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Impact of foliar diseases on photosynthesis, protein content and seed yield of alfalfa and efficacy of fungicide application

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Abstract

Foliar pathogens attack alfalfa wherever the crop is grown, but their impact, especially on seed production, is poorly understood. In greenhouse trials, leaf spot injury caused by inoculation with various pathogens reduced the crude protein content of infected alfalfa leaves by 22% compared with a healthy control. There was a negative relationship between disease injury and the photosynthetic efficiency of alfalfa plants, as determined by measuring chlorophyll fluorescence in leaves from inoculated vs. non-inoculated seedlings. In field trials at two sites in Alberta from 2001 to 2003, inoculation with *Phoma medicaginis* increased disease incidence in four of six trials, *Phoma sclerotioides* increased incidence in four of five trials, and *Leptosphaerulina trifolii* and *Stemphylium botryosum* increased incidence in two of six trials. There was a trend for inoculation treatments to reduce seed yield, despite high levels of background infection by indigenous pathogens. The fungicides benomyl and propiconazole inhibited radial growth of *Phoma* spp. *in vitro* and reduced disease incidence in inoculated greenhouse experiments. In field trials, applications of benomyl and propiconazole reduced disease incidence, but did not always increase seed yield.

Introduction

Alfalfa (*Medicago sativa*) is the most important forage legume in North America, but reductions in yield and quality caused by foliar pathogens occur wherever the crop is grown (Stuteville and Erwin, 1990). Spring black stem (caused by *Phoma medicaginis* Malbr. & Roum. in Roum.) is the most common foliar disease of alfalfa in western Canada, followed, in decreasing order of importance, by common leaf spot (*Pseudopeziza medicaginis*), yellow leaf blotch (*Leptotrochila medicaginis*), lepto leaf spot (*Leptosphaerulina trifolii*), and stemphylium leaf spot (*Stemphylium* spp.) (Gossen and May, 1996; Wang et al., 2000, 2002a, 2003); Gossen et al., 2002, 2003). *Phoma sclerotioides* has recently been shown to produce leaf symptoms similar to *P. medicaginis* (Wang et al., 2004), and this species may contribute to the importance of spring black stem in the region. These pathogens generally occur as a disease complex in fields, or even on individual leaves (Thal and Campbell, 1987a, b), and symptoms can be difficult or impossible to differentiate under field conditions.

Leaf spot diseases cause losses in forage yield of 6–7% in Alberta (Berkenkamp, 1971, 1972, 1974),

resulting in economic losses of CAN$5–9 million per year. Even higher yield losses have been reported from New Zealand (16%) (Hart and Close, 1976), Australia (40%) (Morgan and Parbery, 1980), and the United States (19%) (Nutter et al., 2002). Leaf spot injury in alfalfa reduces photosynthesis (Nan et al., 2001) and affects forage quality by reducing carbohydrate and protein content (Mainer and Leath, 1978; Nan et al., 2001), with reduced protein levels generally having the greatest negative impact on feed value. Control of foliar diseases can reduce losses in forage yield (Wilcoxson et al., 1973; Hart and Close, 1976; Gray and Fernandez, 1987). However, no estimates of the impact of foliar diseases on alfalfa seed production are available. In addition, blossom blight on alfalfa flowers can have a dramatic impact on alfalfa seed yield in western Canada (Gossen et al., 1994).

Measurement of chlorophyll fluorescence provides a sensitive and non-destructive assay of photosynthetic performance. In this assessment, plant leaves are dark-adapted to ensure that all photosynthetic electron acceptors are fully oxidized (Judy et al., 1991). A plant leaf is placed in a spectrofluorometer fitted with a photon-counting photomultiplier. The leaf is then exposed to a single, saturating beam of light at 435 nm (actinic light), after which fluorescence is detected at an emission wavelength of 695 nm. Relative fluorescence intensity is recorded, and the resulting fluorescence trace is used to estimate chlorophyll fluorescence reduction parameters. This method can be used under laboratory and field conditions to monitor plant response to factors that affect photosynthetic metabolism and the effects of environment on plant growth (Judy et al., 1991).

The current study was conducted to: (i) quantify the relationship between leaf spot injury and photosynthetic activity using the chlorophyll fluorescence assay; (ii) quantify the effect of leaf spot injury on protein content in alfalfa leaves; (iii) assess the impact of foliar fungicides on foliar diseases caused by *Phoma* spp. under controlled conditions, and (iv) assess the impact of fungicide application on disease severity and seed production under field conditions. A range of pathogens was assessed because they all contributed to the leaf spot complex.

