

Article

Differential Physiological Characteristics and Fungal Composition of Alfalfa under Salt Stress in Degraded Grasslands

Qiang Lu ^{1,2}, Duowen Sa ^{1,*}, Zhen Wang ¹ , Zhijun Wang ³, Gentu Ge ³, Yushan Jia ³, Tingyu Liu ⁴ and Lin Sun ⁵

¹ Grassland Research Institute, Chinese Academy of Agricultural Sciences, Hohhot 010010, China

² College of Agriculture, Ningxia University, Yinchuan 750021, China

³ College of Grassland and Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010010, China

⁴ College of Agriculture, Inner Mongolia University for Nationalities, Tongliao 028000, China

⁵ Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences, Hohhot 010010, China

* Correspondence: saduowen@caas.cn

Abstract: Alfalfa (*Medicago sativa* L.) is an important source of livestock feed used to address the imbalance between livestock and forage production in China. However, much of the grasslands have a high salt content, which seriously affects the quality and yield of alfalfa. There are many kinds of fungi that play an important role in alfalfa growth and nutrient synthesis. The response of the fungi of alfalfa to salinity is poorly understood. In this study, the physiological characteristics and the fungal community composition of alfalfa under different salt stress conditions were investigated. Salt stress had a great impact on the physiological characteristics and the fungal community composition of alfalfa. The activity of invertase increased significantly under salt stress; the content of water and starch decreased; and the content of crude protein (CP) and soluble sugar increased under mild salt stress. With the increase in salt stress, the relative abundance of Ascomycetes increased, while the relative abundance of basidiomycetes decreased. This showed that the changes in the fungal community may be related to the adaptability of alfalfa plants to salt stress. These findings contribute to a better understanding of alfalfa physiological characteristics and nutrient synthesis under salt stress and deepen our understanding of alfalfa–fungi interactions in the saline soil environment of grasslands.

Keywords: alfalfa; salt stress; physiological characteristics; fungi



Citation: Lu, Q.; Sa, D.; Wang, Z.; Wang, Z.; Ge, G.; Jia, Y.; Liu, T.; Sun, L. Differential Physiological Characteristics and Fungal Composition of Alfalfa under Salt Stress in Degraded Grasslands. *Agriculture* **2022**, *12*, 1636. <https://doi.org/10.3390/agriculture12101636>

Academic Editor: Fadi Chen

Received: 31 July 2022

Accepted: 26 September 2022

Published: 8 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Due to natural and human factors, the phenomenon of grassland salinization is serious. The ecological environment is deteriorating day by day, and biodiversity and productivity are reduced, including the ecological functions of grasslands such as windbreak and sand fixation. This directly affects the grass industry and the living standards and quality of life of the local people. Salinization has become an important obstacle to ecological environment construction and agricultural development. Salinity, a type of abiotic stress, has a strong impact on plant establishment and growth. Previous studies have shown that between 20% and 33% of the total cultivated grasslands worldwide are acutely affected by salinity [1].

Alfalfa (*Medicago sativa* L.), one of the most important forage crops [2], is widely grown throughout the world due to its high biomass production, longevity, and ability to fix nitrogen [3]. In China, alfalfa is cultivated across an area of more than 4×10^6 hm² annually, and it is mainly distributed in northwestern inland saline–alkaline areas, the Middle Yellow River semi-arid saline–alkaline area, the Huang–Huai–Hai Plain, semi-arid saline–alkaline depression areas, northeastern semi-humid and semi-arid low-lying saline–alkaline areas,

and coastal semi-humid saline–alkaline areas [4]. The quality and yield of alfalfa produced in these areas cannot meet the increased demands for the development and requirement of animal husbandry in China.

Salinity can directly affect plant growth and productivity by triggering osmotic stress, nutrient imbalances, and toxicity due to excessive Na^+ and Cl^- ion concentrations. Salt triggers changes in the osmotic pressure in plants by limiting their uptake of water from the soil, which leads to physiological drought; i.e., plants must maintain a low internal osmotic potential to prevent water loss of the cell membrane [5]. Mild salt stress can stimulate the activities of SOD and other enzymes, thereby improving the defense system composed of these protective enzymes, and it can also enhance the salt resistance of plants, promoting the synthesis of photosynthetic pigment, soluble sugars, proteins, and other nutrients, as well as improving the quality of the plants [6,7]. Excessive salt ions can cause toxicity in plants, leading to the loss of cell membrane system composition, channel activity, and regulation mechanisms; reductions in the electron conduction system; decreases in enzyme activity; and strong inhibition of plant metabolism—thereby affecting the formation of a chlorophyll protein complex. This not only prevents the photosynthetic synthesis of organic matter but also causes the decomposition of soluble sugars and proteins [8].

Fungi, including both Ascomycetes and Basidiomycetes, live symbiotically within leaves and play a major role in improving ecosystem functions [9]. Fungi can contribute to the decomposition of leaf litter and can promote carbon and nutrient cycling within ecosystems [10]. On the other hand, fungi are vital to the adaptation of plants to salt stress, which promotes plant growth and resistance to biotic and abiotic stresses (e.g., pathogens, drought, and salinity) [11–13]. Further research has shown that plant identity drives changes in the diversity and community composition of fungi [14,15]. Different kinds of fungi play an active role in promoting the synthesis of soluble sugars and soluble proteins. The growth of fungi depends on the nutrients deposited on host plant tissues [16]. Therefore, elucidating the relationship between plants and fungi can provide a better understanding of the ecological functions of fungi in plant communities under salt stress.

Thus, the aim of the field experiment that we conducted was (1) to assess the changes in the physiological characteristics of alfalfa and the fungal community composition in response to salt stress in grasslands and (2) to comprehensively determine the relationships among the plant physiological characteristics, the endophyte community, and salt stress. This study provides a theoretical basis for comprehending the impact of salt stress on alfalfa.

2. Materials and Methods

2.1. Study Sites

This experiment was conducted in the experimental field of Baotou Xintai Agricultural Science and Technology Co., Ltd., located in the town of Harlinger, Jiuyuan district, Baotou city, Inner Mongolia. The geographical location of the field is longitude: $110^{\circ}37''$ – $110^{\circ}27''$ east and latitude: $40^{\circ}5''$ – $40^{\circ}17''$ north. The region experiences a northern temperate continental climate and dry, windy conditions. Specifically, the region is dry, rainy, and windy in the spring; the summers are short and mild; the temperature varies dramatically in autumn; and winters are long and cold. Northwestern winds are predominant throughout the year. The annual average temperature is 6.8°C ; the average temperature in July ranges from 22.5 to 23.1°C ; and the average temperature in January is -13.7°C . The frost-free period is approximately 165 d, and the maximum depth of frozen soil is 1.4 m. The annual average rainfall is 330 mm; the annual average evaporation is 2094 mm; and the daily average wind speed is 3 m/s. The annual duration of sunshine hours is 3177 h, and the annual percentage of sunny days is 70%. This region is one of the sunniest areas in China.

