs may be useful as indicators of soil functionality [23].

In general, the diversity of AMF communities is strongly influenced by soil pH, phosphorus (P) availability, salinity, soil disturbance, and vegetation or hydrologic condition of the soil [24,25]. Another factor influencing AMF community diversity is seasonality. In this sense, the abundance of certain AMF taxa may increase in one season and may decrease or even disappear in the next season. Interactions between seasonal effects and soil conditions can result in AMF community diversity being dominated by generalist species able to occur in a wide range of soil conditions and seasons, or by exclusive species associated with a particular soil condition, grazing management, or season [24,26,27]. In association with soil conditions and grazing management, GRSP values and hyphal density in the soil would also show a seasonal pattern in grassland environments [28,29]. In this sense, AMF taxa and GRSP and hyphal density can act as indicators of soil functionality of temperate grasslands

in marginal environments subject to grazing over the years. This information is crucial to evaluate the impact of changes in grazing intensity on grasslands with a long history of high grazing intensity or overgrazing, such as the Argentinian temperate grasslands.

The aim of this study was to evaluate how the AMF community composition and GRSP and hyphal density vary in response to grazing intensity and seasonality in soils of Argentinian temperate grasslands. This study was conducted in grasslands on sodic soils subject to low or high grazing intensity in two contrasting seasons: spring and autumn. We hypothesized that AMF diversity, spore and hyphal densities, and GRSP concentration are higher in grasslands with low grazing intensity in spring than in those with high grazing intensity in autumn, due to increased plant growth and improved soil properties.

2. Materials and Methods

2.1. Selected Sites and Soil Sampling Procedure

This study was carried out in six grassland sites with sodic soils and different grazing management of beef cattle in two localities of Argentina: two grasslands in the area of Chascomús in Buenos Aires province (35°43′47″ S, 58°03′18″ W) (Sites 1 and 2) and four grasslands in the area of San Cristobal in Santa Fe province (30°20′46″ S, 61°15′39″ W) (Sites 3 to 6) (Table 1). The distance between these two localities is about 610 km.

Table 1. Main characteristics of the grasslands studied.

Grassland Site	Grazing Intensity	Location	Soil Classification	Na^+ (cmol/kg)	Dominant Plant Species	Stocking Rate (LU/ha)	Period of Management (Years)
1L	low	Chascomús	Typic Natraquoll	7.07	*Cynodon dactylon* (L.) Pers. *Festuca arundinacea* Schreb.	1	13
2H	high	Chascomús	Typic Natraquoll	5.99	*Cynodon dactylon* *Festuca arundinacea*	0.85	35
3L	low	San Cristobal	Typic Natraquoll	7.03	*Cynodon dactylon* *Festuca arundinacea*	1.23	26
4H	high	San Cristobal	Typic Natraquoll	4.56	*Cynodon dactylon*	0.57	30
5L	low	San Cristobal	Typic Natraqualf	6.69	*Sporobolus spartinus* (Trin.) P.M. Peterson & Saarela *Cynodon dactylon*	1.23	26
6H	high	San Cristobal	Typic Natraqualf	5.71	*Sporobolus spartinus* *Cynodon dactylon*	0.57	30

The common scenario in these grasslands is poorly controlled grazing with high livestock density, short rest periods, or even no rest at all (high grazing intensity). However, in several grasslands, livestock management has been modified, decreasing grazing intensity, with cattle remaining in the grassland for only 2 to 5 days with rest periods of 30 to 70 days (low grazing intensity). In particular, three of the selected grasslands (Sites 1, 3, and 5) are currently subject to this low grazing intensity, separated by a fence from their counterparts with high grazing intensity (Sites 2, 4, and 6) (Table 1). Site 1 reduced the grazing intensity 13 years ago, whereas Sites 3 and 5 did so 26 years ago (Table 1). Sites 2, 4, and 6 have been continuously subjected to high grazing intensity for more than 30 years.

The grassland sites were classified as having low or high grazing intensity according to the stocking rate (Table 1). The stocking rate is the number of cows per ha (LU, livestock units). The grasslands were named by the number of each site followed by the initial of the grazing intensity (L, low and H, high intensity). The soil of Sites 1 to 4 is a typic Natraquoll and the soil of Sites 5 and 6 is a typic Natraqualf. The mean annual rainfall in Buenos Aires and Santa Fe grasslands is 980 mm and 1020 mm, respectively, whereas the mean annual temperature is 15 °C and 19 °C, respectively. Plant communities are dominated by perennial and seasonal grasses, with a decrease in the presence of legumes, a fact that has led to a decrease in the forage quality of these grasslands (Table 1).

The samplings were performed in two different seasons: spring (December 2022) and autumn (June 2023). At each grassland site, three random rectangular plots were located

and separated by 30 m. Each plot consisted of two overlapping rectangular areas (subplots) measuring 10 × 8 m and 18 × 16 m, respectively. The inner subplot (10 × 8 m) was used to determine the seasonal floristic composition [30], whereas the outer subplot (i.e., the area of the plot defined by the 10 and 18 m subplots) was used for seasonal soil sampling to determine the soil chemical properties, AMF spore diversity, hyphal density, and glomalin concentration. Ten soil core samples were taken from each plot and mixed homogeneously to form a composite sample. Each soil core sample was 12 cm deep. The top 2 cm were removed to eliminate part of the top leaf litter. The three composite soil samples (one from each plot) were thoroughly mixed and divided into two portions to determine the soil chemical properties and AMF variables. The soil samples were kept in plastic bags at 4 °C until processed. The percentage of bare soil was obtained by calculating the number of bare soil records out of the total number of records obtained, multiplied by 100.

