

Article

Integrating Disease Severity and Growth Retention Reveals Distinct Resistance Responses to Spring Black Stem and Leaf Spot in Alfalfa

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Abstract

Alfalfa spring black stem and leaf spot (ASBS), caused by *Ascochyta medicaginicola*, threatens alfalfa yield, forage quality, and stand persistence. Conventional resistance assessments of alfalfa cultivars generally rely on disease incidence, disease severity, or the percentage of healthy plants. Although these measures describe symptom occurrence and development, they do not capture biomass retention or root growth after infection. In this study, 12 alfalfa cultivars were spray-inoculated with *A. medicaginicola* under greenhouse conditions and evaluated 14 days after inoculation. Disease incidence was positively correlated with the disease severity index and negatively correlated with relative fresh weight, relative dry weight, and relative root length. We integrated these five indicators using a membership-function-based standard-deviation coefficient weighting method to derive a comprehensive response score (D value). D values ranged from 0.0340 to 0.9611. Magnum 2 had the highest D value, followed by Gannong No. 3, Adrenalin, and Dryland, whereas Thunder, Zhongmu No. 1, and Aohan had the lowest values. Compared with the National Alfalfa and Forage Alliance classification based on the percentage of healthy plants, the D-value ranking provided additional discrimination among cultivars within the same resistance class under the present greenhouse conditions. The first two principal components explained 89.24% of the total variation and separated resistant, intermediate, and susceptible response types. Thus, combining disease suppression with growth retention yielded a complementary quantitative description of cultivar performance under the conditions tested.

Keywords: alfalfa; *Ascochyta medicaginicola*; disease resistance; growth retention; membership function; spring black stem and leaf spot



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1. Introduction

Alfalfa (*Medicago sativa* L.) is a globally important perennial legume forage crop, valued for its high nutritional quality, broad ecological adaptability, and contribution to ruminant livestock production [1,2]. Persistent, productive stands support forage supply, soil improvement, and sustainable agriculture. Alfalfa production, however, is frequently limited by fungal diseases, particularly foliar and stem diseases that reduce photosynthetic capacity, accelerate defoliation, and ultimately diminish forage yield and quality [3–7]. Among these, alfalfa spring black stem and leaf spot (ASBS), caused by *Ascochyta medicaginicola*, is a major disease of alfalfa in many production regions [8].

ASBS was historically attributed to *Phoma medicaginis*, which has recently been reclassified as *Ascochyta medicaginicola* on the basis of morphological characters and multilocus phylogenetic evidence [9,10]. The disease causes black stem lesions, leaf spots, premature defoliation, seedling blight, crown rot, and root rot, thereby impairing aboveground biomass accumulation and stand persistence [11–17]. Its occurrence and development are strongly influenced by environmental conditions, particularly temperature and humidity. Warm, humid conditions in spring and autumn favor conidial germination, infection, disease progression, and pathogen dispersal [8,18–20]. ASBS can substantially reduce hay yield and forage quality, and greater disease severity has been associated with marked yield losses [11]. Infection by *A. medicaginicola* may also reduce crude protein content and promote the accumulation of coumestrol, a phytoestrogen that can impair reproductive performance in grazing animals [21,22]. Effective ASBS management is therefore important for maintaining alfalfa yield and quality and sustaining forage–livestock systems.

The deployment of resistant or tolerant cultivars is widely regarded as an economical, environmentally sound, and effective strategy for managing alfalfa diseases [3,23]. Reliable resistance assessment is therefore essential for germplasm utilization, cultivar deployment, and disease management. Alfalfa screening systems commonly use disease incidence, disease severity index, or the percentage of healthy plants. Each measure captures a distinct aspect of disease development, and its suitability depends on the purpose of the screening program. Disease incidence and the percentage of healthy plants primarily indicate whether symptoms occur, whereas disease severity reflects lesion development and disease progression. For example, the National Alfalfa and Forage Alliance (NAFA) classification assigns resistance levels according to the percentage of healthy plants and provides a simple, interpretable benchmark for large-scale screening [24]. None of these variables alone, however, quantifies post-infection biomass retention, root growth, or the capacity to sustain forage production. An integrated assessment can provide complementary information when post-infection growth performance is also of interest.

Cultivar performance under ASBS pressure is multidimensional, encompassing both restriction of disease development and maintenance of growth after infection. Previous studies have evaluated alfalfa resistance to ASBS or related diseases using disease incidence and disease severity index under field or controlled conditions [25,26]. Although these studies revealed cultivar differences, disease and agronomic growth traits have seldom been integrated within a single quantitative framework. In plant stress-resistance research, membership-function analysis combined with objective weighting has been used to integrate indicators that differ in units and direction of response [27,28]. This is one of several possible integration methods and is not presumed to be universally superior to other multivariate or multi-criteria approaches. To our knowledge, it has not been applied systematically to combine disease and relative growth responses to alfalfa spring black stem and leaf spot caused by *A. medicaginicola*.

