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Occurrence and Nutrition Indicators of Alfalfa with*Leptosphaerulina* in Chifeng, Inner Mongolia

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Abstract: Alfalfa *Leptosphaerulina* leaf spot is a common disease of alfalfa, while its effect on alfalfa quality has not been reported. The present study aimed to investigate the alfalfa *Leptosphaerulina* leaf spot in Chifeng City, Inner Mongolia, China and determine the quality of alfalfa plants and leaves with different scales. The incidence and disease index of nine alfalfa cultivars ranged from 12.1% to 59.8% and 10.0 to 51.0, respectively. The incidence of the Optimus cultivar and the disease index of the WL168 cultivar were significantly higher than those of the other cultivars. Therefore, different scales (0–4) of the alfalfa WL168 plant and leaves were used to determine their nutritional levels. Compared with healthy plants and leaves, the severity of alfalfa leaf spot on a scale of 4 decreased by 3.7% to 29.4% or 1.7% to 40.7%, respectively, in 18 nutrients; and increased by 12.0% to 14.5% or 17.8% to 26.9% in the Rumen protein (RUP), acid detergent fiber (ADF) and neutral detergent fiber (NDF), respectively. In addition, the crude protein (CP) content of alfalfa plants or leaves based on a severity scale of 4 decreased by 16.7% and 6.2%, respectively. Correlation analysis revealed a strong negative correlation between 18 nutritional contents and disease severity, except for NDF, ADF and RUP. Conclusively, alfalfa *Leptosphaerulina* leaf spot strongly influences the plant and the leaves' nutrient content in the plant.

Keywords: alfalfa *Leptosphaerulina* leaf spot; incidence; disease index; nutrition

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1. Introduction

Alfalfa is a highly nutritious perennial legume plant with a long history of use as a forage crop [1]. Compared with grasses, legumes such as alfalfa have a lower neutral detergent fiber (NDF) content but contain higher protein, energy and calcium concentrations [2–5]. Alfalfa is a high-quality forage crop that is also used to restore pastures owing to its deep root system and its ability to reduce soil erosion [6,7]. Alfalfa is widely cultivated in China as animal feed [8]. Alukhorqin Banner in Chifeng City, Inner Mongolia, China, is one of the important regions for alfalfa farming [9]. Alfalfa farming in China is restricted to certain regions because of alfalfa diseases [10].

The disease caused by *Leptosphaerulina australis* was found in Inner Mongolia in 2019 [11]. Alfalfa *Leptosphaerulina* leaf spot is a common disease of alfalfa leaves in humid areas [12]. The hyphae and ascocarps of *Leptosphaerulina* fungi overwinter in diseased tissues, and the ascospores are transmitted through the air [13]. This disease is primarily caused by fungi in the *Leptosphaerulina* sp., which typically infect members of *Agrostis*, *Festuca*, *Lolium* and *Poa* [14–16], while it can also infect *Setaria*, *Phleum pratense*, *Pisum sativum*, *Trifolium* and *Vicia faba* [17]. Five *Leptosphaerulina* species have been reported to be associated with *Leptosphaerulina* leaf blight. *Leptosphaerulina americana* has been isolated from the dead leaves of *Phleum pretense*, while it can also infect *Pisum*, *Trifolium* and *Vicia* [17]. *Leptosphaerulina argentinensis* infects creeping bentgrass (*Agrostis stolonifera* L.) [18], whereas

Leptosphaerulina australis has been isolated from members of *Agrostis*, *Festuca*, *Lolium* and *Poa* [14]. *Leptosphaerulina trifolii* has been isolated from *Poa* and *Medicago sativa* and other members of the non-turfgrass genera [19,20]. *Leptosphaerulina briosiana* has been isolated from alfalfa [21]. In China, alfalfa *Leptosphaerulina* leaf spot with *L. briosiana* was first reported in the Gongzhuling area, Jilin Province [13]. *L. trifolii* was then isolated from alfalfa in Heilongjiang Province, whereas *L. australis*on (alfalfa) in Inner Mongolia has also been reported [11,20,22]. In 2021, *Leptosphaerulina* species, including *L. americana, L. argentinensis*, *L. australis* and *L. trifolii,* were isolated from golf turfgrass in Beijing and Hainan [12].

Alfalfa disease seriously affects the yield and quality of alfalfa [23]. Previous studies have shown that leaf spot diseases reduced yield by 6% to 7% in Alberta, Canada [24], 16% in New Zealand [25], 40% in Australia [26] and 19% in the United States [27]. Leaf spot injuries caused by different pathogens can reduce the CP content in alfalfa leaves by up to 22% [28]. Lucerne common leaf spot and alfalfa *Stemphylium* leaf spot losses in alfalfa can be as high as 40–60% [29]. Anthracnose caused the yield reduction of 7% to 37% in alfalfa, but the NDF and ADF contents increased by 21% and 16%, respectively, and the crude fat and CP decreased by 35% and 17%, respectively [30]. *Phoma medicaginis* can reduce alfalfa yields about 50% [31]. *Pseudopeziza medicaginis* infection increased the ash, ADF, NDF, calcium (Ca), tannin and total phenol contents in diseased leaves by 20%, 71%, 40%, 136%, 22% and 51%, respectively, relative to the healthy leaves [32].