Materials and methods

Inoculum

One isolate each of *P. medicaginis*, *P. sclerotioides*, *Leptosphaerulina briosiana*, and *Stemphylium botryosum* were used. These isolates were collected between 1998 and 2000 from symptomatic alfalfa plants in east-central Alberta. The isolates for each species were selected based on maximum aggressiveness toward alfalfa plants. Single-spore cultures were grown on potato dextrose agar (PDA) at 20–25 °C with a 9 h photoperiod for 2 weeks. The cultures were blended with sterilized water for 2–3 min in a sterilized commercial blender at low speed, then filtered through two layers of cheesecloth to remove mycelial fragments. A spore suspension (10^6 conidia ml^{-1}) was prepared using 0.5% (v/v) Tween 80 (EM Science, Gibbstown, NJ) as a wetting agent. Spore concentration was quantified using a haemocytometer, and the spore suspension was sprayed onto alfalfa plants in a moist chamber using an H-set airbrush at 100 kPa, or onto field plots using a 2.5 l Spray-Doc backpack sprayer.

Chlorophyll fluorescence

Relative fluorescence intensity (counts s^{-1}) was recorded using a chlorophyll fluorometer, and the following chlorophyll fluorescence induction parameters were estimated from the fluorescence trace: F_0 (minimal fluorescence), F_m (maximal fluorescence), F_v (the difference between F_0 and F_m), F_v/F_m (photochemical yield of photosystem II), and $t_{1/2}$ (time required for fluorescence to reach a level halfway between F_0 and F_m) (Gensemer et al. 1996). Measurements were performed on leaflets attached to the plant. The impact of the foliar pathogens on chlorophyll fluorescence was assessed in a greenhouse experiment in which six alfalfa cultivars (Absolute, Blazer XL, Heinrichs, Peace, Ultrastar and Vernal) were inoculated with one isolate each of *P. medicaginis*, *P. sclerotioides*, *L. trifolii* and *S. botryosum*, or with a 0.5% Tween 80 suspension only. Three pots with 3–5 plants each were inoculated for each cultivar × isolate combination. Inoculation was done in the early vegetative stage when the stem length measured less than 15 cm. Ten measurements of chlorophyll fluorescence were recorded 2 weeks after

inoculation for each treatment on leaflets in both the upper and lower portions of the plant canopy. The experiment was repeated once.

The impact of leaf spot injury on chlorophyll fluorescence levels was also evaluated using leaf samples from field trials at Camrose and Vegreville, Alberta in July 2001. The samples were collected at the mid to late vegetative stage (stem length >15 cm, but no flower buds were present). Diseased leaves were collected at each field site and grouped into disease classes (0 = no symptoms, 1 = 1–4% of leaf area with lesions, 2 = 5–19%, 3 = 20–49%, and 4 = 50–100% of leaf area with lesions) using the disease severity descriptions developed by James (1971). Since multiple causal pathogens were involved in the study, a wide range of disease symptoms appeared on the leaf, from chlorosis to various spots. Readings of chlorophyll fluorescence were taken from 10 lower leaves each site at the lower end of each severity category, i.e. 0, 1, 5, 20, and 50% leaf infection.

Protein content

Leaf samples were collected from 20 alfalfa cultivars to evaluate the impact of *P. medicaginis* on protein content (Wang et al., 2002b). Alfalfa seeds were planted in a soil mix (1:1, loam:peat moss) in 350 ml pots (8 cm diam foam cups), and maintained in a greenhouse at 15–25 °C, 270 μE m^{-2} s^{-1} light intensity and 12 h photoperiod for 3 weeks before inoculation using the spore suspension spray method described above. The inoculated seedlings were incubated in a $145 \times 85 \times 90$ cm moist chamber, enclosed with a polyethylene film. Diseased and healthy leaf samples were excised from the lower portion of the plant canopy 30 days after inoculation (Wang et al., 2004). The samples were oven-dried at 70 °C for 24 h and ground to mesh size 20 (opening of 0.84 mm). The nitrogen content of diseased and healthy alfalfa for each cultivar was estimated based on total Kjeldahl nitrogen (Helrich, 1990). The % of crude protein (CP) was calculated as follows: %CP = 6.25 × N concentration × 100.