In this study, 12 Chinese and introduced alfalfa cultivars were inoculated with *A. medicaginicola* under controlled greenhouse conditions. Disease incidence, disease severity index, plant height, root length, fresh weight per plant, and dry weight per plant were measured after inoculation. We hypothesized that combining disease development with post-infection growth retention relative to non-inoculated controls would provide a complementary description of cultivar responses under the conditions evaluated. The membership-function-based standard-deviation coefficient method was selected because it provides transparent, reproducible normalization and data-derived weighting for indicators that differ in scale and direction. The objectives were to (1) quantify differences in ASBS response among 12 alfalfa cultivars under controlled inoculation; (2) identify disease and growth-related indicators associated with cultivar response; (3) apply a standard-

deviation coefficient weighting method based on membership functions for integrated evaluation; and (4) compare the integrated results with conventional classification based on the percentage of healthy plants to identify promising germplasm for further validation.

2. Materials and Methods

2.1. Plant Materials and Fungal Isolate

Twelve alfalfa (*Medicago sativa* L.) cultivars, including six cultivars bred in China and six introduced cultivars from other countries (Table 1), were used in this study. Seeds were obtained from the institutions or companies listed in Table 1.

Table 1. Alfalfa cultivars and seed sources.

No.	Cultivar	Country	Seed Source
1	Magnum 2	United States	Beijing Clover Company
2	Gannong No. 3	China	Gansu Academy of Agricultural Sciences
3	Dryland	United States	Beijing Clover Company
4	Adrenalin	Canada	Xinjiang Agricultural University
5	Thunder	United States	Beijing Clover Company
6	Zhongmu No. 1	China	Chinese Academy of Agricultural Sciences
7	Aohan	China	Inner Mongolia Agricultural University
8	Salt-tolerant star	United States	Beijing Clover Company
9	Xinjiangdaye	China	Xinjiang Agricultural University
10	Gannong No. 6	China	Gansu Academy of Agricultural Sciences
11	Longdong	China	Gansu Agricultural University
12	Vision	United States	Beijing Clover Company

Fungal isolate CS007 was recovered from symptomatic alfalfa leaves collected in an ASBS-affected field in Lintao County, Dingxi City, Gansu Province, China. It was selected for greenhouse resistance assessment because it originated in Gansu Province and consistently produced abundant pycnidia and conidia on potato dextrose agar (PDA), enabling preparation of a uniform conidial suspension for inoculation.

Isolate CS007 was identified as *Ascochyta medicaginicola* based on its morphological characteristics and internal transcribed spacer (ITS) sequence. Morphological identification was based primarily on colony morphology, pycnidial formation, and conidial characteristics, with reference to published descriptions of *Phoma medicaginis* and *A. medicaginicola*. For molecular identification, the ITS region was amplified with primers ITS1 and ITS4 and sequenced; the resulting sequence was then compared with sequences available in public databases. The isolate is maintained in the Biocontrol Engineering Laboratory of Crop Diseases and Pests of Gansu Province, Gansu Agricultural University and is available upon reasonable request.

2.2. Preparation of the Conidial Suspension

After 14 days of incubation, mycelia and pycnidia were gently scraped from the surfaces of PDA cultures with a sterile blade. Sterile distilled water was added to each Petri dish, and the colony surface was rubbed gently to release conidia from the pycnidia. The suspension was filtered through two layers of sterile gauze to remove mycelial fragments. Conidial concentration was determined with a hemocytometer and adjusted to 1×10^6 conidia mL⁻¹ with sterile distilled water.

2.3. Experimental Design and Inoculation

Healthy seeds of each cultivar were selected and rinsed three times with sterile distilled water. They were surface-disinfected with 75% ethanol for 30 s and 1% sodium hypochlorite

for 1 min, rinsed three additional times with sterile distilled water, dried on sterile filter paper, and sown in 15 cm-diameter plastic pots containing sterilized soil. Five seeds were sown per pot.

The experiment comprised two treatments: inoculated plants and non-inoculated controls. For each cultivar and treatment, five biological replicates were established, with one pot serving as one replicate. Greenhouse environmental setpoints were 25 °C and 65% relative humidity, with a 12 h light/12 h dark photoperiod. The pots were not periodically re-randomized within the greenhouse during the experimental period. Eight weeks after seedling emergence, plants of all cultivars had reached comparable vegetative growth stages under the same greenhouse conditions. Plants assigned to the inoculated treatment were sprayed simultaneously with the conidial suspension until runoff. To maintain inoculum uniformity, the suspension was mixed thoroughly before and during application, and the same sprayer and application procedure were used for all inoculated plants. Control plants were sprayed with sterile distilled water using the same procedure. After inoculation, plants were covered with black plastic sheeting for 24 h to maintain near-saturated humidity (approximately 100% relative humidity) and promote infection. The covers were then removed, and plants were returned to routine greenhouse conditions described above. Plants were watered at 3-day intervals, and all disease and growth variables were measured 14 days after inoculation.