2.2. Taxonomic Identification of AMF Spores in the Soil

Spores were isolated from 100 g of dry soil by sieving through 125 and 38 µm meshes, according to Gerdemann and Nicolson [31]. The supernatant was centrifuged with 80% sucrose [32], and the spores were placed in Petri dishes divided into 1 × 1 cm grids. Subsequently, spores were separated by morphotype by mounting on slides with polyvinyl acetate (PVA) and PVA + Melzer's reagent [33] and recognized at the morphospecies level under a light microscope (ECLIPSE E200, Nikon Corporation, Tokyo, Japan) according to the identification manuals of Schenck and Perez [34], Blaszkowski's website [2013, www.zor.zut.edu.pl/Glomeromycota_2/Taxonomy.html] (accessed on 20 March 2025), and the INVAM website [https://invam.ku.edu/species-descriptions] (accessed on 20 March 2025). Total spore density was the total number of spores in 100 g of dry soil, whereas specific spore density was the number of each morphospecies detected in each grassland site.

2.3. Diversity of the AMF Community

The ecological measures of diversity here used to describe the seasonal structure of the AMF community included relative abundance (RA), defined as the percentage of spore number of a species and indicating the sporulation ability of different AMF species; the Shannon–Weaver index of diversity (H), calculated from the equation $H' = -\Sigma$ pi(ln pi), where pi is the relative abundance of the i th species based on its spore counts; the richness index (R), estimated as the total number of species in each sampling grassland; and the evenness (E) index, estimated as the ratio between H′ and ln R [35].

Isolation frequency (IF%) was defined as the percentage of soil samples in which a species occurred, which revealed the extent of distribution of a given AMF species in each site. The importance value (IV) was calculated to assess the dominance of AMF species based on IF% and RA as follows: IV = (IF + RA)/2. Species dominance was classified into four levels: the dominant species (IV $\geq$ 50%), the most common species (30% < IV $\leq$ 50%), common species (10% < IV $\leq$ 30%), and rare species (IV $\leq$ 10%) [36,37]. To evaluate the degree of community similarity of AMF between two grasslands with different grazing intensity, Sorenson's coefficient (CS) was used and calculated according to the following formula: CS = 2j/(a + b), where j is the number of AMF species co-existing in both sites, a is the total number of AMF species in the low-intensity grazing site, and b is the total number of AMF species in the high-intensity grazing site [36].

2.4. Hyphal Density in the Soil

The length of external hyphae was determined after extracting them with a procedure modified from Abbott et al. [38] and measuring on 100 fields per soil sample by using the modified grid line intersect method [39] under ×200 magnification. Hyphal density was expressed as meters of hyphae per gram of dry soil.

2.5. Glomalin

EE-GRSP and T-GRSP were quantified following the method of Wright and Upadhyaya [40]. The EE-GRSP fraction was obtained from 1 g of soil using 8 mL of sodium citrate (20 mM, pH 7.0), followed by autoclaving at 121 °C for 30 min, whereas the T-GRSP fraction was extracted from 1 g of soil with 8 mL of sodium citrate (50 mM, pH 8.0), autoclaved at 121 °C for 60 min. In both cases, the supernatants were collected by centrifugation at 3000 rpm for 15 min. For T-GRSP, the extraction was repeated until the reddish coloration of the solution disappeared, and the extracts from all the cycles were pooled. A 2 mL aliquot of each extract was subsequently centrifuged at 10,000 rpm for 15 min. The GRSP concentrations were determined using the Bradford protein assay (Coomassie-Bradford Protein Assay Kit, Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as the standard [41], and absorbance was measured spectrophotometrically. The GRSP content was expressed as milligrams per gram of dry soil.

2.6. Soil Analyses

Soil pH (soil–solution ratio of 1:2.5 in water), moisture content, exchangeable Na [42], exchangeable sodium percentage (ESP), organic carbon (C) [43], organic nitrogen (N) [44], and P availability [45] were measured in each season and site.

2.7. Statistical Analyses

AMF variables and soil properties were analyzed through a two-way ANOVA with grazing intensity (grassland site) and season as the first and second factors, respectively. The means were compared by the Fisher test. The normality and homogeneity of variances were previously verified. Non-normal distributed data were appropriately transformed to compare means. Spore density was transformed by ln and bare soil by ln(x + 1). Pearson correlation coefficients were calculated to quantify the association between the AMF community vs. soil properties for all sites seasonally. Seasonal relationships between pH, soil moisture, ESP, organic C, organic N, P availability, and spore density of the AMF community at species level, hyphal density, and T-GRSP were evaluated by principal component analysis (PCA). Statistical analyses were performed with Infostat version 2019 [46].

3. Results

3.1. Soil Chemical Properties

With the exception of available P, the remaining soil properties were affected by the grazing intensity, season, and interaction between these two factors (Figure 1; Table S1). In general, the soil pH of the grasslands studied was above neutrality and considered to be either slightly alkaline (pH < 8.0) (2H and 3L in spring, 4H in both seasons), moderately alkaline (pH 8.0–8.5) (1H in spring), or strongly alkaline (pH > 8.5) (5L and 6H in spring and 1L to 6H in autumn) (Figure 1a). The pH value in grasslands 1L to 4H increased in autumn, whereas the pH in grasslands 5L and 6H showed no seasonal differences. Soil moisture was the lowest in 1L and 2H in spring (Figure 1b), and higher than 15% in the other grasslands, regardless of the season. ESP decreased by grazing intensity in both sampling seasons (Figure 1c; Table S1) and increased seasonally, except in grassland 6H. The electrical conductivity values for grasslands 1L to 6H were 2.44 dS m $^{-1}$, 2.77 dS m^{-1}, 3.62 dS m^{-1}, 2.85 dS m^{-1}, 4.00 dS m^{-1}, and 3.19 dS m^{-1}, respectively. The soils of the grasslands studied were classified as non-saline–sodic, with electrical conductivity values lower than 4 dS m^{-1} and ESP values higher than 15% [47].

