DEVELOPMENT AND CONTROL OF BOTRYTIS CINEREA IN ALFALFA FLOWERS

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DEVELOPMENT AND CONTROL OF BOTRYTIS CINEREA

ON ALFALFA FLOWERS

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by
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ABSTRACT

Blossom blight of alfalfa was first identified on the Canadian prairies in 1993. It is caused by two common fungal pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. This disease develops rapidly under wet and cool weather conditions, and can result in serious losses in alfalfa seed yield. Strategies for the management of the disease are needed. This study was conducted to develop a quick method to assess the incidence of the two pathogens in alfalfa flowers in the field, to screen cultivars for resistance to infection by *B. cinerea* and to quantify the relationship between environmental conditions and infection incidence.

Various amendments to potato dextrose agar (PDA) and pretreatments in the dark vs. a 14-h photoperiod were explored to develop a semi-selective medium for rapid identification of the causal agents. Amendment with alfalfa leaves in PDA and pretreatment in the dark promoted sporulation of *B. cinerea* and sclerotial formation of *S. sclerotiorum*. A combination of alfalfa leaves and lactic acid in PDA (AL-PDA) or pimaricin, chloramphenicol and alfalfa leaves in PDA (PCL-PDA) resulted in the best suppression of contamination in field samples. AL-PDA also promoted sporulation of *B. cinerea* and sclerotial formation of *S. sclerotiorum*.

The reaction of 12 alfalfa cultivars to infection by *B. cinerea* was evaluated in several tests, including detached flower and whole plant tests in growth cabinets and field tests. There were consistent differences among cultivars in their susceptibility to infection by *B. cinerea*. Cvs. DK 135, OAC Minto and Iroquois were generally less...
susceptible than cvs. Apollo II, Algonquin and Heinrichs. Upward-facing flowers in cv. Vernal were less susceptible (16%) than downward-facing flowers (86%). Purple flowers were less susceptible than white flowers in cv. Iroquois, but not in cvs. Apollo II and AC Nordica.

The impact of wetness duration and temperature on infection of alfalfa flowers by *B. cinerea* was examined under controlled conditions in a split-plot design. The main-plot treatments were different temperatures (10°, 15°, 20°, 25°, 30°C) and the subplot treatments were surface wetness duration (0, 4, 8, 12, 16, 20, 24, 48 h). The incidence of infection of alfalfa flowers was very low at 0, 4 and 8 h of wetness for all temperatures. Incidence increased sharply after 12 h at 20°C, 16 h at 15°C and 20 h at 10° and 25°C. Infection at 30°C was very low. The optimum conditions for infection were between 15° and 20°C, with a minimum of 12 to 16 h of surface wetness.
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1. INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most widely adapted crop species in the world (Barnes *et al.*, 1988). It has the highest feeding value for farm animals of any hay crop and provides high levels of fiber, protein, vitamins and minerals (Barnes and Gordon, 1972; Barnes and Sheaffer, 1985). Alfalfa is also recognized as an effective source of biological nitrogen fixation for the improvement of soil fertility. These characteristics have made alfalfa the most important forage crop species in Canada and the USA, and production of alfalfa seed and hay is an important industry in North America (Barnes *et al.*, 1988; Barnes and Sheaffer, 1985). In 1995, the Pacific Northwest of the USA exported 1.7 million tonnes of hay, and the value of the production from Washington state alone exceeded $100 million (Ford, 1996). In Canada, production of alfalfa seed and hay has increased in the past 20 years. The acreage of pedigreed seed, for example, has increased approximately 65% during this period; the average acreage devoted to pedigreed production was 11,000 ha from 1980 to 1985, 23,000 ha from 1986 to 1995, and 19,000 ha from 1996 to 1998 (Canadian Seed Growers Association, unpublished reports). Production of common seed, which accounts for about 50% of the total alfalfa seed acreage, also showed a similar trend (B.D. Gossen, personal communication).
The production of this crop can be limited by disease. It has been estimated that about one-fourth of the U.S. alfalfa hay crop and one-tenth of the seed yield are lost annually to diseases (Graham et al., 1972). Alfalfa blossom blight, a flower disease caused by *Botrytis cinerea* Pers.:Fr. or *Sclerotinia sclerotiorum* (Lib.) de Bary, was first reported in 1937 on Prince Edward Island (Hurst, 1937) and 1952 in Wisconsin, USA (Jones, 1952). It was identified in western Canada for the first time in 1993 (Gossen et al., 1994), although it had been present in the region for many years (Gossen et al., 1997a).

Epidemics of blossom blight can result in severe yield losses (Gossen et al., 1994). In Saskatchewan, the total seed yield was about 590 tonnes in 1993, 4,400 tonnes in 1994, 4,100 tonnes in 1995, and 8,100 tonnes in 1996, although the total acreage was relatively constant (Anon., 1997; Canadian Seed Growers Association, unpublished reports); correspondingly, blossom blight was severe across the province in 1993, broke out in localized areas in 1994 and 1995, but occurred at low levels in 1996 (Gossen et al., 1997a). The disease contributed to these fluctuations in the yield, although weather conditions have a dominant and over-riding effect on the yield potential of alfalfa seed production in western Canada.

Management of alfalfa blossom blight is dependent on understanding the factors that affect epidemics of the disease. These factors, such as the amount of inoculum in the field, cultivar resistance and the effect of environmental factors on infection, have not been studied in detail. This study was undertaken to identify a method for rapid estimation of pathogen levels in the field (Chapter 3), to evaluate cultivar reaction to infection by *B. cinerea* (Chapter 4), and to quantify the effect of
key environmental factors on infection (Chapter 5). The overall goal was to contribute to the development of a practical disease management strategy for blossom blight in western Canada.
2. LITERATURE REVIEW

2.1. Economic importance of alfalfa

Alfalfa is an important forage crop species in Canada. It is a basic component of feed for dairy and beef cattle, horses, sheep, and other livestock, and is adapted to a wide diversity of soil and climatic conditions (Barnes et al., 1988).

Production of alfalfa is concentrated in certain regions within the northern hemisphere, e.g. Canada, China, France, Italy, Russia, and the USA, and a few countries in the southern hemisphere, e.g. Argentina, Australia, Chile, South Africa, and New Zealand. (Michaud et al., 1988). Together, the USA, Canada, and Mexico grow nearly 42% of the total alfalfa acreage in the world.

In the USA, alfalfa was grown on about 10.6 million ha in 1988, of which more than 60% was harvested for hay and more than 174,000 ha were devoted to seed production. About 58% (44.5 million tonnes) of the total alfalfa hay crop is produced in the northcentral region (Michaud et al., 1988). Alfalfa hay is the second most important crop in Oklahoma, where it was grown on 202,000 ha, with a value of up to $180 million (Shelton, 1992). In Washington state in 1989, alfalfa was grown on 192,000 ha with a total yield of 2 million tonnes of hay, worth approximately $186 million (Ford, 1990).

In Canada, alfalfa accounted for nearly 50% of the total area devoted to tame hay production in 1981, as compared with 37% in 1961 and 44% in 1971 (Michaud
et al., 1988). The largest alfalfa production in Canada occurs in Saskatchewan, Alberta, and Ontario, which together account for more than 70% of the total acreage.


2.2. Management for alfalfa seed production

Production of alfalfa seed depends on pollination by leafcutting bees, _Megachile rotundata_ (F.). The bees overwinter as pupae in cocoons stored at about 4°C. They are then incubated for about 3 weeks at 30°C. After emerging from her cocoon, the female mates and begins building a cell made from leaf cuttings. She deposits an egg and food reserves in the cell, and constructs successive cells in a tunnel. In commercial production, these tunnels are formed from grooved boards of laminated wood or polystyrene. These boards are placed in shelters, with one shelter for each 2 to 4 ha of seed field to be pollinated. Normally, up to 50,000 bees per ha are used. The bees pollinate the alfalfa flowers as they collect pollen and nectar, which are used as the food reserves for the cells. The eggs hatch quickly, and the young larvae feed on the food reserves within the cell. The full-grown larva spins a
cocoon in its cell and develops into a pupa. At the end of the season, the cells with pupa are removed from the tunnels and are stored over the winter to complete the cycle (Goplen et al., 1980).

Management practices for seed production are different from those normally used in forage production. Stands of seed alfalfa are normally thinner than forage stands, with wider row spacing and fewer plants within the row. Irrigation (where available) is generally applied in early spring to maximize flowering, but is restricted before and during flowering to limit extensive vegetative growth. Seed is harvested by straight-combining after desiccation, or by swathing and drying in windrows.

2.3. *Botrytis cinerea*

2.3.1. Taxonomy

The genus *Botrytis* was validated by Persoon in 1801 (Jarvis, 1980a). It includes many economically important pathogens that primarily affect horticultural crops. It is in the class Hyphomycetes of the subdivision Deuteromycotina (Agrios, 1997). The taxonomic characteristics used to differentiate the species in this genus are mainly based on the length, basal swelling and terminal branch structures of conidiophores, sporulation and sclerotial production in culture, and the size of conidia (Jarvis, 1980a; Morgan, 1971a). The mean number of nuclei in terminal and hyphal cells is another important characteristic (Jarvis, 1980a). Hennebert (1973) recognized 22 species within the genus of *Botrytis*, including *B. aclada* (= *B. allii*), *B. cinerea*, *B. fabae*, and *B. squamosa*. Three more species were subsequently added to the genus by Jarvis (1980a).
Botrytis cinerea produces erect, septate conidiophores. Conidia develop from projections that form at the tip of these conidiophores. Conidia are obovoid to almost globose, light brown to hyaline, finely apiculate at the base, and 8-14 × 6-9 μm (Chand, 1997; Domsch et al., 1980; Rubin, 1952). Colonies on agar media are hyaline at first, then grow rapidly, and become grey to greyish brown. Sclerotia are black and irregular in size and shape (Chand, 1997; Domsch et al., 1980).

Within B. cinerea, different isolates may vary slightly in their morphology. Morgan (1971b) assessed 33 isolates of B. cinerea and found that colony colour, production of sclerotia, and sporulation were the most important characteristics for distinguishing two groups of isolates; 26 isolates had cream or white colonies, sparse sporulation, and abundant sclerotia production on media (group A), and the other 7 isolates developed grey colonies with profuse sporulation and few sclerotia (group B). However, the relationship between these morphological characteristics and the pathogenicity of the groups were not studied.

Isolates of B. cinerea also differ in their tolerance to fungicides. In one study, detached cucumber cotyledons were treated with various fungicides and inoculated with 12 isolates of B. cinerea. Lesion size varied, and the incidence of infection ranged from 0 to 100% (Elad et al., 1992). In a study of 706 isolates of B. cinerea isolated from greenhouse tomato in England and Wales, 63% of the isolates were resistant to benomyl and 43% to iprodione, based on colony growth on PDA supplemented with fungicide (Locke and Eletcher, 1988). Similar variation in fungicide sensitivity among 18 isolates was reported in a study in which the incidence of infection on tomato fruits treated with dicarboximides and
benzimidazoles varied from to 0 to 90% and 0 to 100%, respectively (Pappas, 1997). In the absence of fungicides, isolates with a high tolerance to fungicides often have poor growth and weak sporulation relative to sensitive isolates (Choi et al., 1996; Rubin, 1952; Wang and Coley-Smith, 1986).

Sclerotia of Botrytis usually produce mycelial threads that can infect the host directly. However, sclerotia of some Botrytis species, including B. cinerea, occasionally produce a Botryotinia perfect stage, in which ascospores are produced from apothecia (Agrios, 1997). The perfect stage of B. cinerea is Botryotinia fuckeliana (de Bary) Whetzel, which is not important in the epidemiology of most botrytis diseases (Braun and Sutton, 1987; Jarvis, 1977, 1980a).

2.3.2. Host range

Botrytis species are ubiquitous and function both as saprophytes and plant pathogens. They are important pathogens on stored fruits, vegetables, ornamental crops and nursery stock (Elad and Shtienberg, 1995; Hausbeck and Moorman, 1996; Jarvis, 1980a). Botrytis cinerea has a wide host range that includes over 200 plant species (Jarvis, 1980a). Common hosts include grape, strawberry, tomato, cabbage, lettuce, pepper, eggplant, cucumber, alfalfa, chickpea, pea, lentil, bean, flax, geranium, carnation, chrysanthemum, tulip, rose, and black spruce (Chand, 1997; Domsch et al., 1980; Elad, 1991; Elad et al., 1993; Elad and Volpin, 1993; Gossen et al., 1994; Hausbeck and Moorman, 1996; Zhang and Sutton, 1994).

Some Botrytis species are host specific. For example, B. fabae is restricted to plants in the Leguminosae (Jarvis, 1977), whereas B. sphaerosperma is specific to Allium triquetum (Hennebert, 1973). Within B. cinerea, variation in virulence has
also been reported. In a study of six isolates of *B. cinerea* from chickpea collected at different locations in India, two isolates were strongly virulent on two chickpea cultivars, two were weakly virulent and two showed a differential reaction on the two cultivars (Rewal and Grewal, 1989b). Differences in virulence were also reported among isolates of *B. cinerea* on carrot (Sharman and Heale, 1979).