2.2. Soil Conditions

Soil samples were taken from 10 cm and 20 cm depths at the test site, and the following parameters were measured: pH; conductivity; and total salt, organic matter, and total

nitrogen contents (Table 1). Based on the classification standard for saline–alkaline soil, four positions were selected to represent various salt stress levels, i.e., non-salt stress (CK), mild salt stress (LS), moderate stress (MS), and severe stress (HS). The salt stress contents in the CK, mild, moderate, and severe sites were <1, 1–2, 2–3, and 3–4‰, respectively. Each plot had an area of 20 m², and each treatment was carried out in triplicate.

Table 1. Survey of soil parameters under different salt stresses.

Index	pH	EC (mS/cm)	Total Salt (g/kg)	Organic Matter (g/kg)	Total Nitrogen (g/kg)
CK	7.83	0.21	0.91	5.32	0.28
Mild	8.42	0.59	1.76	5.09	0.24
Moderate	8.46	1.35	2.63	4.89	0.33
Severe	8.64	2.30	3.85	5.29	0.16

2.3. Alfalfa Preparation

The test material was the alfalfa variety Zhongmu No. 3., which was provided by Crovo (Beijing) Ecological Technology Co., Ltd. The alfalfa was sown in May 2018. The sowing was performed using the drill method, and the row-to-row distance was 10 cm. Water was subsequently supplied on 22 May, 12 June, 3 July, 20 July, and 8 August. Due to the slow growth caused by the severely saline–alkaline soil, the bud stage in the severe test plot began on 6 September. During the initial flowering stage, the plant samples in the CK, mild, and moderate test plots were harvested. A 3 × 3 m² sampling area was established in each plot by randomly placing a marker on the ground; each treatment was performed in triplicate. Sampling started at 8:00 a.m., and the plants were cut at a height of 5–8 cm.

A sample weighing 3 kg was obtained from each test area; 1 kg from each sample was transferred into paper bags, and the remaining samples were divided into two parts by chemical composition determination and stored in a liquid nitrogen tank at −80 °C for enzyme activity and fungal community determination.

2.4. Analysis of Physiological Characteristics

The mass of the alfalfa dry matter (DM) was determined after oven drying at 65 °C for 48 h. The dried samples were then ground to a 1 mm particle size, and the CP, soluble sugar, and starch contents of the samples were analyzed by near-infrared reflectance spectroscopy (NIRS). The results were reported as the percent DM (%DM). The spectra were analyzed using a large dataset of calibration samples from different kinds of grasslands from the Institute VDLUFA Qualitätssicherung NIRS GmbH, Kassel, Germany.

Protease activity was determined according to the methods of Peterson and Huffaker (1975) [17]. To measure the protease activity, a 10 g sample and 50 mL of 0.1 M sodium phosphate buffer (precooled with 5 mM hyposulfite, pH 6.0) were homogenized in a mixer for 30 s. The homogenate was filtered through four layers of denim and centrifuged at 8000× g and 4 °C for 10 min. The derived supernatant was stored at −80 °C for enzyme activity analysis. The invertase assay procedure was adapted from that of Sergeeva's research [18].

2.5. DNA Extraction and PCR Amplification

Genomic DNA was extracted from a mix of stems and leaves of the fresh alfalfa with the following procedure. A total of 10 g of plant sample was mixed with 90 mL of sterile water and then treated with a table concentrator at 120 r/m for 2 h. The sample was then filtered with carbasus, and the liquid was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was suspended in 1 mL of sterile water solution. The precipitate was used for DNA extraction.

DNA extraction was performed using an EZNA[®] kit (Omega Biotek, Norcross, GA, US) according to the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington,

USA). DNA extraction quality was measured by 1% agarose gel electrophoresis. The 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers were used for PCR amplification of the V3-V4 variable region. The amplification procedure was as follows: predenaturation at 95 °C for 3 min, 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and extension at 72 °C 10 min (PCR instrument: ABI GeneAmp® 9700). The amplification system volume was 20 µL, including 4 µL of 5 × FastPfu buffer solution, 2 µL of 2.5 mM dNTPs, 0.8 µL of primer (5 µM), 0.4 µL of FastPfu polymerase, and 10 ng of DNA template.

2.6. Illumina Hiseq2500 Sequencing and Microbial Diversity Analysis

Amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions. They were quantified using QuantiFluor-ST (Promega, United States). The purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina platform according to standard protocols. We uploaded the sequence data to the NCBI and obtained the accession number PRJNA731627.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) was used to examine the significance of the different levels of salt stress on the water, CP, soluble sugar, and starch contents and the neutral invertase, sucrose synthase, and neutral protease activity. Significant differences between treatments were confirmed using Tukey's honestly significant difference test at $p < 0.05$. The relationships between fungal alpha diversity and plant physiological characteristics were assessed by Pearson's correlation.

To determine the dissimilarities among fungal communities, Bray–Curtis distance metrics were calculated. To assess how salt stress influenced the fungal communities, a permutational multivariate analysis of variance was carried out using the *adonis* function in the “vegan” package in R. The taxonomic community table for fungi was converted to a distance table through the calculation of Bray–Curtis distances. The phylogenetic dissimilarity between samples was assessed using the weighted UniFrac distance. To examine the relationships between plant physiological characteristics and fungal communities, we performed a redundancy analysis (RDA) [19]. In the RDA, the significance of the effect of each variable based on its eigenvalue was tested using a Monte Carlo permutation test, and the resulting significance level was determined by the F ratio and the p -value [20].

3. Results

3.1. Physiological Characteristics of Alfalfa

The water content of the alfalfa decreased significantly with increasing salt stress ($p < 0.05$, Figure 1a). Alfalfa productivity was lower in the severe salt stress treatment than in the CK, mild, and moderate treatments (Figure 1b). Compared with the CK treatment, the mild salt stress treatment significantly increased the CP content ($p < 0.05$, Figure 1c) and soluble sugar content ($p < 0.05$, Figure 1d) of the alfalfa. With the increase in salt stress, the content of CP decreased significantly ($p < 0.05$). The starch content was lower in the severe salt stress treatment than in the CK treatment (Figure 1e).