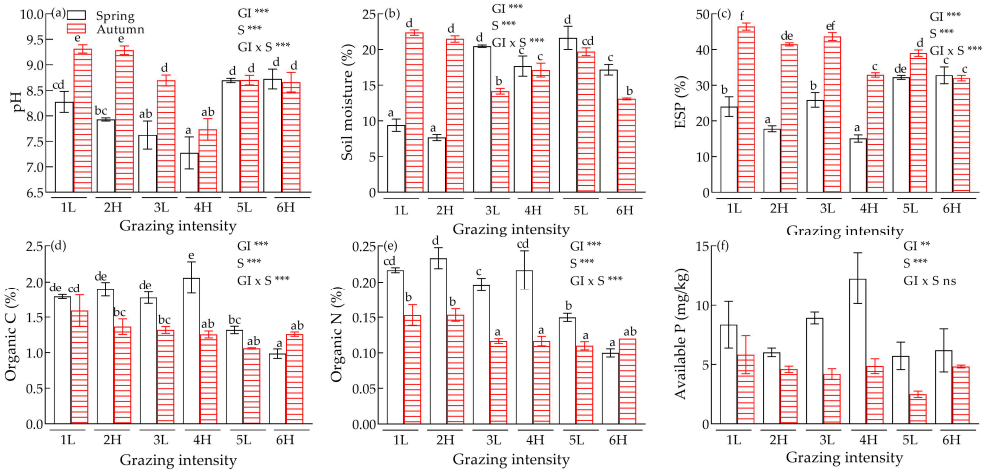

Figure 1. Soil properties in six grasslands under low (L) or high (H) grazing intensities in spring and autumn. (**a**) Soil pH, (**b**) soil moisture, (**c**) exchangeable sodium percentage, (**d**) organic carbon, (**e**) organic nitrogen, (**f**) soil available P. GI: grazing intensity, S: season, ESP: exchangeable sodium percentage. Values are means ± SE. Different letters indicate significant differences among means according to the Fisher test. Statistically significant factor effects are shown in each graph: ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$.

Organic C was highest in grasslands 1L to 4H and lowest in grasslands 5L and 6H in spring. This is because the soil of grasslands 1L to 4H was classified as Natraquoll, while the soil of grasslands 5L and 6H was classified as Natraqualf (Figure 1d). Organic C showed a tendency to decrease in autumn, except in grassland 6H. The pattern of organic N was similar to that of organic C (Figure 1e). The available P ranged from 4.20 to 8.93 mg kg^{-1} (Figure 1f). Soils from all the grasslands studied showed a marked deficiency of P for plant growth (<10 mg P kg^{-1}), except for 4H in spring (12.29 mg kg^{-1}). Available p values decreased in autumn independently of the grazing intensity. The concentrations of organic C, organic N, and available P were negatively correlated with pH (organic C, r = −0.53, $p = 0.0009$; organic N, r = −0.49, $p = 0.0027$; available P, r = 0.54, $p = 0.0007$).

As grazing intensity increased, the percentage of bare soil increased significantly (Figure 2; Table S1), reaching a maximum value in grassland 6H, where the soil type and poorly controlled grazing management led to grassland deterioration. Additionally, the percentage of bare soil showed no seasonal differences. The percentage of bare soil and organic N were negatively correlated (r = −0.34, $p = 0.00407$).

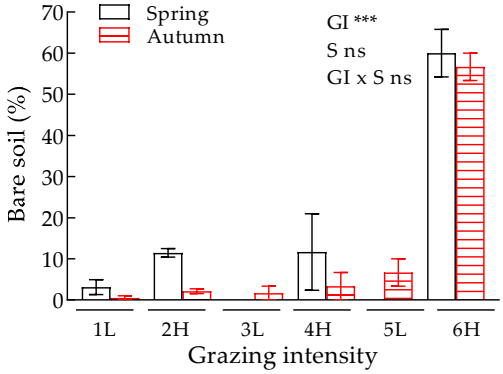

Figure 2. Percentage of bare soil in six grasslands under low (L) or high (H) grazing intensities in spring and autumn. GI: grazing intensity, S: season. Values are means ± SE. Significant differences among means according to the Fisher test. Statistically significant factors effects are shown in the graph: *** $p < 0.001$, ns $p > 0.05$.

3.2. AMF Community

Spore density was significantly affected by the grazing intensity, season, and their interaction (Figure 3a; Table S1). In grasslands 3L, 4H, and 5L, spore density showed a seasonal decline, with the lowest values recorded in autumn. Contrary to expectations, in 6H, despite the high proportion of bare soil, spore density was high (Figure 3a).