Previous research has shown that diseases can reduce alfalfa yield and quality to different degrees [30–32]. A hypothesis was proposed as to whether alfalfa *Leptosphaerulina* Leaf spot could affect alfalfa quality, alfalfa production and livestock health. Only a few studies have reported alfalfa *Leptosphaerulina* leaf spot in the past five years. Therefore, we systematically investigated the *Leptosphaerulina* leaf spot disease in nine (alfalfa) cultivars in Chifeng City Inner Mongolia to determine whether this disease was the most important alfalfa plant disease. Then, the cultivars with the highest disease index were selected to explore the relationship between the disease severity and nutrient content in alfalfa using a near-infrared detection method. Our findings will provide a theoretical basis for alfalfa *Leptosphaerulina* leaf spot disease indexing and control and the effect of this disease on livestock health and production.

2. Materials and Methods

2.1. Sample Cultivation

The nine diseased alfalfa cultivars used in this study were sampled from Aluhorqin Banner, Chifeng City, Inner Mongolia autonomous region of northeast China. The cultivars for the Gansu Yasheng Pastoral Company were planted in 2018, 2019 and 2020, respectively. The longitude of the field is between 120°13′13″ and 120°29′14″, the latitude is between 43°27′52″ and 43°34′54″ and the altitude is between 308.89 m and 357.18 m (Table 1). The sowing was broadcast. Irrigation was sprinkler. Fertilizer was used five times a year. Fertilizer kinds and dosage are shown in Supplementary Table S1.

Table 1. Field cultivars and survey times.

Planting Site No.	Cultivars	Proportion (hm^2)	Planting Years	Survey Years	Longitude	Latitude	Altitude
4	WL525	13.33	2020	2020	120°26′16″	43°32′5″	324.59
52	SW5909	35.07	2020	2020	120°29′14″	43°27′52″	315.13
26	Xinmu No.3	4.53	2019	2020	120°15′20″	43°34′0″	342.95
33	WL440	74.20	2020	2020	120°26′51″	43°29′45″	333.42
50	WL440	43.87	2020	2020	120°28′34″	43°27′56″	281.71
5	WL168HQ	35.07	2018	2019, 2020	120°19′3″	43°32′0″	331.28
35	Optimus Prime	40.80	2018	2019, 2020	120°26′43″	43°29′16″	333.15
2	Zhongmu No.5	37.13	2019	2019	120°18′58″	43°31′56″	340.11
38	Zhongmu No.5	69.20	2019	2019	120°19′3″	43°32′0″	331.28
5	Bright clover	35.07	2019	2019, 2020	120°19′3″	43°32′0″	331.28
20	Bright clover	24.60	2019	2020	120°19′22″	43°34′48″	324.49
25	Bright clover	43.87	2019	2020	120°14′44″	43°34′5″	356.64

Table 1. *Cont.*

Planting Site No.	Cultivars	Proportion (hm^2)	Planting Years	Survey Years	Longitude	Latitude	Altitude
27	Bright clover	5.80	2019	2020	120°15′21″	43°33′56″	349.83
46	Bright clover	54.73	2019	2020	120°13′13″	43°34′54″	350.72
47	Bright clover	59.67	2019	2020	120°13′23″	43°34′34″	343.48
76	Bright clover	38.60	2019	2019	120°18′49″	43°32′23″	360.13
18	Adina	42.40	2019	2020	120°29′10″	43°32′38″	321.18
19	Adina	83.47	2019	2020	120°26′34″	43°30′19″	323.24
25	Adina	43.87	2018	2020	120°13′43″	43°34′6″	356.51
35	Adina	36.33	2019	2019	120°13′43″	43°34′7″	357.18
37	Adina	10.27	2019	2020	120°26′39″	43°29′5″	319.44
41	Adina	35.67	2019	2020	120°26′40″	43°29′6″	331.19
42	Adina	35.40	2019	2020	120°27′52″	43°28′40″	308.89
56	Adina	47.33	2018	2019, 2020	120°13′43″	43°34′6″	356.51
57	Adina	30.27	2019	2020	120°13′43″	43°34′6″	356.51

2.2. Soil Characteristics

The soil type is sandy loam. Soil organic matter content was 5.9 $g \cdot kg^{-1}$. Total nitrogen was 0.5 $g \cdot kg^{-1}$. The content of available phosphorus was 10.17 $g \cdot kg^{-1}$. The available potassium content was 200 $g \cdot kg^{-1}$.

2.3. Climate Information

The climate information (rainfall and the air temperature) of the region during the experimental periods from mid-March to October in 2018, 2019 and 2020 is shown in Supplementary Table S2.