### 2.3.3. Epidemiology of diseases caused by *Botrytis* spp.

#### 2.3.3.1. Disease cycle

In many crop systems, *Botrytis* spp. overwinter as mycelium in soil or dead plant debris, and as sclerotia in the soil (Fold and Haglund, 1963; Jarvis, 1962d). Conidia of *B. cinerea* may be produced from mycelia or sclerotia (Jarvis, 1962a, 1977; Maude, 1980), and are dispersed mainly by air currents, rain splash and insects (Jarvis, 1962a, 1962b, 1962c, 1962d; Silow, 1934). Conidia can germinate and penetrate plant tissue through undamaged epidermal cells and stomata (Clark and Lorbeer, 1976), or through wounds (Jarvis, 1980b). Infected cells eventually collapse and disintegrate. Conidia are produced on these rotted tissues and initiate another infection cycle in plants.

#### 2.3.3.2. Infection by *Botrytis cinerea*

The infection process of *Botrytis* spp. involves three phases: germination, penetration and establishment (Goodman *et al*., 1986). The first two phases are extremely sensitive to microclimatic influences. In the presence of free water, conidia of *B. cinerea* usually initiated germination after 4 h at 22°C, with 50% of the conidia germinated after 7 h (Carre and Coyier, 1984). Germination of *B. cinerea* conidia occurred between 4° and 25°C on petals of gerbera (Salinas *et al*., 1989) and
germination was fastest at 20°C (Rewal and Grewal, 1989b). The process of penetration usually lasts 2-3 h (Elad and Shtienberg, 1995). Therefore, a minimum of 6-7 h is required for conidia to establish infection. After penetration, the infective mycelium is relatively protected by the host tissue and the temperature and RH requirements are less stringent.

Many botrytis diseases may have a latent period (Jarvis, 1977). For example, in fruit rots caused by *B. cinerea*, infection may occur at blooming or early in the development of the fruit, but symptoms do not developed until the fruit ripens (Verhoeff, 1980). Latent infections by *B. cinerea* is relatively common on flowers of strawberry, grape, and apple (Jarvis, 1977).

2.3.3.3. Management of diseases caused by *Botrytis*

Diseases caused by *B. cinerea* can be managed using a variety of approaches in the field and in stored and market products. The first approach is sanitation. Infected plant debris and weed hosts can be removed to reduce sources of inoculum (Agrios, 1997; Hausbeck and Moorman, 1996; Maude, 1980; Peng et al., 1992). Sanitation can be carried out by using crop rotation in the field, or by steaming, heating or applying fungicides and disinfectants in the greenhouse (Agrios, 1997; Elad and Shtienberg, 1995).

Environmental control is also important in disease management. High relative humidity or free water is required for infection from conidia of *B. cinerea*. Measures to reduce the duration of free moisture on plant surfaces may result in reduced levels of disease. These measures can be achieved by increasing the space between plants, by planting crops in rows parallel to the prevailing wind, or by
selecting cultivars with an open canopy architecture (Bond et al., 1994; Chand, 1997; Hausbeck and Moorma, 1996; Peng et al., 1992). However, the quantitative relationship between environmental factors and infection of alfalfa flowers was not known when this study was initiated.

Fungicides can be effective as protectants against infection by *B. cinerea* (Gossen et al., 1997a, 1997b). However, fungicides have to be applied prior to establishment of infection to be effective (Peng et al., 1992). Fungicide efficacy can also be affected by the amount of inoculum, weather conditions, and fungicide resistance in the pathogen population (Brands, 1971; Chand, 1997; Elad and Shtienberg, 1995; Hausbeck and Moorman, 1996; Jarvis, 1966, 1969).

Plants have a variety of natural defenses to protect themselves against infection by *B. cinerea*, including open plant architecture (reduced humidity) (Michailides, 1991; Vail and Marois, 1991), early flowering and maturation (disease escape) (Bajpai et al., 1995), and flower colour (protects the cell membrane from deterioration) (Tamura and Yamagami, 1994). Also, cultivars that are less susceptible to botrytis diseases often have thickened cuticles or cell walls (Commenil et al., 1997; Hammer and Evensen, 1994; Rijkenberg et al., 1980).

2.4. *Sclerotinia sclerotiorum*

2.4.1. Infection cycle

*Sclerotinia sclerotiorum* is the other pathogen involved in the alfalfa blossom blight complex (Holley et al., 1996). This fungus overwinters as sclerotia or as mycelium in infected debris in soil. Sclerotia germinate to produce apothecia or mycelium. Ascospores from apothecia play a major role in diseases caused by *S.*
sclerotiorum on canola and other hosts (Cook et al., 1975; Morrall and Dueck, 1982). Ascospores germinate readily on senescent plant parts such as old blossoms, resulting in infection. In many crops, infection occurs only occasionally from mycelium from germinating sclerotia in soil (Purdy, 1979).

Symptoms caused by S. sclerotiorum vary with the host, the tissues affected, and environmental conditions. Infected flowers develop small, watery, light brown spots on the petals. These spots later enlarge, coalesce, and involve the entire flowers under wet conditions. Eventually the whole flower becomes dark and drops off the plant (Agrios, 1997). A typical symptom on many other tissues is the appearance of white, fluffy mycelial growth.

2.4.2. Management of diseases caused by Sclerotinia sclerotiorum

The management of diseases caused by S. sclerotiorum depends mainly on cultural practices and fungicide application. Since diseases caused by S. sclerotiorum are monocyclic (Morrall et al., 1991), reduction in the levels of initial inoculum is important for disease management. Common cultural practices include crop rotation, tillage, weed control, field sanitation, and soil sterilization (Adams and Ayers, 1979; Agrios, 1997; Steadman, 1979, 1983; Williams and Stelfox, 1980). Initial inoculum (ascospores) of S. sclerotiorum may be transmitted from other fields and sclerotia of the pathogen may survive in the soil for at least three years. As a result, measures such as field sanitation and crop rotation are relatively ineffective in the field (Agrios, 1997; Morrall and Dueck, 1982; Turkington and Morrall, 1993).

Foliar fungicide application can provide effective management of diseases caused by S. sclerotiorum (Morrall et al., 1989; Morrall and Thomson, 1995;
Steadman, 1979). To be economical, application of fungicides needs to occur at the correct time, e.g. prior to ascospore release. To maximum the efficacy of fungicides for stem rot of canola in western Canada, a forecasting system was developed. The system used petal infestation to forecast disease incidence (Morrall and Thomson, 1995; Morrall et al, 1991; Turkington and Morrall, 1993; Turkington et al., 1988, 1991a, 1991b). The rationale underlying the forecasting system is based on the role of petals in the disease cycle. Dead or dying petals provide a saprophytic food base for \textit{S. sclerotiorum}, without which ascospores are not able infect leaves or stems of canola (Morrall et al., 1991). The petal infestation in a field was estimated by sampling 40 petals per site and 5 sites per field. The rates of petal infestation were integrated with weather conditions and crop density to estimate the risk of disease. Fungicide application was not triggered until at least a moderate disease risk was present. Fungicide application rates were also adjusted according to the disease risk.

Genetic resistance to \textit{S. sclerotiorum} has been observed in some host species, e.g. purple flowers of alfalfa are slightly more resistant than white flowers (Lefol et al., 1999), but this resistance is generally weak, and only quantitatively inherited. In bean, resistance to this pathogen was associated with open canopy architecture, which allowed faster drying of leaves and stems after rain or dew and therefore inhibited lesion development (Coyne et al., 1974). In \textit{Brassica carinata}, cultivars with a leaf abscission reaction to infection by \textit{S. sclerotiorum} exhibited a slower rate of lesion expansion on stems since leaf abscission resulted in a reduced inoculum level (Sedun et al., 1989).
2.5. Blossom blight of alfalfa on the Canadian prairies

Alfalfa usually starts to flower around the end of June and flowering generally continues for 5 to 6 weeks. In late July of 1993, an outbreak of blossom blight was noted in alfalfa seed production fields across western Canada (Gossen et al., 1994). *Botrytis cinerea* was isolated from infected flowers and its pathogenicity was confirmed in a greenhouse test. *Sclerotinia sclerotiorum* has been isolated from alfalfa flowers since 1991 and its pathogenicity was also confirmed (Holley et al., 1996). These two pathogens form the pathogen complex that is responsible for blossom blight of alfalfa in this region. Wet, cool weather appears to be the necessary precondition for epidemics of the disease (Gossen et al., 1994).

Symptoms of the disease begin as small, water-soaked spots along petal edges, which coalesce rapidly to affect the whole flower. Infected blossoms abscise prematurely without setting seeds. The mycelium spreads quickly from blossom to blossom within the raceme, and one common symptom of the disease is that infected flowers remain attached to the raceme by strands of mycelium (Gossen, 1997). Since symptoms are often subtle and indistinct in the early infection stage, they generally cannot be used for disease identification and assessment. Therefore, the frequency of flower infestation has been used as the main diagnostic characteristic (Gossen and Wong, 1998).

Field surveys were conducted across the prairies from 1994 to 1998 by sampling the oldest floret in randomly selected racemes and placing them onto nutrient media to estimate the amount of pathogen inoculum. Since samples were not surface-sterilized, the frequency of recovery of the pathogens in flowers
estimates pathogen incidence instead of infection frequency. Severity was also evaluated by visually assessing symptoms on flowers when disease levels were high. The results showed that the disease occurred in localized areas across the prairies in 1994 (Gossen et al., 1994, 1997a). In 1995, the incidence of B. cinerea in alfalfa flowers averaged more than 60% in Saskatchewan in July and remained at high levels in southern and northern areas in August, although a sharp reduction was observed in the central region (Gossen et al., 1996). In northern Alberta, the incidence of B. cinerea was high in July, with a mean of 79% and range of 37 to 98% in 16 fields surveyed, while the incidence of B. cinerea and S. sclerotiorum was low in southern Alberta and Manitoba. Botrytis cinerea was the dominant pathogen across the prairies in 1995 (Gossen et al., 1996).

In 1996, the incidence of the two pathogens on alfalfa flowers was low throughout the season in southern Alberta, although the incidence of S. sclerotiorum was high in a few fields (Gossen et al., 1997a, b, c). In northern Alberta, both B. cinerea and S. sclerotiorum occurred at high levels. In Saskatchewan and Manitoba, pathogen incidence was generally low at early bloom, but increased over time and reached moderate to high levels in most regions by the late-bloom stage. Botrytis cinerea was generally the dominant pathogen whenever the incidence was high (Gossen et al., 1997a, b, c).

In 1997, the incidence of B. cinerea and S. sclerotiorum was generally low in Saskatchewan and southern Alberta. In northern Alberta, the levels of B. cinerea were high during the entire flowering season (Holley et al., 1998).
In 1998, hot and dry weather across most of Saskatchewan resulted in low incidence of the two pathogens. An exception was reported in an area in northeastern Saskatchewan, where heavy rainfall resulted in high levels of infestation at mid and late flowering (Gossen and Platford, 1999). In Alberta, the pathogens occurred at moderate to high levels after cool and wet conditions at early flowering, but the incidence declined with the onset of hot and dry weather at late bloom (Gossen and Platford, 1999).

2.6. Fungicide management of alfalfa blossom blight

Field trials to assess the impact of foliar fungicides on blossom blight incidence and seed yield were conducted across the prairie provinces from 1995 to 1998 (Gossen et al., 1996, 1997a; Gossen and Wong, 1998). Benlate, Bravo, Dithane, Ronilan, and Rovral were evaluated in these trials. In 1995, Benlate reduced the incidence of *B. cinerea* in flowers, but Bravo and Rovral rarely had an impact on the incidence of *B. cinerea*. Both Benlate and Bravo increased seed yield by 50-80% at sites where pathogen levels were high (Gossen et al., 1996).

In 1996, Benlate consistently reduced the incidence of *B. cinerea* and increased seed yield by 25-100% in three commercial fields in Saskatchewan (Gossen et al., 1997a). Ronilan reduced infection by *S. sclerotiorum* and increased seed yield by 16% at one site in Alberta, but had no effect on most sites. Bravo did not reduce flower infection, but improved the yield at one site in Saskatchewan.

In 1997, Benlate, Bravo and Dithane increased seed yield by 18%, 14% and 12%, respectively, at a site in Saskatchewan where *S. sclerotiorum* was the dominant pathogen (Gossen and Lan, 1998). In 1998, Benlate reduced the incidence of *B.
cinerea and increased seed yield by 17% at Ridgedale, SK. At MacDowall, SK, fungicide application increased yield by 7% with Bravo and by 17% with Dithane (Gossen and Wong, 1998).

Overall, Benlate provides the best control of the pathogens and the most consistent increase in alfalfa seed yield. However, when pathogen incidence was low, e.g. at Atwater, SK in 1998, fungicide application did not affect pathogen incidence or seed yield (Gossen and Wong, 1998).
3. ASSESSMENT OF *BOTRYTIS CINEREA* AND *SCLEROTINIA SCLEROTIORUM* FROM ALFALFA FLOWERS

3.1. Introduction

Inoculum is a driving component in the development of plant disease epidemics (Campbell and Madden, 1990). Its interaction with the host and environment influences the rate of disease development, and the duration and intensity of epidemics. When little or no inoculum is present, or if the weather is not conducive to disease increase, control measures such as fungicide application may not be required. However, if both inoculum and favourable weather are present, a timely fungicide application can be critical. Rapid and realistic estimation of pathogen inoculum is, therefore, an important part of the decision-making process.

For alfalfa blossom blight, early disease symptoms on flowers are often subtle and indistinct, and are unsuitable for disease identification and assessment. The presence of inoculum in flowers provides a good diagnostic characteristic, and is considered to be a realistic indicator of available inoculum. Colony morphology of *B. cinerea* and *S. sclerotiorum*, the pathogens that cause alfalfa blossom blight, is sufficiently distinctive that individuals who are familiar with these fungi can distinguish them from most contaminants. However, colony morphology alone may not be a reliable diagnostic tool for producers who have little background in
mycology. Sporulation of *B. cinerea* and sclerotial formation by *S. sclerotiorum* are more reliable characters for identification, although these characteristics normally take much longer to develop.