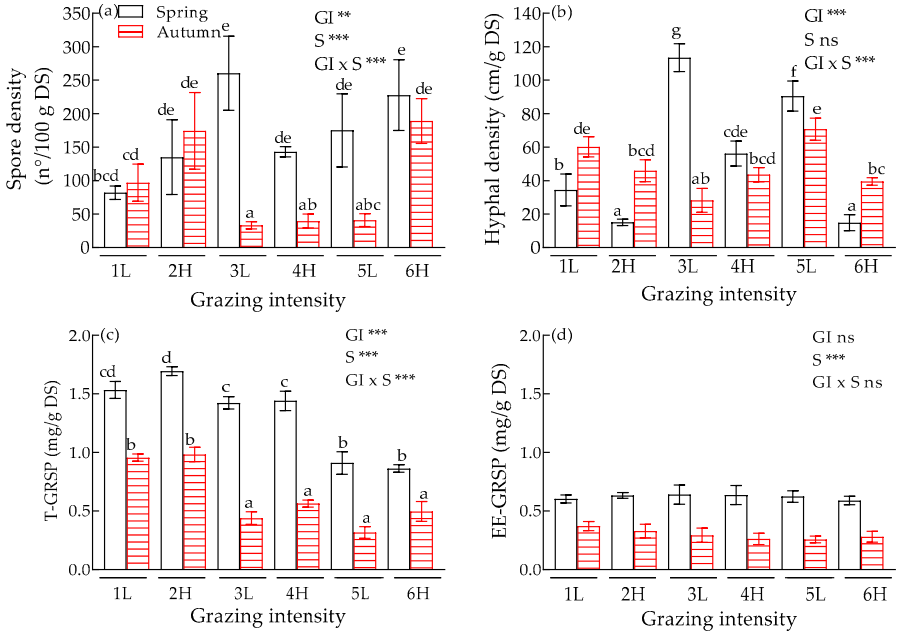

Figure 3. Spore density (**a**), hyphal density (**b**), total glomalin-related soil proteins (T-GRSP) (**c**), and easily extractable glomalin-related soil proteins (EE-GRSP) (**d**) in six grasslands under low (L) or high (H) grazing intensities in spring and autumn. GI: grazing intensity, S: season. Values are means ± SE. Different letters indicate significant differences among means according to the Fisher test. Statistically significant factors effects are shown in each graph: ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$.

Hyphal density was found to be influenced by grazing intensity, as well as by the interaction between grazing intensity and season (Figure 3b; Table S1). In general, hyphal density tended to decrease with increasing grazing intensity, except in 4H during autumn, where values remained high. GRSPs were detected across all the grasslands studied and during both seasons. The T-GRSP concentration was affected by the grazing intensity, season, and their interaction (Figure 3c; Table S1). The T-GRSP concentration was consistently higher in spring than in autumn, regardless of the grazing intensity. The highest values of T-GRSP were recorded in grasslands 1L to 4H (Natraquoll soils) and the lowest in 5L and 6H (Natraqualf soils), with maximum values observed in 1L and 2H during spring (Figure 3c). The EE-GRSP concentration varied significantly only with season (Figure 3d; Table S1), following a pattern similar to that of the T-GRSP concentration, with higher levels in spring than in autumn in all the grasslands studied.

Hyphal density was positively associated with soil moisture (r = 0.63, $p < 0.0001$) and negatively correlated with the percentage of bare soil (r = −0.40, $p = 0.0151$). The concentrations of T-GRSP and EE-GRSP were positively associated with organic C, organic N, and available P (for T-GRSP: organic C, r = 0.77, $p < 0.0001$; organic N, r = 0.88, $p < 0.0001$; available P, r = 0.65, $p < 0.0001$) (for EE-GRSP: organic C, r = 0.53, $p = 0.0009$; organic N, r = 0.62, $p = 0.0001$; available P, r = 0.60, $p = 0.0001$).

Figure 4 shows the percentage of AMF species belonging to each genus and the percentage of genera belonging to each family in each sampling season. In spring, AMF spore diversity was associated with nine genera distributed in five families, whereas in autumn, spore diversity was associated with five genera distributed in two families

(Figure 4a,b). In spring, the dominant genus was *Glomus*, with a species percentage of 28.57%, followed by *Entrophospora* and *Funneliformis* (14.29%). The remaining genera, i.e., *Sclerocystis*, *Rhizoglomus*, *Septoglomus*, *Diversispora*, *Scutellospora*, and *Acaulospora*, had the same percentage (7.14%) (Figure 4a). The family with the greatest number of genera was the Glomeraceae (55.55%), followed by the Entrophosporaceae, Diversisporaceae, Gigasporaceae, and Acaulosporaceae with the same percentage (11.11%) (Figure 4a). In autumn, the leading genus was *Funneliformis*, with a species percentage of 33.33%, followed by *Entrophospora*, *Glomus*, *Sclerocystis*, and *Rhizoglomus*, which had a similar percentage (16.67%) (Figure 4b). In this season, the genera were distributed among only two families: the Glomeraceae (80%) and the Entrophosporaceae (20%) (Figure 4b).

