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Etiology of Alfalfa Blossom Blight Caused by *Sclerotinia sclerotiorum* and *Botrytis cinerea*

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ABSTRACT

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Blossom blight caused by *Sclerotinia sclerotiorum* and *Botrytis cinerea* is an important disease of alfalfa in the forage seed production areas of western Canada. A greenhouse study was conducted to determine the relative importance of these two pathogens in causing blossom blight of alfalfa, and the roles of humidity and floral parts in development of this disease. Tripping alfalfa florets with spores of *S. sclerotiorum* or *B. cinerea* not only caused infection of styles, but also reduced pod formation and seed production. Under humid conditions, the pathogens grew from infected styles, spread rapidly onto pods, and infected seeds. The frequency of infected seeds was higher when humidity was applied at the mature pod stage than when applied at the young pod stage. The rate of seed infection was reduced in the absence of flower petals, suggesting that senescent petals play an important role in movement of the pathogens from infected styles to seeds. Losses in pod formation, seed yield and seed quality were more severe for *S. sclerotiorum* than for *B. cinerea*. Humidity, the presence of senescent petals, and the age of pods all affect the development of alfalfa blossom blight caused by *S. sclerotiorum* and *B. cinerea*.

Key words : Blossom blight, *Sclerotinia sclerotiorum*, white mold, *Botrytis cinerea*, gray mold, *Medicago sativa*, alfalfa, lucerne, etiology

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most important leguminous forage crop in Canada. In 1996, the area for alfalfa tame hay production in Canada was 3.6 million ha (1). Approximately 4,200 ha of seed alfalfa was grown in Alberta in 1996, resulting in a total seed yield of nearly 3000 t (2). This represents more than 50 percent of Canadian alfalfa seed production (Harold Moore, personal communication). The pollinating insect, alfalfa leafcutter bee (*Megachile rotundata* (Fabricius)), is used exclusively in commercial production of alfalfa seed in Canada (5).

Blossom blight, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary and *Botrytis cinerea* Pers.:Fr., has become an important disease of alfalfa in the forage seed production areas in western Canada (6, 7, 8, 9). Both pathogens have been found throughout the Canadian prairies, and their occurrence varies with year and region. The fungicide, benomyl, has recently been registered for control of *S. sclerotiorum* and *B. cinerea* on alfalfa in Canada (3).

Understanding the cause and development of blossom blight of alfalfa is crucial in developing effective control measures for the disease. The objectives of this study were to determine the importance of *S. sclerotiorum* and *B. cinerea* in causing blossom blight of alfalfa and to determine major factors affecting the development of this disease.

MATERIALS AND METHODS

Seeds of alfalfa, cultivar Barrier, were planted in Cornell Peat-Lite Mix™ (4) in Root- trainer books™ (Spencer-Lemaire Industries Ltd., Edmonton, Alberta, Canada) and grown in a greenhouse at 20 ± 5 ℃. After 10 weeks, plants were transplanted into Cornell Peat-Lite Mix™ in plastic pots (16 cm in diam.), watered daily, and used for the experiments during the blossom period.

Effect of pathogens on alfalfa pods, styles and seeds.

Sclerotinia sclerotiorum, isolate LRC 2148, and *Botrytis cinerea*, isolate LRC 2421, were used to study the effects of these pathogens on alfalfa styles, pods and seeds. For *S.*

sclerotiorum, ascospores were obtained from mature apothecia produced on sclerotia according to the method described by Huang and Kozub (10). Ascospore suspensions containing approximately 10^5 spores/ml were made by washing apothecia in sterile distilled water. Alfalfa flowers were simultaneously pollinated and inoculated with the pathogen by tripping the keel petal of each floret, using the flat end of a toothpick covered with a mixture of ascospores and alfalfa pollen from sister plants. For *B. cinerea*, conidia were obtained by scraping the colony surface of a 3-wk-old culture, grown at 20 ℃ on potato dextrose agar (PDA, Difco, Detroit, MI) under fluorescent light at the intensity of 15.2. μE/sec/m^2. Alfalfa flowers were pollinated and inoculated with *B. cinerea* by the same method used for *S. sclerotiorum*. Flowers tripped with pollen alone were used as a control. A total of 100 florets from 10 racemes, at 10 florets per raceme, were tripped per treatment. The plants were kept in the greenhouse and watered daily through a saucer at the bottom of each pot.

