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Potential role of pollen and pollinators in the spread of blossom blight of seed alfalfa caused by *Botrytis cinerea*

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Abstract: The fungal pathogen *Botrytis cinerea* Pers.: Fr. infects flowers of many plant species, including alfalfa (*Medicago sativa* L.). In southern Alberta, blossom blight, caused in part by *B. cinerea*, reduces yields of seed alfalfa in cool, wet growing seasons. Previous studies have suggested that *B. cinerea* uses pollen to begin infection, and that the fungal-infected pollen may be vectored by alfalfa leafcutter bees (*Megachile rotundata* Fab.). A longitudinal greenhouse study was performed to investigate the role of pollen in the infection process of alfalfa florets by *B. cinerea*. The effects of pollination (pollinated vs unpollinated) and inoculation method (dry vs aqueous suspension) on floret and pollen infection was tested. Florets were harvested at 0, 24, 48 and 94 h after inoculation. Pollinated florets that were dry inoculated had significantly greater levels of infection (84%) than similarly inoculated unpollinated florets (38%, $P < 0.001$); however no pollen infection was observed. In contrast, 90% of suspension-inoculated florets were infected and a small (<1%) but significant ($P < 0.0001$) level of infected pollen was observed in these samples. Leafcutter bees were also collected from seed alfalfa fields in 2013 and 2014 to estimate the prevalence of *B. cinerea* on pollen. Pollen removed from field bees showed no growth by *B. cinerea*; however, the bees that were directly plated on agar medium exhibited an increased load of *B. cinerea* as the growing season progressed. Thus, under typical field conditions, alfalfa pollen is not likely to be a significant factor contributing to the establishment and spread of *B. cinerea*.

Keywords: alfalfa, *Botrytis cinerea*, infection process, leafcutter bee, pathogen spread

Résumé: L'agent pathogène fongique *Botrytis cinerea* Pers.: Fr. infecte les fleurs de nombreuses espèces de plantes, y compris celles de la luzerne (*Medicago sativa* L.). Dans le sud de l'Alberta, la brûlure de la fleur, causée en partie par *B. cinerea*, peut réduire les rendements de luzerne graine durant les saisons fraîches et pluvieuses. Des études antérieures ont suggéré que *B. cinerea* utilise le pollen pour amorcer l'infection et que le pollen ainsi infecté par le champignon peut être transporté par des abeilles coupeuses de feuilles (*Megachile rotundata* Fab.). Une étude longitudinale a été menée en serre pour évaluer le rôle joué par le pollen dans le processus d'infection des fleurettes de la luzerne par *B. cinerea*. Les effets de la pollinisation (pollinisés *vs* non pollinisés) et les méthodes d'inoculation (à sec *vs* suspension aqueuse) sur l'infection des fleurettes et du pollen ont été testées. Les fleurettes ont été récoltées à 0, 24, 48 et 94 heures après inoculation. Les fleurettes pollinisées qui avaient été inoculées à sec affichaient un taux d'infection significativement plus élevé (84%) que les fleurettes non pollinisées inoculées de la même manière (38%, $P < 0.001$); toutefois, aucune infection du pollen n'a été observée. En revanche, 90% des fleurettes inoculées avec la solution aqueuse ont été infectées et un faible (< 1%), mais significatif taux ($P < 0.0001$) de pollen infecté a été noté dans ces échantillons. En 2013 et 2014, des abeilles coupeuses de feuilles ont également été collectées dans des champs de luzerne afin d'évaluer la prévalence de *B. cinerea* sur le pollen. Le pollen prélevé sur les abeilles au champ n'a pas affiché de croissance de *B. cinerea*; toutefois, les abeilles qui ont été mises en contact direct avec un milieu d'agar ont affiché une charge croissante de *B. cinerea* à mesure que la saison progressait. Ces données suggèrent que, dans des conditions réelles typiques, il est peu probable que le pollen de la luzerne soit un facteur important quant à l'établissement et à la propagation de *B. cinerea*.

Mots clés: Luzerne, *Botrytis cinerea*, processus d'infection, abeille coupeuse de feuille, propagation des agents pathogènes

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Introduction

The fungal pathogen *Botrytis cinerea* Pers.: Fr. is well documented to infect flowers of a broad range of plant species, causing diseases such as blossom blight, soft rot and blossom-end rot, as well as numerous post-harvest diseases (Elad et al. 2007). Flowers are particularly susceptible to infection because they contain nutrient-rich resources that provide ideal growth conditions for various fungi, including *B. cinerea*. In particular, pollen and nectar contain many proteins, sugars, minerals, and amino acids that stimulate conidia germination and may enhance growth rates of *B. cinerea* (Chou & Preece 1968; Huang et al. 1999; Ngugi & Scherm 2006). Petals, thin-walled and lacking a waxy cuticle, are also easily infected by pathogenic fungi, particularly if they have senesced (Huang et al. 2000; Ngugi & Scherm 2006; Olivier et al. 2008). Infection of flowers by *B. cinerea* has been described in many plants, including strawberry (Bristow et al. 1986), raspberry (McNicol et al. 1985), grape (Viret et al. 2004), geranium (Sirjusingh & Sutton 1996) and rose (Elad 1988).