With the increase in salt stress, neutral invertase activity also increased ($p < 0.05$, Figure 2a). Sucrose synthase activity was higher in the mild salt stress treatment than in the CK treatment ($p < 0.05$, Figure 2b). Compared with severe salt stress treatment, the mild salt stress treatment significantly increased neutral protease activity ($p < 0.05$, Figure 2c). As shown in Table 2, there was no significant correlation between soil N content and water, starch, and soluble sugar contents in this study. However, in general, there was a significant negative correlation between soil total N content and crude protein content ($p < 0.05$).

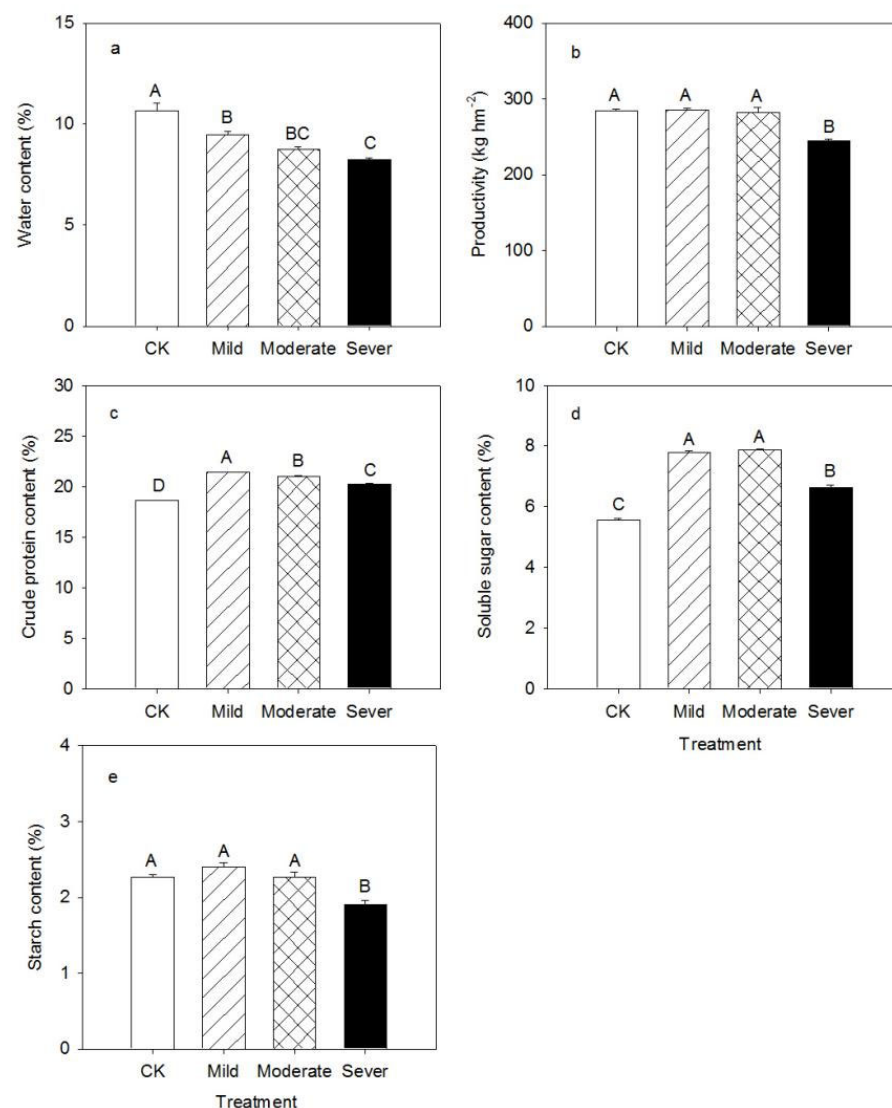


Figure 1. Effects of salt stress (CK, mild, moderate, and severe) on plant water content (a), productivity (b), crude protein content (c), soluble sugar content (d), and starch content (e). Values are means \pm SEs. Significant differences between different levels of salt stress are indicated by uppercase letters ($p < 0.05$).

Table 2. Correlation analysis of physiological characteristics and soil N under different salt stresses.

Index		Water Content	Crude Protein	Starch	Soluble Sugar
Total N	R	0.44	−0.89	0.66	0.19
	p	0.15	<0.0001	0.02	0.55

3.2. Fungal Community Diversity and Composition

The salt stress caused no significant differences in the OTUs (Figure 3a). The moderate salt stress treatment significantly decreased the Shannon–Wiener index ($p < 0.05$, Figure 3b) for the fungi but increased the Simpson index for the fungi ($p < 0.05$, Figure 3c). The ACE index is shown in Figure 3d, and the Chao 1 index is shown in Figure 3e. The fungal community structures at the four levels of salt stress were distinct from each other on the principal coordinate analysis (PCoA) ordinations (Figure 4).

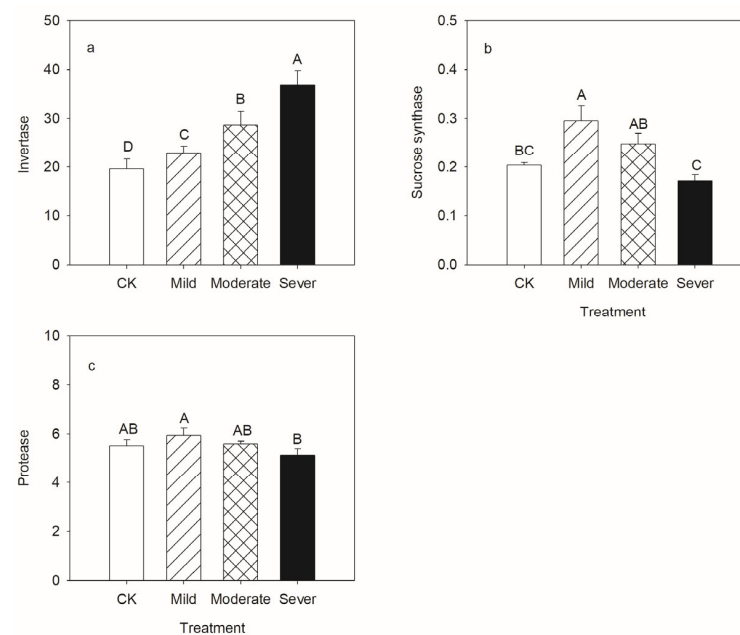


Figure 2. Effects of different levels of salt stress (CK, mild, moderate, and severe) on neutral invertase activity (a), sucrose synthase activity (b), and neutral protease activity (c). Values are means \pm SEs. Significant differences between different levels of salt stress are indicated by uppercase letters ($p < 0.05$).