A diagnostic medium should promote the recovery of the target pathogen. Potato dextrose agar (PDA) has been used routinely for isolation of many fungi. However, diverse populations of fungi and bacteria are present on alfalfa flowers. On a general growth medium such as PDA, it is often difficult to recognize colonies of specific fungi such as *B. cinerea* and *S. sclerotiorum* growing among many other colonies, and a more selective medium is required. Amendments such as antibiotics, acids, salts, and fungicides have been used in media to inhibit or stimulate certain microorganisms, and thereby increase the recovery of the target microorganisms. Although these amendments can effectively inhibit contaminants, they may also inhibit the desired microorganism.

Several semi-selective media have been developed for isolation of these two pathogens. In one study, the fungicides PCNB (pentachloronitrobenzene), Maneb (manganese ethylene bisdithiocarbamate), Rubigan (fenarimol), and CuSO₄ were added to a basic growth medium to inhibit fungal contaminants such as *Penicillium* and *Trichoderma* spp. (Kerssies, 1990). In another study, *B. cinerea* was distinguished from *B. allii* by using sorbose and Euparen \([N,N\text{-dimethyl-}N\text{-phenyl-}(N\text{-fluodichlorethylthio}) \text{ sulfamid}]\) in PDA to inhibit the growth of *B. allii* (Netzer and Dishon, 1967). Low pH and antibiotics such as chloramphenicol, streptomycin and erythromycin were used to reduce populations of bacterial contaminants when isolating *Botrytis* spp. from onions, leeks, and gerbera (Kerssies, 1990; Presly.
1985), and for isolation of *Verticillium* and *Septoria* spp. from celery seed (Maude, 1963; Talboys, 1960). Acidified media are often used when isolating fungi from food (Koburger and March, 1984) and have been used to detect *B. cinerea* and *S. sclerotiorum* in alfalfa flowers (Gossen et al., 1996, 1997c).

Sporulation is an important diagnostic character for *B. cinerea*, so a medium that induces early sporulation would be useful for rapid and reliable identification of the pathogen. Many factors affect the sporulation of *B. cinerea*, including cultural conditions, nutrients, and the osmotic potential of the medium (Elad et al., 1993; Nicot et al., 1996; Verhoeff et al., 1988). Sporulation of *B. cinerea* is stimulated by near ultraviolet (NUV), black and far-red light (Domsch et al., 1980; Elad, 1997; Leach, 1962; Tan and Epton, 1973). When cultures of *B. cinerea* were covered with plastic films containing additives that absorb NUV, sporulation was delayed (Nicot et al., 1996). In another study, *B. cinerea* sporulated best at 15°C under NUV (Presly, 1985). In contrast, incubation in darkness produced more rapid mycelial growth of *B. cinerea* than in continuous light (Rubin, 1952). Initial growth in the dark, following by incubation in the light, has been used to enhance sporulation. For example, after 3 days of incubation in darkness and 2 days with a 12-hr photoperiod, abundant sporulation was obtained from inoculated chickpea leaves (Laha and Grewal, 1983).

Nutrient content of the food source is another factor affecting growth and sporulation of *B. cinerea*. For example, hyphal growth and sporulation is better on freshly prepared PDA than on commercial PDA (Ogawa et al., 1978). Treating young fruits of cucumber with glucose and phosphate increased disease incidence of
*B. cinerea*, whereas washing the fruit decreased incidence (Elad *et al.*, 1993). The wash water contained 25 µg glucose per fruit. This indicates that nutrients on the plant surface are critical for infection or disease development. Addition of sodium nitrate, sodium sulphate and sucrose to bean leaf extract agar promoted sporulation of *B. fabae*. The salts and sucrose increased osmotic stress, which favour sporulation of *B. fabae* (Leach and Moore, 1966). Also, adding trypticase soy to a basic medium increased recovery of heat-injured *B. cinerea* (Day *et al.*, 1987).

Diagnosis of *S. sclerotiorum* is also required for assessment of blossom blight incidence or inoculum potential in the field. PDA amended with ampicillin and streptomycin has been used to detect *S. sclerotiorum* infestation of canola petals (Turkington *et al.*, 1988). These two antibiotics have only minor inhibitory effects on the growth of *S. sclerotiorum*, but provide good control of bacteria (Turkington, 1988). Similarly, a combination of PCNB, penicillin, and streptomycin in PDA was used to reduce bacterial and fungal contaminants when isolating airborne ascospores of *S. sclerotiorum* (Ben-Yephet *et al.*, 1985; Steadman *et al.*, 1994). Light may also affect mycelial growth of *S. sclerotiorum*. When greenhouses were covered with polyethylene films that block the light in the far-red region of the spectrum, the incidence of white mold caused by *S. sclerotiorum* was reduced by 50% to 90% on stems and fruit of cucumber plants (Elad, 1997). This indicates that infection by the pathogen is inhibited in the absence of far-red light.

There are many examples in the literature for selective isolation of two pathogens at the same time. Also, reduction in contamination has frequently been demonstrated. However, these two objectives are rarely combined into one method.
The objective of this study was to develop a rapid and effective method to assess the incidence of *B. cinerea* and *S. sclerotiorum* in the field, by combining measures to promote the growth of *B. cinerea* and *S. sclerotiorum* with measures to inhibit contamination.

### 3.2. Materials and Methods

#### 3.2.1. Pathogen isolation, maintenance and inoculum

Two fungal isolates were used in the study: isolate Nov9543 of *B. cinerea* and Nov9541 of *S. sclerotiorum*. Both isolates were originally obtained from naturally infected florets of alfalfa in Alberta, and maintained on PDA. Inoculum of *B. cinerea* was harvested by washing spores with sterile distilled water plus surfactant (2 drops of Triton X-100 per 100 ml of water) from 10- to 15-day-old cultures grown on PDA. The spore suspension was adjusted to a concentration of $1 \times 10^5$ conidia ml$^{-1}$. Inoculum of *S. sclerotiorum* was grown in potato dextrose broth at room temperature with 14-hr days. After 7 days, the mycelial colonies were homogenized in a blender, and the suspension was diluted to a concentration of $10^5$ mycelial fragments ml$^{-1}$.

#### 3.2.2. Rapid identification of *B. cinerea* and *S. sclerotiorum* in the field

In preliminary experiments, *B. cinerea* and *S. sclerotiorum* were cultured on PDA with various amendments. Growing the pathogens on PDA amended with alfalfa leaves and incubating the cultures in the dark for the first 3 days promoted radial growth and sporulation of *B. cinerea* and had no substantial effect on *S. sclerotiorum* compared to acidified PDA (the standard medium) (Appendix A). In addition, the impact of several anti-bacterial and anti-fungal agents on the growth of
these pathogens was assessed (Appendix B). Based on the results of these preliminary tests, the treatments were chosen for field trials in 1997 and 1998.

In 1997, the following antibiotics/ingredients were added to PDA (per litre) and assessed for their impact on pathogen development: (i) PDA (Control); (ii) PL-PDA: 5 mg pimaricin and 50 g homogenized alfalfa; (iii) CL-PDA: 200 mg chloramphenicol and 50 g alfalfa leaves; (iv) NL-PDA: 50 mg novobiocin and 50 g alfalfa leaves; (v) PNL-PDA: 5 mg pimaricin, 50 mg novobiocin and 50 g alfalfa leaves; (vi) PCL-PDA: 5 mg pimaricin, 200 mg chloramphenicol and 50 g alfalfa leaves; (vii) A-PDA: 3 ml 85% lactic acid; (viii) AL-PDA: 3 ml 85% lactic acid and 50 g alfalfa leaves.

In 1998, four media were selected for further testing based on the results in 1997. They were A-PDA, AL-PDA, PCL-PDA and a control (PDA), with amendments at the same concentration as in 1997. Pimaricin and novobiocin are heat stable, so they were added with the leaves prior to autoclaving. Lactic acid and chloramphenicol were added after autoclaving. Inoculum of the two pathogens was prepared as described previously.

Field trials to evaluate these media were carried out eight times (repetition A to H). In repetitions A (July 14, 1997) and B (July 30, 1997), flowers were collected from a commercial seed field near Watson. These two trials were not inoculated, but samples were taken after a period of wet and cool weather. All of the subsequent repetitions (C to H) were conducted at the AAFC research farm at Saskatoon, SK, and were inoculated with B. cinerea (C, D, F, and G) or S. sclerotiorum (E and H) during flowering. Inoculum was applied until runoff with a backpack air-pressure
sprayer. Inoculation was conducted under various weather conditions; clear skies (C on July 29, 1997, D on August 7, 1997, and E on August 25, 1997), after a heavy rain (F on August 21, 1998), or under cloudy conditions (G on August 29, 1998 and H on September 9, 1998). In order to favour infection, alfalfa plants in repetitions D to H were mist-irrigated for 3 min at 9 pm immediately prior to inoculation and on each day between inoculation and sampling.

The oldest floret from randomly selected racemes (16 florets in repetition F, 8 florets in all others) was collected 2 or 3 days after inoculation from each of five sampling sites (replications) in the field and placed onto media without surface sterilization. The flowers were incubated in the dark at room temperature for 3 days, and the number of contaminant colonies was counted. The plates were then transferred to a culture room and incubated under fluorescent light at room temperature with a 14-h photoperiod. The number of flowers infested with *B. cinerea* (based on sporulation) or *S. sclerotiorum* (based on formation of sclerotia) was recorded daily.

**3.2.3. Statistical analysis**

Analysis of variance of the number of contaminant colonies and the incidence of flower infestation for each repetition was performed using the Statistical Analysis System (SAS Institute, 1985). The means were compared using Duncan’s Multiple Range Test at $P \leq 0.05$.

**3.3. Results**

The incidence of *S. sclerotiorum* recovered from alfalfa flowers was high in repetitions A and B at Watson in 1997. In repetition A, incidence was 90% on A-
PDA by Day 8, but only 40% on PDA (Table 3.1). A similar pattern of results was observed at Day 10, with 93% incidence on A-PDA and 55% on PDA. In repetition B, the incidence of *S. sclerotiorum* on Day 14 was higher ($P \leq 0.05$) on A-PDA (70%) and PCL-PDA (73%) than on the other media (Table 3.1).

*Botrytis cinerea* was recovered only on PNL-PDA (3%) and A-PDA (3%) in repetition A (data not shown). It was present on all of the media in repetition B, with incidence ranging from 3% (PDA) to 35% (PCL-PDA) at Day 14. Recovery of *B. cinerea* was higher ($P \leq 0.05$) on PCL-PDA than on the other media except A-PDA (Table 3.1).

The incidence of contaminants on the various media was assessed on Day 3. In repetition A, all of the amendments reduced the number of bacterial colonies relative to PDA ($P \leq 0.05$). PCL-PDA also reduced ($P \leq 0.05$) the number of fungal contaminant colonies compared to PDA and PL-PDA. The other amendment combination produced intermediate numbers of fungal contaminants. The total number of contaminant colonies was lower ($P \leq 0.05$) on PCL-PDA, A-PDA, NL-PDA and CL-PDA than on PDA (Table 3.2). In repetition B, CL-PDA resulted in lower number of bacterial colonies than PDA, PL-PDA and PNL-PDA. PCL-PDA, A-PDA, PL-PDA and PNL-PDA reduced ($P \leq 0.05$) the number of colonies of fungal contaminants relative to PDA; PCL-PDA had lower numbers of fungal contaminants than any other media. The total number of contaminants on PCL-PDA and A-PDA was lower ($P \leq 0.05$) than on PDA, PL-PDA, NL-PDA and PNL-PDA (Table 3.2).
Table 3.1. Recovery of *Sclerotinia sclerotiorum* (S. *s*) and *Botrytis cinerea* (B. *c.*) from alfalfa flowers on PDA with various amendments at two sampling dates (repetitions A and B) in a commercial alfalfa seed field at Watson, SK in 1997 (n = 5)

<table>
<thead>
<tr>
<th>Media†</th>
<th>Repetition A (S. <em>s</em> only*)</th>
<th>Repetition B‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6</td>
<td>Day 8</td>
</tr>
<tr>
<td>PDA</td>
<td>18 ab</td>
<td>40 b</td>
</tr>
<tr>
<td>A-PDA</td>
<td>28 a</td>
<td>90 a</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>15 ab</td>
<td>23 bc</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>8 ab</td>
<td>33 bc</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>13 ab</td>
<td>30 bc</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>5 b</td>
<td>15 c</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>10 ab</td>
<td>28 bc</td>
</tr>
<tr>
<td>Mean</td>
<td>13.9</td>
<td>32.3</td>
</tr>
</tbody>
</table>

† PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimaricin.

‡ The mean frequency of *B. cinerea* in repetition A was less than 1%, so the data are not shown.