2.4. Alfalfa Leptosphaerulina Leaf Spot Disease Survey

Planting site, cultivars, proportion, planting years, survey years, longitude, latitude and altitude data are shown in Tables 1 and S3. In each field, five locations of approximately 25 m^2 (5 m×5 m) were randomly selected to assess the disease incidence. The plants were visually assessed for foliar alfalfa *Leptosphaerulina* leaf spot symptoms. The disease incidence was the proportion of the diseased plants in a given field. The disease severity index was the proportion of diseased leaves on each plant. The severity scale ranged 0–4, where0was a healthy plant; 1 was lesions covering 0–25% of the leaf; 2 was lesions covering 26–50% of the leaf; 3 was lesions covering 51–75% of the leaf; and 4 was lesions covering 76–100% of the leaf.

$$\text{Disease severity index (DSI)} = [\sum(\text{no. of plants/scale} \times \text{scale value})/(\text{highest scale value} \times \text{total no. of plants})] \times 100$$

2.5. Nutrient Sampling and Extraction

The nutrient content in the alfalfa plants and leaves with different disease severity in June 2019 was determined using WL168. A total of 10 alfalfa plants and 500 alfalfa leaves were selected for each severity scale. The samples were dried naturally, crushed and analyzed at the Quality Testing Laboratory of LandLakes Feed and Forage Testing Laboratory (China). For each severity scale, 10 alfalfa plants or 500 leaves were mixed and then divided into 3 samples for nutrient determination, respectively. A total of 21 nutrients were measured using rapid near-infrared (NIR) spectroscopy, and this equipment had been producted by FOSS in Denmark (FOSS DS2500, Hilleroed, Denmark). Crude protein (CP) was calculated by CP% = 6.25 N (%) [33]. Details of this process are shown in the Supplementary Table S4.

2.6. Statistical Analysis

The incidence data, disease index, and nutrition indices were analyzed using IBM SPSS Statistics 25 (Version 25.0; SPSS Inc, Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) and Duncan's test. Statistical significance was set at 0.05 or at 0.01.

3. Results

3.1. Incidence and Disease Index

We found significant differences in disease incidence among the cultivars ($p < 0.05$). The alfalfa *Leptosphaerulina* leaf spot incidence in nine alfalfa cultivars ranged from 12.1% to 59.8%. The highest incidence was observed on the Optimus Prime cultivar, whereas the lowest was on Adina. There was a significant difference in the disease incidence on Adina, which ranged from 12.1% to 52.5%. The index ranged from 10.0% to 51.0% ($p < 0.05$). The highest index was observed on cultivar WL168HQ. Thus, WL168HQ was used to measure the nutritional index (Table 2).

Table 2. The incidence and disease index of alfalfa *Leptosphaerulina* leaf spot.

Planting Site No.	Cultivar	Incidence (%)	Index
4	WL525	49.19 ± 3.97^{bc}	32.58 ± 2.05^{bc}
52	SW5909	43.77 ± 2.40^{cd}	27.79 ± 2.85^{bcd}
26	Xinmu No.3	36.44 ± 2.41^{e}	27.89 ± 1.04^{bcd}
33	WL440	42.87 ± 2.63^{d}	22.51 ± 1.91^{cde}
50	WL440	43.35 ± 2.67^{d}	33.64 ± 2.44^{b}
5	WL168HQ	24.73 ± 1.59^{hij}	51.00 ± 6.99^{a}
35	Optimus Prime	59.76 ± 2.07^{a}	16.50 ± 1.84^{ef}
2	Zhongmu No.5	29.04 ± 1.77^{fghi}	18.49 ± 1.82^{def}
38	Zhongmu No.5	21.11 ± 1.06^{jkl}	15.76 ± 0.96^{ef}
5	Bright clover	30.92 ± 1.88^{efgh}	17.51 ± 3.03^{def}
20	Bright clover	33.42 ± 1.59^{ef}	18.15 ± 1.52^{def}
25	Bright clover	25.31 ± 2.15^{ghij}	17.21 ± 1.30^{def}
27	Bright clover	25.30 ± 1.33^{ghij}	20.52 ± 3.29^{def}
46	Bright clover	26.21 ± 1.78^{ghij}	18.54 ± 1.36^{def}
47	Bright clover	23.57 ± 2.65^{ijk}	17.92 ± 1.31^{def}
76	Bright clover	31.38 ± 2.09^{efg}	14.86 ± 1.94^{ef}
18	Adina	35.45 ± 1.71^{e}	17.98 ± 2.35^{def}
19	Adina	18.02 ± 0.84^{klm}	14.74 ± 6.30^{ef}
25	Adina	15.10 ± 1.34^{lm}	22.73 ± 4.60^{cde}
35	Adina	23.74 ± 1.33^{ijk}	16.33 ± 1.74^{ef}
37	Adina	52.54 ± 2.32^{b}	18.81 ± 5.95^{ef}
41	Adina	$17.28 \pm 0.77l^{m}$	21.64 ± 4.09^{de}
42	Adina	17.08 ± 0.94^{lm}	19.41 ± 4.71^{def}
56	Adina	15.44 ± 1.10^{lm}	14.46 ± 3.48^{def}
57	Adina	12.13 ± 0.89^{m}	9.94 ± 1.08^{f}
		$p < 0.05$	$p < 0.05$

Different letters in the column indicate a significant difference ($p < 0.05$; Duncan's test).