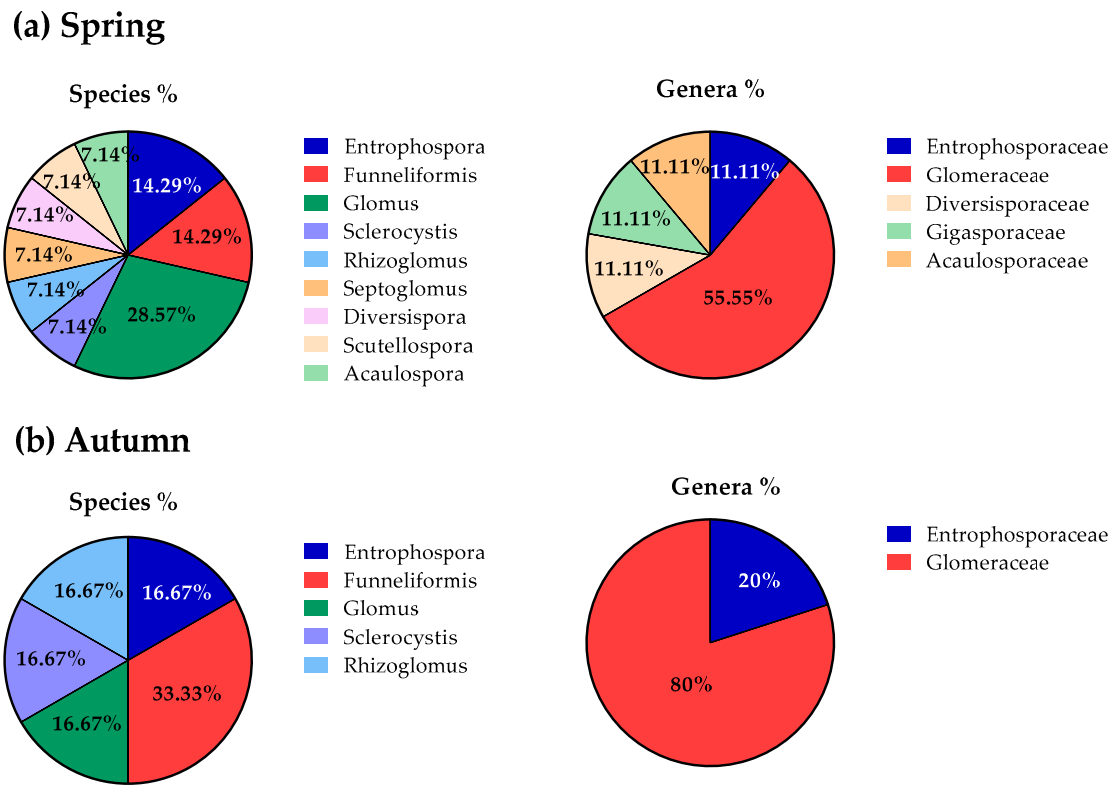

Figure 4. Percentage of species classified for each genus and percentage of genera corresponding to each family in all the grasslands studied under low or high grazing intensities in spring (**a**) and autumn (**b**), respectively.

Within the Glomeraceae, *Funneliformis geosporus* and *Glomus brohultii* were the dominant and generalist species (Table 2 and Figure S1) in all the sites and seasons, each with importance values > 50% (dominant species) (Table S2). *Funneliformis mosseae* was frequent and dominant in grasslands 1L, 2H, 3L, 5L, and 6H (Table S2 and Figure S1). *Glomus* sp. 1 and sp. 2 were exclusive to 3L and 5L, respectively, and considered common species according to the importance value (Table 2 and Table S2). *Glomus fuegianum* was found only in 3L and 4H during spring, with low relative abundance (Table 2 and Figure S1). *Sclerocystis sinuosa* appeared in 1L, 2H, and 4H in spring (Table S2), with higher frequency under high grazing intensity. *Rhizoglomus intraradices* was present in grasslands 1L to 3L in both seasons, and in 4H to 6H only in autumn (Figure S1). *Septoglomus constrictum* was present only in spring across all the sites, with higher abundance under high grazing (Figure S1).

A total of 14 AMF species were identified in spring and 6 in autumn across all the grasslands (Table 2 and Table S2). Within the Entrophosporaceae, *Entrophospora etunicata*

was exclusive to spring and present in 1L, 2H, 3L, and 6H, while *E. claroidea*, classified as a generalist species, was present in all the grasslands and both seasons (Table 2 and Table S2).

Table 2. Relative abundance of AMF species, diversity (H), richness (R), and evenness (E) indexes in six grasslands under low (L) or high (H) grazing intensities in spring and autumn.

AMF Species	1L		2H		3L		4H		5L		6H	
	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn
Family Entrophosporaceae												
Entrophospora etunicata	2.33	0	4.67	0	1.33	0	0	0	0	0	0.67	0
Entrophospora claroidea	0	5	1.67	0.33	12	7.33	11.33	6	5	22.67	23.33	7
Family Glomeraceae												
Funneliformis geosporus	29.67	38.67	22.33	56.33	23.33	37.67	33.67	51.67	41.67	52	34	39
Funneliformis mosseae	0	13.33	0.67	7.33	3.67	0	0	0	7.33	0	6.67	10.67
Glomus brohultii	31.66	33	16.67	28	34.67	30	32.33	32.33	29.33	10	22	36.67
Glomus sp. 1	0	0	0	0	2.33	0	0	0	0	0	0	0
Glomus sp. 2	0	0	0	0	0	0	0	0	0	0	1.67	0
Glomus fuegianum	0	0	0	0	0.67	0	1.33	0	0	0	0	0
Sclerocystis sinuosa	12.33	0	16.33	0	0	0	2.67	6.33	0	0	0	0
Rhizoglomus intraradices	13.00	9.67	0	1.67	7.67	0.67	25.33	0	3	14.67	0	6.67
Septoglomus constrictum	10.66	0	26	0	6.67	0	14.33	0	1.33	0	3.67	0
Family Diversisporaceae												
Diversispora spurca	0	0	0	0	13	0	4	0	15.67	0	5.67	0
Family Gigasporaceae												
Scutellospora sp.	0	0	0	0	1.33	0	0	0	0	0	0	0
Family Acaulosporaceae												
Acaulospora sp.	0	0	2.33	0	7.50	0	0	0	0	0	0	0
H	1.83	1.65	2.18	1.50	2.35	1.62	1.98	1.38	1.91	1.47	2.29	1.91
R	4.33	4.67	5.67	4.67	7.33	3.33	4.67	3.33	4.67	3.33	6.67	5.33
E	0.93	0.73	0.89	0.72	0.82	0.94	0.90	0.83	0.86	0.90	0.85	0.79

Within the Diversisporaceae, *Diversispora spurca* was found in 3L to 6H during spring, with variable dominance in 3L and 5L, and was a common species in 4H and 6H, respectively (Table 2 and Table S2).

Finally, within the Gigasporaceae, *Scutellospora* sp. was exclusive to 3L in spring as a common species (Table S2), and within the Acaulosporaceae, *Acaulospora* sp. appeared in 2H and 3L in spring.

No specific pattern of the presence of AMF species was associated with a particular grazing management. However, various species, such as *E. etunicata*, *G. fuegianum*, *S. constrictum*, and *Acaulospora* sp., were associated only with the spring season, and no species were associated only with autumn.