Fungicide efficacy in vitro *and greenhouse trials*

The growth of *P. medicaginis*, *P. sclerotioides* and *Phoma exigua* (syn. *P. solanicola*) (one isolate each) was examined on agar modified with benomyl (Benlate 50WP, DuPont Canada Inc., Mississauga, ON), iprodione (Rovral 50 WP, Bayer Canada, Etobicoke ON), chlorothalonil (Bravo 500 F, Syngenta Crop Protection Canada Inc., Guelph, ON), trifloxystrobin (Flint 125 EC, Syngenta Crop Protection Canada Inc.), azoxystrobin (Quadris 250 SC, Syngenta Crop Protection Canada Inc.), or propiconazole (Tilt 250 EC, Syngenta Crop Protection Canada Inc.). Each fungicide was suspended in sterile distilled water and added to autoclaved, cooled (ca. 50 °C) PDA, to produce final concentrations of 0, 0.01, 0.1, 0.5, 1.0, and 5.0 mg a.i. ml^{-1}. The amended media was then dispensed into 9 cm diam Petri dishes. Each fungal isolate was grown on unamended PDA for 2 weeks. Agar disks (5 mm diam) were cut from a colony and inverted onto the centre of each Petri dish in the fungicide concentration series, and incubated at 20–25 °C. Colony size (diameter from the edge of the agar plug to the farthest edge of mycelial growth) was measured every 24 h until mycelium in the control treatment reached the edge of the dish. Each treatment was replicated 10 times, and the test was repeated.

Propiconazole and benomyl were selected for further evaluation in a greenhouse test. Seedlings of cvs Beaver and Absolute alfalfa were grown as previously described. There were four fungicide treatments, applied at 1.3 ml per pot: (i) pre-inoculation application of propiconazole at 6.3×10^{-2} mg a.i. ml^{-1}, (ii) post-inoculation application of propiconazole at 6.3×10^{-2} mg a.i ml^{-1}, (iii) pre-inoculation with benomyl at 0.38 mg a.i. ml^{-1}, and (iv) a control sprayed with water. Each pot of 3 week-old seedlings was inoculated with 1.3 ml of spore suspension of *P. medicaginis* (10^6 conidia ml^{-1} + 0.5% Tween 80, applied using an airbrush). Pre- and post-inoculation treatments were applied 1 week before or after inoculation, respectively. The inoculated seedlings were maintained in a moist chamber in a greenhouse for 2 months after inoculation. The treatments were arranged in a randomized complete block design with five replicates, and the experiment was repeated once. Disease ratings (incidence and severity) were made at 15-day intervals for up to 2 months. Disease incidence was defined as the % of leaves with symptoms, and severity as the % leaf area affected (James, 1971).

Field trials

Field experiments were conducted from 2001 to 2003 at the Alberta Research Council site at Vegreville, AB and the Battle River Research Group site near Camrose, AB. Alfalfa cv. Algonquin was planted at 0.1 g seed m^{-1} row in 1998 at both sites in a split-plot design with four replications. Each main plot consisted of eight rows, each 5.0 m long, with 20 cm between rows, a 0.4 m buffer zone between plots, and a 2 m buffer between replications. The main plot treatments consisted of inoculation with a spore suspension of *P. medicaginis*, *P. sclerotioides*, *S. botryosum*, or *L. trifolii*, and a non-inoculated control. Spores were suspended in 0.5% Tween 80 (10^6 conidia ml^{-1}) and applied at 1.0 l per plot. Each main plot was split into four sub-plots with four rows, each 2.5 m long. The sub-plot treatments were foliar fungicides applied in 360 l ha^{-1} of water using a backpack sprayer. Disease assessments (% area affected) were made on leaves (20 leaves each from the upper and lower portions of the canopy), and stems (20 upper and 20 lower stems) for each treatment, and disease severity was assessed as described previously. Seed yield was evaluated by harvesting one linear meter from each of two centre rows in each sub-plot. The harvested crop was air-dried, threshed and weighed. Seed was harvested from both sites in 2001, and at Vegreville in 2002 and 2003. No seeds were harvested at Camrose in 2002 and 2003 because the crop grew slowly due to severe drought, and most plots failed to produce mature seeds.

In 2001, the inoculation treatments were applied in mid-July. The fungicide treatments (benomyl at 750 g a.i. ha^{-1}, propiconazole at 209 g a.i. ha^{-1}, and a non-treated control), were applied 2 weeks after inoculation with *P. medicaginis*, *P. sclerotioides*, *S. botryosum* or *L. trifolii*, and to a non-inoculated control. A single disease assessment was made 2 weeks after fungicide application.