Previous histopathological studies on flower-infecting fungi have documented the growth of fungi down the style towards the ovaries, resulting in infected seed (McNicol et al. 1985; Shinners & Olson 1996; Yang et al. 2010). In a study of the aetiology of blossom blight of seed alfalfa (*Medicago sativa* L.) (Huang et al. 2000), *B. cinerea* could infect developing seeds, and researchers hypothesized a similar mechanism of infection as above. This hypothesis was supported by the observation that *B. cinerea* grows more rapidly in the presence of alfalfa pollen (Chou & Preece 1968; Huang et al. 1999).

Less well documented, however, is the potential for *B. cinerea* to be transmitted vertically – that is, from parent to offspring – via seed and to exist as an asymptomatic endophyte in the offspring. *Botrytis cinerea* infection of flax seed (*Linum usitatissimum* L.) (Harold et al. 1997), primula seed (*Primula* × *polyantha* Mill.) (Barnes & Shaw 2003), lettuce seed (*Lactuca sativa* L.) (Sowley et al. 2009), and alfalfa seed (Huang et al. 2000) results in poor seed germination or increased seedling death. Surviving seedlings may give rise to plants harbouring *B. cinerea* as a symptomless endophyte, which could become pathogenic only once its host is stressed or environmental conditions are conducive for disease development (Barnes & Shaw 2003; Sowley et al. 2009).

Southern Alberta is Canada's largest producer of seed alfalfa, a highly lucrative crop that involves the management of leafcutter bees (*Megachile rotundata* Fab.) for pollination (Pitts-Singer & Cane 2011; Kosinski 2012; CSGA 2014). As in other flowering plants, alfalfa grown for seed is susceptible to blossom blight caused, in part, by *B. cinerea*, which has been identified as a major constraint to seed production on the Canadian prairies in moist growing seasons (Gossen et al. 1997, 1998). Following floret pollination, petals senesce and provide a nutrient-rich base for *B. cinerea* establishment (Huang et al. 2000; Olivier et al. 2008). Under warm (18–22°C), humid (>93% RH) conditions, *B. cinerea* can rapidly colonize a whole raceme (Broome et al. 1994; Sirjusingh & Sutton 1996). A severe blossom blight epidemic could result in conditions suitable for infection of pollen, ovaries and seed; however, infection of these tissues under field conditions has not been documented.

Insect vectors, particularly leafcutter bees, could be contributing to the spread of *B. cinerea* in fields. Insects transmit many economically important phytopathogenic fungi over a range of production systems (Hatcher 1995). Fungus gnat larvae transmit *Pythium aphanidermatum* (Edson) Fitzp. in greenhouse cucumber production (Jarvis et al. 1993), and adults transmit *B. cinerea* in conifer seedling production (James et al. 1995). Fruit fly (*Drosophila melanogaster* Meigen) is capable of transporting viable *B. cinerea* conidia for many days after foraging on infected grape (Louis et al. 1996). Bee pollinators and other insects are known to carry fungal spore loads of both beneficial and pathogenic fungi, including *Verticillium albo-atrum* Reinke & Berth. (Huang et al. 1986), *Sclerotinia sclerotiorum* (Lib.) de Bary (Stelfox et al. 1978), *Trichoderma harzianum* Rifai (Kovach et al. 2000) and *Chlonostachys rosea* (Link) Schroers, Samuels, Seifert & Gams (Kapongo et al. 2008). Consequently, insects have also been implicated as vectors of infected pollen (Stelfox et al. 1978; Huang et al. 1986). It remains unclear, however, whether and under what conditions bee pollinators are significant contributors to disease spread in field contexts, whether they disperse fungal spores or fungal-infected pollen, and whether these methods of dispersal increase the likelihood of seed infection.

The objectives of this study, therefore, were to: (i) describe the infection process of *B. cinerea* on alfalfa florets under greenhouse conditions; (ii) describe the frequency of infected pollen under greenhouse conditions; (iii) determine whether *B. cinerea* could infect the ovaries of flowers under greenhouse conditions; and (iv) evaluate potential for pollinators to vector *B. cinerea* conidia under field conditions.

Materials and methods

Plant and fungal material

Alfalfa 'AC BlueJ' plants were grown from seed in Cornell potting mix (Boodley & Sheldrake 1977) in one-gallon

(3.8 L) pots. Greenhouse conditions were: 16/8 h photoperiod and 22/18°C temperature (day/night), and plants received 600 mL of water a day by drip irrigation.

Colonies of *B. cinerea* isolated from geranium were grown in the dark on potato dextrose agar (PDA) for 5–7 days and then transferred to an incubation chamber and grown under a 12 h dark, 12 h near-UV cycle at 22°C to induce sporulation (16–21 days). For the second repetition of the experiment, *B. cinerea* colonies were reisolated from infected florets in the first experiment, and these isolates were used for inoculations. Plants were cut back to ~5 cm height 2–3 times prior to the experiment to stimulate stem and flower production. The plants were 12 and 14 months old for the first and second repetitions of the study, respectively.