2.4. Disease Assessment

Symptoms on leaves and stems were recorded 14 days after inoculation. Disease severity was rated on a five-grade scale adapted, with minor modifications, from Zhang et al. [25]: grade 0, no visible lesions; grade 1, lesions covering 1–5% of the leaf or stem surface; grade 2, lesions covering 6–20%; grade 3, lesions covering 21–50%; and grade 4, lesions covering more than 50%. All symptom assessments were performed by a single evaluator. Disease incidence and the disease severity index were calculated as follows:

$$\text{Disease incidence (\%)} = \frac{\text{number of diseased plants}}{\text{total number of plants}} \times 100\%$$

$$\text{Disease severity index} = \frac{\sum(\text{number of plants at each disease grade} \times \text{corresponding grade value})}{\text{total number of plants investigated} \times \text{highest grade value}} \times 100$$

Following the NAFA criteria [24], each cultivar was assigned a preliminary resistance class according to the percentage of healthy plants: highly resistant (HR), >50%; resistant (R), 31–50%; moderately resistant (MR), 15–30%; low resistance (LR), 6–14%; and susceptible (S), 0–5%. This established classification was retained as a readily interpretable benchmark for conventional alfalfa resistance screening, whereas the comprehensive index was used to integrate disease responses with relative growth performance.

2.5. Measurement of Growth Traits and Calculation of Relative Growth Performance

At 14 days after inoculation, plant height, root length, fresh weight per plant, and dry weight per plant were measured in both inoculated plants and non-inoculated controls. Plant height and root length were measured with a ruler. Fresh weight per plant was recorded immediately after harvest using an electronic balance. The harvested plants were then dried at 80 °C to constant weight, cooled to room temperature, and weighed to determine dry weight per plant.

To account for inherent growth differences among cultivars, each relative growth trait was expressed as the value for inoculated plants divided by the value for the corresponding

non-inoculated controls and multiplied by 100. Relative growth performance was calculated as follows:

$$\text{Relative growth performance (\%)} = \frac{\text{Trait inoculated}}{\text{Trait control}} \times 100\%$$

2.6. Indicator Selection for Comprehensive Resistance Evaluation

Pearson correlations were calculated among disease incidence, disease severity index, and relative growth traits. Indicators significantly associated with disease response or post-inoculation growth retention were selected for the comprehensive evaluation. Disease incidence and disease severity index were treated as negative indicators, whereas the relative growth traits were treated as positive indicators.

2.7. Membership-Function-Based Comprehensive Evaluation

To integrate disease development and post-infection growth retention, indicators with different units and directions of response were converted to comparable, dimensionless values using membership functions. The responses of the 12 alfalfa cultivars to ASBS were then evaluated using the standard-deviation coefficient weighting method. For positive indicators, the membership-function value was calculated as follows:

$$\mu(X_j) = \frac{X_j - X_{\min}}{X_{\max} - X_{\min}}$$

For negative indicators, the inverse membership function was used:

$$\mu(X_j) = 1 - \frac{X_j - X_{\min}}{X_{\max} - X_{\min}}$$

where $\mu(X_j)$ denotes the membership-function value of the j th indicator, X_j is the observed value of that indicator, and $X_{\min}$ and $X_{\max}$ are its minimum and maximum values, respectively.

The standard deviation coefficient V_j was calculated as follows:

$$V_j = \frac{\sqrt{\sum_{i=1}^n (X_{ij} - \bar{X}_j)^2}}{\bar{X}_j}$$

where V_j denotes the standard-deviation coefficient of the j th indicator, $\bar{X}_j$ is the mean value of that indicator, and X_{ij} is the membership-function value of the j th indicator for the i th cultivar. A larger V_j indicates greater relative differentiation among cultivars in the present dataset.

The weight of each indicator was calculated as follows:

$$W_j = \frac{V_j}{\sum_{j=1}^m V_j}$$

The comprehensive response score, namely the D value, was calculated as follows:

$$D = \sum_{j=1}^n [\mu(X_j) \times W_j]$$

where D denotes the comprehensive response score of the i th cultivar. Higher D values indicate stronger integrated resistance responses to ASBS, whereas lower values indicate weaker responses.

2.8. Comparison Between Conventional Classification and Integrated Evaluation

To determine whether the integrated D value provided information beyond the conventional disease-incidence-based classification, the NAFA resistance class of each cultivar was compared with its D-value ranking. Cultivars whose NAFA classes and D-value rankings differed were examined further to assess whether post-infection growth retention contributed to the discrepancy.

2.9. Principal Component Analysis

Principal component analysis (PCA) was conducted using disease incidence, disease severity index, relative fresh weight, relative dry weight, and relative root length. Variables were standardized before analysis to remove scale effects. The first two principal components were used to visualize cultivar distribution, and loadings were examined to identify the traits contributing most strongly to differentiation among cultivars.

2.10. Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY, USA) and Origin 2024b (OriginLab Corporation, Northampton, MA, USA). Before one-way analysis of variance (ANOVA), the normality of the residuals and homogeneity of variances were assessed using the Shapiro–Wilk test and Levene’s test, respectively. The residuals of all variables included in the ANOVA met the assumptions of normality and homoscedasticity. Differences among cultivars were tested by one-way ANOVA, followed by Duncan’s multiple range test for mean separation. Pearson correlation analysis was performed to examine the relationships between disease indicators and relative growth traits. Principal component analysis (PCA) was conducted using standardized values of the selected indicators. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Symptom Development and Disease Responses After Inoculation

At 14 days after inoculation with *A. medicaginicola*, susceptible cultivars such as Thunder showed typical symptoms of spring black stem and leaf spot. The adaxial leaf surfaces were chlorotic and bore numerous small, circular to subcircular black spots. Premature leaf abscission also occurred, but stems showed no obvious symptoms at this assessment time. Non-inoculated control plants remained symptomless (Figure 1). Colonies reisolated from symptomatic plants were morphologically consistent with the original CS007 isolate, whereas no colonies resembling *A. medicaginicola* were recovered from non-inoculated controls.