§ Assessed on Day 14.

|| Means within a column followed by the same letter do not differ based on Duncan’s Multiple Range Test at *P* ≤ 0.05.
Table 3.2. Mean number of contaminant colonies per dish from alfalfa flowers on PDA with various amendments at two sampling dates (repetitions A and B) in a commercial alfalfa seed field at Watson, SK in 1997 (n = 5)

<table>
<thead>
<tr>
<th>Media</th>
<th>Repetition A</th>
<th></th>
<th></th>
<th>Repetition B</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fungi</td>
<td>Bacteria</td>
<td>Total</td>
<td>Fungi</td>
<td>Bacteria</td>
</tr>
<tr>
<td>PDA</td>
<td>10 b</td>
<td>14 b</td>
<td>24 b</td>
<td></td>
<td>29 e</td>
<td>18 b</td>
</tr>
<tr>
<td>A-PDA</td>
<td>6 ab</td>
<td>2 a</td>
<td>7 a</td>
<td></td>
<td>13 b</td>
<td>7 ab</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>10 b</td>
<td>6 a</td>
<td>15 ab</td>
<td></td>
<td>20 cd</td>
<td>34 c</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>7 ab</td>
<td>1 a</td>
<td>8 a</td>
<td></td>
<td>27 de</td>
<td>11 ab</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>5 ab</td>
<td>1 a</td>
<td>6 a</td>
<td></td>
<td>23 cde</td>
<td>3 a</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>8 ab</td>
<td>4 a</td>
<td>12 ab</td>
<td></td>
<td>18 bc</td>
<td>20 b</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>1 a</td>
<td>1 a</td>
<td>2 a</td>
<td></td>
<td>5 a</td>
<td>5 ab</td>
</tr>
<tr>
<td>Mean</td>
<td>6.5</td>
<td>4.1</td>
<td>10.6</td>
<td></td>
<td>19.2</td>
<td>13.7</td>
</tr>
</tbody>
</table>

‡ PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimaricin.
† Values in a column, followed by the same letter, did not differ based on Duncan’s Multiple Range Test at P ≤ 0.05.
In repetition C, conducted at the AAFC research farm at Saskatoon in 1997, hot dry conditions after inoculation limited infection, so recovery of the two pathogens was low (≤ 20%, Appendix C). A-PDA consistently reduced contamination and increased recovery of the two pathogens compared to PDA, so an AL-PDA treatment was added in subsequent repetitions of the study. Also, the field plots were irrigated as described in Section 3.2.3 to provide a conductive environment for infection in subsequent repetitions.

In repetition D, which was inoculated with *B. cinerea* and irrigated, AL-PDA produced the highest recovery of the pathogen (*P* ≤ 0.05), which approached its maximum value by Day 5 (90%). In contrast, the maximum recovery of *B. cinerea* was only 70% on PDA by Day 10 and 83% on A-PDA by Day 9 (Table 3.3). AL-PDA and PCL-PDA reduced (*P* ≤ 0.05) the number of fungal and bacterial contaminant colonies relative to PDA (Table 3.4). A-PDA, NL-PDA, CL-PDA also resulted in lower (*P* ≤ 0.05) number of bacterial colonies than PDA.
Table 3.3. Recovery of *Botrytis cinerea* from alfalfa flowers on PDA with various amendments in an inoculated trial at Saskatoon, SK in 1997 (in repetition D, n = 5)

<table>
<thead>
<tr>
<th>Media</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA</td>
<td>10 d†</td>
<td>35 c</td>
<td>53 bc</td>
<td>58 bc</td>
<td>63 bc</td>
<td>70 abc</td>
</tr>
<tr>
<td>A-PDA</td>
<td>8 d</td>
<td>53 bc</td>
<td>68 bc</td>
<td>80 ab</td>
<td>83 ab</td>
<td>83 ab</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>13 d</td>
<td>33 c</td>
<td>45 c</td>
<td>50 c</td>
<td>53 c</td>
<td>58 bc</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>23 cd</td>
<td>40 bc</td>
<td>45 c</td>
<td>55 bc</td>
<td>63 bc</td>
<td>53 c</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>33 c</td>
<td>53 bc</td>
<td>63 bc</td>
<td>65 bc</td>
<td>65 bc</td>
<td>63 bc</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>33 c</td>
<td>45 bc</td>
<td>55 bc</td>
<td>55 bc</td>
<td>55 c</td>
<td>58 bc</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>55 b</td>
<td>60 b</td>
<td>75 ab</td>
<td>75 abc</td>
<td>78 abc</td>
<td>78 ab</td>
</tr>
<tr>
<td>AL-PDA</td>
<td>90 a</td>
<td>93 a</td>
<td>93 a</td>
<td>93 a</td>
<td>93 a</td>
<td>93 a</td>
</tr>
<tr>
<td>Mean</td>
<td>33.1</td>
<td>51.5</td>
<td>62.1</td>
<td>66.4</td>
<td>69.1</td>
<td>69.5</td>
</tr>
</tbody>
</table>

† PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimarcin.
† Values in a column, followed by the same letter, did not differ based on Duncan’s Multiple Range Test at $P \leq 0.05$. 
Table 3.4. Number of contaminant colonies on PDA with various amendments (per dish) from alfalfa flowers at two sampling dates (repetitions D and E) at Saskatoon, SK in 1997 (n = 5)

<table>
<thead>
<tr>
<th>Media(^1)</th>
<th>Repetition D</th>
<th>Repetition E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fungi</td>
<td>Bacteria</td>
</tr>
<tr>
<td>PDA</td>
<td>15 c(^1)</td>
<td>16 bc</td>
</tr>
<tr>
<td>A-PDA</td>
<td>8 abc</td>
<td>0 a</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>12 bc</td>
<td>18 c</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>15 c</td>
<td>4 a</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>12 bc</td>
<td>1 a</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>13 bc</td>
<td>8 ab</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>2 a</td>
<td>1 a</td>
</tr>
<tr>
<td>AL-PDA</td>
<td>5 ab</td>
<td>0 a</td>
</tr>
<tr>
<td>Mean</td>
<td>10.0</td>
<td>5.9</td>
</tr>
</tbody>
</table>

\(^1\) PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimarcin.

\(^\dagger\) Values in a column, followed by the same letter, do not differ based on Duncan’s Multiple Range Test at \(P \leq 0.05\).
Repetition E was inoculated with *S. sclerotiorum*. The incidence of the pathogen on AL-PDA was 28% on Day 5 and 38% on Day 9, which was higher (*P* ≤ 0.05) than on PDA (5 and 10%, respectively) (Table 3.5). The incidence of *B. cinerea* was higher (*P* ≤ 0.05) on AL-PDA and PCL-PDA than on the other media from Day 5 to Day 7 (Table 3.5). The maximum recovery on AL-PDA was 93% compared with only 35% on PDA. The number of fungal and bacterial contaminant colonies on A-PDA and PCL-PDA was lower (*P* ≤ 0.05) than on PDA. AL-PDA, NL-PDA, CL-PDA and PNL-PDA also reduced the number of bacterial colonies relative to PDA (*P* ≤ 0.05) (Table 3.4).

In repetitions F and G in 1998, alfalfa plots were inoculated with *B. cinerea*. The incidence of *B. cinerea* on Day 6 in repetition F was higher (*P* ≤ 0.05) on AL-PDA (40%) than on PDA (14%) (Fig 3.1). The maximum recovery was 45% on AL-PDA, compared with 21% on PDA. Recovery on A-PDA was also higher (*P* ≤ 0.05) than on PCL-PDA and PDA from Day 5 to Day 8. In repetition G, the results showed a similar trend to those from repetition F, so they are presented in Appendix D. Recovery of *B. cinerea* on AL-PDA was 85% on Day 6 and reached a maximum value of 88% on Day 7 (Table D1).

Repetition H was inoculated with *S. sclerotiorum*. The recovery of the pathogen was higher (*P* ≤ 0.05) on AL-PDA than on PCL-PDA from Day 5 to Day 7, than on A-PDA from Day 5 to Day 9 and on PDA from Day 5 to Day 10 (Fig. 3.2). The maximum incidence of *S. sclerotiorum* was 93% on AL-PDA by Day 8, 88% on PCL-PDA by Day 9, 78% on A-PDA and 55% on PDA by Day 10.
Table 3.5. The effect of medium amendment on recovery of *Sclerotinia sclerotiorum* and *Botrytis cinerea* from alfalfa flowers in an inoculated trial at Saskatoon, SK in 1997 (repetition E, n = 5)

<table>
<thead>
<tr>
<th>Media</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sclerotiorum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>5 b†</td>
<td>10 c</td>
<td>13 a</td>
<td>10 a</td>
<td>10 c</td>
<td>8 c</td>
</tr>
<tr>
<td>A-PDA</td>
<td>10 b</td>
<td>13 bc</td>
<td>15 a</td>
<td>23 a</td>
<td>20 abc</td>
<td>23 abc</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>3 b</td>
<td>5 c</td>
<td>10 a</td>
<td>10 a</td>
<td>13 c</td>
<td>15 bc</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>10 b</td>
<td>13 bc</td>
<td>15 a</td>
<td>23 a</td>
<td>23 abc</td>
<td>20 abc</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>13 b</td>
<td>13 bc</td>
<td>13 a</td>
<td>10 a</td>
<td>23 abc</td>
<td>20 abc</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>0 b</td>
<td>3 c</td>
<td>10 a</td>
<td>20 a</td>
<td>15 abc</td>
<td>15 bc</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>10 b</td>
<td>23 ab</td>
<td>28 a</td>
<td>30 a</td>
<td>35 ab</td>
<td>35 ab</td>
</tr>
<tr>
<td>AL-PDA</td>
<td>28 a</td>
<td>30 a</td>
<td>28 a</td>
<td>33 a</td>
<td>38 a</td>
<td>38 a</td>
</tr>
<tr>
<td>Mean</td>
<td>9.9</td>
<td>13.8</td>
<td>15.4</td>
<td>19.9</td>
<td>22.4</td>
<td>21.8</td>
</tr>
<tr>
<td>B. cinerea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>3 C</td>
<td>8 E</td>
<td>10 D</td>
<td>25 D</td>
<td>25 C</td>
<td>35 D</td>
</tr>
<tr>
<td>A-PDA</td>
<td>3 C</td>
<td>20 CDE</td>
<td>28 CD</td>
<td>63 BCD</td>
<td>68 AB</td>
<td>69 AB</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>10 C</td>
<td>15 CDE</td>
<td>25 CD</td>
<td>33 CD</td>
<td>40 C</td>
<td>45 BCD</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>13 C</td>
<td>30 CD</td>
<td>33 CD</td>
<td>48 CD</td>
<td>43 BC</td>
<td>43 CD</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>8 C</td>
<td>35 C</td>
<td>38 CD</td>
<td>38 CD</td>
<td>45 BC</td>
<td>50 BCD</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>3 C</td>
<td>13 DE</td>
<td>18 D</td>
<td>38 CD</td>
<td>35 C</td>
<td>40 CD</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>33 B</td>
<td>58 B</td>
<td>70 AB</td>
<td>73 AB</td>
<td>75 A</td>
<td>73 AB</td>
</tr>
<tr>
<td>AL-PDA</td>
<td>60 A</td>
<td>83 A</td>
<td>90 A</td>
<td>88 A</td>
<td>93 A</td>
<td>90 A</td>
</tr>
<tr>
<td>Mean</td>
<td>16.6</td>
<td>32.8</td>
<td>39.0</td>
<td>50.8</td>
<td>53.0</td>
<td>55.5</td>
</tr>
</tbody>
</table>

† PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimarcin.

† Values in a column, followed by the same letter and case, did not differ based on Duncan’s Multiple Range Test at $P \leq 0.05$. 

Mean
Figure 3.1. Effect of medium amendments on recovery of *Botrytis cinerea* from alfalfa flowers collected in an inoculated field trial at Saskatoon, SK in 1998 (repetition F, n = 5). Amendments are described in Section 3.2.2.
Figure 3.2. Effect of medium amendments on recovery of *Sclerotinia sclerotiorum* from alfalfa flowers collected in an inoculated field trial at Saskatoon, SK in 1998 (repetition H, n = 5). Amendments are described in Section 3.2.2.
The pattern of differences among media for frequency of contaminant colonies in the three repetitions in 1998 was also similar. Therefore, only the results from repetition F are shown (Fig. 3.3). Repetitions G and H are presented in Appendix D (Tables D2 and D3). In general, no differences in the number of contaminant colonies were observed among AL-PDA, A-PDA, and PCL-PDA. Contamination on these three media was lower than on PDA in all three repetitions, except for fungal colonies on PCL-PDA in repetition F. The number of bacterial colonies was always lower than that of fungal contaminants. Also, contamination in repetition F was much higher than in repetitions G and H.
The pattern of differences among media for frequency of contaminant colonies in the three repetitions in 1998 was also similar. Therefore, only the results from repetition F are shown (Fig. 3.3). Repetitions G and H are presented in Appendix D (Tables D2 and D3). In general, no differences in the number of contaminant colonies were observed among AL-PDA, A-PDA, and PCL-PDA. Contamination on these three media was lower than on PDA in all three repetitions, except for fungal colonies on PCL-PDA in repetition F. The number of bacterial colonies was always lower than that of fungal contaminants. Also, contamination in repetition F was much higher than in repetitions G and H.
Figure 3.3. Effect of medium amendments on the number of contaminant colonies from alfalfa flowers collected in an inoculated field trial at Saskatoon, SK in 1998 (repetition F, n = 5). Amendments are described in Section 3.2.2.
3.4. Discussion

The primary objective of this experiment was to develop a method to shorten the time required to diagnose *B. cinerea* and *S. sclerotiorum* infestation in alfalfa seed production fields. Based on the results in the preliminary tests (Appendices A and B), a total of eight media, together with a 3-day pretreatment in the dark, were assessed in the field trials. AL-PDA consistently produced the highest recovery of both pathogens among media, and only 6 days were needed to approach the maximal recovery rate (Table 3.3 and 3.5, Fig. 3.1 and 3.2). PCL-PDA and A-PDA usually also produced high recoveries of the two pathogens, but they required more time to approach the maximum values compared with AL-PDA (Table 3.3 and 3.5, Fig. 3.1 and 3.2). Also, the maximum recovery of the two pathogens was not consistently higher than on PDA.