Experimental design

Treatments tested the effects of pollination (untripped vs tripped) and inoculation method (dry vs suspension) in a 2 × 2 factorial design, with a control for each treatment, for a total of 8 treatments. The treatments were: (i) not tripped; (ii) tripped; (iii) not tripped and sprayed with H_2O; (iv) tripped and sprayed with H_2O; (v) not tripped and dry inoculated; (vi) tripped and dry inoculated; (vii) not tripped and suspension inoculated; and (viii) tripped and suspension inoculated. An individual alfalfa plant served as the experimental unit, and seven unpollinated racemes per plant were marked to receive the treatment assigned to that plant. At each sampling time (0, 24, 48 and 96 h post inoculation (hpi)), a single raceme was removed from each plant and the florets were subdivided into vials for processing. An additional sample at 12 hpi was taken for confocal and electron microscopy only. A final raceme was harvested 6 weeks after inoculation to test for seed yield and infection. Each treatment was replicated three times, and plants were placed on a greenhouse bench in a complete randomized block design. The study was performed twice.

Inoculation methods

For treatments in which plants were both pollinated and inoculated, the florets of the racemes were tripped prior to inoculation to expose the pollen to the conidia. A metal dissecting tool was used to pollinate florets; it was loaded with pollen by tripping florets from one unmarked raceme prior to tripping the florets receiving treatment. For dry inoculated plants, a Petri dish of sporulating *B. cinerea* was held inverted over the raceme and the dish was tapped to release the spores. To determine the number of spores released while tapping, the lid of a sterile Petri dish was held below the raceme while tapping the conidia. Two lids were used for each plant (=7 racemes per plant), and the number of spores on the lids were estimated by viewing the Petri dishes under a light microscope (Olympus CK40, 20× magnification), and counting three randomly selected fields of view. The average of the top and bottom Petri dish counts was used to determine the number of conidia per mm^2 per inoculation event. This method determined that between 0.7 to 10.7 conidia mm^{-2} were deposited per raceme inoculation.

For plants receiving inoculation with a suspension, Petri dishes of sporulating *B. cinerea* were flooded with water, scraped with a scalpel, and transferred to a Waring 2-Speed Blender (Cole-Parmer). The solution was mixed on high speed for 60 s, strained through cheesecloth, and the concentration of spores was adjusted to 6 x 10^5 spores mL^{-1} using a hemocytometer. Two drops of Silwet L-77 (Momentive Performance Materials Inc.) were added to the spore suspension, and an atomizer was used to apply the suspension until the raceme glistened.

To maintain 100% relative humidity conditions, a clear plastic bag was placed over each treated raceme and twist-tied to the stem immediately after the raceme received treatment. Plastic bags were removed after 24 h and plants were maintained under normal greenhouse conditions, as described previously.

Floret and pollen infection

At each sampling time, five florets were surface-sterilized with 0.05% NaOCl for 60 s, rinsed in distilled water three times, and plated on *Botrytis* semi-selective medium (BSM) (Edwards & Seddon 2001). Plates were incubated for 7 days in the dark and were rated positive for *B. cinerea* if both (i) the plate colour changed from pink to brown, and (ii) spreading hyphae (>2 cm) and/or conidia were present.

To harvest the pollen from the florets, three florets were placed in a 1.5 mL vial, 1 mL of 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and adjusted to pH 7.4 with HCl) was added to the vials, and the vials were vortexed for 30 s. The florets were removed with forceps and the vials were placed in a centrifuge at 13 000 rpm for 3 min. After centrifugation, the supernatant was removed, 100 µL PBS added to the vial, and the pellet resuspended. 10 µL of the suspension were counted on a hemocytometer to estimate number of pollen grains per floret. In addition, the number of fungal-infected pollen grains out of 100 randomly counted pollen grains was recorded. Both counts were performed twice for each sample.

Pollen viability

To determine the proportion of viable pollen at each sampling period, florets were placed in a 2 mL vial and 1 mL of Carnoy's fixative (6 ethanol: 3 chloroform: 1 glacial acetic acid, v:v:v) was added. Samples were set for at least 2 h but no more than 24 h. Pollen was then isolated in the same manner as above, except that after centrifugation, approximately 900 µL of supernatant was removed, leaving approximately 100 µL in the vial. The pellet was resuspended and 10 µL of the suspension was added to a glass slide. Two to three drops of differential stain for pollen viability (10 mL 95% EtOH, 1 mL malachite green (1% w:v in 95% EtOH), 54.5 mL distilled H_2O, 25 mL glycerol, 5 mL acid fuchsin (1% w:v in H_2O), 0.5 mL Orange G (1% w:v in H_2O), and 4 mL glacial acetic acid) (Peterson et al. 2010) were added and the slide was heated over a flame for 20 s. The slides were then viewed using a light microscope (Olympus CX41) under 10× objective magnification, and 100 pollen grains were counted and assessed for viability. Pollen stained red or orange was considered viable, and pollen stained blue was considered not viable.

Scanning electron microscopy

At each sampling time, three florets from each treatment were fixed in 1 mL of FAA (10 EtOH (95%):1 glacial acetic acid:5 formalin:4 deionized H_2O) until processing for scanning electron microscopy (SEM). Samples were rinsed twice with distilled water, dehydrated through a graded series of ethanol washes (30, 50, 70, 85, 95%, and three changes of 100% EtOH for 15 min each), and critical-point dried in CO_2. Samples were then mounted on aluminium platforms, sputter coated with gold, and viewed on a scanning electron microscope (Hitachi S-3400N) at 5.0 kV at 400× magnification.