Disease incidence differed significantly among the 12 cultivars and ranged from 6.67% to 100.00% (Figure 2A). Thunder and Zhongmu No. 1 had the highest incidence (100.00%), followed by Aohan (93.33%), and were significantly more susceptible than most other cultivars. Magnum 2 had the lowest incidence (6.67%), followed by Gannong No. 3 (20.00%), Dryland (26.67%), and Adrenalin (26.67%).

The disease severity index also differed significantly among cultivars, ranging from 1.67 to 64.44 (Figure 2B). Thunder had the highest value (64.44), followed by Zhongmu No. 1 (48.33). By contrast, the indices for Magnum 2, Gannong No. 3, Dryland, and Adrenalin were all below 10.00.

Based on the NAFA classification using the percentage of healthy plants, Magnum 2, Gannong No. 3, Dryland, and Adrenalin were highly resistant. Aohan was assigned to the low-resistance class, whereas Thunder and Zhongmu No. 1 were susceptible. These results indicate substantial variation in ASBS response among the cultivars tested.

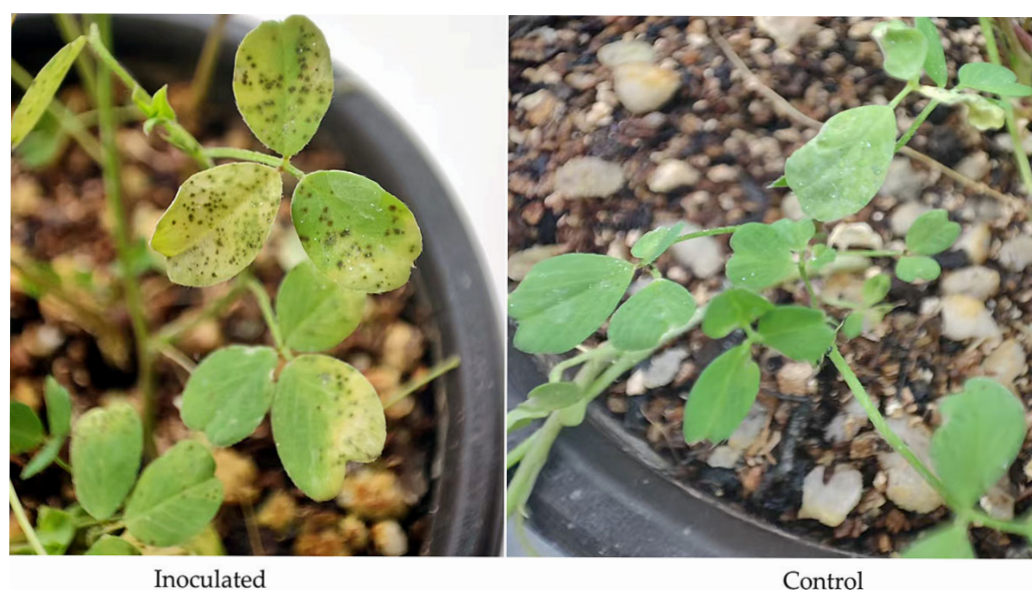


Figure 1. Symptom development on leaves of inoculated Thunder plants and non-inoculated controls 14 days after inoculation.

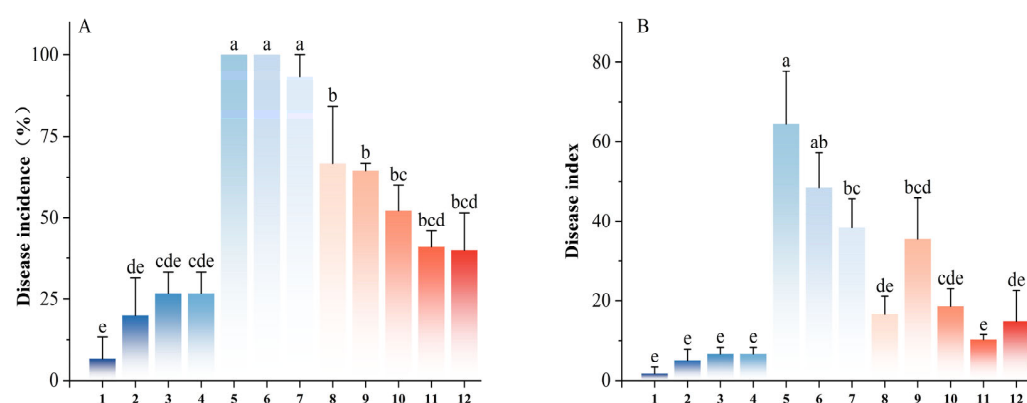


Figure 2. Disease incidence (A) and disease severity index (B) among the tested alfalfa cultivars. Note: Numbers on the x-axis correspond to the cultivar numbers in Table 1. Different lowercase letters indicate significant differences among cultivars at $p < 0.05$ according to one-way ANOVA followed by Duncan's multiple range test.