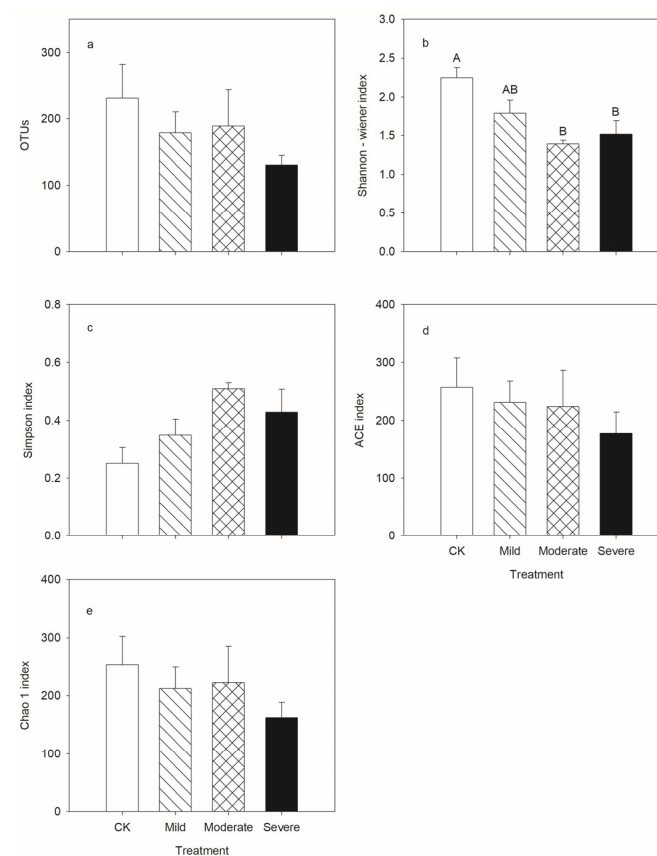


Figure 3. Effects of different levels of salt stress (CK, mild, moderate, and severe) on the endophytic fungal diversity of alfalfa, including the OTU number (a), Shannon–Wiener index (b), Simpson index (c), ACE (d), and Chao 1 estimator (e). Values are means \pm SEs. Significant differences between different levels of salt stress are indicated by uppercase letters ($p < 0.05$).

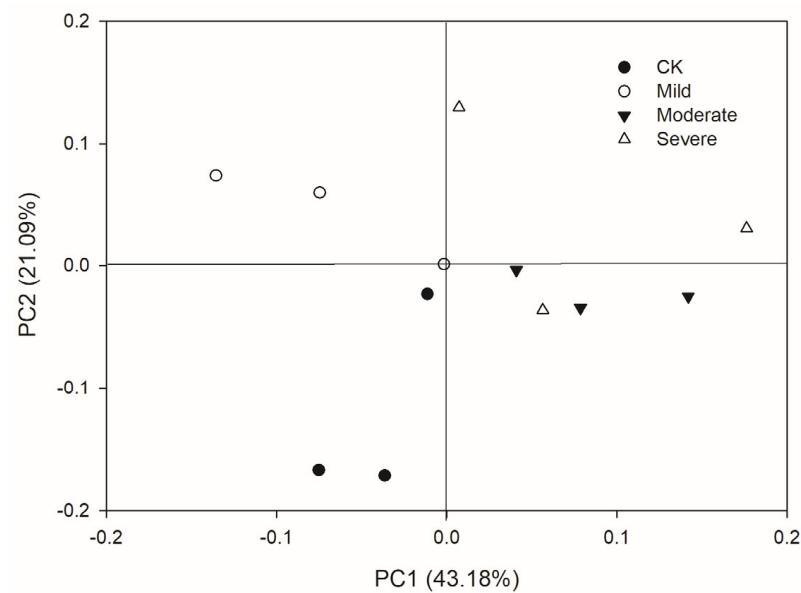


Figure 4. Principal coordinate analysis (PCoA) of endophytic fungal dissimilarity (Bray–Curtis).

The Ascomycota and Basidiomycota phyla were the dominant fungi and showed relative abundances ranging from 78.99% to 93.10% and from 6.70% to 20.61% across all the samples, respectively (Figure 5). Compared with the CK treatment, the severe salt stress treatment significantly increased the relative abundance of Ascomycota ($p < 0.05$, Figure 2a) and decreased the relative abundance of Basidiomycota ($p < 0.05$, Figure 2b). Compared with the CK treatment, the mild, moderate, and severe salt stress treatments significantly increased the relative abundance of *Davidiellaceae*, whereas the severe salt stress treatment significantly decreased the relative abundance of *Tremellales* and *Sporidiobolales* (Table 3).