Confocal scanning microscopy

As with SEM, at each sampling time, three florets from each treatment were placed in 1 mL of FAA until processing for confocal scanning microscopy (CSM). Processing the samples was similar to SEM preparation, with two alterations: first, after the 85% wash, samples were stained with a 0.005% aniline blue in 95% EtOH (w:v) solution for 25 min and the dehydration was completed as per SEM; second, after the three 100% EtOH washes, samples were cleared using methyl salicylate (Sigma-Aldrich). Samples were viewed with 455 and 633 nm lasers on an Olympus Fluoview FV1000 laser microscope under 10× and 20× magnification.

Data collection of SEM and CSM samples

Data were collected for the 96 hpi for inoculated treatments only. For each sample, two florets were dissected and one calyx, one keel petal, one banner petal, and two staminal columns (stamens + style) were examined. Each tissue sample was scanned for conidia. If conidia were found, the following data were recorded: estimated number of conidia, whether the conidia had germinated, an estimate of the length of hyphal growth, whether the hyphae had infected pollen, and an estimate of the proportion of pollen infected.

Seed counts and viability

Seed pods were harvested 6 weeks after inoculating plants. Pods were surface-sterilized as described previously, split open to expose seeds, and the seeds were plated on BSM and examined for infection, as described previously.

Collection and processing of leafcutter bees from seed alfalfa fields

To investigate whether leafcutter bees may transmit *B. cinerea* spores and/or infected pollen, leafcutter bees were collected from three seed alfalfa fields in southern Alberta throughout the 2013 and 2014 growing seasons. In each season, fields were surveyed on three dates and on each date 10 bees from 10 nests were collected in Falcon tubes, though numbers of bees collected varied depending on weather conditions. Female bees with a large pollen load returning from the field were preferentially collected, and, once caught, were transported in a cooler and stored at −20°C until processing.

To process the samples, 5 mL of 1× PBS were added to the tubes and the tubes were vortexed for 60 s. The concentration of pollen in the suspension was estimated by counting two sides on a hemocytometer, and the suspension was adjusted to 1×10^4 pollen grains mL^{-1}. From each sample, 1000 pollen grains were plated on each of three BSM plates. Following pollen removal, the bees were removed from the vial, patted dry, and plated on BSM. Plates were observed after 7 days for evidence of *B. cinerea* growth.

Statistical analyses

Data from both repetitions of the experiment were tested for homogeneity of variance and were combined for final analysis. Control plants showed trace levels of infection by *B. cinerea* not statistically different from zero, and were

therefore excluded from all analyses, with the exception of the preplanned contrasts described below. Analysis was performed using the GLM procedure in SAS (SAS Institute Inc.), testing for treatment, time and interaction effects. Means were separated by Tukey's HSD. There were not enough pods and seeds produced at the end of 6 weeks for analysis of seed yields, so these data were not analysed.

Preplanned orthogonal contrasts were used to test the effects of (i) pollination and (ii) inoculation on both pollen viability and pollen number. The first contrast tested all pollinated treatments (including controls) against all unpollinated treatments. The second contrast tested all inoculated treatments against all controls. Because there were significant time effects on pollen viability, these contrasts were repeated for each sampling time.

Results

Floret and pollen infection

There was a significant effect of time ($P < 0.05$) on floret infection only, so these data were separated by time (Table 1). Overall, florets collected at 0 hpi had lower infection levels than those collected at 24, 48 and 96 hpi. There were also significant effects of both inoculation method and pollination. Within the dry inoculated samples, florets that were not tripped had significantly ($P < 0.0001$) lower levels of infection (48%) than florets that were tripped (80%). Within the wet inoculated samples, however, there was no significant effect of tripping on infection (87% and 93% for untripped and tripped treatments, respectively).

Pollen infection occurred at levels significantly greater than zero ($P < 0.001$), but was still rare (<1% pollen infection) and occurred in wet inoculated treatments only (Table 2). Under light microscopy, infection was typically observed on small clusters of pollen grains and less frequently on single pollen grains (Fig. 1*a*).

Table 2. The proportion of alfalfa pollen infected by *B. cinerea* over 96 hours and four collection times.

Treatment[a]	% Infected[b]	
DNT	0.013	(0.25)
DT	0.083	(0.24)
SNT	0.861*	(0.25)
ST	0.875*	(0.24)

[a]DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped.
[b]Data are the means (SE) of 100 pollen grains counted under light microscopy at four collection times for six replicates. Means followed by * are significantly different from zero at $P < 0.001$.

Pollen counts and viability

There was a large variation in the number of pollen grains per floret (ranging from 200 to 7500, data not shown) within and between plants, and therefore no significant effects of treatment or time were found on pollen number ($P > 0.05$). Neither pollination nor inoculation method significantly affected pollen viability ($P = 0.5$). However, when contrasts were run including the controls, all treatments exhibited a similar decrease of about 12 percentage points in pollen viability from 0 hpi to 96 hpi ($P < 0.05$, Table 3).

Histopathology

By 96 hpi, conidia were found germinated with hyphae growing on all parts of the floret observed, including the calyx, banner and keel petals, anthers, filament, style and stamen (Table 4; Figs 1*b*, *c* and 2*a*–*c*). The majority of conidia (germinated or ungerminated) and hyphal growth were observed on the keel and banner petals in regions of

Table 1. The effect of inoculation method and pollination on the infection of alfalfa florets by *B. cinerea* over 96 hours.