In 2002, the study was modified to focus on the impact of pre-inoculation fungicide application to explore the effect of foliar spraying on disease development. Identical experiments were repeated in 2003. In 2002 and 2003, the plots were inoculated at the end of July. *P. sclerotioides* was not included in 2003. The fungicide treatments in 2002 and 2003 were: benomyl applied 1 week before inoculation, propiconazole 1 week before inoculation, propiconazole 1 week after inoculation (at the same rates as in 2001), and a non-treated control. The fungicide application rates were the same as were used in 2001. Disease ratings (leaves only) were taken at 15 day intervals for 2 months to monitor disease development. To enhance alfalfa seed production in 2003, a leafcutting bee shelter and nest boxes were placed at the edge of each field trial, and leafcutting bees (*Megachile rotundata Fabricus*) were released at the 50% bloom stage.

Statistical analysis

The impact of leaf spots on chlorophyll fluorescence levels was assessed using analysis of variance in SAS (SAS Institute, Cary, NC). Curve fitting for F_v/F_m and $t_{1/2}$ verses leaf spot severity was performed using non-linear regression in SigmaPlot (SigmaPlot 2001 for Windows 7.0, SPSS Inc., Chicago, IL), in which % change of F_v/F_m or $t_{1/2}$ was calculated as a % value relative to the measured value from the healthy leaves. Fungicide concentrations that inhibited mycelial growth by 50% (EC_{50}) and 90% (EC_{90}) were estimated using linear regression. For disease assessments over time, the area under the disease progress curve (AUDPC) was calculated according to Shaner and Finney (1977). Values for AUDPC were normalized by dividing the AUDPC by the total area of the graph to produce relative AUDPC values (Fry, 1978) prior to analysis. Data from the field trials were examined using the mixed model procedure in SAS, with the Satterthwaite option to determine degrees of freedom. Fungicide treatment and inoculation were considered as fixed factors and years, locations and sampling location within the plant were treated as random factors. Treatment means were compared using Tukey's honestly significant difference (HSD) test and differences were significant at $P \leq 0.05$ unless specified.

Results

Chlorophyll fluorescence

In the greenhouse assessment, variances were homogenous and there were no significant interactions between pathogen and canopy position (upper vs. lower) for any cultivar, so data from the two repetitions of the trial were pooled for

Table 1. Levels of chlorophyll fluorescence (F_v/F_m) as a measure of photosystem II activity in leaves of six alfalfa cultivars inoculated with four fungal pathogens in a greenhouse test

Treatment	Cultivar					
	Absolute	Blazer XL	Heinrichs	Peace	Ultrastar	Vernal
Pathogens						
P. medicaginis	0.70 b	0.71 a	0.73 a	0.74 ab	0.71 b	0.74 a
P. sclerotioides	0.71 b	0.71 a	0.71 b	0.72 b	0.71 b	0.72 a
Leptosphaerulina trifolii	0.70 b	0.71 a	0.70 b	0.75 ab	0.72 b	0.72 a
Stemphylium botryosum	0.75 a	0.74 a	0.73 a	0.74 ab	0.74 a	0.74 a
Control	0.76 a	0.74 a	0.74 a	0.76 a	0.76 a	0.74 a
SE	0.008	0.010	0.006	0.005	0.007	0.008
Canopy position						
Upper leaves	0.73 a	0.73 a	0.74 a	0.75 a	0.74 a	0.74 a
Lower leaves	0.72 b	0.71 b	0.71 b	0.74 b	0.72 b	0.72 b
SE	0.005	0.006	0.004	0.003	0.005	0.005

Measurements were done on leaves while still attached to seedlings 1 week after inoculation using a chlorophyll fluorometer.
SE = standard error. Values within a column and category followed by the same letter do not differ based on Tukey's HDS test at $P \leq 5\%$.

subsequent analysis. Three of the six cultivars (Absolute, Heinrichs and Ultrastar) inoculated with *P. medicaginis*, *P. sclerotioides* and *L. trifolii* had lower F_v/F_m values than the non-inoculated control, except that the F_v/F_m value was not reduced in the cv. Heinrichs inoculated with *P. medicaginis* (Table 1). Inoculation with *S. botryosum* did not reduce F_v/F_m values for any cultivar. F_v/F_m values in upper leaves were higher than in lower leaves.