3.2. Relative Growth Performance of Alfalfa Cultivars After Inoculation

Relative growth traits differed significantly among the 12 cultivars after inoculation with *A. medicaginicola* (Table 2). Relative fresh weight ranged from 21.70% to 86.11%; Adrenalin and Magnum 2 retained the highest proportions of fresh weight, whereas Zhongmu No. 1, Thunder, and Aohan retained comparatively little fresh biomass. Relative dry weight ranged from 42.93% to 93.22%. Vision, Gannong No. 6, and Longdong retained the most dry biomass, whereas Thunder retained the least.

Relative plant height ranged from 69.75% to 91.79%, with the highest value in Vision and the lowest in Thunder. Differences in relative plant height were smaller than those in biomass and root traits. Relative root length ranged from 52.96% to 92.08%; Gannong No. 6 had the highest value, followed by Magnum 2 and Adrenalin, whereas Zhongmu No. 1 and Thunder had the lowest values. Overall, Gannong No. 6, Magnum 2, Adrenalin, Vision, and Longdong retained relatively strong growth after infection, whereas growth was markedly suppressed in Zhongmu No. 1 and Thunder.

Table 2. Relative growth performance of 12 alfalfa cultivars after inoculation with *A. medicaginicola*.

No.	Cultivar	Relative Fresh Weight (%)	Relative Dry Weight (%)	Relative Plant Height (%)	Relative Root Length (%)
1	Magnum 2	83.73 ± 5.27 a	65.49 ± 6.83 ab	79.97 ± 7.49 ab	85.29 ± 2.90 ab
2	Gannong No. 3	75.74 ± 4.53 ab	83.68 ± 28.51 ab	75.55 ± 1.23 ab	76.10 ± 11.59 abcd
3	Dryland	65.48 ± 20.41 abc	80.27 ± 5.68 ab	73.35 ± 1.61 ab	78.66 ± 7.15 abcd
4	Adrenalin	86.11 ± 4.19 a	82.30 ± 14.29 ab	90.13 ± 2.72 ab	84.29 ± 1.76 abc
5	Thunder	29.24 ± 2.05 cd	42.93 ± 8.82 b	69.75 ± 1.89 b	58.15 ± 4.93 de
6	Zhongmu No. 1	21.70 ± 5.47 d	61.43 ± 13.44 ab	74.99 ± 8.59 ab	52.96 ± 3.53 e
7	Aohan	37.57 ± 9.42 bcd	61.73 ± 3.39 ab	89.30 ± 0.72 ab	69.12 ± 11.11 bcde
8	Salt-tolerant star	74.69 ± 15.80 ab	70.14 ± 4.86 ab	82.18 ± 8.09 ab	62.77 ± 1.75 cde
9	Xinjiangdaye	61.86 ± 15.57 abc	67.00 ± 3.49 ab	79.75 ± 8.59 ab	82.42 ± 6.47 abc
10	Gannong No. 6	66.82 ± 1.50 abc	92.62 ± 16.96 a	78.40 ± 10.57 ab	92.08 ± 5.66 a
11	Longdong	56.12 ± 18.39 abcd	89.73 ± 2.62 a	83.62 ± 6.57 ab	73.07 ± 2.43 abcde
12	Vision	65.92 ± 15.16 abc	93.22 ± 12.36 a	91.79 ± 4.59 a	77.63 ± 9.31 abcd

Note: Different lowercase letters within the same column indicate significant differences at $p < 0.05$.

3.3. Correlation Analysis Between Disease Response and Relative Growth Performance

Pearson correlation analysis showed significant associations between disease indicators and relative growth traits after inoculation with *A. medicaginicola* (Figure 3). Disease incidence was positively correlated with the disease severity index ($p < 0.001$). Relative fresh weight was negatively correlated with both disease incidence and disease severity index ($p < 0.001$). Relative dry weight was also negatively correlated with disease incidence ($p < 0.01$) and disease severity index ($p < 0.05$). Relative root length was negatively correlated with disease incidence ($p < 0.01$) and disease severity index ($p < 0.001$). Relative plant height, however, was not significantly correlated with either disease indicator.