In particular, *E. claroidea* was positively associated with bare soil ($r = 0.56$, $p < 0.0004$) and *F. mosseae* with pH ($r = 0.43$, $p = 0.0089$). *Rhizoglomus intraradices* was positively associated with both pH ($r = 0.47$, $p = 0.0036$) and ESP ($r = 0.40$, $p = 0.0156$). *Glomus brohultii* was positively associated with hyphal density ($r = 0.40$, $p = 0.0144$). *Sclerocystis sinuosa* was positively associated with organic N ($r = 0.44$, $p = 0.0078$) and available P ($r = 0.37$, $p = 0.0257$) and negatively associated with hyphal density ($r = -0.41$, $p = 0.0134$), moisture percentage ($r = -0.59$, $p = 0.0002$), and ESP ($r = -0.50$, $p = 0.0017$).

The diversity (H) and richness (R) indexes were significantly affected by the season, while the evenness (E) index was influenced by both the season and the interaction between season and grazing intensity (Table 2 and Table S1). Overall, the AMF community exhibited low diversity ($H < 2$), low species richness, and similar distribution in both sampling seasons, with different grazing intensities. The diversity index showed a positive correlation with available P ($r = 0.50$, $p = 0.0019$) and a negative correlation with ESP ($r = -0.37$, $p = 0.0247$). The similarity of the AMF community was assessed using Sorenson's coefficient. This analysis showed higher similarity in autumn than in spring for all low and high grazing intensity grasslands pairs: 1L and 2H (1.00), 3L and 4H (0.89), and 5L and 6H (0.80) in autumn versus 1L and 2H (0.80), 3L and 4H (0.63), and 5L and 6H (0.75) in spring. These results align with those of diversity indexes, suggesting similar AMF community structure

between grasslands with contrasting grazing intensities, with greater homogeneity in the AMF species composition in autumn than spring.

3.3. Relationship Between AMF Community and Soil Properties

Seasonal relationships among soil properties (pH, soil moisture, ESP, organic C, organic N, P availability), AMF spore density (at the species level), hyphal density, and T-GRSP were evaluated through a PCA (Figure 5). The first two PCA axes explained 64.3% of the total variance, with PC1 explaining 40.7% of the variance and PC2 explaining 23.6%. In spring, grasslands with Natraquoll soils (Sites 1 to 4) were located on the left side of the figure, while Natraqualf soils (Sites 5 and 6) were on the right side. This differentiation was not observed in autumn. However, grasslands 1L and 3L were separated from 2H and 4H, and all four grasslands were associated with higher levels of organic C, organic N, and available P. T-GRSP showed a positive correlation with both organic C and organic N. *Sclerocystis sinuosa* was positively associated with organic N, organic C, available P, and T-GRSP and negatively associated with pH, hyphal density, and ESP. *Entrophospora claroidea*, *F. geosporus*, and *G. brohultii* were positively associated with hyphal density, while *F. mosseae* was associated with increased soil moisture, especially in grasslands 5L and 6H. *Rhizoglomus intraradices* was associated with higher pH and ESP values. Additionally, grasslands 1L, 2H, 3L, and 4H were located in opposite quadrants of the PCA diagram depending on the season, while grasslands 5L and 6H were located on the right side of the diagram independently of the season.

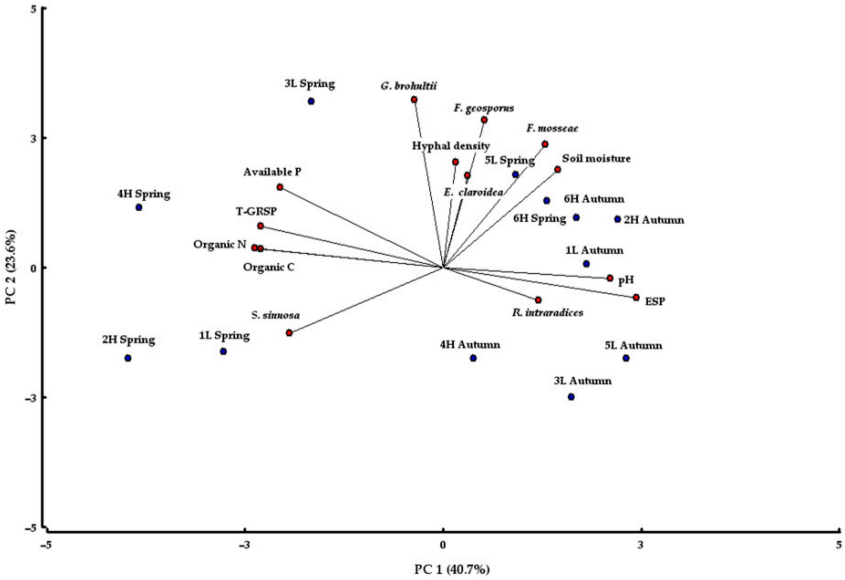

Figure 5. Principal component analysis (PCA) linking soil properties with spore density of AMF community at species level, hyphal density, and T-GRSP, determined in six grasslands under low (L) or high (H) grazing intensities in spring and autumn. ESP: exchangeable sodium percentage. T-GRSP: total glomalin-related soil proteins. Sites were named by the number of each grassland site followed by the initial of the grazing intensity (L, low and H, high intensity).

4. Discussion

This study provides a seasonal assessment of the impact of decreasing the grazing intensity on the AMF community in temperate grasslands with a long history of poorly controlled grazing in marginal environments. The results suggest that spore and hyphal densities, AMF species composition, and GRSP levels can be used as effective indicators of soil functionality and are valuable parameters to evaluate the degree of deterioration or recovery of grassland soils.