The effect of disease severity on chlorophyll fluorescence in alfalfa leaf samples is shown in Figure 1; $t_{1/2}$ and F_v/F_m declined, indicating that photosynthetic activity was reduced as disease severity increased. The $t_{1/2}$ decreased considerably when the leaf was infected, even at the 1% severity level, while F_v/F_m decreased only slightly between the 1% and the 20% severity levels. The relationship between severity and % change in $t_{1/2}$ best fitted in a non-linear exponential decay model with three parameters, $Y = Y_0 + ae^{-bX}$ (Figure 1a), where Y = % change in F_v/F_m or $t_{1/2}$, X = disease severity, Y_0 = a constant, a = the intercept, and b = the slope. The relationship between severity and % change in F_v/F_m fitted an exponential decay model with two parameters, $Y = ae^{-bX}$ (Figure 1b). R^2 values for F_v/F_m and $t_{1/2}$ were significant at $P \leq 0.01$ and 0.05, respectively.

Protein content

The crude protein level was 22% lower ($P \leq 0.0003$) in alfalfa leaves inoculated with *P. medicaginis* than in healthy leaves, when assessed across all 20 alfalfa cultivars (Figure 2a). Infected leaves had a lower CP content than healthy leaves in 19 of 20 cultivars. Seven cultivars showed up to an 11% reduction in CP content, and another seven showed a 12–23% reduction from *P. medicaginis* infection. CP content was reduced 35–60% in five cultivars compared to the healthy control (Figure 2b).

Fungicide efficacy

In a preliminary assessment of fungicides, the relationship between fungicide concentrations and % inhibition of mycelial growth was described using a linear regression model ($Y = a + b \log X$) with R^2 values ranging from 77% to 99%. Benomyl and propiconazole inhibited the growth of all three *Phoma* species, with an EC_{50} of 1–6 ng ml^{-1} and an EC_{90} of 38–54 ng ml^{-1} for benomyl, and an EC_{50} of 1–14 ng ml^{-1} and an EC_{90} of 49–121 ng ml^{-1} for propiconazole. Chlorothalonil and iprodione inhibited growth at higher concentrations, but the EC_{90} was over 500 ng ml^{-1}. Trifloxystrobin and azoxystrobin did not inhibit the isolates; the latter even slightly stimulated growth at low concentrations (data not shown).

In the greenhouse test, symptoms of spring black stem caused by *Phoma* spp. developed rapidly after inoculation. Disease incidence on leaves was high in the non-treated control for Absolute (50%) and Beaver (42%), but was lower ($P \leq 0.01$) in the benomyl and propiconazole treatments (17–21%).

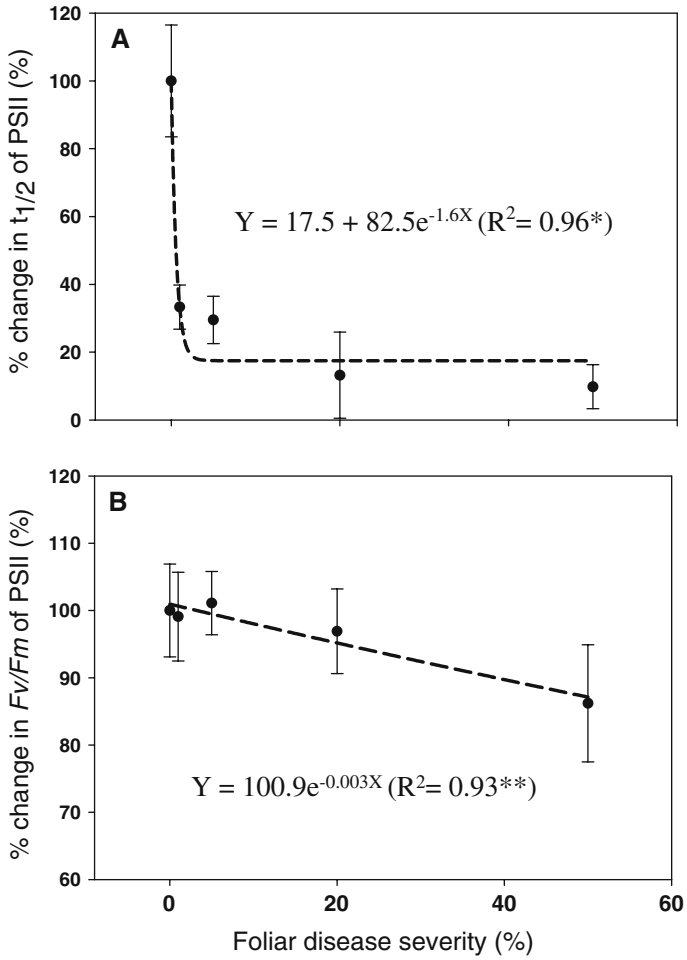

Figure 1. Relative changes (%): (A) $t_{1/2}$ (time required for fluorescence to reach a halfway level between minimal and maximal fluorescence), and (B) F_v/F_m (photochemical yield of chlorophyll-α of photosystem II) in alfalfa leaves in relation to foliar disease severity under the field conditions. Black dots represent actual measurements and dashed lines are predicted curves from non-linear regression. Capped bars represent one standard error. In the models, Y = % change in F_v/F_m or $t_{1/2}$, X = disease severity, Y_0 = a constant, a = the intercept, and b = the slope. R^2 values for F_v/F_m and $t_{1/2}$ were significant at $P \leq 0.01$ and $P \leq 0.05$, respectively.