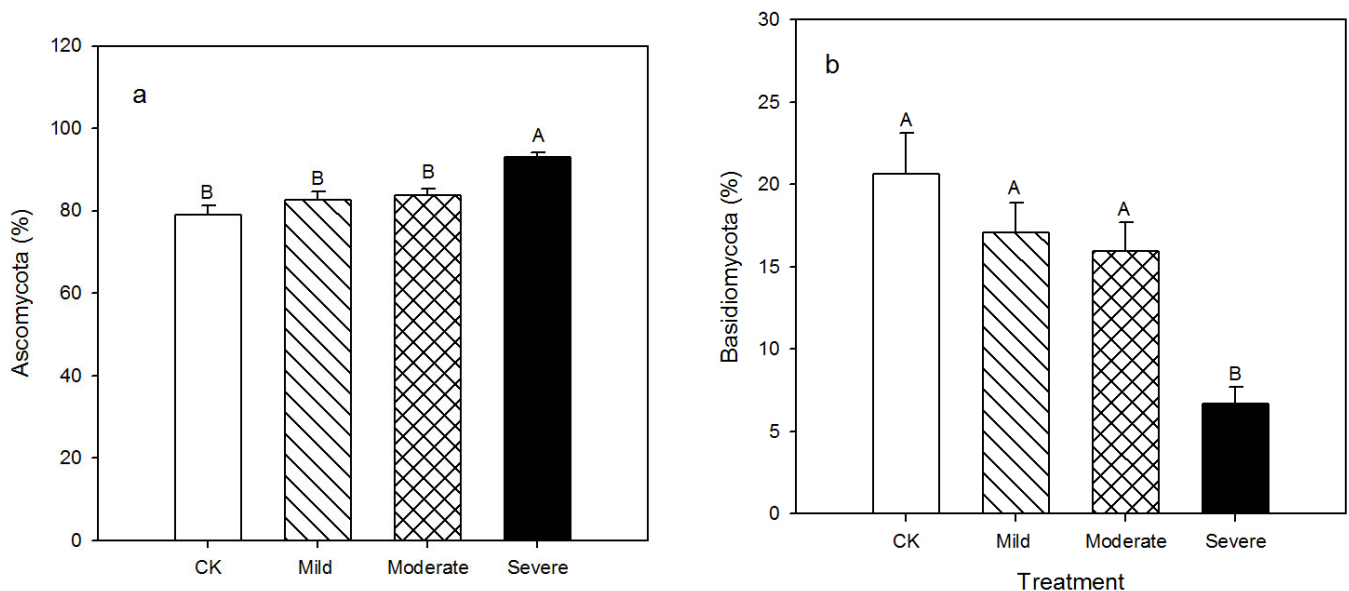


Figure 5. Examples of various responses of endophytic fungi to salt stress. The phyla included Ascomycota (a) and Basidiomycota (b). Values are means \pm SEs. Significant differences between different levels of salt stress are indicated by uppercase letters ($p < 0.05$).

Table 3. The relative abundances (%) of the families of different fungi under salt stress.

Phylum	Family	CK	Mild	Moderate	Severe
<i>Ascomycota</i>	<i>Davidiellaceae</i>	52.36 ± 10.74	59.40 ± 5.71	61.42 ± 10.51	63.07 ± 6.47
	<i>Pleosporaceae</i>	6.27 ± 0.93	7.69 ± 3.47	7.71 ± 1.72	8.48 ± 0.42
	<i>Mycosphaerellaceae</i>	11.43 ± 8.37	8.67 ± 5.36	1.88 ± 0.82	9.48 ± 7.15
	<i>Pleosporales</i>	1.32 ± 0.77	0.87 ± 0.08	2.65 ± 2.17	7.31 ± 6.93
	<i>Nectriaceae</i>	0.77 ± 0.22	1.84 ± 0.62	6.21 ± 4.07	2.24 ± 1.29
	<i>Plectosphaerellaceae</i>	2.00 ± 1.60	0.52 ± 0.15	0.44 ± 0.10	0.47 ± 0.25
	<i>Xylariales</i>	0.54 ± 0.44	0.92 ± 0.74	0.32 ± 0.24	0.31 ± 0.18
	<i>Dothideomycetes</i>	0.16 ± 0.10	0.97 ± 0.54	0.77 ± 0.30	0.24 ± 0.09
	<i>Pleosporales</i>	0.06 ± 0.01	1.05 ± 1.01	0.38 ± 0.33	0.13 ± 0.12
<i>Basidiomycota</i>	<i>Tremellales</i>	17.38 ± 1.99	14.54 ± 1.64	11.30 ± 1.28	5.78 ± 0.90
	<i>Sporidiobolales</i>	1.83 ± 0.51	1.31 ± 1.64	2.58 ± 1.28	0.43 ± 0.19
	<i>Filobasidiaceae</i>	0.23 ± 0.06	0.56 ± 0.24	0.65 ± 0.31	0.10 ± 0.05

3.3. Fungal Functional Groups

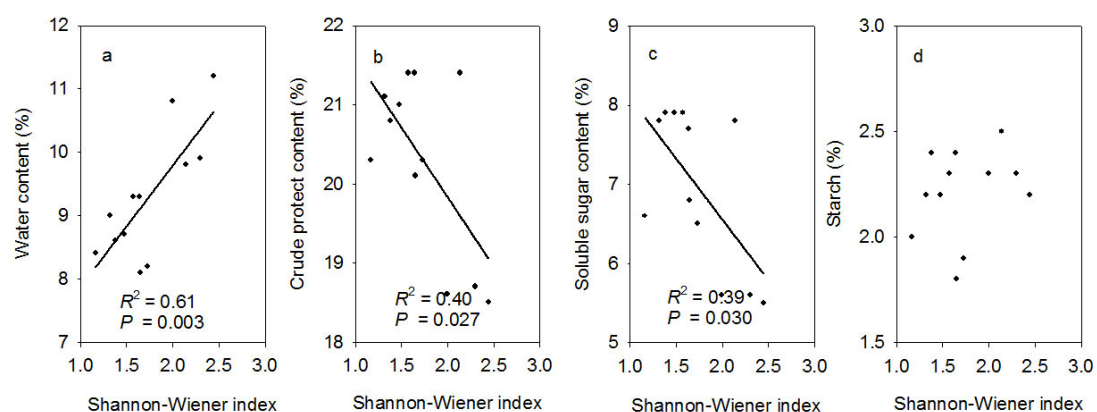
Among the 620 operational taxonomic units (OTUs) detected in this study, 268 OTUs (43.22% of the total OTUs) were annotated in five functional groups based on the FUNGuild database (Table 4). The relative abundances of animal pathogens, fungal parasites, and plant pathogens differed significantly among the salt stress treatments ($p < 0.05$, Table 4). Compared with the CK treatment, the severe salt stress treatment significantly increased the relative abundance of animal and plant pathogens ($p < 0.05$, Table 4).

Table 4. The relative abundance (%) of different fungal functional guilds under salt stress.

Guild	OUTs	CK	Mild	Moderate	Sever
Animal pathogen	58	7.99 ± 0.81	8.12 ± 0.77	8.61 ± 1.33	23.04 ± 6.51
Dung saprotroph	21	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.02
Endophyte	59	1.14 ± 0.29	3.80 ± 3.17	1.03 ± 0.27	2.88 ± 1.22
Fungal parasite	67	16.31 ± 2.84	9.92 ± 4.33	11.93 ± 4.54	25.18 ± 6.89
Plant pathogen	63	3.75 ± 1.07	14.63 ± 4.86	23.52 ± 7.76	30.53 ± 3.26

3.4. Multivariate Statistical Analyses of Plant Physiological Characteristics, Microbial Diversity, and Community Composition

The Shannon–Wiener index for the fungi showed positive linear relationships with the plant water content ($R^2 = 0.61$, $p = 0.003$; Figure 6a), CP content ($R^2 = 0.40$, $p = 0.027$; Figure 6b), soluble sugar content ($R^2 = 0.39$, $p = 0.030$; Figure 6c). There was no correlation between the Shannon–Wiener index for the fungi and starch content of alfalfa.

**Figure 6.** Spatial dependence of the endophytic fungal diversity of alfalfa on the water content (a), crude protein content (b), soluble sugar content (c), and starch (d) across twelve plots.