	% Floret infection[a]				
Treatment[b]	0 hpi	24 hpi	48 hpi	96 hpi	Overall[c]
DNT	30.0 (14.4)	60.0 (18.2)	43.3 (8.0)	56.7 (13.7)	47.5 a
DT	63.3 (11.5)	90.0 (6.8)	83.3 (10.0)	83.3 (6.1)	80.0 b
SNT	76.7 (15.8)	90.0 (9.5)	96.7 (3.3)	83.3 (13.1)	86.7 b
ST	80.0 (13.1)	100.0 (6.1)	100.0 (13.1)	93.3 (4.2)	93.3 b
Overall[c]	62.5 a	85.5 b	80.8 ab	79.2 ab	

[a]Data are the mean (SE) of six replicates over two trials, with five florets plated per treatment per harvest time per trial.
[b]DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped.
[c]Means followed by different letters are significantly different at $P = 0.05$.

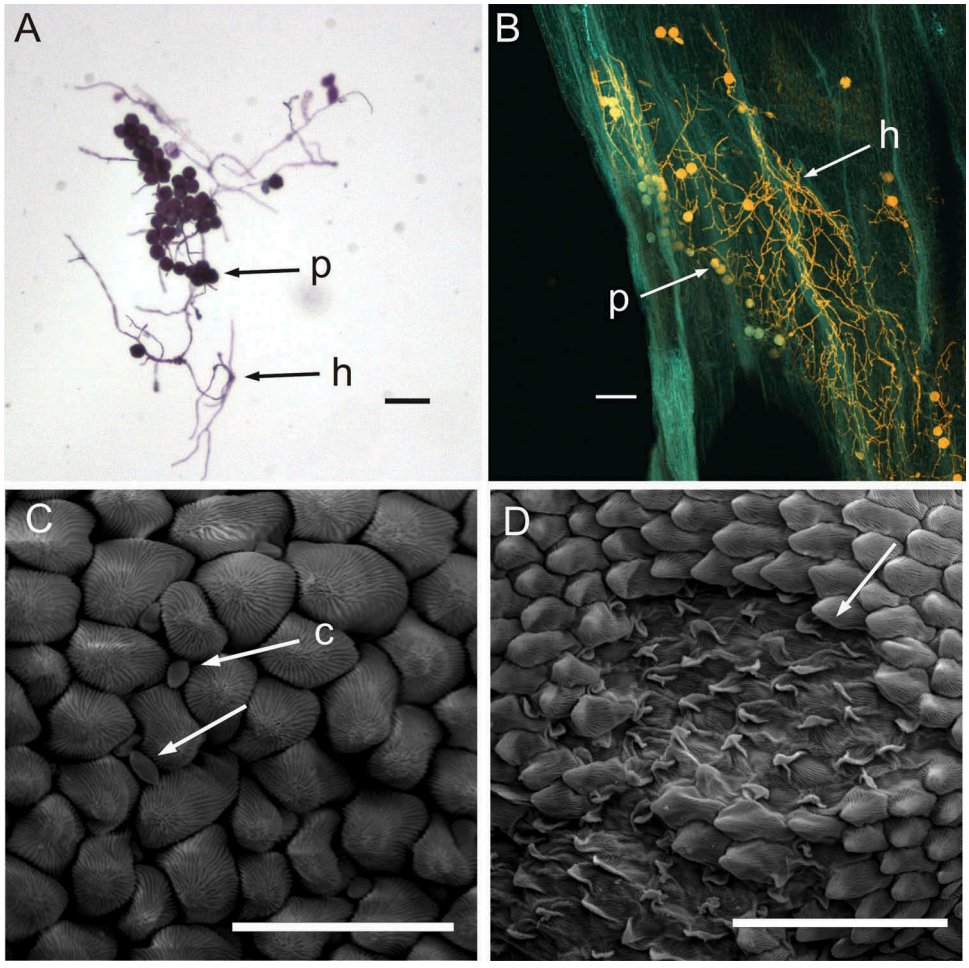

Fig. 1 (Colour online) **a**, Pollen infection by *B. cinerea* as observed by light microscopy 48 hpi (treatment: not tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae; scale bar = 100 µm. **b**, A severely infected banner petal as observed by CLM 48 hpi (treatment: tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae; scale bar = 100 µm. **c**, Conidia of *B. cinerea* are lodged in the grooves of the banner petal but none have germinated, as observed by SEM 96 hpi (treatment: not tripped, dry inoculated); c = *B. cinerea* conidia; scale bar = 50 µm. **d**, Collapsed cell walls on the banner petal associated with *B. cinerea* infection and observed by SEM 96 hpi (treatment: not tripped, dry inoculated); arrow indicates boundary of collapsed cells; scale bar = 100 µm.

Table 3. The effect of inoculation method of *B. cinerea* and pollination on the viability of alfalfa pollen over 96 hours as indicated by a differential stain.

	% Viable pollen[b]			
Contrast[a]	0 hpi	24 hpi	48 hpi	96 hpi
Inoculated	75.8 a	74.9 a	67.7 a	68.2 a
Not inoculated	73.6 a	74.0 a	63.7 a	60.2 a
Tripped	74.5 a	74.0 a	64.6 a	61.7 a
Not tripped	75.0 a	74.9 a	66.9 a	66.7 a

[a]Orthogonal contrasts were run for each collection period and are the mean (SE) of six replicates, with 100 pollen grains counted per harvest time under a light microscope. Inoculated vs Not inoculated tests all inoculated florets (dry and suspension) against all controls; Tripped vs Not tripped tests all tripped florets (treatments and controls) against all not tripped florets.
[b]Means within each set of contrasts followed by the same letter in the same column are not significantly different at P = 0.05.