There were no differences among the fungicide treatments for either cultivar, or between pre- and post-inoculation application treatments with propiconazole (Table 2). The relative AUDPC for disease incidence is presented because disease severity was highly correlated to disease incidence in the experiments. Disease incidence was higher ($P \leq 0.001$) on lower leaves than upper leaves in all treatments and for both cultivars.

Field trials

Both lower leaves and lower stems had a 100% incidence of symptoms for all treatments. Combined results from upper leaves and upper stems for 2001 are presented (Table 3) because plant parts were treated as a random factor in the statistical analysis. Disease incidence was higher at Camrose than at Vegreville. At Camrose, inoculation with *P. medicaginis* or *P. sclerotioides* produced a 7–8% increase ($P \leq 0.001$) in disease incidence compared to the non-inoculated control, but there were no differences among inoculation treatments at Vegreville. Incidence was lower for all fungicide treatments than for the non-treated control at both sites. Fungicide application reduced disease incidence by 20–26% compared to the non-treated control at Camrose, and by 48–

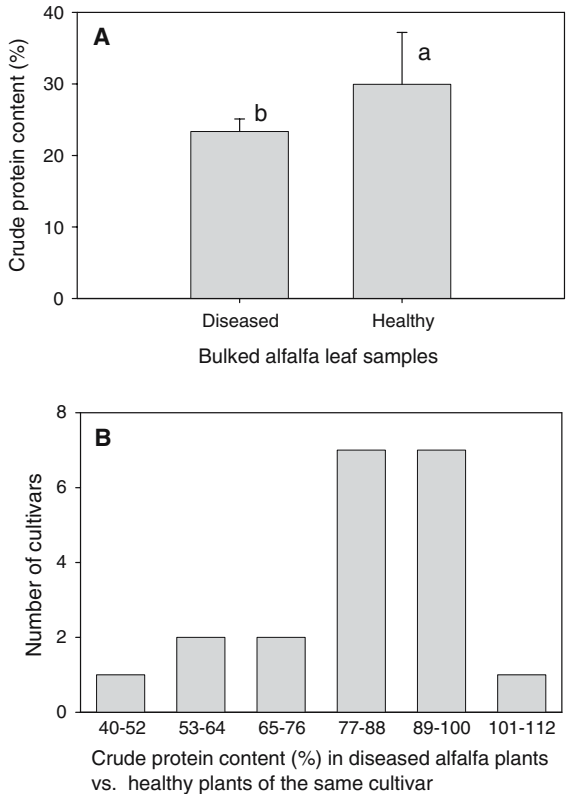

Figure 2. Effect of foliar disease injury (caused by multiple pathogens) on crude protein content in alfalfa leaf tissue. (A) N content of diseased and healthy leaves from 20 alfalfa cultivars in a greenhouse test. Capped bars represent one standard error and bars marked by the same letter do not differ based on Tukey's HSD test at $P \leq 5\%$, and (B) percentage of crude protein content in diseased plants compared to healthy plants of the same cultivar.

52% at Vegreville. Incidence was similar among the fungicide applications at Vegreville, while application of benomyl resulted in a lower incidence than propiconazole at Camrose (Table 3).

At both sites in 2002 and 2003, no stem infection was observed during the early stages of plant growth. Therefore, AUDPC values of disease incidence were calculated only from leaves; the results of AUDPC from upper and lower leaves are combined for presentation (Table 4). Disease severity was quite low in these trials, but was highly correlated to disease incidence (data not shown. AUDPC for disease incidence was increased by inoculation with *P. medicaginis* in three of six trials, by *P. sclerotioides* and *S. botryosum* in two trials, and by *L. trifolii* in one trial. Inoculation had no effect on disease development at Camrose in 2003. Disease severity was higher in 2003 than in 2002 (restricted by severe drought). Disease incidence was lower for all fungicide treatments compared to the non-treated control at both sites, but there were no differences among fungicide treatments (Table 3). Fungicide application reduced AUDPC by 22–27% at Vegreville and by 8–36% at Camrose.