AL-PDA, A-PDA and PCL-PDA also reduced fungal and bacterial contamination compared to PDA in these field trials (Table 3.2 and 3.4, Fig. 3.3). CL-PDA consistently reduced bacterial contamination, but PL-PDA, NL-PDA, and PNL-PDA had no consistent effect on bacterial contamination. Similarly, CL-PDA, PL-PDA, NL-PDA, and PNL-PDA had no effect on fungal contamination.

Overall, AL-PDA, in combination with a 3-day pretreatment in the dark, was the best combination for diagnosing *B. cinerea* and *S. sclerotiorum* in the field trials. The superiority of this method may be due to various factors. Firstly, alfalfa leaves provide nutrients to facilitate mycelial growth of the two pathogens and conidial sporulation of *B. cinerea*. Secondly, the low pH in the medium, contributed by amendment with lactic acid, slows the growth of some bacterial and fungal
contaminants (Day et al., 1987; Presly, 1985), but *B. cinerea* and *S. sclerotiorum* can tolerate highly acidic media (Harrison and Heilbronn, 1988; Jarvis, 1977; Willetts and Wong 1980). Other media, such as PL-PDA, CL-PDA, NL-PDA and PNL-PDA, did not provide adequate control of contamination, bacterial and/or fungal contaminants often inhibit the growth of *B. cinerea* and *S. sclerotiorum*. Even on APDA and PCL-PDA, where contamination was reduced, the two pathogens still developed slowly, since they did not have factor such as nutrient amendment to promote growth.

Another factor that contributed to the success of this method was the dark pretreatment. Incubation in the dark increases subsequent sporulation of *B. cinerea* (Laha and Grewal, 1983; Tan and Epton, 1973).

The recovery of the pathogens varied substantially between repetitions in the same field and year. For example, the maximum recovery of *B. cinerea* at Saskatoon in 1998 was 45% in repetition F vs. 88% in repetition G. This variation may be related to differences in weather conditions. Repetition F was conducted under windy conditions, which would speed drying within the plant canopy and reduce the frequency of infection. Calm weather during repetition G may have slowed drying, resulting in relatively longer periods of wet or humid conditions after irrigation each evening. Furthermore, windy weather resulted in more contamination on the sampling media. There was a total of 36 colonies per dish observed on PDA in repetition F (Fig. 3.3), but only 6 in repetition G (Fig. D2). High levels of contamination can reduce the recovery of these pathogens on isolation media.
The combination of AL-PDA plus pretreatment in the dark has been selected for further evaluation in the field (B.D. Gossen, personal communication). The method is being assessed by growers for diagnosis of blossom blight in their fields and may provide an effective tool helping them for timing fungicide application.
4. RESISTANCE OF ALFALFA CULTIVARS TO FLORAL INFECTION

BY *BOTRYTIS CINEREA*

4.1. Introduction

Many natural defense strategies are employed by plants to limit infection by *Botrytis cinerea*, including physical and chemical barriers to penetration and growth of the pathogen. No gene-for-gene resistance to *B. cinerea* has been identified (Elad et al., 1995), but isolates of *B. cinerea* have shown differential virulence on chickpea cultivars (Rewal and Grewal, 1989b). However, genetic variation in resistance has been observed within host species in some studies (Hammer and Evensen, 1994; Marois et al., 1986; Rijkenberg et al., 1980).

The first barrier to infection by *B. cinerea* is the cuticle. In addition to providing a physical and chemical barrier to penetration of the pathogen, intact cuticles also prevent diffusion of cellular solutions from plant tissues and reduce retention of water on plant surfaces. This limits availability of nutrients and water on tissue surfaces, which are required for germination of conidia of *B. cinerea* (Jarvis, 1962a; Verhoeff, 1980). The cuticle can vary in structure (Marois et al., 1986), thickness (Commenil et al., 1997; Hammer and Evensen, 1994; Rijkenberg et al., 1980) and composition (Commenil, 1997; Marois et al., 1986). In grape, berries often touch each other during growth. The epicuticular wax at the points of contact is
amorphous, with numerous shallow depressions, whereas non-contact surfaces have well-defined wax platelets. When inoculated with conidia of *B. cinerea*, the frequency of infection at points of contact on mature berries was higher than that on non-contact areas (Marois *et al.*, 1986).

Cutin constitutes the framework of the cuticle and forms a physical barrier against pathogens. Cuticle thickness may be related to mechanical resistance to penetration by the pathogen, or resistance to breakdown by fungal enzymes. The cutin content of grape berries among cultivars was correlated to increased resistance to *B. cinerea*; cultivars with more cutin were less susceptible (Commenil *et al.*, 1997). Cultivars with thicker cuticles were also generally less susceptible to *B. cinerea* than those with relatively thin cuticles in rose petals (Hammer and Evensen, 1994) and tomato fruits (Rijkenberg *et al.*, 1980).

The cell wall of the host is the second barrier to infection by *B. cinerea*. In some cases, the wall thickens and produces papillae at the site of penetration, which restricts the growth and penetration of hyphae of *B. cinerea* (Mansfield and Hutson, 1980; Rijkenberg *et al.*, 1980). *Botrytis cinerea* secretes hydrolytic enzymes to degrade the cell wall (Verhoeff, 1980), but this degradation is inhibited when calcium content in host tissues is high (Elad and Volpin, 1988; Elad *et al.*, 1993; Volpin and Elad, 1991).

Calcium is also important to the integrity of cell membranes (Simon, 1978). Preventing membrane deterioration reduces leakage of nutrients to the surface of the host tissue, and makes these nutrients less available to *B. cinerea* (Volpin and Elad, 1991). Calcium is also associated with delayed senescence of plant tissues by
reducing the respiration rate and ethylene production (Ferguson, 1984). This may also make plants less susceptible to degradation by enzymes produced by B. cinerea. High levels of calcium reduce B. cinerea severity in crops such as rose (Volpin and Elad, 1991), tomato (Elad et al., 1993; Stall, 1963), pepper and eggplant (Yunis et al., 1991).

Other genetic traits involved in resistance to infection by B. cinerea are diverse and vary among cultivars and crops. They include canopy architecture, flower colour, timing of flowering and even the amount of pollen in flowers. An open canopy dries quickly and humidity in the canopy is generally lower (Bond et al., 1994), while a dense canopy retains more moisture and dries more slowly (Michailides, 1991; Vail and Marois, 1991). In grapes, cultivars with tight fruit clusters develop severe bunch rot, but those with loose clusters are less affected (Gulber et al., 1987; Vail and Marois, 1991). Similarly, in pistachio (Michailides, 1991), castor bean (Thomas and Orellana, 1963) and almond (Ogawa and English, 1960), compact inflorescences are more susceptible to botrytis diseases than open inflorescences. Pollen may also play a role by providing nutrients that are important for spore germination of B. cinerea (Blakeman, 1980; Fourie and Holz, 1998). The susceptibility of one cultivar of pistachio has been associated with increased production of pollen in the flowers (Michailides, 1991). Increased amounts of pollen provide a larger nutrient source and predispose the flowers to infection by the pathogen. Early flowering may result in disease escape (Bond et al., 1994). For example, an early flowering and maturing pigeonpea cultivar had lower infection levels than late-maturing cultivars (Bajpai et al., 1995). Finally, flowers colour may
affect the disease reaction in some host species. Purple colour flowers is often produced by anthocyanin pigments (Ruberto et al., 1997). Anthocyanins have antioxidant activity, which reduces the detrimental effects of free radicals and reactive oxygen species. This protects the host from peroxidation damage and membrane deterioration (Hipskind et al., 1996; Ruberto et al., 1997; Tamura and Yamagami 1994).

Genetic resistance to *B. cinerea* has been introduced into several crops. For example, pistillate plants of strawberry have a lower incidence of necrosis on flowers and fruits than hermaphrodite plants. The difference is primarily due to the absence of anthers in the pistillate flowers (Simpson, 1991). Anthers in hermaphrodite flowers provide an important pathway for *B. cinerea* to enter the receptacle, and the presence of pollen also enhances conidial germination. In chickpea and kenaf, resistance to *B. cinerea* appears to be controlled by two major genes. One gene is dominant for resistance, the other dominant for susceptibility. The resistance gene is not expressed when the dominant susceptible allele is present. Therefore, the genotype R_ss is resistant to botrytis blight, while genotypes R_S_, rrS_ and rrss are susceptible (Campbell, 1984; Rewal and Grewal, 1989a).

In the present study, the disease reaction of locally-adapted alfalfa cultivars to blossom blight caused by *B. cinerea* was examined in greenhouse and field trials and the effect of flower architecture and colour on infection incidence was evaluated in greenhouse trials.
4.2. Materials and Methods

4.2.1. Host plants

The reaction of 12 alfalfa cultivars to infection by *B. cinerea* was evaluated in several trials, including inoculation of detached flowers, whole-plant inoculation in growth cabinets, and in inoculated field trials in 1997-98. These cultivars represent the range of winter hardiness. Seven of them are grown primarily in eastern Canada: OAC Minto, Iroquois, Apollo II, Apica, DK 135, Oneida-VR, Saranac. The other five cultivars are grown primarily on the prairies: AC Nordica, Algonquin, Heinrichs, Vernal and Beaver. In general, the western cultivars have very high winter hardiness, while eastern cultivars have high winter hardiness (Melton *et al.*, 1988). All 12 cultivars are highly resistant to bacterial wilt (*Clavibacter michiganense* subsp. *insidiosum* [McCullach] Davis, Gillaspie, & Harris) (Barnes and Sheaffer, 1985; Goplen and Gossen, 1994; Lawrence and Irvine, 1984; Moutary *et al.*, 1983; Viands *et al.*, 1990). In addition, AC Nordica has moderate resistance to winter crown rot (*Coprinus psychromorbidus* Redhead & Traquair) (Goplen and Gossen, 1994) and Oneida-VR is highly resistant to verticillium wilt (*Verticillium albo-atrum* Reinke & Berth) and fusarium wilt (*Fusarium oxysporum* f. *locopersici*) (Viands *et al.*, 1990).

For the tests under controlled environments, alfalfa plants were grown in a growth chamber at 20°C under a 14-h photoperiod with light intensity of about 300 μmol s⁻¹ m⁻² around the canopy. The alfalfa plants were seeded in roottrainers (2 × 2.5 × 10.5 cm) containing a soilless mix (Appendix E) and transferred to plastic pots (20 cm dia. × 15.5 cm high) after 4 weeks of growth. Plants were watered once each day.
4.2.2. Preparation of inoculum

A single isolate of *B. cinerea*, Nov9603, originally isolated from an alfalfa floret collected from southern Alberta in 1996, was used for the study. It was maintained in culture on PDA. Conidia of the pathogen were produced on PDA cultured under cool-white fluorescent lamps at room temperature. Spores were harvested from petri dishes using sterile distilled water plus surfactant (0.1 ml Triton X-100 per 100 ml), shaken vigorously, and filtered through four layers of sterile cheesecloth. The spore concentration was estimated using a haemacytometer and diluted to $1 \times 10^5$ spores per ml.

4.2.3. Surface sterilization

Flowers were surface sterilized by immersion in 70% ethanol for 30 sec. followed by 0.6% NaOCl for 90 sec. and ten rinses in sterile distilled water. This method was developed based on the preliminary trials described in Appendix F.

4.2.4. Detached-flower tests

Ten racemes from two plants per cultivar were picked, inoculated by spraying the flower clusters with a spore suspension to run-off using an atomizer, and immediately placed in small moist chambers (8.5 cm dia. × 2.7 cm high) with two racemes per chamber. The chambers were constructed by sealing the bottom halves of two 9-cm plastic petri dishes together with parafilm to prevent moisture loss. Each chamber contained a layer of wet filter paper on the bottom, covered by a layer of sterile plastic mesh. The samples were incubated at room temperature in the dark. At 12 h or 24 h, four flowers were removed from each raceme. A total of 40 flowers for each cultivar × time combination were surfaced-sterilized, incubated for
10 to 14 days, and infection incidence was assessed by microscopic examination of flowers for sporulation of *B. cinerea*. The experiment was repeated four times.

4.2 5. **Whole-plant inoculation**

A total of 24 vigorously flowering plants (two per cultivar) were inoculated to run-off as described in Section 4.2.4. Each plant was then covered with a clear plastic bag and incubated for 48 h under a 14-h photoperiod with fluorescent lighting at 20°C. The four youngest flowers were removed from each of 10 racemes per cultivar, surface-sterilized and plated onto PDA. The flowers were incubated and assessed as described above. In order to ensure that all of the alfalfa flowers were at the same stage of development, all of the unopened flower buds on each plant were trimmed off immediately before inoculation. The youngest flowers available were selected, rather than the oldest flowers (as in Chapter 3), to provide samples of more uniform age and development stage for these tests. The experiment was repeated four times, using new plants for each repetition.

4.2.6. **Cultivar reaction in the field**

In the field, plants of each cultivar were grown in microplots (1 m²) in a randomized complete block design with five replications. The plots were established in 1996 by transplanting seedlings, with nine plants in each plot. Some of the original plants died in the winter of 1997, and were replaced with new transplants of the same age in the spring of 1998. Flowering plants were inoculated with a spore suspension (as described previously) in the early evening of August 21, 1998, after a heavy rain. The inoculated plants were mist-irrigated for 3 min. at 9 pm on the following two evenings to maintain high levels of humidity. On the third day, the
two youngest flowers that had been fully open at the time of inoculation from each of 20 racemes per cultivar were collected from each plot, surfaced-sterilized and assessed as described in Section 4.2.4.

