Nitrogen

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Effects of Sheep Grazing and Nitrogen Addition on Dicotyledonous Seedling Abundance and Diversity in Alpine Meadows

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Abstract: Seedling is a crucial stage in the growth and development of plants, and the expansion and persistence of plant populations can be achieved through seed regeneration. Sheep grazing, fertilization, light, soil moisture, vegetation diversity and biomass, and litter all have potential impacts on species regeneration. We measured vegetation diversity, annual net primary productivity (ANPP), litter, ground photosynthetically active radiation (PAR), and soil moisture of alpine meadows under sheep grazing and nitrogen addition treatments, and studied their effects on the dicotyledonous seedling abundance and diversity using linear regression models (LMs) and structural equation models (SEMs). We found that sheep grazing reduced ANPP, increased vegetation diversity and PAR, and decreased soil moisture. Fertilization increased ANPP and litter, decreased vegetation diversity and PAR, but had no effect on soil moisture. Sheep grazing and fertilization both reduced the abundance of dicotyledonous seedlings, and simultaneously fertilization can reduce the diversity of dicotyledonous seedlings, while sheep grazing had no effect on the diversity of dicotyledonous seedlings. LMs showed that vegetation diversity, ANPP, and litter, rather than light and soil moisture, affected dicotyledonous seedling abundance and diversity. SEMs revealed that sheep grazing and fertilization indirectly influenced seedling regeneration through vegetation diversity rather than ANPP and litter. Our research will increase our understanding of the dicotyledonous plant regeneration process in alpine grasslands and facilitate the development of strategies for management and protection of alpine grassland.

Keywords: annual net primary productivity; litter; photosynthetically active radiation; vegetation diversity; seedling regeneration

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1. Introduction

Seedlings are an important stage in the life history of plants [1,2], and seedling regeneration can affect population size, persistence, and genetic variability by altering plant survival and establishment, ultimately affecting the structure, dynamics, and succession direction of plant communities [1,3–5]. Abiotic and biotic environmental factors (e.g., light, soil moisture, litter, aboveground biomass) can affect species regeneration [1].

Light is one of the main environmental factors in the growth process of plants, and it is the energy source that plants rely on for survival [6–8]. Plants regulate their growth, development, and morphological changes by sensing light signals to adapt to changes in the external environment. Due to differences in plant physiological characteristics, the demand for light also varies. Different light conditions affect the processes of plant germination and seedling establishment and influence subsequent stages of plant life history [8,9]. Therefore, light plays an important role in the entire plant life cycle of plants. Soil moisture is another important environmental factor for plant growth and survival. In addition, appropriate soil moisture can regulate plant carbon distribution and stress resistance and provide necessary growth conditions for plant regeneration [1]. As the initial stage of plant growth, the seedling stage of plants is extremely sensitive to water, and excessive or insufficient water can affect the normal growth and physiological processes of seedlings [1,10]. Soil moisture also indirectly affects the germination of seeds and the growth of seedlings by influencing factors such as soil aeration and nutrient availability [11].

Grassland aboveground biomass is an important indicator of grassland vegetation status and reflects the annual net primary productivity level of grasslands, playing an important role in the carbon cycle of terrestrial ecosystems [12]. The aboveground biomass of grasslands can affect the photosynthetically active radiation (PAR) on the ground, as well as soil moisture, which in turn affects seedling regeneration [1]. Litter is a collective term for organic matter produced by biological components in terrestrial ecosystems and returned to the ground [13–15]. As the first physical environment that plant seeds come into contact with after being released, it can have a significant impact on plant seed germination and seedling survival through environmental effects such as blocking light, changing soil temperature, and increasing water content [16,17]. In addition, vegetation diversity may have a more direct impact on seedling regeneration by affecting the species diversity and abundance of seed output.

Soil fertility reflects the potential ability of soil to supply nutrients to plant roots and affects the species regeneration and survival in plant communities. Nitrogen is a key nutrient element that restricts plant growth in many ecosystems [18,19]. Nitrogen addition can cause an increase in the available nitrogen content in the soil, thereby reducing or breaking the nutrient limitations of nitrogen in the region [20], allowing plant seedlings to absorb and utilize more nutrients for growth. Nitrogen addition in grassland ecosystems typically increases aboveground biomass and litter [19,21], thereby affecting the physical environment required for seedling regeneration.