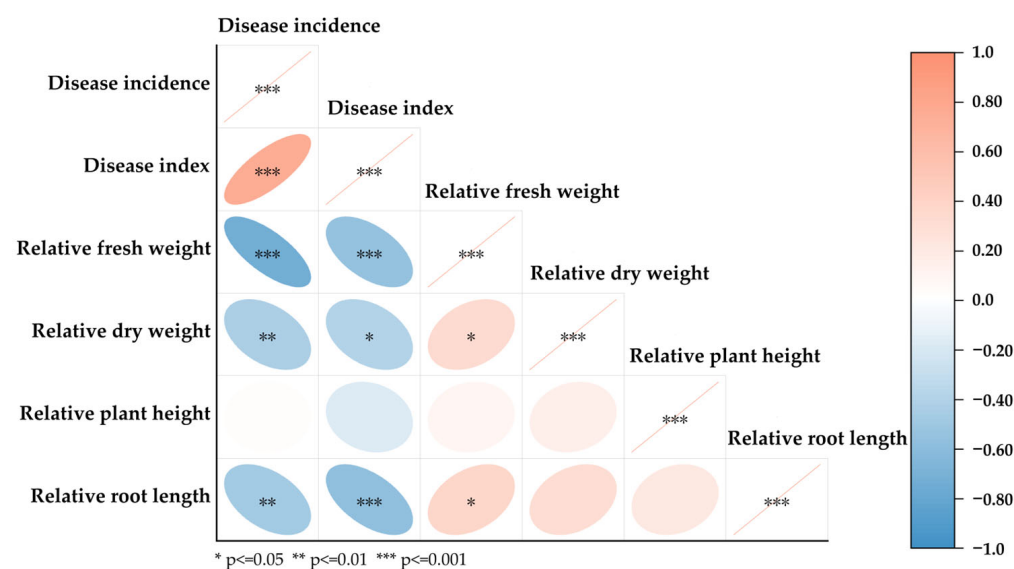


Figure 3. Correlation heatmap for disease indicators and relative growth traits after inoculation. Note: Color intensity is proportional to the absolute magnitude of the Pearson correlation coefficient. Brown denotes positive correlations, and blue denotes negative correlations.

Among the relative growth traits, relative fresh weight was positively correlated with relative dry weight and relative root length ($p < 0.05$); all other pairwise correlations were nonsignificant. Disease incidence, disease severity index, relative fresh weight, relative dry weight, and relative root length were therefore retained for the comprehensive evaluation, whereas relative plant height was excluded.

3.4. Comprehensive D-Value Ranking

Disease incidence, disease severity index, relative fresh weight, relative dry weight, and relative root length were used to calculate a comprehensive D value for each cultivar. Values ranged from 0.0340 to 0.9611 (Table 3), indicating substantial variation in integrated responses among the cultivars.

Table 3. Membership function values and comprehensive D values of the 12 alfalfa cultivars.

Cultivar	Membership-Function Value					D Value	Rank
	μ (1)	μ (2)	μ (3)	μ (4)	μ (5)		
Magnum 2	1.000	1.000	0.963	0.449	0.826	0.9611	1
Gannong No. 3	0.857	0.947	0.839	0.810	0.592	0.8548	2
Dryland	0.786	0.920	0.680	0.742	0.657	0.7970	4
Adrenalin	0.786	0.920	1.000	0.783	0.801	0.8410	3
Thunder	0.000	0.000	0.117	0.000	0.133	0.0340	12
Zhongmu No. 1	0.000	0.257	0.000	0.368	0.000	0.1484	11
Aohan	0.071	0.416	0.246	0.374	0.413	0.3101	10
Salt-tolerant star	0.357	0.761	0.823	0.541	0.251	0.5727	8
Xinjiangdaye	0.381	0.460	0.623	0.478	0.753	0.4944	9
Gannong No. 6	0.512	0.730	0.701	0.988	1.000	0.7068	7
Longdong	0.631	0.863	0.534	0.931	0.514	0.7075	6
Vision	0.643	0.788	0.687	1.000	0.631	0.7241	5

Note: μ (1), μ (2), μ (3), μ (4), and μ (5) denote the membership-function values for disease incidence, disease severity index, relative fresh weight, relative dry weight, and relative root length, respectively.

Magnum 2 had the highest D value (0.9611), followed by Gannong No. 3 (0.8548), Adrenalin (0.8410), and Dryland (0.7970). Vision (0.7241), Longdong (0.7075), and Gannong No. 6 (0.7068) also had comparatively high values, whereas Salt-tolerant star (0.5727) and Xinjiangdaye (0.4944) were intermediate. Aohan (0.3101), Zhongmu No. 1 (0.1484), and Thunder (0.0340) had the lowest D values, indicating weaker integrated responses to *A. medicaginicola*.

3.5. Comparison Between NAFA Classification and Integrated D-Value Evaluation

NAFA classes based on the percentage of healthy plants were compared with the integrated D-value ranking (Table 4). The two approaches agreed for the cultivars at the extremes of the response range. Magnum 2 was highly resistant according to NAFA and ranked first by D value, whereas Thunder and Zhongmu No. 1 were susceptible and had the two lowest D values. Aohan was assigned to the low-resistance class and also ranked near the bottom of the integrated evaluation.

The D-value evaluation also distinguished cultivars within the same NAFA class. Among the highly resistant cultivars, Magnum 2, Gannong No. 3, Adrenalin, and Dryland ranked above Vision and Longdong. Among the resistant cultivars, Gannong No. 6 had a higher D value than Salt-tolerant star and Xinjiangdaye. Thus, combining disease response with relative growth performance after inoculation provided additional resolution within NAFA classes.

Table 4. Comparison of NAFA classification and integrated D-value ranking.

Cultivar	NAFA Class	D-Value	Rank	Comparative Interpretation
Magnum 2	HR	0.9611	1	Concordant; highest comprehensive resistance
Gannong No. 3	HR	0.8548	2	HR; high D value within the same NAFA category
Adrenalin	HR	0.8410	3	HR; high D value within the same NAFA category

Table 4. Cont.