Table 4. Percentage of alfalfa floret parts on which conidia were observed at 96 hpi.

	% With conidia present[a]			
Treatment[b]	Calyx	Banner	Keel	Pistil/Stamen
DNT	12.5	55.6	28.6	5.6
DT	11.1	18.2	22.2	22.7
SNT	40.0	80.0	44.4	45.0
ST	54.5	100.0	55.6	47.6

[a]Data are pooled results of samples viewed under confocal and electron microscopy, and include both germinated and non-germinated conidia.
[b]DNT – dry inoculated, not tripped; DT = dry inoculated, tripped; SNT = suspension inoculated, not tripped; ST = suspension inoculated, tripped.

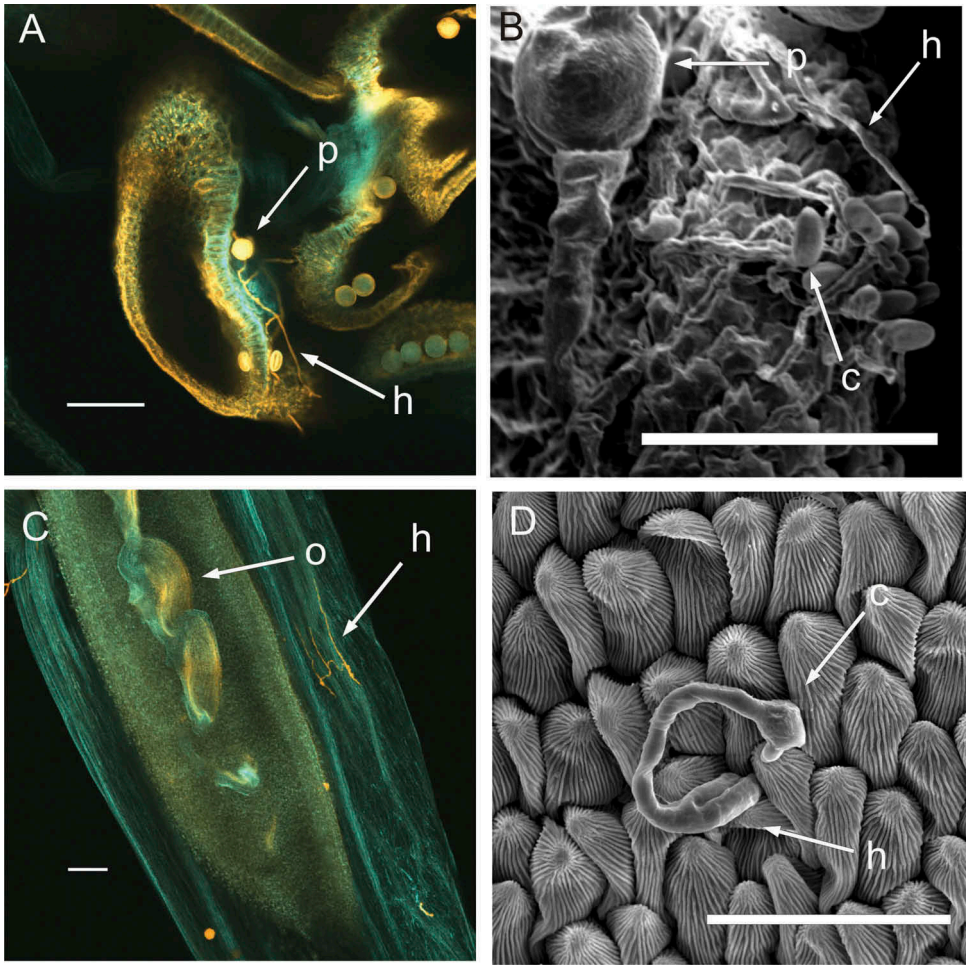

Fig. 2 (Colour online) **a**, Hyphal growth of *B. cinerea* on alfalfa anthers as observed by CLM 96 hpi (treatment: tripped, suspension inoculated); p = alfalfa pollen, h = *B. cinerea* hyphae; scale bar = 100 µm. **b**, Conidia of *B. cinerea* germinate on the anthers of an alfalfa floret, 96 hpi (treatment: tripped, dry inoculated), as observed by SEM; p = alfalfa pollen, c = *B. cinerea* conidia, h = *B. cinerea* hyphae; scale bar = 50 µm. **c**, Hyphae of *B. cinerea* germinates directly on the style, directly over top of the ovaries 96 hpi (treatment: tripped, suspension inoculated), as observed by CSM; o = alfalfa ovary, h = *B. cinerea* hyphae; scale bar = 100 µm. **d**, A conidium of *B. cinerea* penetrates the banner petal directly, 96 hpi (treatment: not tripped, suspension inoculated), as observed by SEM; c = conidium of *B. cinerea*, h = hypha of *B. cinerea*; scale bar = 50 µm.

rougher texture, particularly in suspension inoculated treatments (Figs 1*b*, *c* and 2*d*). Collapsed cells, indicative of lesions, were observed frequently on keel and banner petals (Fig. 1*d*). Hyphae were also seen growing directly on the style, on top of the ovaries (Fig. 2*c*). Appressoria were observed infrequently across all treatments. While inoculations had deposited conidia on all flower parts, by 96 hpi not all conidia had germinated, and this was true for all treatments (Fig. 1*c*).