In grasslands, grazing by domestic herbivores is a very important land use and a major driver of biodiversity and function [22–24]. The recent changes in grassland utilization patterns, such as fertilization and overgrazing, have had a significant impact on local grassland productivity and plant diversity [18,19,23,25]. Grazing directly affects plant growth through trampling [26,27] and can also affect the dynamics of the grass population through selective feeding, leading to a decrease in seeds and changes in community structure [18,28], indirectly reducing the abundance and diversity of seedlings.

The Qinghai–Tibet Plateau is one of the highest, largest, and most unique alpine ecosystems in the world. Researchers have found that nitrogen deposition is becoming increasingly evident in this region [29]. In alpine meadows, harsh habitats constrain seed germination and seedling establishment [30–32]. Studies have found that grazing and nitrogen input can lead to differences in light, water, vegetation diversity and biomass, and litter in alpine meadow communities [33–36], which can potentially affect seed germination in communities [37,38]. However, the combined effects of light, soil moisture, aboveground biomass, and litter on seedling abundance and diversity in natural communities have been less studied. Here, in an alpine meadow of the Qinghai–Tibet Plateau, we investigated the effects of sheep grazing and fertilization on the establishment of dicotyledonous seedlings in plant communities, as well as the effects of vegetation diversity and biomass, litter accumulation, light, and soil moisture on the establishment of dicotyledonous seedlings. Specifically, the objectives of our study were to test the following hypothesis: sheep graz-

ing and fertilization will influence the dicotyledonous seedling abundance and diversity directly and indirectly through PAR, soil moisture, vegetation diversity, ANPP, and litter.

2. Materials and Methods

2.1. Study Site

The experimental site is located at the Qinghai Haibei National Field Research Station of Alpine Grassland Ecosystem in China. The station is situated in the northeast of the Qinghai Tibet Plateau. The region has a typical plateau continental climate, with long and cold winters and short and cool summers [39]. The average annual precipitation is 488 mm, mainly distributed in short and cool summers. The annual average temperature is −1.1 °C, ranging from the lowest in January (−15.2 °C) to the highest in July (9.9 °C).

2.2. Experimental Design

The experiment was designed in June 2018 using a split-plot design (sixteen 10 m × 15 m plots), with sheep (*Ovis aries* L.) grazing (non-grazing and grazing) and fertilization (non-fertilization and fertilization) as the main treatment factors (Figure S1). There were four treatments [non-grazing + non-fertilization (G_0F_0); non-grazing + fertilization (G_0F_+); grazing + non-fertilization (G_+F_0); grazing + fertilization (G_+F_+)] with each treatment repeated four times. The grazing intensity was 4 sheep units per hectare (SU/hm^2). The source of the fertilization was urea ($CO(NH_2)_2$) and the amounts applied corresponded to 10 g m^{-2} of elemental nitrogen.

2.3. Measurement of Abundance and Diversity of Dicotyledonous Seedling and Adult Plants

In late August 2022, for each plot, three sample circles (40 cm diameter) were randomly selected to count species diversity (i.e., number of species) and abundance (i.e., number of individuals) of all dicotyledonous seedlings within each sample circle. This study only focused on dicotyledonous seedlings, firstly because dicotyledonous plants are easy to determine whether they were unearthed in the same year, and secondly because they are easy to identify as species.

We also counted species diversity (i.e., number of species) and abundance (i.e., number of individuals) of adult plants in the three sample circles.

2.4. Measurement of Aboveground Biomass, Litter, and Environmental Factors

We harvested all plants from 4 quadrants (25 cm × 25 cm) in each plot, separated the litter, dried them at 60 °C for 48 h to a constant weight, and weighed them. Aboveground biomass was taken as a proxy for ANPP. At the same time, we used a soil drill to take 4 cylindrical soil cores (3.4 cm diameter, 15 cm deep), weighed the fresh weight, dried it at 105 °C for 24 h, weighed it, and then calculated soil moisture content. In August, the LightScout Quantum PAR Full Spectrum Meter (Spectrum Technologies, Chicago, IL, USA) was used to measure the PAR (PAR_0) at the ground level and the PAR (PAR_5) at 5 cm aboveground level three times.