At two weeks after flower tripping, the number of pods formed on the racemes of individual treatments, control and pathogen-inoculated, was recorded. At seven weeks after inoculation, racemes were harvested. Styles were excised from the pods on each raceme, surface-sterilized in 70% ethanol for 90 sec, and air-dried. They were plated on PDA amended with 200 ppm streptomycin sulfate (Sigma, St. Louis, MO) in Petri dishes, incubated at room temperature (20 ± 2 ℃) for 10 days, and examined for growth of microorganisms. The number of seeds in each pod was determined, and seeds were examined for presence of the pathogens using the same aseptic plating technique as described for styles.

Effect of humidity on infection of alfalfa styles and seeds.

Individual florets on alfalfa plants were simultaneously pollinated and inoculated with *S. sclerotiorum* or *B. cinerea* using the same hand-tripping method as described above. Plants were then exposed to high humidity (95-100%) by covering individual plants with transparent plastic bags (45 x 80 cm in size). The humidity treatment was applied at either 2 weeks after inoculation when the pods were young, or 5 weeks after inoculation when the pods were maturing, and the treatment lasted for 14 days. Corresponding low humidity (25-35%) treatments were set up by leaving the plants with pollinated and pathogen-inoculated florets in the greenhouse without the plastic cover for 14 days.

At the end of the humidity treatment, bags were removed, and the racemes were harvested. Styles were excised, surface sterilized and examined for presence of the pathogens by the same plating method described above. The number of seeds in each pod was determined, and seeds were examined for presence of the pathogens using the same techniques described above.

Effect of senescent flower petals on infection of alfalfa seeds.

Florets on alfalfa plants were pollinated and inoculated with *S. sclerotiorum* and *B. cinerea*, using the same hand-tripping methods as described above. After keeping the plants in the greenhouse for 14 days, senescent petals were removed from the pods on half of the plants, while the petals were left intact on the other half. Plants were then exposed to humidity treatments using the same plastic covering methods as described above. At the end of the humidity treatments, pods were harvested and the number of seeds was determined for each pod. Seeds were surface sterilized and plated on PDA using the same procedure described above for styles and then examined for presence of the pathogens.

Statistical Analysis

Each experiment was conducted three times, and the data were analyzed using a completely randomized design with 3 replications. Analyses of variance were carried out to determine significant differences among treatments in percent formation of pods, percent infection of styles by each pathogen, number of seeds produced, and percent infection of seeds by each pathogen. Treatment means were compared using Fisher's least-significant difference test. All statistical analyses were conducted using SAS/STAT® software (15).

RESULTS

Effect of pathogens on alfalfa pods, styles and seeds.

Tripping alfalfa florets with *S. sclerotiorum* or *B. cinerea* significantly ($P<0.05$) reduced pod formation. While 90% of tripped florets produced pods in the control, only 47% and 67% of tripped florets produced pods in the treatments of *S. sclerotiorum* and *B. cinerea*, respectively (Fig. 1). *S. sclerotiorum* caused more reduction in pod formation than *B. cinerea* ($P<0.05$). In contrast to white or light green colored styles in the control, tripping of alfalfa florets with *S. sclerotiorum* or *B. cinerea* resulted in brown to dark brown discoloration at the distal end of each style (Fig. 2). While no infection of styles by the pathogens was found in the control, the frequency of infection of stylar tissues by *S. sclerotiorum* and *B. cinerea* was 90% and 92%, respectively. The two pathogens induced the same level of infection ($P>0.05$) in the stylar tissues (Table 1).

Tripping of alfalfa florets with *S. sclerotiorum* or *B. cinerea* significantly reduced the formation of seeds in each pod ($P<0.0001$). The mean number of seeds produced per pod was 5.7 for the control, 4.6 for the treatment of *B. cinerea*, and 3.5 for the treatment of *S. sclerotiorum* (Table 1). The difference between the two pathogens in seed production was significant ($P<0.0001$). No infection of seeds was observed in any of the treatments, under low humidity (25-35%) conditions used for this experiment (Table 1).