3.2. Plant Nutrients

Alfalfa *Leptosphaerulina* leaf spot disease significantly affected the nutrient levels in the plants (Table 3) ($p < 0.05$). Compared with healthy plants, except for ADF, NDF and RUP, the nutrient content in alfalfa with scale 4 alfalfa *Leptosphaerulina* leaf spot decreased by 3.7% to 29.4%. The calcium (Ca), phosphorus (P), potassium (K), magnesium (Mg), ash, fat, CP, lignin, net milk production, maintenance net energy, net weight gain, relative feeding value, relative forage quality, total digestible nutrients, milk production,30h NDF digestible rate,48h NDF digestible rate and nonfibrous carbohydrate decreased by 29.4%, 25.0%, 2.5%, 20.0%, 7.2%,18.8%,16.7%, 9.0%, 5.0%, 7.0%, 14.3%, 10.3%,16.3%, 6.2%, 8.0%, 11.1%, 8.9% and 3.7%, respectively. However, the decrease in the 18 nutrients did not decrease continuously with the disease severity. For example, the P content was the same in grade 2 and grade 3 alfalfa *Leptosphaerulina* leaf spot, and Mg content was the same in grade 3 and grade 4 alfalfa *Leptosphaerulina* leaf spot. The highest disease severity in cultivar ash was grade 2.

Table 3. Nutrients in alfalfa plants with different severity levels.

Nutrient	Disease Severity Index				
	0	1	2	3	4
Calcium (Ca %)	1.7 ± 0.01 ^{a}	1.5 ± 0.01 ^{b}	1.5 ± 0.00 ^{c}	1.3 ± 0.01 ^{d}	1.2 ± 0.00 ^{e}
Phosphorus (P %)	0.4 ± 0.00 ^{a}	0.3 ± 0.00 ^{b}	0.3 ± 0.00 ^{b}	0.3 ± 0.00 ^{b}	0.3 ± 0.00 ^{c}
Potassium (K %)	2.4 ± 0.01 ^{ab}	2.4 ± 0.01 ^{a}	2.3 ± 0.03 ^{ab}	2.3 ± 0.02 ^{c}	2.3 ± 0.04 ^{bc}
Magnesium (Mg %)	0.4 ± 0.00 ^{a}	0.3 ± 0.00 ^{b}	0.3 ± 0.00 ^{b}	0.3 ± 0.00 ^{c}	0.3 ± 0.00 ^{c}
Ash (%)	7.8 ± 0.06 ^{c}	7.6 ± 0.04 ^{c}	8.4 ± 0.03 ^{a}	8.0 ± 0.05 ^{b}	7.2 ± 0.03 ^{d}
Fat (%)	1.8 ± 0.01 ^{a}	1.6 ± 0.01 ^{b}	1.6 ± 0.00 ^{c}	1.5 ± 0.01 ^{d}	1.5 ± 0.00 ^{d}
Crude protein (CP %)	21.5 ± 0.07 ^{a}	20.0 ± 0.14 ^{b}	19.1 ± 0.08 ^{c}	18.4 ± 0.07 ^{d}	17.9 ± 0.05 ^{e}
Lignin (%)	6.6 ± 0.01 ^{a}	6.6 ± 0.07 ^{a}	6.4 ± 0.02 ^{b}	6.3 ± 0.03 ^{c}	6.0 ± 0.02 ^{d}
Rumen protein (RUP %)	17.9 ± 0.07 ^{c}	18.0 ± 0.27 ^{c}	19.9 ± 0.22 ^{ab}	19.3 ± 0.21 ^{b}	20.5 ± 0.32 ^{a}
Acid washing fiber (ADF %)	30.2 ± 0.01 ^{d}	30.9 ± 0.32 ^{c}	33.0 ± 0.25 ^{b}	32.3 ± 0.17 ^{b}	33.9 ± 0.16 ^{a}
Neutral detergent fiber (NDF %)	39.1 ± 0.07 ^{c}	40.0 ± 0.34 ^{c}	42.4 ± 0.27 ^{b}	42.4 ± 0.28 ^{b}	43.8 ± 0.16 ^{a}
Net milk production (NEL %)	1.4 ± 0.00 ^{a}	1.4 ± 0.01 ^{b}	1.3 ± 0.01 ^{c}	1.3 ± 0.00 ^{c}	1.3 ± 0.01 ^{c}
Maintain net energy (NEM %)	1.4 ± 0.00 ^{a}	1.4 ± 0.01 ^{b}	1.3 ± 0.01 ^{c}	1.3 ± 0.01 ^{c}	1.3 ± 0.01 ^{c}
Net weight gain (NEG %)	0.7 ± 0.00 ^{a}	0.6 ± 0.01 ^{b}	0.6 ± 0.01 ^{c}	0.6 ± 0.00 ^{c}	0.6 ± 0.01 ^{c}
Relative feeding value (RFV %)	155.7 ± 0.33 ^{a}	150.7 ± 1.67 ^{b}	138.3 ± 1.45 ^{c}	132.7 ± 0.88 ^{d}	139.7 ± 1.20 ^{c}
Relative forage quality (RFQ %)	141.3 ± 0.33 ^{a}	128.3 ± 2.33 ^{b}	118.0 ± 1.53 ^{c}	114.3 ± 0.67 ^{c}	118.3 ± 1.45 ^{c}
Total digestible nutrients (TDN %)	59.0 ± 0.00 ^{a}	56.3 ± 0.33 ^{b}	55.3 ± 0.33 ^{c}	55.0 ± 0.00 ^{c}	55.3 ± 0.33 ^{c}
Milk production (kg/milk) (MT DM %)	1415.0 ± 1.53 ^{a}	1324.0 ± 9.00 ^{b}	1279.7 ± 6.77 ^{c}	1284.7 ± 2.85 ^{c}	1289.3 ± 6.84 ^{c}
30 h NDF digestible rate (NDF30 %)	14.9 ± 0.08 ^{a}	14.5 ± 0.10 ^{b}	14.0 ± 0.04 ^{c}	14.0 ± 0.05 ^{c}	13.2 ± 0.13 ^{d}
48 h NDF digestible rate (NDF48 %)	16.6 ± 0.08 ^{a}	16.1 ± 0.03 ^{b}	15.9 ± 0.07 ^{bc}	15.8 ± 0.06 ^{c}	15.1 ± 0.05 ^{d}
Nonfibrous carbohydrate (NFC %)	32.4 ± 0.20 ^{a}	31.9 ± 0.04 ^{b}	31.9 ± 0.17 ^{b}	30.8 ± 0.19 ^{c}	31.2 ± 0.08 ^{c}