Cultivar	NAFA Class	D-Value	Rank	Comparative Interpretation
Dryland	HR	0.7970	4	HR; high D value within the same NAFA category
Vision	HR	0.7241	5	HR; slightly lower D value than top HR cultivars
Longdong	HR	0.7075	6	HR; slightly lower D value than top HR cultivars
Gannong No. 6	R	0.7068	7	R; higher D value among R cultivars
Salt-tolerant star	R	0.5727	8	R; intermediate comprehensive resistance
Xinjiangdaye	R	0.4944	9	R; intermediate comprehensive resistance
Aohan	LR	0.3101	10	LR; low integrated resistance
Zhongmu No. 1	S	0.1484	11	S; consistently susceptible
Thunder	S	0.0340	12	S; consistently susceptible

3.6. PCA-Based Differentiation of Cultivar Responses to ASBS

PCA was conducted with disease incidence, disease severity index, relative fresh weight, relative dry weight, and relative root length to characterize response patterns among the 12 cultivars. The first two components explained 89.24% of the total variation: PC1 accounted for 79.68% and PC2 for 9.56% (Figure 4).

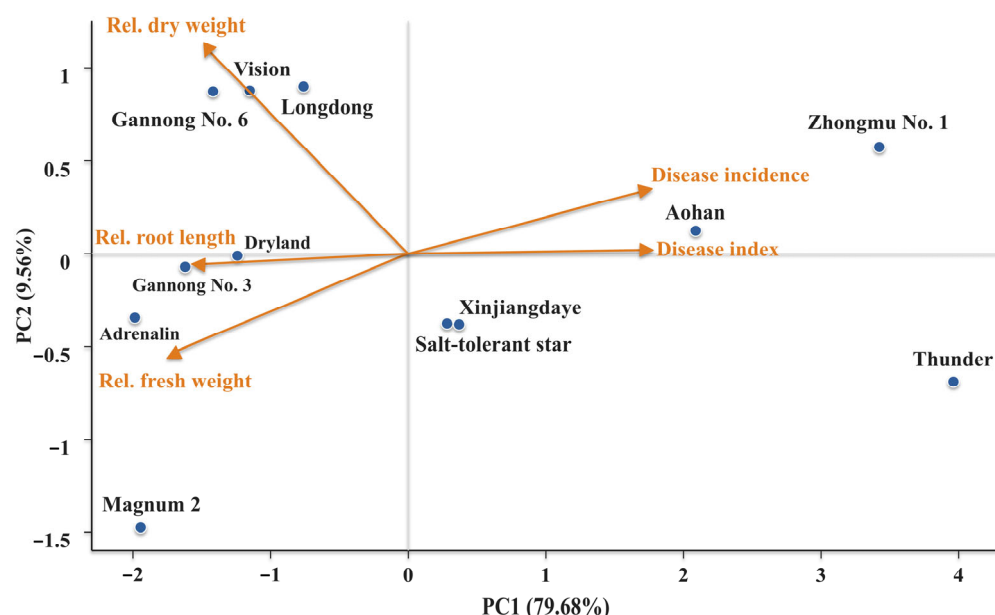


Figure 4. Principal component analysis (PCA) biplot of five resistance-related traits for 12 alfalfa cultivars after inoculation with *A. medicaginicola*. PC1 and PC2 explained 79.68% and 9.56% of the total variation, respectively (89.24% combined). Points represent cultivars, and arrows show the magnitude and direction of variable loadings. Disease incidence and disease severity index loaded positively on PC1, whereas relative fresh weight, relative dry weight, and relative root length loaded negatively. Cultivars on the negative side of PC1 therefore had lower disease levels and greater relative growth retention than cultivars on the positive side.

PC1 was positively associated with disease incidence and disease severity index and negatively associated with relative fresh weight, relative dry weight, and relative root length. This axis therefore represented the overall resistance–susceptibility gradient. Thunder, Zhongmu No. 1, and Aohan occurred on the positive side of PC1, consistent with high disease levels and poor relative growth after inoculation. Magnum 2, Gannong No. 3, Adrenalin, Dryland, Gannong No. 6, Longdong, and Vision were positioned on the negative side, reflecting lower disease severity and better post-inoculation growth retention. Salt-tolerant star and Xinjiangdaye were near the origin and showed intermediate responses.

PC2 was associated primarily with relative dry weight and, to a lesser extent, relative fresh weight, further separating cultivars with similar PC1 scores. Gannong No. 6, Longdong, and Vision had higher PC2 scores than Magnum 2, Gannong No. 3, Dryland, and Adrenalin, revealing differences in post-inoculation biomass retention among cultivars with relatively strong overall responses. The PCA pattern was broadly consistent with the D-value ranking and separated resistant, intermediate, and susceptible response types.

4. Discussion

ASBS is an important constraint on alfalfa production, and reliable identification of resistant cultivars is central to disease management and germplasm utilization [29]. Under the greenhouse conditions used here, the 12 cultivars differed markedly in symptom expression 14 days after inoculation. Susceptible cultivars developed chlorosis, numerous small black leaf spots, and premature leaf abscission, whereas obvious stem symptoms were absent. Because symptom expression depends on host genotype, pathogen aggressiveness, environmental conditions, and assessment timing, the foliar phenotype observed here represents an early response under controlled conditions rather than the full spectrum of ASBS symptoms observed in the field.