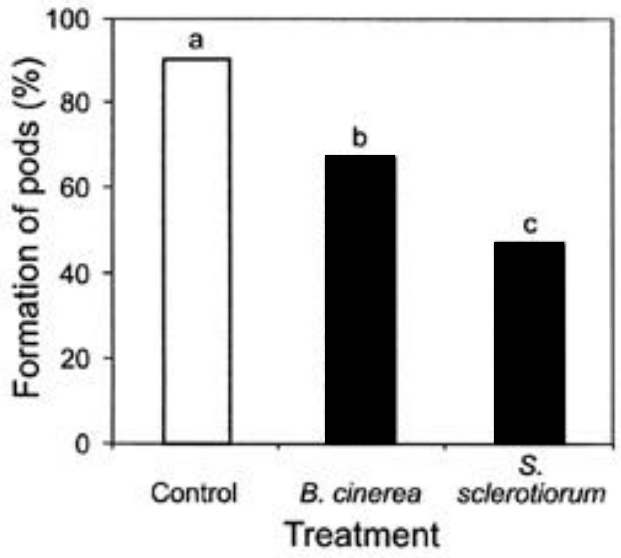

Fig. 1. Effect of *Sclerotinia sclerotiorum* and *Botrytis cinerea* on pod formation in alfalfa, as observed at 2 weeks after flower tripping. Columns with the same letter are not significantly different (P<0.0001; standard error = 0.5)

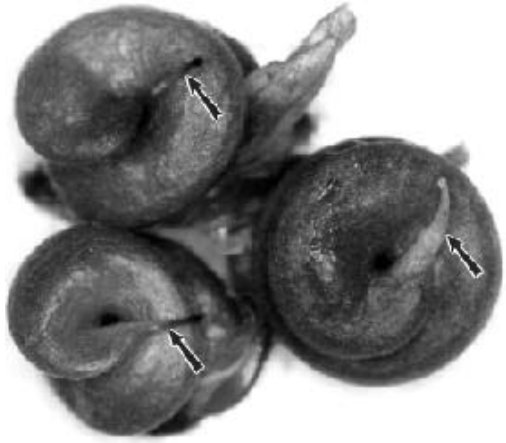

Fig. 2. Young alfalfa pods developed from tripping of florets with pollen (right), pollen and *Sclerotinia sclerotiorum* (top left) and pollen and *Botrytis cinerea* (bottom left). Note the whitish-green color of healthy style (right, arrow) and the discoloration of *S. sclerotiorum*-infected (top left, arrow) and *B. cinerea*-infected (bottom left, arrow) styles. The pods were excised at 10 days after flower tripping. Magnification 5X.

Effect of humidity on infection of alfalfa styles and seeds.

The frequency of infection of styles ranged from 89% to 95% for *B. cinerea* and from 86% to 96% for *S. sclerotiorum*. The rate of stylar infection was not affected by pathogen, level of humidity, or timing of humidity (P>0.05) (Table 2). High humidity caused a significant (P<0.0001) reduction in the number of seeds produced per pod for the florets treated with either pathogen, but the timing of humidity had no significant effect (P>0.05) (Table 2). The mean number of seeds produced per pod for the high humidity treatment was 3.7 and 3.0 for *B. cinerea* and *S. sclerotiorum*, respectively, while the means for pods treated with the same pathogens were 5.7 and 4.1 seeds per pod, respectively, with low humidity.

Table 1. Effect of *S. sclerotiorum* and *B. cinerea* on infection of alfalfa styles and seeds, and number of seeds produced.[1]

Pathogen	% infection		No. seeds
	styles	Seeds	per pod
Control	0	0	5.7 a[2]
B. cinerea	92 a[2]	0	4.6 b
S. sclerotiorum	90 a	0	3.5 c
Standard Error	± 0.4	± 0.0	± 0.1

1. Florets simultaneously pollinated and inoculated with pathogens, and rated two weeks later. A total of 100 florets were tripped per treatment (10 racemes at 10 florets per raceme). Data represents the means of three repetitions of the experiment.
2. Means in each column followed by same letter are not significantly different at 0.05 level (Fisher's least-significant difference test).

Infection of alfalfa seeds by *S. sclerotiorum* or *B. cinerea* occurred under conditions of high humidity, but not low humidity (Table 2). For both pathogens, there was a significant (P<0.0001) effect on infection of alfalfa seeds by timing of humidity. For the high humidity treatments of *S. sclerotiorum*, the frequency of infected seeds was 46% for the young pod stage (2 weeks after flower tripping) and 75% for the pod maturing stage (5 weeks after flower tripping) (Table 2). For the high humidity treatments of *B. cinerea*, the frequency of infected seeds was 28% for the young pod stage and 45% for the pod maturing stage (Table 2). Infection of seeds by *S. sclerotiorum* was significantly (P<0.0001) higher than infection by *B. cinerea*.

Effect of senescent flower petals on infection of alfalfa seeds.