RDA biplots were used to assess which plant physiological characteristics influenced the fungal community at the phylum level. Overall, a combination of physiological variables explained 89.9% of the variance in the fungal community structure (Figure 7). The water content ($F = 18.33$, $p = 0.008$) and starch content ($F = 6.67$, $p = 0.030$) were found to be determining factors for the fungal communities and explained 65% and 15% of the variance in the fungal community structure, respectively.

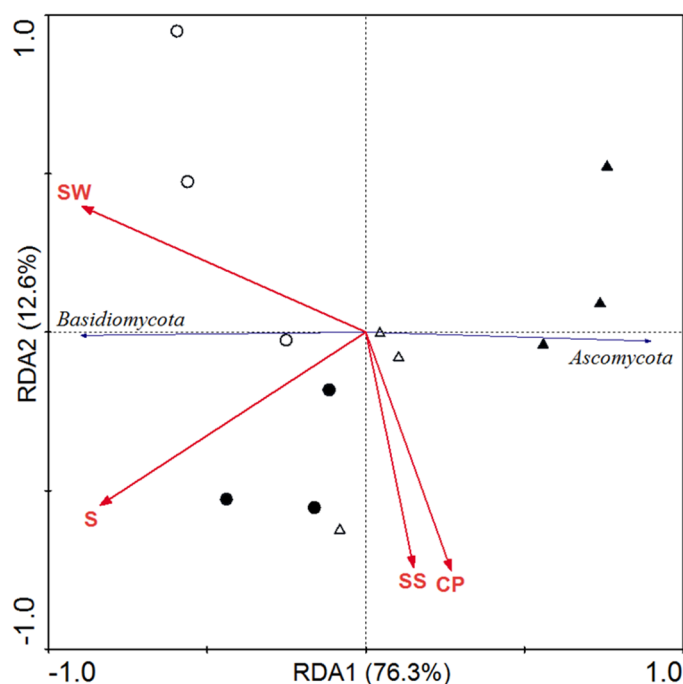


Figure 7. Redundancy analysis (RDA) plots showing the influence of physiological characteristics (water, CP, soluble sugar, and starch contents) on endophytic fungal communities.

4. Discussions

4.1. Physiological Characteristics of Alfalfa

Salt stress affects the normal growth and development of plants and affects the physiological characteristics of alfalfa. The water content can be used to indicate plant physiological characteristics under salt stress. Kusvuran et al. believed that the water content of plants would decrease with the increase in soil salt content under salt stress [21]. As a result, alfalfa grows slowly or even die due to poor water content. Under salt stress, the plants' water content decreases with the increase in salinization degree, which is a way for plants to adapt to salinization [22]. This also explains why the water content of the alfalfa under severe salt stress was lower than that of the other treatment groups in this study. Alfalfa needs to absorb a large amount of water from the soil to grow and develop normally. From another perspective, by taking Na^+ , K^+ , and other ions from the soil, alfalfa can increase its cell concentration and reduce its water content. CP is an important factor in relieving osmotic pressure in response to salt stress [2]. The CP content in a low- NaCl group increased significantly [3]; this is congruent with the findings of Lu et al., who reported that the CP content in a high-salinity growth environment was decreased. Studies have shown that with increases in salt stress, the biomass and harvest index of plants decreased significantly, while the CP content increased [23]. The CP content in a low- NaCl treatment increased significantly. This is congruent with the findings of Agastian et al., (2000), who reported that the soluble protein content in a high-salinity growth environment was decreased [3]. Moreover, the lower soil N content under severe salt stress may also be the reason for the lower crude protein content. Mao believed that a high N content would lead to an increase in the crude protein content of plants, while a low N content would also lead to a decrease in protein content [24]. The main storage form of energy in plants is

non-structural carbohydrates (NSC), which include soluble carbohydrates (e.g., sucrose and fructose) and starch, which are important sources of energy in the process of plant growth and metabolism. The soluble sugar content in CK was significantly lower than that in the other groups because appropriate salt stress promotes the metabolism and accumulation of carbohydrates in plants [7]. In this study, salt stress was found to have a certain influence on the soluble sugar synthesis of plants because salt stress can be alleviated by increasing the soluble sugar content. Lu et al. believed that with the enhancement of the salinization degree, soluble sugar content increases [25].

The severe salt stress treatment in this study inhibited plant growth and productivity. Salt stress leads to a large extracellular Na^+ content in plants, thereby forming a large osmotic pressure outside the cells, resulting in a decrease in water potential, which, in turn, inhibits the photosynthesis of plants, and ultimately leads to the inhibition of plant growth and development [11]. During the process of growth and development, due to the accumulation of a large amount of Na^+ , the plant itself needs to spend more energy on absorbing water and other mineral elements to maintain the balance of water potential and osmotic pressure in the body, resulting in a decrease in the crop growth rate and productivity [21]. Our results agree with a previous study in which high salinity amplified the ionic imbalance inside plant cellular organs and created a water deficit [26]. Although severe salt stress limited the plant water content, the mild salt stress treatment significantly increased the plant water, CP, soluble sugar, and starch contents (Figure 1). There are several explanations for the results of this study. First, most of the detected enzymes were invertase in alfalfa; the other insoluble invertase isoforms were either absent or present in negligible amounts in the alfalfa. Second, under salt stress, sucrose synthase and protease activity were also significantly increased due to the endogenous defense mechanisms initiated by plants so as to alleviate oxidative stress-associated damage [27]. The increased invertase activity due to salt stress may have resulted in the increased content of soluble sugar and starch in the alfalfa. These results agree with previous studies on wheat and maize [4,28]. Moreover, the invertase activity regulated enzyme secretions at higher pH values [29], and higher pH values significantly increased invertase activity (Table 1 and Figure 2).

4.2. Alfalfa Fungi and Response to Salt Stress

Fungi have been reported to ameliorate salt stress in plants by influencing biotic and abiotic factors [30]. Our results are consistent with those of previous studies, in which the Shannon–Wiener index for fungi was related to the plant water, CP, soluble sugar, and starch contents (Figure 1). We observed a higher Shannon–Wiener index for fungi in the CK treatment than in the severe salt stress treatment. This might have resulted from the high relative abundance of *Ascomycota* (Figure 1). In this study, the alfalfa fungal communities under severe salt stress were dominated by *Ascomycota* ($93.10 \pm 1.06\%$), which primarily drove the fungal community composition.