Table 2. Impact of fungicide application on area under the disease progress curve (AUDPC) for alfalfa cvs Beaver and Absolute inoculated with *P. medicaginis* in a greenhouse study

Treatment	AUDPC for disease incidence	
	Absolute	Beaver
Fungicide treatment		
Propiconazole, pre-inoculation	24 b	24 b
Propiconazole, post-inoculation	27 b	25 b
Benomyl, pre-inoculation	26 b	25 b
Control	58 a	57 a
SE	0.88	
Canopy position		
Upper leaves	27 b	25 b
Lower leaves	40 a	40 a
SE	0.63	

Means in a column and category followed by the same letter do not differ based on Tukey's honestly significant difference at $P \leq 0.05$. AUDPC values were normalized (see text). SE = standard error. There was no interaction between fungicide treatment and canopy position in analysis of variance.

Table 3. Proportion of alfalfa plants (%) with symptoms after inoculation and fungicide application at Camrose and Vegreville AB in 2001 ($n = 4$)

Treatment	Disease incidence (%)	
	Camrose 2001	Vegreville 2001
Inoculation		
P. medicaginis	69 a	33 a
Phoma sclerotioides	68 ab	32 a
Leptosphaerulina trifolii	62 c	30 a
Stemphylium botryosum	63 bc	34 a
Control	61 c	31 a
SE	2.3	2.5
Fungicide applied		
Benomyl	60 c	25 b
Propiconazole	64 b	27 b
Non-treated control	80 a	51 a
SE	1.9	2.1

Means within a column and category followed by the same letter do not differ based on Tukey's HSD test at $P \leq 5\%$.

Table 4. Area under the disease progress curve (AUDPC) for disease incidence of alfalfa plants after inoculation and fungicide application at Camrose and Vegreville AB, 2002 and 2003 (n = 4)

Treatment	AUDPC			
	Camrose 2002	Vegreville 2002	Camrose 2003	Vegreville 2003
Inoculation				
P. medicaginis	56 a	25 a	93 a	69 a
Phoma sclerotioides	56 a	23 ab	90 a	nd
Leptosphaerulina trifolii	54 a	19 c	92 a	67 ab
Stemphylium botryosum	57 a	22 b	94 a	65 ab
Control	51 b	19 c	90 a	63 b
SE	3.4	1.8	2.3	3.1
Fungicide treatment				
Benomyl (pre-inoculation)	48 b	19 b	88 b	61 b
Propiconazole (post-inoc.)	50 b	19 b	90 b	61 b
Propiconazole (pre-inoc.)	49 b	20 b	90 b	64 b
Non-treated control	74 a	26 a	98 a	80 a
SE	3.3	1.7	2.2	3.1

Means within a column and category followed by the same letter do not differ based on Tukey's HSD test at $P \leq 0.05$. nd – not done.

Inoculation reduced alfalfa seed yield in two of the four trials for *L. trifolii* (Vegreville in 2002 and 2003), and in one of four trials for the other three pathogens (Table 5). Inoculation with *L. trifolii* produced the lowest yield (68% of the control) at Vegreville in 2003, and inoculation with *P. sclerotioides* resulted in the lowest yield (74% of the non-treated control) at Camrose in 2001. Inoculation with three of the four pathogens reduced seed yield at Vegreville in 2002, even though crop growth and seed yield was restricted by severe drought. In contrast, inoculation had no impact on seed yield at Vegreville in 2001, when conditions during the growing season were also very dry and seed yield was low.

Benomyl applied before inoculation increased yield in inoculated treatments at two of four sites (Table 5). Application of benomyl increased seed yield over the fungicide control at Vegreville by 42% in 2003. Both propiconazole, applied before

Table 5. Effect of inoculation with four foliar pathogens and fungicide application on seed yield of alfalfa at Camrose AB in 2001 and Vegreville AB in 2001–2003 (n = 4)