After pollination of alfalfa, numerous senescent flower petals remained firmly attached to coiled pods on each raceme (Fig. 2). Regardless of the timing of high humidity at young pod (2 weeks after flower tripping) or mature pod (5 weeks after flower tripping) stages, the rate of infection of alfalfa seeds by *S. sclerotiorum* or *B. cinerea* was significantly lower (P<0.0001) in the absence of senescent petals on the pods than for those treatments with the senescent petals present (Table 3). In the case of *S. sclerotiorum*, when the young pods were subjected to high humidity for two weeks, the rates of infected seeds were 64% and 27% for the presence and the absence of petals, respectively. When the mature pods were

Table 2. Effect of humidity on infection of alfalfa styles and seeds by *S. sclerotiorum* and *B. cinerea*, and number of seeds produced.

Humidity		*B. cinerea*			*S. sclerotiorum*		
		% infection		No. seeds	% infection		No. seeds
Level	Timing[1]	styles	Seeds	per pod	styles	seeds	per pod
High	2 wk	89 a[2]	28 a[2]	3.7 a[2]	88 a[2]	46 a[2]	3.3 a[2]
	5 wk	90 a	45 b	3.7 a	92 a	75 b	2.7 a
Low	2 wk	93 a	0	6.3 b	86 a	0	4.1 b
	5 wk	95 a	0	5.0 b	96 a	0	4.1 b
Standard Error		± 0.6	± 0.7	± 0.1	± 0.6	± 0.7	± 0.1

1. High humidity = 95-100%, low humidity = 25-35%. High humidity treatments were achieved by covering plants with plastic bags, whereas plants in low humidity treatments were not covered.
2. Interval between tripping of florets and application of humidity treatments. 2 wk = young pods; 5 wk = mature or maturing pods.
3. Means in each column followed by same letter are not significantly different at 0.05 level (Fisher's least-significant difference test)

Table 3. Effect of flower petals on infection of alfalfa seeds by *S. sclerotiorum* and *B. cinerea*.

	% seeds infected					
	Control		*Botrytis cinerea*		*Sclerotinia sclerotiorum*	
Petals	2 wk[1]	5 wk	2 wk	5 wk	2 wk	5 wk
Present	0	0	40 a[2]	52 a[2]	64 a[2]	86 a[2]
Absent	0	0	16 b	38 b	27 b	64 b

1. Interval between tripping of florets and application of humidity treatments. 2 wk = young pods; 5 wk = mature or maturing pods.
2. Means in each column followed by same letter are not significantly different at 0.05 level (Fisher's least-significant difference test). Standard Error = 2.2 (Controls excluded from analysis)

subjected to high humidity for two weeks, the rates of infected seeds were 86% and 64% for the presence and the absence of petals, respectively (Table 3). No pathogen-infected seeds were found in the controls regardless of the presence or absence of senescent petals on the racemes.

DISCUSSION

This greenhouse study reveals that both *S. sclerotiorum* and *B. cinerea* are important pathogens for blossom blight of alfalfa, since tripping of florets with these organisms caused reduction of pods and seeds, as well as infection of styles and seeds. This study also indicates that under the same greenhouse conditions, the loss in seed yield and seed quality is more severe for *S. sclerotiorum* than for *B. cinerea*. Previous reports show that pollen grains of alfalfa are susceptible to infection by *S. sclerotiorum* (12) and *B. cinerea* (13). It is possible that the reduced formation of pods and seeds by *S. sclerotiorum* and *B. cinerea* observed in the present study may have been related to the negative effects of these pathogens on pollen viability and pollination efficiency in alfalfa.

Alfalfa is a cross pollinated crop, and to produce seeds, it requires tripping of florets by physical means, such as hand-tripping or foraging of pollinating insects. The infection of stigma and stylar tissues observed in the present study of *S. sclerotiorum* and *B. cinerea*, and in a previous report of *Verticillium albo-atrum* Reinke and Berthold (11), indicates that transmission of these pathogens can readily occur through the flower tripping process, resulting in infection and discoloration of stigma and styles. Huang et al. (14) observed transmission of *V. albo-atrum* by leafcutter bees foraging in an alfalfa field naturally infested with the Verticillium wilt pathogen. Stelfox et al. (16) reported transportation of *S. sclerotiorum*-contaminated pollen to rapeseed (*Brassica napus* L.) flowers by honeybees (*Apis mellifera* L.), resulting in the development of head blight under greenhouse conditions. Therefore, the risk of transmission of spores of *S. sclerotiorum* and *B. cinerea* to alfalfa flowers by pollinating insects is high because blossom blight is widespread in seed alfalfa fields in the Canadian prairies (6, 7, 8, 9).