The RDA results showed that the water content determined the changes in fungal community composition. The reason is that water content fluctuations induce a rapid turnover of fungal populations, thereby conferring a high community plasticity [31]. A decrease in water content may be a stressful process for some microorganisms due to physical constraints that affect bacterial and fungal habitats [32].

We also found clear differences in the fungal communities among the different salt stress treatments, mostly with regard to the relative abundance of *Ascomycota*. The high salt stress treatment limited the plant water content in this study, and the dominance of *Ascomycota* may reflect a unique fungal distribution pattern in response to salt stress [33]. The increased relative abundance of *Ascomycota* associated with salt stress in alfalfa may have occurred because *Ascomycota* intervenes in stress-related physiological processes and improves the ability of plants to tolerate, avoid, or prevent stressful situations. Moreover, the effects of high salt stress also led to differences in the fungal communities in terms of their dominant families (Table 3), including *Davidiellaceae* (*Ascomycota*), *Tremellales* (*Basidiomycota*), and *Sporidiobolales* (*Basidiomycota*). Compared with CK, mild salt stress

significantly increased the relative abundance of the *Davidiellaceae* family. A high relative abundance of the *Davidiellaceae* family enhances the resistance of plants to salt stress.

According to preliminary studies, fungal endophyte–plant host interactions are characterized by a balance between fungal virulence and plant defenses [34]. If this balance is disturbed by a decrease in plant defenses or an increase in fungal virulence, diseases will occur in the plant. The relative abundances of animal and plant pathogens were higher under the severe salt stress treatment than under the CK treatment (Table 4). These functional groups belong to the *Ascomycota* phylum according to the FUNGuild database. The relative abundance of the fungal parasite functional group, which belongs to Basidiomycota, was lower under mild and moderate salt stress than under severe salt stress. The change in the abundance of fungal functional groups resulted in a marked shift in fungal community composition due to the changes in the relative abundances of the *Ascomycota* and Basidiomycota phyla in the fungal communities under salt stress. Our results suggest that fungal communities are responsible for the ability of plants to adapt to the environment under highly selective pressures.

5. Conclusions

In summary, salt stress altered the physiological characteristics of alfalfa plants and their fungal community structure and decreased the fungal alpha diversity. Salt stress affected the relative abundance of animal pathogens, fungal parasites, and plant pathogens. The water content and starch content, both of which were significantly affected by salt stress, were significantly correlated with the fungal community structure. Taken together, our results contribute to a better understanding of plant physiological characteristics and indicate that fungi control the response of alfalfa to salt stress.

Author Contributions: Data curation, G.G.; Methodology, T.L. and Z.W. (Zhijun Wang); Software, D.S., and Z.W. (Zhen Wang); Writing—original draft, Q.L. and D.S.; Writing—review and editing, Z.W. (Zhen Wang), Q.L., Y.J., and L.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Mining and Utilization of Microbial Resources in Saline-alkali Land (2022NMRC011), and the High Quality Raw Feed Resources Development and Utilization Demonstration (16200157-10) and the National Technical System of Forage Industry for Dry Grass Storage (CARS-34). The funding bodies had no role in the design of the study, collection, analysis, or interpretation of the data or in the writing of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Available upon request.

Acknowledgments: We sincerely thank the Key Laboratory of Grassland Resources of the Ministry of Education and the Key Laboratory of Forage Cultivation, Processing, and High-Efficiency Utilization of the Ministry of Agriculture for their technical support for this research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cao, C.; Tao, S.; Cui, Z.; Ying, Z. Response of Soil Properties and Microbial Communities to Increasing Salinization in the Meadow Grassland of Northeast China. *Microb. Ecol.* **2021**, *82*, 722–735. [[CrossRef](#)] [[PubMed](#)]
2. Hu, Y.; Kang, S.; Ding, R.; Zhao, Q. A crude protein and fiber model of alfalfa incorporating growth age under water and salt stress. *Agric. Water Manag.* **2021**, *255*, 107037. [[CrossRef](#)]
3. Lu, Q.; Ge, G.T.; Sa, D.W.; Wang, Z.; Hou, M.; Jia, Y.S. Effects of salt stress levels on nutritional quality and microorganisms of alfalfa-influenced soil. *PeerJ* **2021**, *9*, e11729. [[CrossRef](#)] [[PubMed](#)]
4. Wang, Y.; Liu, G.; Zhao, Z.; Wu, C.; Yu, B. Using soil erosion to locate nonpoint source pollution risks in coastal zones: A case study in the Yellow River Delta, China. *Environ. Pollut.* **2021**, *283*, 117117. [[CrossRef](#)]
5. Gabrijel, O.; Santosha, R.; Kallakeri, K.M.; Channappa, G.; Madhyavenkatapura, S.A.; Akshay, S.S.; Brajendra, P.; Brahamdeo, K.Y.; Nirmala, B.; Farzana, R.; et al. Salt Stress in Plants and Mitigation Approaches. *Plants* **2022**, *11*, 717. [[CrossRef](#)]