2.5. Statistical Analyses

Two-way ANOVAs were used to analyze the effects of sheep grazing and fertilization on the abundance and diversity of dicotyledonous seedlings, PAR_0, PAR_5, soil moisture, vegetation diversity, ANPP, and litter in alpine meadows. Linear regression models were used to analyze the relationships of dicotyledonous seedling abundance and diversity with PAR_0, PAR_5, soil moisture, vegetation diversity, ANPP, and litter under sheep grazing and fertilization treatments. Linear regression models were also used to test the relationships of PAR_0, PAR_5, soil moisture, vegetation diversity with ANPP and litter, and the relationship between ANPP and litter.

We used SEMs to explore indirect and direct effects of sheep grazing and fertilization on the dicotyledonous seedling abundance and diversity. We made hypothesized path diagrams (Figure S2) including factors significantly affecting seedling abundance or diversity in linear regression models. The outputs of the SEMs were produced based on the *p*-values

of conditional independence tests combined into a single Fisher's C statistic. To release the degrees of freedom, we removed non-significant pathways except for the pathways from sheep grazing/fertilization to seedling abundance/diversity. Two-way ANOVAs, standard linear regression models, and SEMs were conducted using the packages base and piecewiseSEM [40] in R environment (https://www.r-project.org/, accessed on 6 April 2023) [41], respectively.

3. Results

We found that sheep grazing and fertilization significantly affected PAR_0 and PAR_5, with their interaction not significantly affecting PAR_0 and PAR_5 (Table 1). Sheep grazing significantly increased PAR_0 and PAR_5, regardless of fertilization or not (Figure 1A,B). Fertilization significantly reduced PAR_5, regardless of sheep grazing or not (Figure 1A,B). Compared to G_+F_0 treatment, G_+F_+ treatment significantly reduced PAR_0 (Figure 1A). Sheep grazing significantly affected soil moisture (Table 1), and both G_+F_0 and G_+F_+ treatments significantly reduced soil moisture compared to G_0F_+ treatment (Figure 1C).

Table 1. Effects of sheep grazing and fertilization on ground photosynthetically active radiation (PAR_0) at the ground level and the PAR (PAR_5) at 5 cm above ground, soil moisture, vegetation diversity, annual net primary productivity (ANPP), litter, and dicotyledonous seedling abundance and diversity based on results from two-way ANOVAs (*F* values).

	PAR_0	PAR_5	Soil Moisture	Vegetation Diversity	ANPP	Litter	Dicotyledonous Seedling Abundance	Dicotyledonous Seedling Diversity
Grazing	76.70 ***	156.34 ***	9.74 **	6.95 *	24.29 ***	1.17	6.25 *	0.00
Fertilization	12.34 **	18.85 ***	1.01	111.42 ***	34.81 ***	45.85 ***	167.89 ***	16.95 ***
Grazing × Fertilization	0.47	0.18	0.83	0.19	2.78	0.36	13.70 ***	2.85

Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Sheep grazing and fertilization both significantly affected vegetation diversity (Table 1). Sheep grazing significantly reduced vegetation diversity, regardless of fertilization or not (Figure 1D). Compared to G_0F_0, G_+F_0 treatment significantly increased vegetation diversity (Figure 1D). Fertilization significantly reduced vegetation diversity, regardless of sheep grazing or not (Figure 1D).

Sheep grazing and fertilization both significantly affected ANPP (Table 1). Sheep grazing significantly reduced ANPP, regardless of fertilization or not (Figure 1E). Fertilization significantly increased ANPP, regardless of sheep grazing or not (Figure 1E). Fertilization significantly increased litter (Table 1), regardless of sheep grazing or not (Figure 1F).