4.2.7. Flower orientation and colour

Four greenhouse-grown plants of cv. Vernal were inoculated and incubated as described in Section 4.2.5. In each of three replicates, 10 upward-facing and 10 downward-facing racemes were collected after 48 h incubation and four flowers were removed from each raceme. The flowers were surface sterilized and assessed as described in Section 4.2.4.

In a second experiment, greenhouse-grown plants from each of cvs. AC Nordica, Apollo II and Iroquois were selected based on their flower colour, two plants per cultivar with purple flowers and two with white or yellow flowers. Ten racemes of each colour (purple vs. yellow or white) per cultivar were picked, inoculated, incubated and assessed as described in Section 4.2.4. The experiment was repeated four times, using new plants for each repetition.

4.2.8. Statistical analysis

Analysis of variance of infection incidence was performed using SAS. When a test was repeated, each repetition was included as a replicate in analysis of variance. The means were compared using Duncan's Multiple Range Test at $P \leq 0.05$. Kendall's coefficient of rank correlation ($\tau$) was employed to assess the correlation between pairs of tests and Kendall's coefficient of concordance ($W$) was used to assess the association among all of the tests in the field and greenhouse. The
significance level for $\tau$ and $W$ was estimated using a t-test or chi-square test, respectively.

4.3. Results

4.3.1. Detached-flower tests

After 12 h incubation, the incidence of infection among cultivars ranged from 12% to 33% (Fig. 4.1). OAC Minto, Beaver, and DK 135 had a lower ($P \leq 0.05$) incidence of infection (mean of 12%) than Apollo II, Heinrichs and AC Nordica (mean of 30%). In addition, Iroquois had a lower incidence of infection than Apollo II (17% vs. 33%). After 24 h incubation, the incidence of infection had a somewhat different pattern than at 12 h (Fig. 4.1). DK 135 had a lower ($P \leq 0.05$) incidence of infection (54%) than the other cultivars. The incidence in Iroquois (68%) was lower than the most heavily infected group (Algonquin, Heinrichs, Apollo II, and Vernal, mean 90%), and OAC Minto had lower ($P \leq 0.05$) incidence than Vernal (72% vs. 92%). Analysis indicated that the rank order of the cultivars was not correlated between the two sampling intervals ($\tau = 0.41$).

4.3.2. Whole-plant inoculation

There were consistent differences among cultivars in infection incidence by *B. cinerea* in this trial. The mean incidence of flower infection in OAC Minto, Iroquois, and DK 135 (37%) was lower ($P \leq 0.05$) than in Apica, Algonquin, Heinrichs, AC Nordica and Apollo II (73%, Fig. 4.2). Saranac, Vernal and Oneida-VR (mean 47%) also had a lower incidence of infection than AC Nordica and Apollo II (mean 77%), but they were not different from Apica, Algonquin and Heinrichs.
Fig. 4.1. Incidence of flower infection by *Botrytis cinerea* among alfalfa cultivars in a detached flower test after 12 and 24 h of incubation (n = 4).
Fig. 4.2. Incidence of flower infection by *Botrytis cinerea* among alfalfa cultivars in a whole-plant inoculation test (n = 4).
4.3.3. Cultivar tests in the field

In general, infection incidence was lower in the field test than under controlled conditions (Fig. 4.3). The incidence of flower infection in DK 135 (3%) and OAC Minto (4%) was lower ($P \leq 0.05$) than in Heinrichs (22%) and Apollo II (22%). DK 135 also had a lower ($P \leq 0.05$) incidence of infection than Algonquin (20%).

4.3.4. Correlation in the incidence of infection among tests

Analysis of $\tau$ showed that the rank order of cultivars was correlated ($P \leq 0.01$) between the whole-plant inoculation test and the detached-flower test at 12 h ($\tau = 0.78$), but not at 24 h ($\tau = 0.32$, Table 4.1).

The rank of cultivars in the field test was correlated with each of the other assessments (Table 4.1). The $\tau$ value was 0.69 ($P \leq 0.01$) with the detached-flower test at 12 h, 0.61 ($P \leq 0.05$) at 24 h, and 0.78 ($P \leq 0.01$) with the whole-plant inoculation test.

The overall similarity among the tests was assessed using Kendall’s coefficient of concordance ($W$). The value of $W$ was 0.84 ($P \leq 0.001$), which demonstrates a strong similarity in cultivar response among the tests.
Fig. 4.3. Incidence of flower infection by *Botrytis cinerea* among alfalfa cultivars in a micro-plot field test at Saskatoon, SK in 1998 (n=5).
Table 4.1. Kendall’s coefficient of rank correlation of the incidence of flower infection by *Botrytis cinerea* among 12 alfalfa cultivars in four inoculated tests.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Detached flowers, 12-h</th>
<th>Detached flowers, 24-h</th>
<th>Whole-plant</th>
<th>Field trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detached, 12-h</td>
<td>0.42 n.s.</td>
<td>0.78 **</td>
<td>0.69 **</td>
<td></td>
</tr>
<tr>
<td>Detached, 24-h</td>
<td>0.32 n.s.</td>
<td>0.61 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-plant</td>
<td></td>
<td></td>
<td></td>
<td>0.78 **</td>
</tr>
<tr>
<td>Field</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*, ** Significant at $P \leq 0.05$ and 0.01 respectively based on a t-test of Kendall’s coefficient of rank correlation ($\tau$), respectively.

n.s. – not significant.
4.3.5. **Flower orientation and colour**

In whole-plant inoculation tests, upward-facing flowers had a lower ($P \leq 0.001$) incidence of infection (16%) than downward-facing flowers (86%). The effect of flower colour on infection frequency was not consistent among the three cultivars. Purple flowers had a lower incidence of infection than white flowers in Iroquois (20% vs. 38% at 12 h, $P \leq 0.05$; 64% vs. 85% at 24 h, $P \leq 0.01$). However, there were no differences in incidence between purple and white flowers in Apollo II (43% vs. 33% at 12 h; 83% vs. 81% at 24 h) or between purple and yellow flowers in AC Nordica (38% vs 26% at 12 h; 84% vs. 83% at 24 h) (Fig. 4.4).
Fig. 4.4. Effect of flower colour on infection of alfalfa flowers by Botrytis cinerea in a whole-plant inoculation study at 12 and 24 h of incubation (n = 4).
4.4. Discussion

The incidence of flower infection was used as a quantitative measure of the disease reaction of alfalfa cultivars to *B. cinerea* in this study. Cvs. OAC Minto and DK 135 were generally less susceptible than cvs. Apollo II, Heinrichs, and AC Nordica in the different tests.

Most cultivars demonstrated a consistent disease reaction among the tests. The correlation in cultivar reaction over the four tests was strong (*W* = 0.84, *P* ≤ 0.001). DK 135, OAC Minto and Iroquois were consistently less susceptible than the other cultivars in the trials, while Apollo II, Heinrichs and Algonquin were more susceptible. The reaction of a few cultivars varied among tests. For example, Beaver was in the less-susceptible group in the detached-flower test at 12 h of incubation, but was one of the most-susceptible cultivars when incubated for 24 h. However, the results were sufficiently consistent across the various tests to conclude that the differences in susceptibility among cultivars were real.

The infection incidence for individual cultivars was lower in the field test (range 3-22%, Fig. 4.4) than in the whole-plant (36-74%, Fig. 4.3) and detached-flower tests (12-33% at 12-h incubation, Fig. 4.1; 54-92% at 24-h, Fig. 4.2). The lower incidence of infection in the field was probably associated with dry conditions during the test. Surface wetness on the flowers may not have persisted long enough to establish a high level of infection, in spite of periodic mist irrigation.

In the experiment examining the impact of flower architecture on infection, flower orientation influenced the susceptibility of flowers to *B. cinerea*. Upward-facing flowers had a substantially lower incidence of infection than downward-
facing flowers. This difference might have been associated with a difference in wetness duration on the flower surfaces. After 48 h of incubation, the surface of upward-facing flowers appeared to be dry, but the downward-facing flowers were still wet, based on casual observation. In other studies, plant architectures that result in longer period of wetness have been associated with increased susceptibility to \textit{B. cinerea}. For example, longer retention of surface wetness and increased disease severity has been observed in tighter grape cluster and more compact racemes of pistachio, almond and castor bean (Michailides, 1991; Ogawa and English, 1960; Thomas and Orellana, 1963; Vail and Marois, 1991).

Another factor involved in alfalfa susceptibility to \textit{B. cinerea} may be flower colour. Purple flowers were less susceptible to \textit{B. cinerea} than white flowers in cv. Iroquois, but not in AC Nordica or Apollo II. In another study, infection of alfalfa flowers by ascospores of \textit{S. sclerotiorum} occurred more slowly on purple flowers than on white flowers of alfalfa (Lefol \textit{et al.}, 1999).

The flower colour of an alfalfa cultivar depends on its genetic background. Lines with \textit{Medicago sativa} ssp. \textit{sativa} parentage have purple flowers, and the pigments are primarily anthocyanins. In contrast, cultivars with predominantly \textit{Medicago sativa} ssp. \textit{falcata} background have yellow flowers due to the presence of anthoxanthins and carotenoids (Barnes \textit{et al.}, 1985). Anthocyanins may play an antifungal role (Coley and Aide, 1989; Hoagland and Boyette, 1994). It is known that oxygen species and free radicals are produced by the host and by \textit{B. cinerea} during infection (Edlich \textit{et al.}, 1989). These chemicals attack polyunsaturated fatty acids in cell membranes and cause lipid peroxidation, which leads to deterioration of
the cell membrane and promotes pathogen development (Hipskind et al., 1996; Tsuda et al., 1994a, b). Anthocyanins reduce the formation of oxygen radicals and inhibit lipid peroxidation, thereby protecting the host from peroxidation damage and membrane deterioration (Hipskind et al., 1996; Ruberto et al., 1997; Tamura and Yamagami 1994; Tsuda et al., 1994a). In variegated flowers, the purple pigments (anthocyanins) are usually present in buds and newly opened flowers, but the colour quickly fades (Barnes et al., 1985). More yellow xanthophyll pigments are produced as the flowers develop, resulting in an off-white colour.

Differences in disease reaction to *B. cinerea* among alfalfa cultivars may be affected by many characteristics. In this study, differences in flower architecture and colour influenced infection incidence, but other factors, such as floret density, or cuticle thickness and composition, may also have an impact. Individual cultivars may carry numerous characteristics that influence susceptibility, but the relative contribution of these characters may be highly variable. For example, a cultivar with purple flowers may be more susceptible than one with white flowers, if the purple racemes are downward-facing and the white racemes face upward. Therefore, it is unlikely that less-susceptible lines can be identified solely on the basis of one or two simple traits, such as flower colour and orientation.
5. INFECTION OF ALFALFA FLOWERS BY *BOTRYTIS CINerea *
UNDER CONTROLLED CONDITIONS

5.1. Introduction

Blossom blight is an important constraint to alfalfa seed production in western Canada, especially when conditions during flowering are cool and wet (Gossen et al., 1994). Seed production may be reduced from 25-100% if disease severity is high (Gossen et al., 1997c). Fungicide application can reduce blossom blight incidence and increase seed yield (Gossen et al., 1997c). Fungicides can be an unnecessary expenditure in years when the environment is not conducive to the disease, and a reduction in application frequency would represent significant saving to growers. An understanding of the relationship between environmental conditions and infection of alfalfa flowers by *B. cinerea* is one of the components required in order to reduce the frequency of fungicide application.

*Botrytis cinerea* is often the dominant pathogen in the blossom blight complex in Saskatchewan and Manitoba (Gossen et al., 1997c) and initially infects alfalfa flowers via airborne conidia. The basic requirement for infection is the availability of surface wetness for a minimum period, which is a function of precipitation, temperature and relative humidity. Infected petals provide a food base for *B. cinerea* and established mycelium can directly infect other petals or adjacent flowers (Verhoeff, 1980). After colonization of petals, the fungus
produces abundant mycelium that can produce large number of conidia (Agrios, 1997). In alfalfa, the mycelium from an infected floret often invades the rest of the inflorescence. The infected inflorescence quickly becomes covered with gray mycelium and dies under cool, wet conditions. However, when conditions are less conducive for mycelial growth, the pathogen spreads more slowly and infected flowers generally abscise prematurely without setting seed (Gossen, personal communication).

Surface wetness and air temperature are the most important factors that affect the infection success of *B. cinerea*. The highest percentage germination of *B. cinerea* was observed at 20°C (Rewal and Grewal, 1989b). Germination rate was reduced when the temperature moved away from 20°C. Higher germination percentage was also found at higher RH 20°C (Rewal and Grewal, 1989b). The reason may be that higher RH maintained an increased duration of surface wetness than lower RH. No germination occurs at 0° and 35°C or at less than 75% RH. Temperature and surface wetness may interact to limit the initial infection from conidia (Jarvis, 1980). For example, infection of geranium flowers required a surface wetness period of at least 4 h at temperatures from 21° to 30°C, but more than 20 h at 5° or 10°C (Sirjusingn and Sutton, 1996). Infection by *B. cinerea* was most rapid around 20°C on flowers and fruits of grape (Nair and Allen, 1993) and strawberry (Bulger et al., 1987; Wilcox and Seem, 1994). At temperatures above 22°C, infection was negatively correlated with temperature on macadamia racemes (Hunter *et al.*, 1972).
Surface wetness and temperature also affect infection through their effects on sporulation and latent period (the time between initiation of infection and the beginning of sporulation). Spore production of *B. cinerea* on dead strawberry leaves was highest at 17 and 18°C and the latent period was less than 3 days when the temperature was between 15-22°C. As temperatures shifted away from this range, the latent period increased (6 to 7 days at 5°C and 5 days at 27°C) and sporulation decreased (Sosa-Alvarez *et al.*, 1995). Sporulation may be completely inhibited when temperature is too low or too high. For example, sporulation of *B. cinerea* was observed on tomato stems 3 days after inoculation and incubation at 15°C, but sporulation was not initiated after 20 days at 5° or 26°C (O’Neill *et al.*, 1997).