Different letters in the line indicate a significant difference ($p < 0.05$; Duncan's test).

In addition, the RUP, ADF and NDF contents increased with the disease severity. They increased significantly because of the change in the severity of the alfalfa *Leptosphaerulina* leaf spot from grade 2 to 3.

3.3. Leaves Nutrients

Alfalfa *Leptosphaerulina* leaf spot affected the nutrient in leaves (Table 4). The content of 18 nutrients, including Ca, P, K, ash, fat, CP, lignin, net milk production and the net energy, among others, decreased significantly ($p < 0.05$). When the alfalfa *Leptosphaerulina* leaf spot severity 4 decreased by 1.7% to 40.7%, the Ca, P, ash, lignin and relative forage quality decreased by more than 20%. These findings demonstrate that several nutritional indices varied significantly at any of the alfalfa *Leptosphaerulina* leaf spot severities(0–4), but the degree of variation in the nutrient concentration was smaller in the 0–3 scales. The difference in the Ca, K, fat, net milk production, net weight gain and relative feeding value was larger, at disease scale 3–4.

Table 4. Nutrients in alfalfa leaves with different severity levels.

Nutrient	Disease Severity Index				
	0	1	2	3	4
Calcium (Ca %)	2.7 ± 0.01 ^{a}	2.5 ± 0.00 ^{b}	2.2 ± 0.01 ^{c}	2.0 ± 0.00 ^{d}	1.6 ± 0.01 ^{e}
Phosphorus (P %)	0.4 ± 0.00 ^{a}	0.4 ± 0.00 ^{b}	0.4 ± 0.00 ^{c}	0.4 ± 0.00 ^{d}	0.3 ± 0.00 ^{e}
Potassium (K %)	2.4 ± 0.02 ^{a}	2.2 ± 0.01 ^{b}	2.1 ± 0.01 ^{c}	1.9 ± 0.02 ^{e}	2.0 ± 0.01 ^{d}
Magnesium (Mg %)	0.6 ± 0.00 ^{a}	0.6 ± 0.00 ^{b}	0.5 ± 0.00 ^{c}	0.5 ± 0.00 ^{d}	0.5 ± 0.00 ^{e}
Ash (%)	12.3 ± 0.03 ^{a}	10.5 ± 0.12 ^{b}	10.3 ± 0.01 ^{bc}	10.2 ± 0.04 ^{c}	9.5 ± 0.05 ^{d}
Fat (%)	2.4 ± 0.01 ^{a}	2.4 ± 0.01 ^{a}	2.3 ± 0.01 ^{b}	2.3 ± 0.01 ^{c}	2.1 ± 0.00 ^{d}
Crude protein (CP %)	27.4 ± 0.01 ^{a}	26. 5 ± 0.01 ^{bc}	26.5 ± 0.08 ^{b}	26.3 ± 0.07 ^{c}	25.7 ± 0.10 ^{d}
Lignin (%)	4.2 ± 0.02 ^{a}	3.8 ± 0.01 ^{b}	3.1 ± 0.01 ^{c}	2.9 ± 0.03 ^{d}	2.7 ± 0.04 ^{e}
Rumen protein (RUP %)	13.0 ± 0.04 ^{c}	12.4 ± 0.12 ^{d}	13.4 ± 0.23 ^{c}	14.1 ± 0.22 ^{b}	16.5 ± 0.26 ^{a}
Acid washing fiber (ADF %)	16.1 ± 0.03 ^{d}	16.2 ± 0.13 ^{d}	16.6 ± 0.10 ^{c}	17.2 ± 0.12 ^{b}	19.8 ± 0.10 ^{a}
Neutral detergent fiber (NDF %)	21.4 ± 0.03 ^{d}	21.6 ± 0.19 ^{d}	22.1 ± 0.12 ^{c}	22.6 ± 0.10 ^{b}	25.2 ± 0.06 ^{a}
Net milk production (NEL %)	1.7 ± 0.00 ^{a}	1.6 ± 0.00 ^{b}	1.6 ± 0.00 ^{c}	1.6 ± 0.00 ^{d}	1.5 ± 0.00 ^{e}
Maintain net energy (NEM %)	1.7 ± 0.00 ^{a}	1.7 ± 0.00 ^{b}	1.7 ± 0.00 ^{c}	1.7 ± 0.00 ^{d}	1.6 ± 0.00 ^{e}
Net weight gain (NEG %)	1.0 ± 0.00 ^{a}	1.0 ± 0.00 ^{b}	1.0 ± 0.00 ^{c}	0.9 ± 0.00 ^{d}	0.9 ± 0.00 ^{e}