We found that sheep grazing and fertilization, as well as their interaction, significantly affected dicotyledonous seedling abundance (Table 1). G_+F_0, G_0F_+, and G_+F_+ treatments all significantly reduced dicotyledonous seedling abundance (Figure 1G). Compared to G_+F_0, G_0F_+, and G_+F_+ treatments both significantly reduced dicotyledonous seedling abundance (Figure 1G). Compared to G_0F_0, G_0F_+, and G_+F_+ treatments both significantly reduced dicotyledonous seedling diversity (Table 1 and Figure 1H). Compared to G_+F_0, G_0F_+ significantly reduced dicotyledonous seedling diversity.

We found that PAR_0, PAR_5, and soil moisture did not significantly affect dicotyledonous seedling abundance and diversity (Table S1), while vegetation diversity was positively correlated with dicotyledonous seedling abundance ($R^2 = 0.613$) and diversity ($R^2 = 0.401$), and litter and ANPP were negatively correlated with dicotyledonous seedling abundance ($R^2 = 0.347$; $R^2 = 0.214$) and diversity ($R^2 = 0.161$; $R^2 = 0.134$) (Figure 2 and Table S1). In addition, ANPP and litter both significantly reduced PAR_0 ($R^2 = 0.355$; $R^2 = 0.149$), PAR_5 ($R^2 = 0.422$; $R^2 = 0.159$), and vegetation diversity ($R^2 = 0.336$; $R^2 = 0.436$), but had no significant effect on soil moisture (Table S2). ANPP significantly increased litter (Table S2).