The quantitative relationship between surface wetness duration and temperature with infection has not been examined in flowers of alfalfa. In the present study, the impact of a range of temperature (10° to 30°C) and wetness duration (0 to 48 h) treatment on infection of alfalfa flowers was examined. The objective of this study was to identify the conditions required for infection of alfalfa flowers under controlled conditions.

### 5.2. Methods

#### 5.2.1. Host plants

Alfalfa plants of cv. Vernal were seeded in rootainers (2 × 2.5 × 10.5 cm) containing a soil-less mix (Appendix E) and maintained at about 20°C under 14-h photoperiod in a greenhouse. The seedlings were transferred to plastic pots (20 cm
dia \times 15.5 \text{ cm high}) four weeks after seeding. The tests were conducted when the plants were at the stage of middle flowering.

5.2.2. Pathogen isolation, maintenance and inoculum

Isolate Nov9603 of *B. cinerea* from alfalfa was used in preliminary trials in this study. It was replaced with isolate Nov9703 in later experiments due to poor sporulation. Nov9703 was isolated from the chickpea cv. Arizona near Saskatoon, SK in 1996, and had been maintained on PDA. Conidia of the pathogen were produced on PDA and harvested as described in Section 3.2.2.

5.2.3. Experimental design

This experiment was conducted using a split-plot design with four replications. The main-plot treatments were incubation temperatures; each temperature (10°, 15°, 20°, 25°, and 30°C) was assigned at random to an incubator (in the dark) in each replication. The exception was the 30°C treatment, which was always assigned to a small oven. The subplot treatments were surface wetness duration (0, 4, 8, 12, 16, 20, 24, 48 h). In each replicate, a total of 216 inflorescences were collected (6 inflorescences for each treatment combination) and inoculated to runoff with a spore suspension (1 \times 10^5 spores per ml) of *B. cinerea* in the laboratory. Inoculated inflorescences were immediately placed into small moist chambers, described in Section 3.2.5. Each chamber contained three inflorescences. At each time interval, two dishes were selected at random and removed from each incubator. Five florets were then removed from each inflorescence (30 florets for each wetness duration \times temperature combination),
surface-sterilized and assessed for infection as described in Sections 4.2.3 and 4.2.4.

5.2.4. Statistical analysis

Analysis of variance of infection incidence was performed using SAS. The effect of surface wetness duration was treated as a split-plot-in-time. Where appropriate, differences among means within wetness periods or temperatures were examined using Duncan’s Multiple Range Test at $P \leq 0.05$.

5.3. Results

Analysis of variance (Table 5.1) demonstrated that temperature, wetness duration, and their interaction all affected the infection of alfalfa flowers by *B. cinerea* ($P \leq 0.001$). The highest incidence of infection occurred at 20°C, and the lowest at 30°C ($P \leq 0.05$) (Fig. 5.1). Incidence at 15°C was higher ($P \leq 0.05$) than at 25°C or 10°C. Low levels of infection were observed when surface wetness duration was less than 8 h (Fig. 5.2). The percentage of infection at 0 h of wetness was treated as a background level of infection. After 12 h of surface wetness, infection incidence increased over the background as wetness duration increased ($P \leq 0.05$, Fig. 5.2). Incidence increased sharply after 12 h at 20°C, 16 h at 15°C, 20 h at 10 and 25°C (Fig. 5.3). Infection at 30°C was very low at 0 and at 8 to 48 h of wetness, but highest ($P \leq 0.05$) at 4 h of wetness. The optimum temperature for infection in this trial was between 15° and 20°C, with a minimum of 12 to 16 h of surface wetness for infection under optimum temperature conditions.
Table 5.1. Effect of temperature and wetness duration on infection of alfalfa flowers by *Botrytis cinerea* under conditions in incubators in the dark (ANOVA).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>4</td>
<td>8332</td>
<td>92.3 ***</td>
</tr>
<tr>
<td>Replication</td>
<td>3</td>
<td>911</td>
<td>10.1 ***</td>
</tr>
<tr>
<td>Error a</td>
<td>12</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>7</td>
<td>13838</td>
<td>153.7 ***</td>
</tr>
<tr>
<td>Time x Temp</td>
<td>28</td>
<td>1731</td>
<td>19.2 ***</td>
</tr>
<tr>
<td>Residual</td>
<td>105</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>159</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** significant at P ≤ 0.001.
Figure 5.1. Effect of temperature on infection of detached alfalfa flowers by *Botrytis cinerea* under controlled conditions, calculated over all wetness durations (n = 32).
Figure 5.2. Effect of surface wetness duration on infection of detached alfalfa flowers by *Botrytis cinerea* under controlled conditions, calculated over all temperatures (n = 20).
Figure 5.3. Interaction of temperature and surface wetness duration on infection of detached alfalfa flowers by *Botrytis cinerea* under controlled conditions (n = 4).
5.4. Discussion

Surface wetness and temperature have been recognized as important variables affecting infection by *B. cinerea* (Jarvis, 1980b; Verhoeff, 1980). Their quantitative relationship to infection has been studied in several host systems (Bulger *et al*., 1987; Nelson, 1951; Sirjusingh and Sutton, 1996; Sosa-Alvarez *et al*., 1995; Thomas *et al*., 1988; Wilcox and Seem, 1994; Zhang and Sutton, 1994).

The minimum requirements for wetness duration for infection of flowers differ on hosts. This study showed that a minimum of 12 h of surface wetness was required for infection of alfalfa flowers at 20° C, which was the optimum temperature for infection. By comparison, the pathogen required 4 to 6 h of wetness at 21° C to 30° C to infect geranium flowers (Sirjusingh and Sutton, 1996), 6 to 12 h of wetness at 21° C to infect onion florets (Ramsey and Lorbeer, 1986), 5 to 7 h of wetness at 18 to 25° C to infect gerbera florets (Salinas *et al*., 1989), and 6 to 12 h at 25° C to 30° C to infect flowers and fruit of strawberry (Bulger *et al*., 1987).

Differences in the requirements for surface wetness may be due, at least in part, to differences in methodology in the various studies, rather than to intrinsic differences in host-pathogen interaction among host species. In the trials in geranium, gerbera and strawberry, after inoculation and incubation under wet conditions, flowers were held at moderate RH conditions (50 – 70% RH) for 24 h or more (Bulger *et al*., 1987; Salinia *et al*., 1989; Sirjusingh and Sutton, 1996). In these trials, the duration of surface wetness may have been substantially longer than the incubation period in the humid environment; under still air conditions,
liquid water on the plant surface may have required several hours to evaporate. Also, the minimum requirements for wetness were based on measures of different variables in these studies. In the trials on strawberry, gerbera and onion, the development of a single lesion was used to indicate successful infection, whereas infection statistically higher than background levels was used in the trial on geranium and in the current study.

Other factors may also affect the minimum requirement of wetness among these trials. The age or development stage of the host is an important factor influencing host susceptibility (Elad and Evensen, 1995). Physiological changes in aging or senescing plant tissues, such as increased permeability of cell membranes (Droillard et al., 1987; Mayak et al., 1983), reduction in secondary metabolites with antifungal activity (Pezet et al., 1992) and reduction in cuticle thickness (Commenil et al., 1997) contribute to increase susceptibility to the pathogen. The effect of flower development on infection was evident in several studies. For example, open flowers of onion were more susceptible to *B. cinerea* than unopened flowers (Ramsey and Lorbeer, 1986). Mature flowers in geranium showed a higher rate of infection than younger flowers (Sirjusingh et al., 1996). Also, in macadamia racemes, botrytis blight did not develop in immature buds, but did develop on senescent flower parts (Harrison, 1984).

Nutrient availability may also contribute to variation in the minimum requirement for surface wetness among various hosts. Tissue exudates and pollen diffusates are important sources of nutrients on plant surfaces. Nutrients on the plant surface stimulate conidial germination, hyphal growth, and appressorium
formation of *B. cinerea* (Sirjusingh *et al*., 1996) and increase penetration of the host by the pathogen (Blakeman, 1980; Jarvis 1977). In the trials on strawberry (Bulger *et al*., 1987) and onion flowers (Ramsey and Lorbeer, 1986), whole plants were inoculated, while detached flowers were used in the trials on geranium (Sirjusingh and Sutton, 1996), gerbera (Salinas *et al*., 1989) and in the present study. Inadvertent wounding caused by detaching the flowers may increase exudate levels and result in a reduced requirement for wetness duration in these studies.

When surface wetness is present, infection is a function of temperature (Elad and Yunis, 1993). In this study, infection was highest from 15° to 20°C and very little infection occurred at 30°C. This is consistent with observations in many other studies (Bulger *et al*., 1987; O'Neil *et al*., 1997; Sirjusingh and Sutton, 1996; Thomas *et al*., 1988). Temperature influences various components of infection, including sporulation, germination and latent period.

Wetness duration may be affected by several factors in the field, including wind speed (Hammer and Evensen, 1994; Thomas *et al*., 1988), plant spacing or density (English *et al*., 1989), and canopy architecture (Michailides, 1991; Vail and Marois, 1991). Increased wind speed hastens drying and results in shorter periods of wetness by breaking up the air boundary layer at the plant surface. Wind may also affect surface temperature by increasing transpiration cooling and convective heat transfer between host tissue and air (Nobel, 1983). The result is that wind may reduce infection level. An open plant architecture facilitates air
movement around the plants, which may also reduce infection (English et al., 1989).

In contrast to the effect of optimal temperature on the pathogen, temperatures that are sub-optimal may make the host more susceptible to *B. cinerea*. For example, when cucumber leaves and fruit were exposed to sub-optimal temperatures (8° or 30°C) prior to inoculation and incubation, the incidence and severity of grey mold was higher than for pretreatment at 20°C (Elad and Yunis, 1993). This phenomenon may help to explain the small peak of infection at 30°C and 4 hours of wetness in the present study (Fig. 5.3). A temperature of 30°C may make alfalfa flowers more susceptible, but also inhibit growth of the pathogen at some subsequent point in development.

This study provides an insight into the relationship between environmental factors and flower infection by *B. cinerea* in alfalfa. The results were obtained from experiments under controlled environment, and there is a need to validate the information under field conditions. Nevertheless, used in conjunction with weather data and inoculum monitoring, this information may help growers determine the risk of disease outbreak in a given alfalfa field. This in turn may ultimately result in more effective management of the disease complex.
6. GENERAL DISCUSSION

The primary objectives of this study were to examine several aspects of the epidemiology of blossom blight of alfalfa and to assess the disease reaction of a range of cultivars. To accomplish these objectives, a method to rapidly quantify inoculum levels in the field was developed, the impact of temperature and surface wetness on infection of alfalfa flowers by *B. cinerea* was quantified, and cultivars were screened for susceptibility to this pathogen.

Assessment of inoculum levels is the first step towards management of alfalfa blossom blight. In other host-pathogen systems, high levels of inoculum or development of symptoms are factors that trigger fungicide application. For example, onion growers in New York initiate fungicide application when botrytis leaf blight (*B. squamosa*) reaches one lesion per leaf (Andaloro and Lorbeer, 1982; Shoemaker and Lorbeer, 1977). Weather-based indices of disease severity or inoculum production are also used to schedule fungicide application to control onion leaf blight (Sutton *et al*., 1986; Vincelli and Lorbeer, 1989b).

Blossom blight of alfalfa, caused by *B. cinerea* and *S. sclerotiorum*, was identified for the first time in western Canada in 1993 (Gossen *et al*., 1994). Epidemics of this disease usually occur after a period of rain or humid weather (Gossen *et al*., 1994; Jones, 1952). The early symptoms are often subtle and indistinct, but outbreaks can develop quickly if weather conditions are conducive for
disease development, which makes it difficult to manage the disease based on the occurrence of symptoms. Furthermore, the epidemiology of the disease has not been studied, so weather-based indicators are not available to trigger fungicide application.

When this study was initiated, the best way to assess the incidence of blossom blight in a field was to observe the oldest flowers in an alfalfa raceme. Infected flowers pull away easily from the stem, and are often matted together with other flowers by fungal mycelium. Based on the occurrence of this symptoms, growers could determine if the disease was active in a field. However, it was difficult for even the most observant growers to accurately assess disease severity, and impossible to determine which pathogen was causing the damage.

Alfalfa blossom blight has two causal agents, *B. cinerea* and *S. sclerotiorum*. Blossom blight caused by *B. cinerea* is polycyclic and may increase very quickly after short periods of conducive weather conditions, whereas *S. sclerotiorum* is monocyclic. The control strategy for the two pathogens is also different; The fungicides Benlate, Dithane and Bravo have activity against *B. cinerea* (Gossen and Lan, 1998; Gossen *et al.*, 1997a), but Dithane and Bravo are relatively ineffective against *S. sclerotiorum* (Gossen and Wong, 1998). However, Dithane and Bravo are much less expensive than Benlate. Therefore, it is important to identify the predominant pathogen in a field to ensure cost-effective disease management.