Table 4. *Cont.*

Nutrient	Disease Severity Index				
	0	1	2	3	4
Relative feeding value (RFV %)	332.7 ± 0.33 [a]	328.3 ± 3.18 [a]	319.3 ± 2.03 [b]	310.3 ± 1.76 [c]	271.0 ± 1.00 [d]
Relative forage quality (RFQ %)	326.0 ± 3.46 [a]	317.0 ± 0.58 [b]	305.0 ± 1.73 [c]	289.0 ± 2.08 [d]	255.7 ± 0.67 [e]
Total digestible nutrients (TDN %)	70.0 ± 0.00 [a]	69.0 ± 0.00 [b]	68.3 ± 0.33 [c]	67.0 ± 0.00 [d]	65.0 ± 0.00 [e]
Milk production (kg/milk) (MT DM %)	1830.0 ± 3.79 [a]	1783. 7 ± 0.88 [b]	1768.3 ± 4.41 [c]	1727.7 ± 2.60 [d]	1616.0 ± 2.08 [e]
30 h NDF digestible rate (NDF30 %)	11.3 ± 0.10 [a]	10.9 ± 0.02 [b]	10.4 ± 0.05 [c]	9.9 ± 0.03 [d]	9.7 ± 0.04 [e]
48 h NDF digestible rate (NDF48 %)	12.3 ± 0.03 [a]	11.2 ± 0.05 [b]	10.4 ± 0.07 [c]	10.0 ± 0.03 [d]	10.0 ± 0.06 [d]
Nonfibrous carbohydrate (NFC %)	42.6 ± 0.06 [a]	41.0 ± 0.07 [b]	40.7 ± 0.09 [c]	39.6 ± 0.02 [d]	34.3 ± 0.07 [e]

Different letters in the line indicate a significant difference ($p < 0.05$; Duncan's test).

In addition, the RUP, ADF and NDF contents increased gradually with the alfalfa *Leptosphaerulina* leaf spot severity. The increases ranged from 17.8% to 26.9%. However, the RUP content was significantly lower in grade 1 disease leaves than in healthy leaves ($p < 0.05$).

3.4. The Relationship between Disease Severity and Nutritional Content

We found a negative correlation between the alfalfa *Leptosphaerulina* leaf spot severity and the nutritional index, except for RUP, ADF and NDF. The alfalfa *Leptosphaerulina* leaf spot severity showed a strong negative correlation with the Ca, CP, NDF and 30h NDF digestible rate ($p < 0.01$) and a moderate correlation with Mg, fat, lignin and 48h NDF digestible rate (Table 5).

Table 5. Correlation between the severity of alfalfa *Leptosphaerulina* leaf spot and alfalfa plant nutrient content.

Nutrient	Pearson	Decision Coefficient	Regression Equation
Calcium (Ca %)	−0.986 **	$R^2 = 0.970$	y = −0.115x + 1.645
Phosphorus (P %)	−0.866	$R^2 = 0.677$	y = −0.012x + 0.348
Potassium (K %)	−0.747	$R^2 = 0.586$	y = −0.028x + 2.386
Magnesium (Mg %)	−0.933 *	$R^2 = 0.916$	y = −0.016x + 0.337
Ash(%)	−0.247	$R^2 = 0.061$	y = −0.073x + 7.952
Fat(%)	−0.906 *	$R^2 = 0.821$	y = −0.077x + 1.738
Crude protein(CP %)	−0.976 **	$R^2 = 0.952$	y = −0.883x + 21.160
Lignin(%)	−0.939 *	$R^2 = 0.880$	y = −0.156x + 6.691
Rumen protein(RUP %)	0.876	$R^2 = 0.769$	y = 0.618x + 17.850
Acid washing fiber(ADF %)	0.925 *	$R^2 = 0.855$	y = 0.882x + 30.270
Neutral detergent fiber(NDF %)	0.960 **	$R^2 = 0.921$	y = 1.177x + 39.220
Net milk production(NEL %)	−0.800	$R^2 = 0.650$	y = −0.018x + 1.379
Maintain net energy(NEM %)	−0.839	$R^2 = 0.690$	y = −0.023x + 1.391
Net weight gain(NEG %)	−0.839	$R^2 = 0.673$	y = −0.023x + 0.670
Relative feeding value(RFV %)	−0.835	$R^2 = 0.698$	y = −5.000x + 153.400
Relative forage quality(RFQ %)	−0.865	$R^2 = 0.748$	y = −6.000x + 136.000
Total digestible nutrients(TDN %)	−0.834	$R^2 = 0.695$	y = −0.866x + 57.930
Milk production(kg/milk) (MT DM %)	−0.811	$R^2 = 0.657$	y = −29.060x + 1376.000
30 h NDF digestible rate(NDF30 %)	−0.976 **	$R^2 = 0.950$	y = −0.386x + 14.890
48 h NDF digestible rate(NDF48 %)	−0.951 *	$R^2 = 0.903$	y = −0.320x + 16.550
Nonfibrous carbohydrate(NFC %)	−0.877	$R^2 = 0.769$	y = −0.371x + 32.380

"Pearson" means Pearson product–moment correlation coefficient, the correlation coefficient for *Leptosphaerulina* leaf spot severity and nutritional contents in the plant. "*" indicates significance at 5% ($p < 0.05$; Duncan's test). "**" indicates very significance at 1% ($p < 0.01$; Duncan's test).