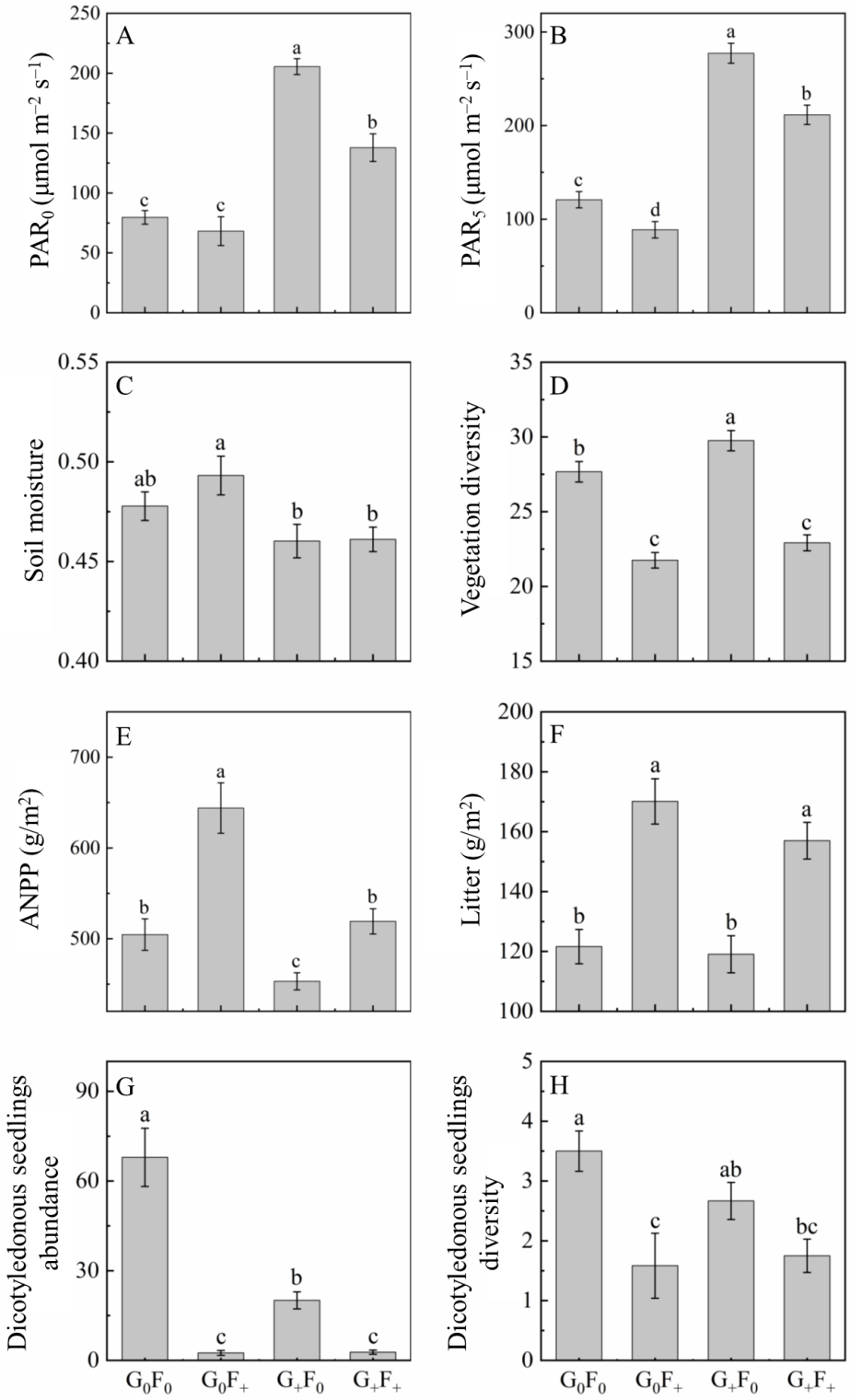

Figure 1. Ground photosynthetically active radiation (PAR_0, (**A**)) at the ground level and the PAR (PAR_5, (**B**)) at 5 cm above ground, soil moisture (**C**), vegetation diversity (**D**), annual net primary productivity (ANPP) (**E**), litter (**F**), and dicotyledonous seedlings abundance (**G**) and diversity (**H**) under treatments (G_0F_0, non-grazing + non-fertilization; G_0F_+, non-grazing + fertilization; G_+F_0, grazing + non-fertilization; G_+F_+, grazing + fertilization). The error bars indicate ± SE (n = 12). The different lowercase letters indicate significant differences between treatments based on LSD (least significant difference) tests.