6. Papon, N.; Courdavault, V. Arresting cytokinin signaling for salt-stress tolerance. *Plant Sci.* **2022**, *314*, 111–116. [\[CrossRef\]](#)
7. Jia, X.M.; Wang, H.; Svetla, S.; Zhu, Y.F.; Hu, Y.; Cheng, L.; Zhao, T.; Wang, Y.X. Comparative physiological responses and adaptive strategies of apple *Malus halliana* to salt, alkali and saline-alkali stress. *Sci. Hortic.* **2019**, *245*, 154–162. [\[CrossRef\]](#)
8. Liao, Q.; Gu, S.; Kang, S.; Du, T.; Tong, L.; Wood, J.D.; Ding, R. Mild water and salt stress improve water use efficiency by decreasing stomatal conductance via osmotic adjustment in field maize. *Sci. Total Environ.* **2022**, *805*, 150364. [\[CrossRef\]](#)
9. Zhou, Y.; Sun, Y.; Liu, J.; Ren, X.; Zhang, Z.; Wang, Q. Effects of microplastics on humification and fungal community during cow manure composting. *Sci. Total Environ.* **2022**, *803*, 150029. [\[CrossRef\]](#)
10. Jiang, S.; Xing, Y.; Liu, G.; Hu, C.; Wang, X.; Yan, G.; Wang, Q. Changes in soil bacterial and fungal community composition and functional groups during the succession of boreal forests. *Soil Biol. Biochem.* **2021**, *161*, 108393. [\[CrossRef\]](#)
11. Al-Farsi, S.M.; Nawaz, A.; Aneesur, R.; Nadaf, S.K.; Farooq, M. Effects, tolerance mechanisms and management of salt stress in lucerne (*Medicago sativa*). *Crop Pasture Sci.* **2020**, *7*, 411–428. [\[CrossRef\]](#)
12. Bouzouina, M.; Kouadria, R.; Lotmani, B. Fungal endophytes alleviate salt stress in wheat in terms of growth, ion homeostasis and osmoregulation. *J. Appl. Microbiol.* **2021**, *130*, 913–925. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Magdalena, K.; Barbara, K. Influence of drought and salt stress on the growth of young *Populus nigra* 'Italica' plants and associated mycorrhizal fungi and non-mycorrhizal fungal endophytes. *New For.* **2022**, *53*, 679–694. [\[CrossRef\]](#)
14. Řezáčová, V.; Řezáč, M.; Gryndler, M.; Hrelová, H.; Gryndlerová, H.; Michalová, T. Plant invasion alters community structure and decreases diversity of arbuscular mycorrhizal fungal communities. *Appl. Soil Ecol.* **2021**, *167*, 104039. [\[CrossRef\]](#)
15. Wang, C.; Cheng, H.; Wang, S.; Wei, M.; Du, D. Plant community and the influence of plant taxonomic diversity on community stability and invasibility: A case study based on *Solidago canadensis* L. *Sci. Total Environ.* **2021**, *768*, 144518. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Mello, I.N.K.; Moreira, B.C.; Kasuya, M.C.; Arajo, J.V.; Freitas, L.G. *Nematophagus* fungi increasing phosphorus uptake and promoting plant growth. *Biol. Control* **2018**, *23*, 71–75. [\[CrossRef\]](#)
17. Peterson, L.W.; Huffaker, R.C. Loss of ribulose 1,5-Diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. *Plant Physiol.* **1975**, *55*, 1009–1015. [\[CrossRef\]](#)
18. Sergeeva, L.I.; Keurentjes, J.J.B.; Bentsink, L. Vacuolar invertase regulates elongation of *Arabidopsis thaliana* roots as revealed by QTL and mutant analysis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2994–2999. [\[CrossRef\]](#)
19. Dong, W.Y.; Liu, E.K.; Yan, C.R.; Tian, J.; Zhang, H.H.; Zhang, Y.Q. Impact of no tillage vs. conventional tillage on the soil bacterial community structure in a winter wheat cropping succession in northern China. *Eur. J. Soil Biol.* **2017**, *80*, 35–42. [\[CrossRef\]](#)
20. Bostrom, C.; O'Brien, K.; Roos, C.; Ekebom, J. Environmental variables explaining structural and functional diversity of seagrass macrofauna in an archipelago landscape. *J. Exp. Mar. Biol. Ecol.* **2006**, *335*, 52–73. [\[CrossRef\]](#)
21. Kusvuran, A.; Bilgici, M.; Kusvuran, S.; Bilgici, M.; Kuvuran, V.; Nazli, R. The effect of different organic matters on plant growth regulation and nutritional components under salt stress in sweet sorghum [*Sorghum bicolor* (L.) Moench.]. *Maydica* **2021**, *66*, 1–9.
22. Yang, X.; Li, Y.; Chen, H.; Huang, J.; Zhang, Y.; Qi, M.; Liu, Y.; Li, T. Photosynthetic response mechanism of soil salinity-induced cross-tolerance to subsequent drought stress in tomato plants. *Plants* **2020**, *9*, 363. [\[CrossRef\]](#)
23. Hedayati, F.A.; Kazemeini, S.A.; Pirasteh-Anosheh, H.; Ghadiri, H.; Mohammad, P. Forage yield and quality as affected by salt stress in different ratios of *Sorghum bicolor*—*Bassia indica* intercropping. *J. Plant Nutr.* **2020**, *43*, 2579–2589. [\[CrossRef\]](#)
24. Mao, X.; Li, Q.; Ren, L. Application of molybdenum fertilizer enhanced quality and production of alfalfa in northern China under non-irrigated conditions. *J. Plant Nutr.* **2018**, *41*, 1009–1019. [\[CrossRef\]](#)
25. Lu, Q.; Zhen, W.; Sa, D.; Hou, M.; Ge, G.; Wang, Z.; Jia, Y. The potential effects on microbiota and silage fermentation of alfalfa under salt stress. *Front. Microbiol.* **2021**, *12*, 688695. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Nounjan, N.; Nghia, P.T.; Theerakulpisut, P. Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. *J. Plant Physiol.* **2012**, *169*, 596–604. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Guo, Q.; Liu, L.; Rupasinghe, T.; Rupasinghe, T.W.T.; Barklaet, B.J. Salt stress alters membrane lipid content and lipid biosynthesis pathways in the plasma membrane and tonoplast. *Plant Physiol.* **2022**, *189*, 805–826. [\[CrossRef\]](#)
28. Qiu, Z.B.; Guo, J.L.; Zhu, A.J.; Zhang, L.; Zhang, M.M. Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. *Ecotoxicol. Environ. Saf.* **2014**, *104*, 202–208. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Li, K.; Li, M.; He, Y.; Gu, X. Effects of pH and nitrogen form on *Nitzschia closterium* growth by linking dynamic with enzyme activity. *Chemosphere* **2020**, *249*, 126154. [\[CrossRef\]](#)
30. Gupta, A.K.; Verma, J.; Srivastava, A.; Srivastava, S.; Prasad, V. *Pseudomonas aeruginosa* isolate PM1 effectively controls virus infection and promotes growth in plants. *Arch. Microbiol.* **2022**, *204*, 1–18. [\[CrossRef\]](#)
31. Kaisermann, A.; Maron, P.A.; Beaumelle, L. Fungal communities are more sensitive indicators to non-extreme soil moisture variations than bacterial communities. *Appl. Soil Ecol.* **2015**, *86*, 158–164. [\[CrossRef\]](#)
32. Fabiana, C.; Laura, Z.; Claudia, P.; Laura, S.; Silvano, O.; József, G. Vegetation, pH and water content as main factors for shaping fungal richness, community composition and functional guilds distribution in soils of Western Greenland. *Front. Microbiol.* **2019**, *10*, 2348. [\[CrossRef\]](#)
33. Waller, F.B.; Achatz, H.; Baltruschat, J.; Fodor, K.; Becker, M. The endophytic fungus *Piriformis indica* reprograms barley to salt-stress tolerance, disease resistance and higher yield. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13386–13391. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Schulz, B.; Boyle, C. The endophytic continuum. *Mycol. Res.* **2005**, *109*, 661–686. [\[CrossRef\]](#)