The contents of 21 nutrients strongly or moderately correlated with the severity of alfalfa *Leptosphaerulina* leaf spot, except for RUP, ADF and net milk production (Table 6).

Table 6. Correlation between the severity of alfalfa *Leptosphaerulina* leaf spot and alfalfa leaf nutrient content.

Nutrient	Pearson	Decision Coefficient	Regression Equation
Calcium (Ca %)	−0.987 **	R^2 = 0.972	y = −0.273x + 2.728
Phosphorus (P %)	−0.926 *	R^2 = 0.859	y = −0.026x + 0.422
Potassium (K %)	−0.913 *	R^2 = 0.834	y = −0.118x + 2.349
Magnesium (Mg %)	−0.994 **	R^2 = 0.986	y = −0.030x + 0.572
Ash(%)	−0.899 *	R^2 = 0.806	y = −0.583x + 11.710
Fat(%)	−0.895 *	R^2 = 0.802	y = −0.078x + 2.437
Crude protein(CP %)	−0.925 *	R^2 = 0.853	y = −0.354x + 27.170
Lignin(%)	−0.969 **	R^2 = 0.937	y = −0.376x + 4.104
Rumen protein(RUP %)	0.895	R^2 = 0.738	y = 0.857x + 12.170
Acid washing fiber(ADF %)	0.861	R^2 = 0.741	y = 0.821x + 15.520
Neutral detergent fiber(NDF %)	0.891 *	R^2 = 0.793	y = 0.876x + 20.830
Net milk production(NEL %)	−0.874	R^2 = 0.799	y = −0.030x + 1.670
Maintain net energy(NEM %)	−0.940 *	R^2 = 0.916	y = −0.034x + 1.750
Net weight gain(NEG %)	−0.956 *	R^2 = 0.901	y = −0.033x + 1.026
Relative feeding value(RFV %)	−0.907 *	R^2 = 0.821	y = −14.130x + 340.600
Relative forage quality(RFQ %)	−0.963 **	R^2 = 0.928	y = −16.860x + 332.200
Total digestible nutrients(TDN %)	−0.979 **	R^2 = 0.958	y = −1.200x + 70.260
Milk production(kg/milk) (MT DM %)	−0.945 *	R^2 = 0.893	y = −48.400x + 1841.000
30 h NDF digestible rate(NDF30 %)	−0.993 **	R^2 = 0.986	y = −0.431x + 11.290
48 h NDF digestible rate(NDF48 %)	−0.947 *	R^2 = 0.894	y = −0.580x + 11.930
Nonfibrous carbohydrate(NFC %)	−0.900 *	R^2 = 0.810	y = −1.801x + 43.210

"Pearson" means Pearson product–moment correlation coefficient, the correlation coefficient for *Leptosphaerulina* leaf spot severity and nutritional contents in the plant. "*" indicates significance at 5% ($p < 0.05$; Duncan's test). "**" indicates very significance at 1% ($p < 0.01$; Duncan's test).

4. Discussion

4.1. Occurrence of Leptosphaerulina Leaf Spot

Previous studies showed that from June to September, alfalfa anthracnose [23], alfalfa downy mildew, alfalfa spring black stem, alfalfa *Stemphyllium* leaf spot and alfalfa rust occurred in Inner Mongolia, and their incidences were 34%, 26%, 23%, 22% and 28%, respectively [30]. In this study, the incidence of *Leptosphaerulina* leaf spot is about 60%, which is much higher than other diseases. In addition, this disease also occurred in alfalfa Heilongjiang province in 2015 [20]. Therefore, we must pay attention to prevention and control of this disease, especially in cultivation areas.

4.2. Quality of Alfalfa with Leptosphaerulina sp.

The quality of hay directly affects the feed intake and production in livestock, and nutrient content and digestibility of feeds can indicate the quality of hay [34,35]. The quality standard of alfalfa hay is mainly based on the national standard of alfalfa hay guidelines set by the United States market. Parameters analyzed include CP, NDF, ADF and relative feeding value (RFV) among others [35,36]. In this study, the CP content of healthy alfalfa and alfalfa with grade 1 and 2 alfalfa *Leptosphaerulina* leaf spot was higher than 19%. In addition, the alfalfa belonged to super-grade grass. For grade 3 and grade 4 disease, the CP content was 17–19%, and the fodder belonged to grade 1 grass. The ADF and NDF content in both healthy and diseased alfalfa plants was less than 31% and less than 40%, respectively, to which alfalfa belonged to the super-grade grass category. RFV is an important forage quality index [37]. In this study, the RFV of diseased alfalfa plants and leaves was reduced by 3% to 15% and 1% to 18%, respectively. The RFV of alfalfa plants in this research was 133–156%, and thus was classified into the first-grade grass category. In conclusion, based on different nutritional indicators, alfalfa grass with alfalfa *Leptosphaerulina* leaf spot still belonged to the grade 1 grass or super-grade grass category, consistent with export alfalfa hay quality. Alfalfa is an important livestock fodder in China [8]. Agricultural industry standards (NY/T1170-2006) for alfalfa hay quality grading based on CP, NDF and ash