4.1. Seasonality of Soil Properties Under Different Grazing Intensities

Previous studies have shown that pH, organic C and N, P availability, and exchangeable ions vary with different grazing intensities. However, the effect of grazing on soil properties depends on the history of grazing management and seasonality and climate conditions [18,25]. In general, nutrient availability in the soil increases under higher grazing intensity [48,49], as dung is rich in organic C and P, and urine is rich in N. In the present study, the values of organic C, organic N, and P availability were higher in spring than in autumn, which was associated with increased soil microbial activity in spring. These results are similar to previous studies in several grasslands [3,50]. In addition, changes in soil pH influence activities of soil microorganisms involved in the mineralization of soil organic C and thus in nutrient cycling (particularly N), thereby affecting the transformation of organic matter [48]. In our study, both pH and ESP showed a stronger seasonal effect than grazing intensity, especially in grasslands 1L to 4H (Natraquoll soil) compared to grasslands 5L and 6H (Natraqualf soil). This seasonal pattern may be associated with the soil salinization process facilitated by the movement of the saline water table due to an increase in soil water content [50]. In this sense, grasslands 5L and 6H also exhibited an increased percentage of bare soil and a marked decrease in organic C and N, showing the vulnerability of these grasslands and the need to implement other strategies to prevent further loss of vegetation cover. In all the grasslands studied, the soil conditions showed no clear improvement despite the long period since the change in the grazing management.

4.2. Seasonal Effect on the Diversity of the AMF Community Under Different Grazing Intensities

In the present study, spore density did not follow a consistent pattern across all the grasslands, with a marked decrease observed in grasslands 3L, 4H, and 5L in autumn. This seasonal pattern is consistent with findings from previous studies in temperate grasslands [3,50,51]. Seasonal variation in spore density can be explained by the interaction between soil properties, plant growth rate, and climate conditions, which together regulate the balance between sporulation and disappearance of spores in the soil. In the present study, since no significant relationship was found between spore density and soil properties, the decrease in the number of spores in autumn may be associated with a decrease in AMF activity and plant growth rate. Therefore, spore density was not considered a good indicator of the effects of changes in grazing intensity on the grasslands analyzed. In contrast, hyphal density was a good indicator of changes in grazing intensity, that is, hyphal density was higher in grasslands where livestock management was changed to an environmentally friendly approach (low-intensity grazing grasslands). This finding is in line with previous studies in grasslands subject to different grazing intensities [18,51]. In addition, hyphal density was positively correlated with soil moisture, in agreement with previous findings [29]. Hyphal density was also negatively associated with the percentage of bare soil, a good indicator of grassland degradation, particularly in grassland 6H, which presented limiting soil conditions with high ESP values and reduced levels of organic C and organic N.

GRSPs are sensitive to soil changes resulting from land use and management [21,22]. Therefore, GRSPs have been proposed as indicators of AMF functionality and soil fertility [23,52]. In the present study, EE-GRSPs showed a strong seasonal effect across all the grasslands, with higher concentrations in spring than in autumn. However, EE-GRSPs were not significantly affected by changes in grazing intensity. Contrary to previous studies, EE-GRSPs would not be good indicators of soil fertility or high anthropogenic activity in these grasslands. In the case of T-GRSPs, a similar seasonal effect was observed, with higher values in spring. Grasslands 1L and 2H showed the highest T-GRSPs concentrations independently of the season, and T-GRSPs were positively associated with

organic N and C. In these grasslands, T-GRSPs could be used as indicators of better soil conditions (i.e., higher fertility) but not as indicators of a change in grazing intensity. In this regard, Lutgen et al. [28] proposed that a small seasonal change in GRSP pools provides further support to the concept that glomalin is relatively stable in soils and suggest that one-time sampling may be sufficient to satisfactorily capture this variable response. In contrast, in our study, T-GRSPs and EE-GRSPs showed seasonal variation independently of the grassland, and this pattern may be associated with greater root growth and AMF activity in spring. It is important to note the need to study these AMF variables seasonally and their relationship with soil properties to evaluate their use as indicators of soil functionality.

The knowledge of AMF community composition in response to grazing is essential to understand the maintenance of AMF diversity and community stability in sodic soils between contrasting seasons. In the present study, neither AMF diversity nor richness were affected by reduced grazing intensity. In line with diversity indexes, Sorenson's coefficient indicated a high degree of species overlap between grasslands under low and high grazing intensities, with greater homogeneity in AMF community composition in autumn than in spring. Soil properties, particularly P availability and pH, are the main contributors to AMF distribution and diversity [24,53,54]. Matinizadeh et al. [25] reported that the main reason for the lack of significant differences in AMF diversity between differently grazed sites could be the absence of differences observed in soil organic C and pH. In our study, the AMF diversity index was positively associated with available P and negatively correlated with ESP, aligning with previous findings [4] in temperate grasslands characterized by a wide range of soil pH, very low P availability, and high ESP values. The lack of significant differences in AMF diversity indexes with decreasing grazing intensity suggests that AMF species are highly adapted to different soil conditions and grazing pressures. We suggest that the environmental selection of AMF species adapted to specific soil and management conditions represents the flexibility of AMF to survive across a range of degraded soil conditions and managed grassland systems.

In the grasslands studied, AMF spore diversity included nine genera within five families in spring but decreased to five genera within two families in autumn. In particular, the Glomeraceae was the most represented family in both seasons, consistent with previous studies identifying it as dominant in grazed grasslands [4,5,55]. Species in this family are typically ruderal, characterized by rapid growth, high spore production, and tolerant to most types of disturbance [9,56]. In the present study, most species identified belonged to the Glomeraceae, but the results show no specific pattern of AMF species associated with a particular grazing intensity. However, certain AMF species showed a seasonal pattern associated with a marked decrease in AMF diversity in autumn.