In some instances, *B. cinerea* colonized the anthers (Fig. 2*a*, *b*), in which case a large proportion of infected pollen was observed. The percentage of pollen infection was greater when estimated by confocal and electron microscopy (1.5 to 2.5% of pollen infected, data not shown) than when estimated by light microscopy (<1%). No pollen infection of dry inoculated treatments was observed.

Leafcutter bees

Pollen collected from leafcutter bees from the field and plated on semi-selective media showed very low levels of pollen infection (<0.001%, data not shown). The bees, however, had a much higher proportion of *B. cinerea* growth, particularly those collected at the end of the season, from which *B. cinerea* was detected almost 100% of the time (Table 5).

Table 5. Average percentage of bees carrying *B. cinerea* conidia over 2 years of collections from four commercial seed alfalfa fields.

	2013[a]			2014		
Collection date	Field 1	Field 2	Field 4	Field 1	Field 2	Field 21
15–23 July	.[b]	.	.	19.4 (9.0)	17.5 (8.8)	0.0 (0.0)
29 Jul–5 Aug	36.0 (11.9)	4.0 (2.7)	8.0 (4.4)	5.0 (3.3)	65.0 (11.6)	12.5 (6.7)
19–21 Aug	100.0 (0.0)	.	100.0 (0.0)	.	96.0 (2.7)	.

[a]Data are the mean (SE) of about 10 bees sampled from 10 nests in each field for each collection period. Following pollen removal, bees were plated on *Botrytis* semi-selective media and examined for *Botrytis* growth 7 days after plating.
[b]No bees collected for the date specified.

Discussion

The goal of this project was to investigate whether, and to what extent, *Botrytis cinerea* can infect alfalfa pollen under conditions that are likely to be experienced in fields of southern Alberta. The region is semi-arid in climate, and, consequently, fields in this area typically experience warm, dry growing seasons. Most seed alfalfa fields, however, are irrigated throughout the growing season, and this added moisture may contribute to conditions conducive for *B. cinerea* growth (Broome et al. 1994).

The greenhouse experiment tested the effects of pollination (tripped vs untripped) and inoculation method (dry vs suspension) on the infection process of alfalfa florets by *B. cinerea* following 24 h of high humidity. Florets collected at 0 hpi had significantly lower infection levels than those collected subsequently, which suggests that the conidia may have been rinsed off during the surface-sterilization procedure prior to full attachment to the tissue. Other studies have shown that adhesion is a passive process and is enhanced by moisture or humidity (Doss et al. 1993; Elad et al. 2007). Both living and dead conidia of *B. cinerea* adhered to a tomato cuticle following dry inoculations, similar to the dry inoculation procedure reported here. The levels of conidia adhesion, however, were greater if the conidia were subsequently hydrated by exposing the tomato tissue to 100% RH (Doss et al. 1993). Thus, the greater levels of floret infection detected at 24, 48 and 96 hpi in this study may be a result of conidia hydration resulting from exposure to 100% RH conditions applied immediately after floret sampling at 0 hpi.

Within dry inoculated florets, there was a significantly greater proportion of infection on tripped than on untripped treatments. Conidia counts from Petri dishes did not reveal any obvious differences between numbers of conidia deposited between the dry inoculated treatments, and, consequently, these results could be explained by either the presence of pollen (Chou & Preece 1968) or of senescing petals on tripped treatments (Huang et al. 2000). Because no infected pollen was observed in dry inoculated treatments, the greater infection levels of pollinated florets support the hypothesis that the senescing petals are more readily infected by *B. cinerea*. This conclusion supports findings by Bristow et al. (1986), who detected no significant increase in virulence of strawberry flowers by *B. cinerea* in the presence of pollen under field conditions.

Within the suspension inoculated samples, there was no statistically significant difference in the proportion of florets infected between treatments that had been tripped and those that had not been tripped. That the overall infection levels were high (87 and 93%) in both treatments suggests that this inoculum delivery method is highly effective at initiating disease. These results are to be expected, given that the high concentration of spores in the suspension (10^5 conidia mL^{-1}) likely resulted in each floret receiving 2–3 orders of magnitude more conidia than dry inoculated florets. Although there was no significant effect of tripping on floret infection, it was observed that, when plated, *B. cinerea* grew more rapidly from those florets that were pollinated than those that were not (data not shown). This observation suggests that conidia deposited on tripped florets had already germinated and colonized the tissue to a greater extent than conidia deposited on untripped florets, which is consistent with the hypothesis that senescing petals are more readily colonized than non-senescing petals (Elad 1997; Li et al. 2004).