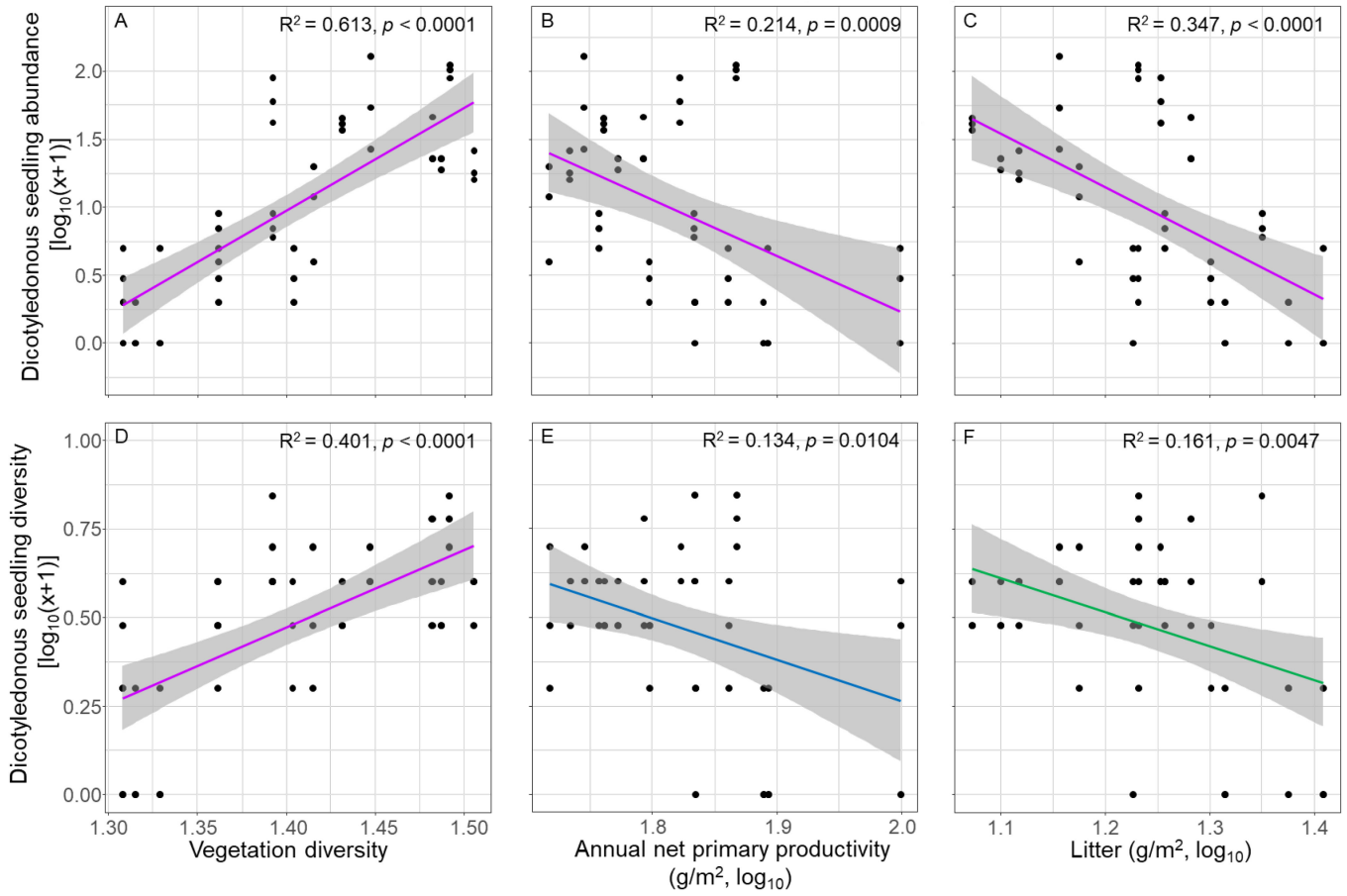

Figure 2. Relationships of vegetation diversity, annual net primary productivity (ANPP), and litter with dicotyledonous seedling abundance (**A**–**C**) and diversity (**D**–**F**). The R^2 and p values are shown in the figure. Significant relationships are denoted with blue ($p < 0.05$), green ($p < 0.01$), and pink ($p < 0.001$) solid lines, respectively, and gray areas indicate the 95% confidence interval of the fit.

According to the parameter values, our SEMs adequately fitted the data (a: Fisher's C = 9.25, $p = 0.6810$, df = 12; b: Fisher's C = 7.79, $p = 0.8020$, df = 12). The main results of SEMs (see Figure 3 for details) include: (1) sheep grazing indirectly increased dicotyledonous seedling abundance and diversity by directly promoting vegetation diversity; (2) fertilization indirectly reduced dicotyledonous seedling abundance and diversity by directly decreasing vegetation diversity; (3) sheep grazing and fertilization both directly reduced dicotyledonous seedling abundance; (4) sheep grazing directly decreased ANPP, and fertilization directly increased ANPP and litter; (5) SEMs explained 80% and 42% variance in dicotyledonous seedling abundance and diversity, respectively; (6) SEMs explained 73%, 56%, and 50% variance in vegetation diversity, ANPP, and litter, respectively.