In this sense, fourteen AMF species and six AMF species were identified in all the grassland soils in spring and autumn, respectively. *Funneliformis geosporus* and *Glomus brohultii* (Glomeraceae) were the most abundant in both seasons and classified as generalists. In non-grazed, restored, and over-grazed grasslands in Inner Mongolia, *F. geosporus* was also identified with high importance values independently of the different management system [55]. On the other hand, in the present study, *E. claroidea*, a species classified as generalist, was present in all the grasslands and seasons but was considered a common species according to the importance value. Contrary to our expectations, *F. mosseae* was not identified in any of the grassland soils, as recorded in previous studies in environments with alkaline and saline or sodic soils [4,57]. *Septoglomus constrictum* was only present in spring in all the grasslands, with highest relative abundance values in high-intensity grazing situations. In this sense, *S. constrictum* has been previously reported as an indicator species for soils under agricultural use and disturbed by agricultural machinery [58]. Although in the present study no AMF species were associated with

a particular grazing intensity, *E. etunicata*, *G. fuegianum*, *S. constrictum*, and *Acaulospora* sp. were associated only with the spring season, and no species were only associated with the autumn season. We propose that root growth is greatest in spring (growing season), favoring spore formation of certain AMF species. In contrast, in autumn, temperatures are lower and root growth rates decrease, thus favoring the disappearance of certain species in the soil that could experience unfavorable climatic periods inside plant roots [59]. Higher pH and ESP values in autumn could also be detrimental to AMF species diversity.

A previous study reported that AMF abundance declined consistently after more than 5 years of grazing, suggesting that this duration is a sensitive period for grazing disturbance [17]. In addition, van der Heyde et al. [9] showed that AMF communities may show delayed responses to changes in livestock grazing. In the grasslands studied, characterized by a long history of poorly controlled grazing and a subsequent change in grazing intensity, longer periods would be necessary to observe improved soil conditions and increased AMF diversity. It is important to consider that soils are finite resources; therefore, their restoration will take more time than the time invested in their formation. Consequently, the time elapsed since the change in grazing intensity would be insufficient to observe changes in AMF species diversity in the grasslands studied. In line with our results, van der Heyde et al. [9] found that changes in the AMF community took time to manifest after the change in grazing management.

4.3. Seasonal Relationships Between Soil Properties and AMF Variables Under Grazing

Previously, Singh et al. [60] showed a strong correlation between GRSP and soil properties, such as organic C, N, P, and pH under different land uses. This suggests that improving GRSP and organic matter is necessary for soil organic C sequestration and soil functionality. The PCA diagram performed here showed a positive relationship between T-GRSP and higher soil fertility, i.e., higher organic N and C and available P, as well as with *S. sinuosa*. This suggests that *S. sinuosa* may significantly contribute to soil glomalin content, particularly in grasslands 1L to 4H during spring. The PCA also showed a negative relationship between high pH values and glomalin formation. This result is consistent with those published by Šarapatka et al. [23]. Conversely, *R. intraradices* was found to be positively related to grasslands with higher pH and ESP and negatively related to T-GRSP, suggesting that this species may contribute, to a lesser extent, to the accumulation of these proteins in the soil. This finding may be associated with the fact that *R. intraradices* mainly produces spores within the roots [61]. In this sense, AMF species have different tolerance efficiency and competitive ability under different grazing pressure or soil conditions [9]. In our study, *F. mosseae* was associated with high pH and ESP values, consistent with previous publications [4,57]. However, a previous study in grasslands of Inner Mongolia reported that *F. geosporus*, *R. intraradices*, and *F. mosseae* were sensitive to overgrazing [55]. Similarly, in Namibia, *F. geosporus* and *F. mosseae* were found only in a lightly grazed site and not in moderately or heavily grazed sites [62]. In contrast, our results show that these species were present in all the grasslands, independent of the grazing management. In addition, *G. brohultii*, *F. geosporus*, and *E. claroidea* were positively associated with hyphal density, suggesting that these AMF species may contribute, to a greater extent, to the formation of the soil hyphal network.

5. Conclusions

The results of the present study allow concluding that the lower grazing intensity in grasslands with a long history of poorly controlled grazing showed no changes in AMF diversity. The species diversity of the AMF community was shaped by poorly controlled livestock management and by limiting soil conditions, particularly highly alkaline pH

and high ESP values, which further reduced P availability. This community was mainly composed of species of the family Glomeraceae, known as ruderal species with a generalist distribution in all grasslands. The AMF community showed high adaptation to soil conditions, denoting great resilience, with a marked seasonal effect on species diversity. Hyphal density can be considered an indicator of soil functionality, and glomalin concentration (total or easily extractable) showed only a seasonal pattern. We hypothesized that AMF diversity, spore and hyphal densities, and GRSP concentration are higher in grasslands with low grazing intensity during spring than in grasslands with high grazing intensity during autumn due to increased plant growth rate and improved soil properties. Based on the results, this hypothesis was partly accepted. More time and controlled grazing management are necessary to improve soil conditions and, consequently, change the AMF species composition. In the case of grasslands with highly limiting conditions, such as 5L and 6H, grazing exclusion is recommended to restore the soil environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems9030087/s1, Table S1. Results of two-way ANOVA (*F* and *p* values) for grazing intensity (GI), season (S), and the interaction between GI and S on different variables. Table S2. Isolation frequency (IF) and importance value (IV) of AMF species in six grasslands under low (L) or high (H) grazing intensities in spring and autumn. According to importance value criteria, importance values highlighted in green indicate dominant species (IV $\geq$ 50%), those highlighted in orange indicate most common species (30% < IV $\leq$ 50%), and those highlighted in blue indicate common species (10% < IV $\leq$ 30%). Figure S1. The most dominant and generalist AMF morphospecies identified across all grasslands. A. *Funneliformis geosporus*. B. *Glomus brohultii*. C. *Funneliformis mosseae*. D. *Glomus fuegianum*. E. *Rhizoglomus intraradices*. F. *Septoglomus constrictum*. [Bars = 50 µm].

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Abbreviations

The following abbreviations are used in this manuscript:

AMF	Arbuscular mycorrhizal fungi
T-GRSPs	Total glomalin-related soil proteins
EE-GRSPs	Easily extractable glomalin-related soil proteins
ESP	Exchangeable sodium percentage

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