Humidity is a major factor affecting the development of alfalfa blossom blight. Under dry conditions, *S. sclerotiorum* and *B. cinerea* remained in the infected tissues of the stigma and style, and were unable to grow and spread onto pods and seeds (Table 2). Under high humidity, however, the pathogens in the infected styles rapidly grew and spread onto pods and seeds. Senescent flower petals and old pod tissues may also play a role in the movement of *S. sclerotiorum* and *B. cinerea* from the infected style to the seeds. Under humid conditions, the senescent petals or old pod tissues can provide nutrients for the saprophytic growth of the pathogens, and thereby enhance the colonization and infection of seeds by the pathogens. This greenhouse study suggests that blossom blight of alfalfa will likely have the greatest impact on seed production under wet weather conditions, especially during the latter part of the growing season when pods are maturing or matured. This possibility appears to coincide with the

observations from a disease survey conducted in Manitoba in 1998, where blossom blight of alfalfa occurred in moderate to high levels in most of the surveyed areas due to cool, wet weather conditions (6). Another survey in Saskatchewan and Manitoba in 1996 showed that the incidence of blossom blight of alfalfa was low at early bloom, but increased with time to moderate or high levels in most regions (7). This increase in disease incidence during the late bloom period may be associated with senescent tissues, such as flower petals and matured pods, which serve as a nutrient source for the pathogens. Since most seed alfalfa crops are grown either under irrigation in southern Alberta or in high rainfall areas across the Canadian prairies, the risk of blossom blight is high, especially if the crops are grown in fields infested with *S. sclerotiorum* and/or *B. cinerea*.

Recent studies showed that alfalfa pollen is susceptible to infection by *S. sclerotiorum* (12) and *B. cinerea* (13). Stelfox et al. (16) observed contamination of rapeseed pollen grains with ascospores of *S. sclerotiorum* under growth room and field conditions. Huang et al. (14) reported that alfalfa pollen grains infected by *V. albo-atrum* were carried by leafcutter bees foraging in an alfalfa field with high incidence of Verticillium wilt. Since leafcutter bees are important pollinators for commercial production of alfalfa seed, transmission of spores of *S. sclerotiorum* and *B. cinerea*, or of pathogen-infected pollen grains, to alfalfa flowers by this pollinating insect may occur under natural field conditions. Therefore, blossom blight will likely create a negative impact on both the alfalfa seed industry and the leafcutter bee industry.

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摘 要

黃鴻章[1,2]. Acharya, S. N. [1], & Erickson, R. S. [1] 2000. *Sclerotinia sclerotiorum* 及 *Botrytis cinerea* 所引起苜蓿花腐病之病原學研究. 植病會刊 9:11-16. ([1.] Agriculture and Agri-Food Canada, Lethbridge Research Centre, PO Box 3000, Lethbridge, Alberta, T1J 4B1 Canada.；[2.] 聯絡作者：電子郵件 huangh@em.agr.ca，傳真 403-3823156)

苜蓿花腐病是加拿大西部苜蓿種子生產區的重要病害。田間調查發現是由 *Sclerotinia sclerotiorum* 或者 *Botrytis cinerea* 所引起。本研究旨在比較兩種病原菌在溫室環境下的相對重要性以及探討溫度與凋謝花瓣對病害發生的影響。實驗結果顯示，於開花期用牙籤將嫡系花粉與 *S. sclerotiorum* 或 *B. cinerea* 孢子混合物觸壓龍骨瓣或所謂解鉤法(tripping)接種每一小花，不但會造成每一形成的果莢之花柱(style)受到病原菌的侵染，而且引起很多小花不孕，導致果莢與種子形成的數目減少。在潮濕的環境下，花柱內的病原菌會蔓延到果莢，進而危害種子。如果花穗上沾有帶殘菌的花瓣，則果莢及種子受害的情形更加嚴重。實驗証明，在相同環境下 *S. sclerotiorum* 所造成的危害比 *B. cinerea* 為嚴重。本研究証實 *S. sclerotiorum* 及 *B. cinerea* 均可藉受粉過程而造成苜蓿花腐病，而且高濕、凋謝花瓣及果莢成熟度等是影響本病發生的重要因子。

關鍵詞 : 花腐病、 *Sclerotina sclerotiorum* 、白黴病 (菌核病)、 *Botrytis cinerea* 、灰黴病、苜蓿、病原學