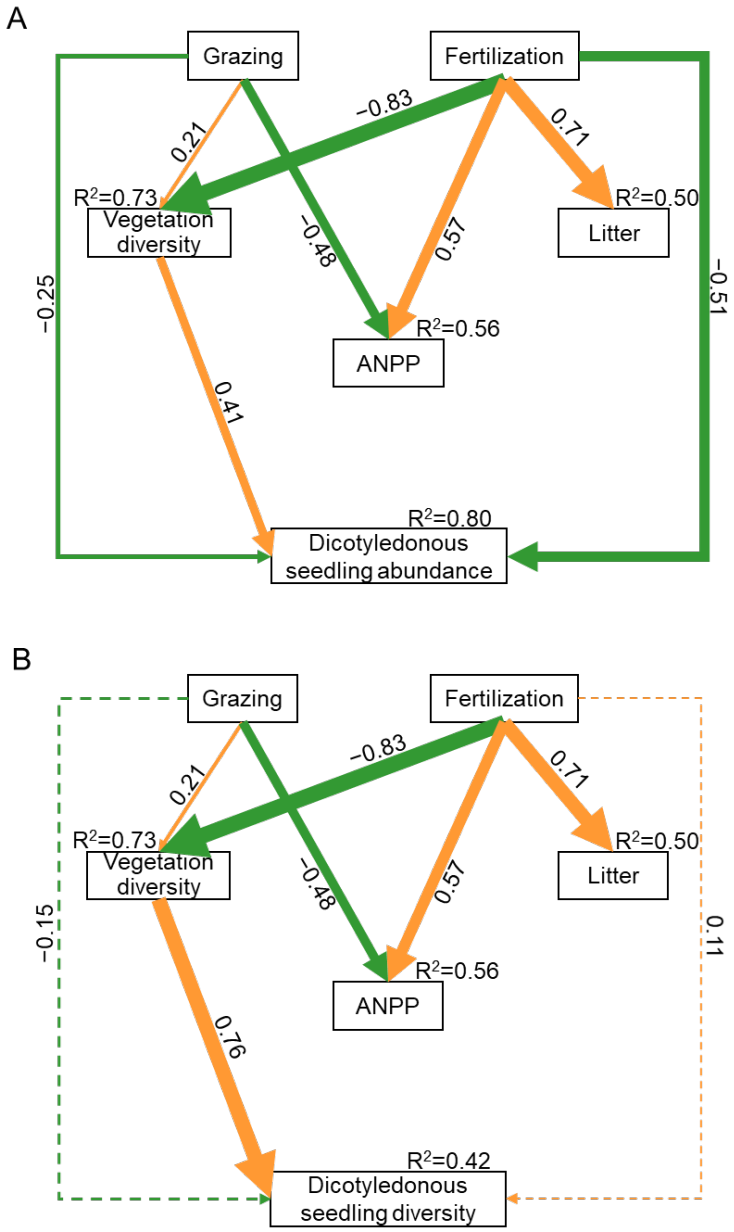

Figure 3. The structural equation models considered all plausible pathways of direct and indirect effect of sheep grazing and fertilization on dicotyledonous seedling abundance (**A**) and diversity (**B**). Solid yellow and green arrows indicate significant negative and positive effects ($p < 0.05$), respectively. The dashed arrow indicates a non-significant effect ($p > 0.05$). Numbers above arrows indicate path coefficients. Width of arrows indicates the strength of the causal influence. R2 represents the proportion of variance explained for each dependent variable in the model (**A**): Fisher's C = 9.25, $p = 0.681$, df = 12; (**B**): Fisher's C = 7.79, $p = 0.802$, df = 12). ANPP, annual net primary productivity.

4. Discussion

Grazing and nitrogen input are important disturbances in alpine meadows [25,42–44]. We investigated the effects of sheep grazing and nitrogen input, as well as changes in PAR, soil moisture, vegetation diversity, ANPP, and litter, on dicotyledonous seedling abundance and diversity. Our research found that sheep grazing, fertilization, vegetation diversity, ANPP, and litter affected dicotyledonous seedling regeneration. More importantly, sheep grazing and fertilization indirectly influenced seedling regeneration through vegetation diversity rather than PAR, soil moisture, ANPP, and litter, in natural communities, which will increase our understanding of the plant regeneration process in alpine grasslands.

4.1. Influences of Sheep Grazing on the Dicotyledonous Seedling Based on ANOVAs and Linear Regression Models

Previous studies have found that sheep grazing can alter the size of soil seed banks through livestock grazing and trampling [45,46], thereby affecting seedling emergence [47,48]. In this study, sheep grazing increased vegetation diversity and PAR and decreased soil moisture, ANPP, and dicotyledonous seedling abundance, but had no effect on the dicotyledonous seedling diversity. Grazing may reduce the output of seeds into the soil [18], thereby reducing dicotyledonous seedling abundance, which actually has the potential to reduce dicotyledonous seedling diversity. But we did not observe a decrease in the dicotyledonous seedling diversity due to sheep grazing (Figure 1G). Sheep grazing reduced vegetation biomass on the ground (Figure 1E), resulting in large environmental fluctuations in light, water, and temperature on the ground [49]. The appropriate environment fluctuations may have the potential to create more niche differentiation in time and space [50], thereby promoting high seedling diversity. The two processes may counteract each other, resulting in little effect of sheep grazing on the dicotyledonous seedling diversity.