Treatment	Seed yield (kg ha^{-1})			
	Camrose 2001	Vegreville 2001	Vegreville 2002	Vegreville 2003
Inoculation				
P. medicaginis	187 bc	78 a	46 b	220 ab
Phoma sclerotioides	170 c	83 a	59 a	nd
Leptosphaerulina trifolii	236 a	60 a	41 bc	182 b
Stemphylium botryosum	184 bc	78 a	37 c	249 ab
Control	231 ab	93 a	65 a	269 a
SE	12.2	8.3	4.3	16.5
Fungicide treatment				
Benomyl (post-inoculation)	220 a	82 a	nd	nd
Benomyl (pre-inoc.)	nd	nd	62 a	271 a
Propiconazole (post-inoc.)	187 a	71 a	53 b	219 ab
Propiconazole (pre-inoc.)	nd	nd	49 b	238 ab
Non-treated control	188 a	70 a	34 c	191 b
SE	10.9	7.4	4.2	16.5

Values within a column and category followed by the same letter do not differ based on Tukey's HSD test at $P \leq 5\%$. nd = not done.

or after inoculation, and benomyl increased seed yield at Vegreville in 2002, but yields were so low that the treatment was unlikely to be cost effective.

Discussion

Beside the obvious physical damage, leaf spot diseases affect leaf metabolism in alfalfa, which can affect productivity and quality. Protein content is an important component of forage quality in alfalfa hay. Although some early reports indicate that protein levels are not affected by foliar disease or fungicide application (Willis et al., 1969; Leath et al., 1974; Summers and McClellan, 1975), the present study demonstrated that foliar disease reduced crude protein in leaves. This supports the conclusions of more recent studies (Mainer and Leath, 1978; Nan et al., 2001), which indicate that the crude protein content in the infected leaves was reduced compared with the healthy leaves. Sampling procedure may have an impact on assessment of protein levels in forage samples. To minimize potential differences in leaf age on protein content, leaf samples were collected from the same position in the plant canopy for both infected and healthy leaves.

Chlorophyll fluorescence has been used to measure the energetic state of photosynthesis in plants, especially in studies on environmental stresses such as extreme temperature, drought, or exposure to chemical agents (Judy et al., 1990, 1991). Leaves were carefully selected to represent the lower end of each severity category. This was especially important to the most severe disease category because fluorescence could not be measured reliably from severely chlorotic leaves. The diminished values for $t_{1/2}$ and F_v/F_m (two important fluorescence induction parameters) observed in infected leaves indicated that infection had an inhibiting effect on photosystem II. The present study demonstrated that the overall photochemical yield (F_v/F_m) of chlorophyll-α in photosystem II within intact leaves tended to decline slightly as foliar disease severity increased (Figure 1). Similar results were also observed in the cultivar study (Table 1), where some of the pathogens significantly reduced F_v/F_m for four of the six cultivars evaluated. Inoculation with *S. botryosum* had little or no effect on F_v/F_m values, while inoculation with *P. sclerotiodes* reduced F_v/F_m values in four of six cultivars. In a previous study, F_v/F_m values within the leaf were lower in the area occupied by the lesion than in undamaged areas (Daley, 1995). Even low levels of infection dramatically reduced $t_{1/2}$. Since $t_{1/2}$ is proportional to the size and accessibility of electron acceptors available to PSII (Judy et al., 1990), this finding suggests that highly localized infections may hamper the efficiency of PSII, even when the overall photochemical yield resembles that found in healthy leaves.

Studies at two sites over three years indicated that artificial inoculation can increase disease levels in field trials. The four foliar pathogens caused different levels of disease on alfalfa plants under the unusually dry weather conditions experienced in Alberta over the course of this study; *S. botryosum* was the least aggressive pathogen (foliar disease levels increased only in one of six tests), and *P. medicaginis* was the most aggressive. However, disease incidence in the non-inoculated control treatment was high in each trial. Even in inoculated treatments, pathogens other than the one that was applied were consistently recovered by isolation from symptomatic leaves (unpublished), which indicates that background levels of endemic pathogens were high in all of the field trials.

In the field studies, yield reduction associated with inoculation was often not statistically significant. Although disease incidence was high in several of the tests, disease severity was generally low (data not presented), even in the inoculated treatments. The low levels of disease were likely due to dry conditions that prevailed throughout the study. Fungicide application consistently reduced disease incidence and occasionally increased seed yield over the non-treated control; increases were not as dramatic as in a previous study where seed yield was increased 17%–360% by fungicide application (Wilcoxson and Bielenberg, 1972). In our study, propiconazole showed some promise as an alternative to mancozeb for control of spring black stem in alfalfa seed production, but additional work is required to determine if application of propiconazole is cost-effective for producers. Benomyl provided more consistent yield improvement, but was withdrawn from commercial use after the study was completed, and is no longer available. In addition, studies are needed on acceptable levels of fungicide residue in alfalfa forage, so that foliar disease management with fungicides can benefit alfalfa seed producers.

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