The differences between dry and suspension inoculum of *B. cinerea* conidia on the infection process have been the subject of several studies (Williamson et al. 1995; Cole et al. 1996). Similar to those studies, the results presented here show that dry inoculated conidia grew shorter germ tubes than those that were inoculated by suspension, and neither inoculation method resulted in appressoria formation. The presence of water can greatly facilitate tissue colonization by *B. cinerea* (Nair & Allen 1993), which likely explains why extensive growth was observed on treatments inoculated with a suspension

(Fig. 1*d*). Williamson et al. (1995), controlling for condensation at high RH levels, observed high colonization of rose petals by maintaining high humidity (>94%) for 48 h. Sowley et al. (2009), investigating quiescence of *B. cinerea* in lettuce, used a similar method for inoculating lettuce seeds as the dry inoculation method presented here: a Petri dish with a sporulating *B. cinerea* colony was inverted over lettuce seeds, the bottom of the Petri dish was tapped to dislodge the conidia, and the inoculated seeds were covered in a plastic bag for 24 h. This method resulted in infected seeds and, subsequently, in asymptomatic plants that hosted *B. cinerea* as an endophyte. These results demonstrate that in some contexts, dry inoculations can result in *B. cinerea* colonization and delayed disease development.

Previous work in alfalfa has documented the possibility of *B. cinerea* to act as a quiescent pathogen (Huang et al. 2000). Quiescence is typically defined as 'the period from host penetration to activation of fungal colonization and symptom appearance' (Prusky et al. 2013), with the main difference from *latency* being that in quiescence, the pathogen halts growth for an extended period of time. In a greenhouse experiment (Huang et al. 2000), alfalfa florets were pollinated with a toothpick dipped in a *B. cinerea* conidia suspension. Plants were maintained under greenhouse conditions and 5 weeks later, the pods were subjected to 100% RH for several days. Grey mould developed following the high humidity treatment. In the present study, *B. cinerea* exhibited extensive growth on alfalfa florets after exposure to 24 h of 100% RH. Infection levels of florets remained steady after a return to ambient conditions, and obvious signs of increased disease (grey mould) were not observed.

Many ungerminated conidia were observed attached to all floret parts when examined under CSM and SEM. In strawberry, Bristow et al. (1986) found that *B. cinerea* conidia were present on anthers, filaments and sepals 6 days after inoculation, and, when these parts were placed in a humidity chamber, *B. cinerea* could still colonize the tissue. While not a quiescent infection, these findings indicate that a large release of conidia on one day may still become infectious several days later when conditions are conducive. Therefore, even if hot or dry conditions halt colonization after initial infection, the quiescent infection may continue to grow incrementally whenever favourable conditions are present.

CSM and SEM revealed that *B. cinerea* hyphae grew more extensively on the anthers and stamens than other parts of the florets. This finding supports many others that pollen acts as a readily available nutrient source (Chou & Preece 1968; Fourie & Holz 1998; Huang et al. 1999). However, it was not obvious that *B. cinerea* grew preferentially toward pollen; in some cases it did, in others, *B. cinerea* hyphae grew directly beside the pollen but no interactions were observed. Thus, as discussed above, pollen alone unlikely explains increased virulence on pollinated treatments (Bristow et al. 1986). Presence of *B. cinerea* did not affect pollen viability, but viability did decrease over time for all treatments (Table 3). This declining trend is likely the result of the florets ageing over the course of the experiment, or due to the high humidity treatment, which has been documented to reduce pollen viability (Hanson 1961).

Ultrastructural results from the confocal and electron microscope show that *B. cinerea* primarily infects the banner and keel petals, and to a lesser extent the style and anthers (Table 4). Viret et al. (2004) observed *B. cinerea* germination to occur on all parts of grape flowers following 24 h of 100% RH. When the style was inoculated, few germ tubes were observed and there was no indication that hyphae grew towards the ovaries. These results agree with our findings and those of Jung (1956, as cited in Jarvis 1977), who found only very limited growth of *B. cinerea* down the style of all florets from 61 plant species that were tested, including alfalfa.

The pollen collected from the leafcutter bees showed very low levels of fungal infection, as indicated by plating on semi-selective medium. However, when the bees were plated following pollen removal, *B. cinerea* was detected at much greater levels (Table 5). This finding indicates that leafcutter bees do carry *B. cinerea* conidia, but that the conidia may be trapped in regions of the body that make them difficult to remove. The number of infected bees increased in the last collection date (19–21 August) of both seasons, which closely follows the trends of *B. cinerea* incidence observed on plants in the alfalfa fields from which the bees were collected (Chatterton & Reich 2014; Reich & Chatterton 2015). By this time, however, few florets remain on the plants, and the majority of pollination has been completed (Frank 2003).

Given the low levels of pollen infection by *B. cinerea* detected in this study, and because *B. cinerea* inoculum is spread primarily by air, it is unlikely that leafcutter bees contribute significantly to the spread of blossom blight – particularly under conditions that are likely to be encountered in the field. In addition, under the tested conditions, it is unlikely that *B. cinerea* infection will lead to seed infection. Indeed, in surveys of seed alfalfa fields, very little *B. cinerea* infection was observed on seed, and these levels dropped further after harvest (Chatterton & Reich 2014; Reich & Chatterton 2015). As previous literature indicates, and these data support, risk of infection is likely to increase as spore load and precipitation increase (Bulger et al. 1987; Sirjusingh & Sutton 1996; Huang

et al. 2000; Xu et al. 2000; de Araujo et al. 2015). Conidia may remain viable for long periods on host tissue and germinate only when conditions are conducive, and the length of this viability period in the field should be determined experimentally. A long viability period could mean that a single large discharge of conidia early in the season would put a crop at high risk of seed losses for the remainder of the season. Methods of describing seasonal discharge of conidia in seed alfalfa fields of southern Alberta are currently being developed.

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