The wide variation among cultivars indicates substantial genotypic differences in response to *A. medicaginicola*, consistent with previous evaluations of alfalfa resistance to spring black stem and leaf spot [11,25]. Deploying resistant cultivars is among the most economical and environmentally sustainable options for managing alfalfa diseases [30]. The cultivars identified here therefore warrant further evaluation for deployment and use in resistance breeding.

An important feature of this study was the inclusion of relative growth performance in the resistance assessment. Resistance and tolerance are related but distinct host responses: resistance restricts infection or disease development, whereas tolerance limits the effect of infection on plant performance [31]. Symptom scores alone may therefore provide an incomplete picture of cultivar performance under pathogen pressure. Expressing each growth trait relative to the corresponding non-inoculated control helps account for inherent differences in plant vigor, but it cannot fully remove cultivar-specific differences in baseline vigor, developmental rate, or biomass allocation. Relative growth retention should therefore be interpreted as a complementary phenotype rather than a direct measure of resistance. Similar relative biomass traits have been used to assess disease resistance and tolerance in other legume crops [32–34]. More broadly, multi-trait evaluations can capture complex biological responses that single traits may overlook [35]. Integrating symptom-based indicators with relative growth traits thus provides a fuller description of cultivar responses after infection.

The observed variation in biomass retention may result from multiple, non-mutually exclusive processes, including the maintenance of photosynthetic function, efficient assimilate allocation, rapid growth recovery, and greater tolerance of tissue damage. However, because these processes were not directly measured in the present study, differences in growth retention should be interpreted as phenotypic response patterns rather than evidence of specific physiological mechanisms. Further physiological validation is therefore required.

Relative fresh weight, relative dry weight, and relative root length were inversely associated with both disease incidence and disease severity index, linking greater disease development to poorer post-infection growth. By contrast, relative plant height varied less among cultivars and was not significantly correlated with either disease variable, indicating limited discriminatory value under the present conditions. Stem elongation may also respond more slowly than biomass accumulation or root growth during the

first 14 days after inoculation. On this basis, relative plant height was excluded from the integrated score.

The membership-function-based D value combined disease response and relative growth performance in a single cultivar-level score. Membership functions transformed indicators with different units and response directions to a common scale, while variability-based weights reflected how strongly each indicator differentiated the cultivars in this dataset. Accordingly, these weights are dataset-specific and should not be interpreted as universal biological priorities. Multi-indicator approaches are widely used in plant stress-resistance studies to reduce dependence on any single trait [36]. Here, the D-value ranking agreed with NAFA classes at the resistant and susceptible extremes and further differentiated cultivars within the same NAFA class. This additional resolution arose from incorporating disease severity and growth retention alongside the percentage of healthy plants. The integrated evaluation should therefore be regarded as a complement to, rather than a replacement for, conventional NAFA classification.

PCA captured the same main gradient in cultivar response reflected by the D-value ranking. Because PCA and the D value were calculated from the same variables, the PCA results represent a consistency check rather than independent validation of the integrated index.

These findings indicate that cultivar responses to ASBS involve two related components: restriction of disease development and maintenance of growth after infection. Gannong No. 6, Longdong, and Vision retained comparatively favorable biomass despite moderate disease expression, a pattern compatible with tolerance or growth maintenance rather than stronger resistance per se. This interpretation and the value of these cultivars as breeding materials require confirmation in repeated and field-based evaluations.

This study has several limitations. The greenhouse experiment used a single pathogen isolate and one assessment time, 14 days after inoculation, when foliar symptoms were evident, but stem symptoms were not. The index should therefore be validated across pathogen isolates, environments, years, and assessment times before being adopted for routine screening. Multi-year field trials that evaluate disease development, yield stability, forage quality, and relevant physiological or molecular traits are needed to establish the practical value of these cultivars for resistance or tolerance breeding.

5. Conclusions

By integrating disease incidence, disease severity index, and relative growth performance after inoculation with *A. medicaginicola*, this study provided a multi-indicator characterization of alfalfa responses to spring black stem and leaf spot (ASBS). Magnum 2 had the highest D value, followed by Gannong No. 3, Adrenalin, and Dryland, whereas Thunder, Zhongmu No. 1, and Aohan had the lowest values. PCA resolved resistant, intermediate, and susceptible response types and was consistent with the D-value ranking. Considering symptom development together with relative growth retention provided a fuller description of cultivar performance under the greenhouse conditions tested. The resulting cultivar rankings and evaluation framework provide a basis for broader germplasm screening and field validation before deployment or use in ASBS management.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16151490/s1>. Figure S1: Pairwise Pearson correlation matrix among disease indicators and relative growth traits of alfalfa after inoculation with *A. medicaginicola*.

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Abbreviations

The following abbreviations are used in this manuscript:

ASBS	Alfalfa spring black stem and leaf spot
ITS	Internal transcribed spacer
NAFA	National Alfalfa and Forage Alliance
PDA	Potato dextrose agar
PCA	Principal component analysis

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