4.2. Influences of Fertilization on the Dicotyledonous Seedling Based on ANOVAs and Linear Regression Models

Soil nutrition can affect the regeneration and survival of seedlings in plant communities [1]. In this study, fertilization reduced dicotyledonous seedling abundance and diversity. Meanwhile, fertilization increased aboveground biomass and litter, while reducing PAR and vegetation diversity. Previous studies generally suggested that low PAR caused by high aboveground biomass limited seedling regeneration [51]. However, this study found that the accumulation of litter, rather than low PAR, limits the abundance and diversity of dicotyledonous seedlings. Litter can reduce seedling establishment by increasing mechanical barriers, producing allelopathic substances, and increasing the risk of fungal infection [4,52]. In addition, fertilization leads to the loss of grassland biodiversity (Figure 1D) [19,44,51], resulting in a decrease in species diversity of seeds input into the soil, which is a reason for the decrease in species diversity of dicotyledonous seedlings.

4.3. Indirect and Direct Effects of Sheep Grazing and Fertilization on the Dicotyledonous Seedling

Based on results of SEMs (Figure 3), we found that sheep grazing indirectly increased dicotyledonous seedling abundance and diversity by directly promoting vegetation diversity. Although sheep grazing reduced ANPP, decreased ANPP did not increase vegetation diversity and dicotyledonous seedling abundance and diversity. It is different from the results of linear regression models (Figure 2) and our expectations (Figure S1). The diverse seed output of vegetation with high species diversity may largely determine the diversity of seedlings, independent of changes in aboveground biomass. The probability of vegetation with high species diversity potentially containing species with high seed yields for seedling establishment also increases. In addition, sheep grazing directly reduced dicotyledonous seedling abundance probably because of the trampling and gnawing of livestock.

In contrast, we found that fertilization indirectly reduced dicotyledonous seedling abundance and diversity through directly decreasing vegetation diversity based on results of SEMs (Figure 3). Fertilization increased ANPP and litter, neither of which reduced dicotyledonous seedling abundance and diversity in SEMs. It is also different from the results of linear regression models (Figure 2) and our expectations (Figure S2). Additionally, fertilization directly reduced dicotyledonous seedling abundance probably because of changes in soil physical and chemical properties (e.g., soil pH) caused by fertilization [19].

There is one limitation of our study, which is that focusing solely on dicotyledonous seedlings cannot fully reflect the seed regeneration process of alpine communities. This study found that sheep grazing decreased the abundance of monocotyledonous plants (Table S1). In general, sheep prefer to eat monocotyledonous plants. Therefore, we predict that sheep grazing will also reduce the abundance of monocotyledonous seedlings like decreasing dicotyledonous seedlings in alpine meadows. We found that fertiliza-

tion increased the abundance of monocotyledonous plants, but decreased their diversity. (Table S1). Thus, it is difficult to predict the effect of fertilization on monocotyledonous seedlings. In short, we need updated methods to break through the bottleneck of difficult identification of monocotyledonous seedlings in order to have a more comprehensive understanding of seed regeneration in alpine meadows containing a large number of perennial and clonal plants.

5. Conclusions

In this study, sheep grazing and fertilization both reduced the abundance of dicotyledonous seedlings, while fertilization reduced the diversity of dicotyledonous seedlings. This indicates that sheep grazing and fertilization both can affect the regeneration of dicotyledonous seedlings. Meanwhile, although sheep grazing and fertilization affected PAR, soil moisture, vegetation diversity, ANPP, and litter, they indirectly influenced seedling regeneration only through vegetation diversity. Maintaining normal plant regeneration is one of the core measures to protect biodiversity, prevent grassland degradation, and restore degraded grasslands on the Qinghai–Tibet Plateau. Our results improve our understanding of the mechanism of the vegetation regeneration process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nitrogen5020032/s1, Figure S1: Experiment design; Figure S2: The hypothetical pathways for how sheep grazing and fertilization influence dicotyledonous seedling abundance and diversity through vegetation diversity, ANPP, and litter; Table S1: The slopes of relationships of PAR0, PAR5, soil moisture, vegetation diversity, ANPP, and litter with dicotyledonous seedling abundance and diversity; Table S2: The slopes of relationships of PAR0, PAR5, soil moisture, vegetation diversity, ANPP, and litter with ANPP and litter.

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