content have been formulated in China [35].In this study, according to the protein content, the disease severity of the sampled alfalfa *Leptosphaerulina* leaf spot was grade 1, grade 2 and grade 3, whereas the corresponding alfalfa hay belonged to grade 1, grade 2 and grade 3, respectively. Therefore, alfalfa *Leptosphaerulina* leaf spot may affect alfalfa pricing in China, and livestock growth, especially for severity, is grade 4. Previous studies have shown that diseased forage grass has poor nutritional quality and is not suitable for the healthy growth of livestock [38].

4.3. Nutrition of Alfalfa with Leptosphaerulina sp.

Alfalfa is the primary livestock feed, owing to its considerable CP [39]. However, high crude fiber negatively affects the digestibility of CP [40]. Alfalfa anthracnose, alfalfa brown spot and alfalfa *Verticillium* wilt decreased the CP of alfalfa by 17%, 16% and 41%, respectively [30,32,41]. Here, the CP of alfalfa with *Leptosphaerulina* sp. decreased by about 17%, which was similar to that of alfalfa anthracnose and alfalfa brown spot, but lower than that of alfalfa *Verticillium* wilt. ADF and NDF increased by 21% and 16% in alfalfa anthracnose [30] and increased by 71% and 40% in alfalfa brown spot, respectively [32]. In this research, the ADF and NDF increased by about 12% in plant, but 18% and 23% in leaves, respectively, and this result is consistent with that of alfalfa anthracnose. Lignin is a complex phenolic polymer and the second most abundant component of secondary plant cell walls [42,43]. Lignin is a major factor limiting the digestibility of dry forage [44,45]. Numerous studies have reported a strong inverse relationship between lignin concentration and forage digestibility [46], while the relationship between lignin and digestion in diseased alfalfa plants has barely been reported. We found that even though the lignin content in the alfalfa leaves decreased with an increase in disease severity, the lowest content was higher than that of the general level in the alfalfa plants. It was proven that the content of lignin in alfalfa could be decreased by alfalfa *Leptosphaerulina* leaf spot. Most previous studies have shown that diseases affect the photosynthesis of alfalfa [32,47,48]. Mg plays an important role in photosynthesis, primarily by participating in chlorophyll synthesis. Mg is the central atom of chlorophyll and the chlorophyll porphyrin ring [49]. In this study, the Mg content in alfalfa decreased significantly after *Leptosphaerulina* infection, particularly in the leaves. Therefore, it can be hypothesized that *Leptosphaerulina* infection decreases photosynthesis in alfalfa by decreasing the Mg in leaves, which then affects chlorophyll synthesis. P, K and Ca are essential nutrients in plants [47]. In this study, the content of Ca and P in alfalfa infected by *Leptosphaerulina* leaf spot decreased by more than 25%, and when the severity of alfalfa anthracnose with *Colletotrichum american-borealis* was grade 4, Ca content decreased by 30% [30]. The results of the two studies were consistent. Most of the proteins in feeds are degraded in the rumen [50]. In this study, the results showed that the RUP content positively correlated with the severity of alfalfa *Leptosphaerulina* leaf spot. The increase in rumen protein over activates fermentation by anaerobic microorganisms in the rumen. The resultant flatulence of livestock reduces protein absorption in the small intestine, affecting the nutrients available to the livestock [50].

In addition, the net milk production (NEL), maintenance net energy (NEM), net weight gain (NEG), total digestible nutrients (TDN), milk production (MT) and nonfibrous carbohydrate (NFC) in this research were decreased when they were infected by *Leptosphaerulina* sp. Therefore, when alfalfa *Leptosphaerulina* disease occurs, it will affect the performance of livestock by affecting the quality of herbage. Most of the studies only measured and studied the main nutritional indexes of alfalfa diseases, such as CP, ADF and NDF [29–32], while this study comprehensively revealed the effects of alfalfa leaf spot disease on 21 conventional nutritional indexes. These results will guide the control of the disease and alfalfa production.

5. Conclusions

Alfalfa leaf spot disease has been prevalent in Inner Mongolia for a long time, and its incidence was much higher than other alfalfa leaf diseases. We suspect that this disease

will continue to be prevalent in the future. In addition, according to the quality standard of alfalfa hay, the occurrence of alfalfa *Leptosphaerulina* leaf spot could affect the quality classification and then affect the economic benefits and feeding value on alfalfa. Therefore, we must pay attention to control this disease in future work, such as with resistant cultivars.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12091465/s1. Table S1: Fertilizer kinds and dosage in the field. Table S2: Climate information. Table S3: Survey method and investigator. Table S4: Reference methods and database for the nutriment measurement.

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