The method developed in this study, which consists of a semi-selective medium (PDA amended with alfalfa leaves and lactic acid) and a 3-day pretreatment in the dark, can help to achieve these goals. Using this method, it took only 5 to 6
days to detect the pathogens, as opposed to 10 days for the standard method using acidified PDA. This method also resulted in much higher rates of pathogen recovery than on PDA (Tables 3.5). By combining estimates of flower infestation in the field, collected over time, with weather forecasts, growers will be able to assess the disease risk and make more informed decisions about fungicide application.

One important problem with this semi-selective medium is that it does not provide adequate control of contamination with *Rhizopus* spp. Colonies of *Rhizopus* rapidly overgrow those of *B. cinerea* and *S. sclerotiorum* on AL-PDA, although their development is much slower than on PDA. Contamination with *Rhizopus* spp. results in reduced recovery of both pathogens. Addition of a fungicide that reduces the growth of *Rhizopus* spp. may increase the efficiency of this assessment.

Information on levels of inoculum provides only one part of the knowledge required for management of blossom blight. For example, when inoculum levels in a field are low, the disease risk is also low and fungicide application is not required. However, when moderate to high levels of inoculum are present, disease management is more complicated; fungicide application may or may not be needed, depending on weather conditions.

The growth cabinet study on the impact of temperature and surface wetness duration on infection of alfalfa flowers by *B. cinerea* gives us additional information towards assessing the risk of blossom blight based on weather conditions. In this study, little or no infection occurred at 30°C, irrespective of surface wetness duration. Similarly, little or no infection by *B. cinerea* occurred at high temperature in geranium and macadamia flowers (Hunter *et al.*, 1972; Sirjusigh and Sutton,
1996), probably because conidial germination does not occur after prolonged exposure to high temperature (Salinas et al., 1989; Shoemaker and Lorbeer, 1977). This suggests that fungicide application may not be required at high temperature, regardless of inoculum level. However, infection may still occur if flowers are only exposed to high temperature for a short period of time. In the current study, incubation of flowers for 4 hr surface wetness at 30°C resulted in significant infection, but the incidence of infection declined with longer exposure to high temperature, regardless of moisture conditions.

The duration of surface wetness is affected by many environmental factors, such as day/night temperature, the time and duration of rainfall, and presence/absence of dew. In a study on geranium, the minimum surface wetness requirement for infection by B. cinerea was 4 hr. As a result, the authors recommended that air circulation in the greenhouse should be adjusted to ensure that the maximum period of surface wetness was less than 4 hr, to prevent infection by B. cinerea (Sirjusingh and Sutton, 1996). In the current study, B. cinerea required at least 12 hr of surface wetness at 20°C, 16 hr at 15°C, and 20 hr at 10° or 25°C to establish infection. Based on these results, we may divide environmental conditions into two categories: (i) conducive: surface wetness duration exceeds the minimum requirement for infection at that temperature; and (ii) non-conducive: less than 12, 16, and 20 hr of wetness duration at 20°, 15°, and 10°/25°C, respectively. These conducive conditions are critical for development of blossom blight caused by B. cinerea. If inoculum is present, flowers can be infected at these critical conducive conditions. If moderate to high levels of inoculum are present in the field in
combination with conducive conditions, fungicide application is needed immediately.

Also, increased duration of surface wetness resulted in increased incidence of infection (Fig. 5.3). For example, to reach 90% incidence of infection in alfalfa flowers, it took about 20 h at 20°C, more than 24 hr at 15°C and about 48 hr at 10° and 25°C. These results are similar to observations from strawberry and geranium, where the incidence of flower infection by *B. cinerea* increased with increases in wetness duration and the optimum temperature for flower infection was 20°C. Infection incidence in both hosts was 100% at 24 hr of wetness (Bulger et al., 1987; Sirjusingh and Sutton, 1996). The optimum temperature for infection was also 20°C for leaf blight of onion caused by *B. squamosa* (Alderman and Lacy, 1983; Ramsey and Lorbeer, 1986).

These quantitative observations may be helpful in controlling blossom blight. When combined with information on levels of inoculum and environmental conditions, we may be able to identify periods when disease risk is high. For example, if the assessment indicates that 40% of flowers are infested with *B. cinerea*, and the forecast predicts a rainy period that will last more than 20 hr with temperature near 20°C, the disease risk is very high. In this case, it would be valuable to apply fungicide before the rain starts. In contrast, if only a short period of showers is forecast for the late morning, followed by a hot, clear afternoon, the disease risk is comparatively low.

In an onion leaf blight predictive system called BOTCAST, various combinations of temperature and moisture, including wetness duration, time of
rainfall, dew period and relative humidity, were employed to estimate the daily disease severity index (DSI). For example, if mean hourly air temperature is more than 30°C for 4 hr on at least one of the preceding five days, DSI = 0. However, if the surface wetness persists for 12 hr, DSI = 1 at 10°C and DSI = 2 at 15°C (Sutton et al., 1986). Similar data sets were used to estimate the inoculum production index in another predictive system, BLIGHT-ALERT (Vincelli and Lorbeer, 1988b).

The work in this study represents the first step towards developing a quantitative forecasting system for blossom blight of alfalfa. More work in detail is needed, not only to quantify the impact of various factors on the development of the disease under controlled conditions, but also to evaluate these quantitative relationships in the field.

Differences in susceptibility to B. cinerea were observed among cultivars in controlled environment and field studies. Cvs. DK 135, OAC Minto and Iroquois generally were less susceptible to infection than cvs. Apollo II, Heinrichs, and AC Nordica. In the field test, the mean incidence of infection among cultivars ranged from 3 to 22%, which was substantially lower than in the controlled environmental studies. This was probably due to the dry conditions that occurred during the test. If the conditions in the field had been more conducive, e.g., rainfall immediately prior to the test, the differences may have been greater. In the greenhouse, the range of incidence was 36-74% in the whole-plant inoculation test and 54-92% in the detached-flower test at 24 hr of incubation.

Consistent differences among cultivars in susceptibility to B. cinerea might be useful for alfalfa seed production. In regions where the climate is often wet and
cool, e.g. the Peace River region of north Alberta, using less susceptible cultivars may reduce disease severity and result in reduced losses in yield. However, the effect of resistance on yield still needs to be confirmed in future field studies.

As shown in this study, evaluation of cultivar reaction to B. cinerea can be conducted in the greenhouse or in the field. The detached-flower test at 12 hr provides a simple and practical assessment method, in which the rank order of cultivars was highly correlated with that of the field test ($\tau = 0.69, P \leq 0.01$) and with the whole-plant inoculation test ($\tau = 0.78, P \leq 0.01$) (Table 5.1). The detached-flower test at 24 hr did not provide as good an evaluation as at 12 hr. It was weakly correlated with the field trial ($\tau = 0.61, P \leq 0.05$), but not correlated with the whole-plant test. The whole-plant test in the greenhouse is also easy and inexpensive. The results of this test were strongly correlated with the field trial ($\tau = 0.78, P \leq 0.01$). This disease occurs sporadically, and often occurs only in localized areas. As a result, field tests for this disease are likely to be relatively time-consuming and expensive, because their success is highly dependent on the weather, especially at sites where irrigation is not available. The whole-plant and detached-flower tests at 12 hr in the greenhouse may provide adequate substitutes for field tests; they are relatively quick and inexpensive, but provide good evaluation for the cultivars.

It was also noted that susceptibility to infection by B. cinerea was associated with genetic traits, such as flower architecture and colour. Upward-facing flowers were less susceptible (16%) than downward-facing flowers (86%). Also, purple flowers were less susceptible than white flowers in Iroquois, but not in Apollo II and AC Nordica. In crops such as pistachio, almond and castor, cultivars with an open
architecture dry quickly and are generally less susceptible to infection than those with a dense growth habit. In addition, many other traits such as thickened cuticles (Hammer and Evensen, 1994; Pie and Brouwer, 1993) and cell walls (Mansfield and Huston, 1980; Rijkenberg et al., 1980) have been associated with reduced susceptibility. Almost all of these studies indicate that cultivar reaction to *B. cinerea* is regulated by the cumulative expression of many genetic characteristics, rather than one or two major genes. None of the cultivars in this study demonstrated a strong resistance reaction. We conclude that efforts to identify major genes for resistance to *B. cinerea* in alfalfa are unlikely to be successful. Even if major genes for resistance were found, cultivars with those genes might not be resistant to *S. sclerotiorum*. In contrast, some of the genetic traits that contribute to reduced susceptibility to *B. cinerea* may also decrease susceptibility to *S. sclerotiorum*. For example, purple flowers were more resistant to *B. cinerea* infection in one alfalfa cultivar in the current study. In another study, purple alfalfa flowers were slightly less susceptible to *S. sclerotiorum* than white flowers (Lefol et al., 1999). Similarly, an open canopy architecture inhibits infection by *S. sclerotiorum* in bean (Coyne et al., 1977).

Some of the traits associated with resistance may also be associated with negative effects on crop production. For example, sparse flower density may reduce infection, but may also reduce seed yield. It would be necessary to evaluate these traits based on their effect on both yield and susceptibility to the two pathogens. Also, various trait evaluations may require different test methods. A whole-plant test in a greenhouse may be effective for evaluating the role of flower architecture,
canopy density and flower colour, while detached-flower studies may provide the best assessment of differences associated with cell walls and cuticles. A field test evaluates the cumulative effect of all of these traits.

In conclusion, the assessment of inoculum levels plus the information from the study of temperature and wetness duration can be used as a first step towards assessing the disease risk in a field. When combined with weather forecasts, this information may be used by growers to reduce the frequency of fungicide application. Identification of cultivars with reduced susceptibility to *B. cinerea* may provide choices for growers who wish to reduce the risk of losses due to blossom blight. However, all of these studies are still at an early stage. In order to develop an effective and comprehensive strategy, more detailed work is required on both *B. cinerea* and *S. sclerotiorum*. 
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Appendix A. Selecting nutrient amendments and incubation conditions

1. Introduction

Inducing sporulation of \textit{B. cinerea} is one possible approach for rapidly assessing the presence of the pathogen. One study showed that an extract of 200 g fresh bean leaf per litre water agar plus 2.36 g NaNO$_3$, 5.75 g Na$_2$SO$_4$ or 40 g sucrose strongly promoted sporulation of \textit{B. fabae} compared to the bean leaf extract agar alone (Leach and Moore, 1966). In another study, chickpea shoots inoculated with \textit{B. cinerea} sporulated abundantly when incubated in darkness for 3 days, followed by exposure to 12-h photoperiod for 2 days (Laha and Grewal, 1983). In the current study, the impact of alfalfa leaves, NaCl and sucrose amendments to commercial PDA on sporulation of \textit{B. cinerea} and sclerotial formation of \textit{S. sclerotiorum} was assessed.

2. Materials and Methods

PDA was used as the basic medium in this study. It contains amount of nutrients such as sugars and salts, so lower concentrations of amendments were assessed in this study than those used in the study on \textit{B. fabae} described above. The concentration of the nutrients per liter of solution of commercial PDA (39 g L$^{-1}$) was as follows: (i) L-PDA: 54 g fresh, homogenized alfalfa leaves; (ii) Na-PDA: 1 g NaCl, (iii) S-PDA: 10 g sucrose; (iv) A-PDA: 3 ml of 85\% lactic acid, (v) PDA: no amendment.

The homogenized alfalfa leaves, NaCl and sucrose were added to PDA prior to autoclaving, whereas lactic acid was added after autoclaving. Experiments were
conducted as a split plot, in which pretreatments in darkness of three days followed
14-h photoperiod (dark pretreatment) or continuously in 14-h photoperiod (light
pretreatment) were the main plots, while amendment with different nutrients were
used as subplots. Twelve 9-cm petri dishes were prepared for each medium. Each
dish was inoculated with a 5-mm mycelial plug taken from the edge of an actively
growing 3- to 4-day-old colony of *B. cinerea*. Six dishes of each medium were
incubated in the dark at room temperature, while the other six dishes were incubated
on a lab bench with 14-h photoperiod. After 3 days, the dishes in the dark treatment
were transferred to the lab bench. The radial growth of each colony (mean of widest
diameter and right angle) and the number of sporulating colonies were assessed each
day. Seven days after inoculation, spores were harvested in sterile distilled water,
filtered through four layers of sterile cheesecloth, and the spore concentration was
estimated with the aid of a haemacytometer.

A similar experiment was conducted for *S. sclerotiorum*. The number of
colonies with sclerotia of *S. sclerotiorum* was recorded daily, starting 3 days after
plating.

Analysis of variance of colony size and spore production was performed
using SAS. Where appropriate, differences among means were examined using
Duncan’s Multiple Range Test at $P \leq 0.05$.

2. Results

Analysis of variance of colony size demonstrated that radial growth of *B.
cinerea* was affected by pretreatments, medium amendments and their interactions.
Dark pretreatment resulted in more ($P \leq 0.05$) rapid growth of *B. cinerea* than in the
light pretreatment from Day 3 to Day 6, but not on Day 7 (ANOVA not shown, Table A1).

After light pretreatment, radial growth on L-PDA occurred more quickly \((P \leq 0.05)\) than A-PDA from Day 3 to Day 5, or than Na-PDA from Day 3 to Day 4, or S-PDA on Day 3 (Table A2). There was no difference in radial growth between PDA and L-PDA, or among the five media from Day 6 to Day 7. Colonies of \(B. \text{cinerea}\) reached the edges of petri dishes by Day 6 on L-PDA, and after Day 7 on other media.

After dark pretreatment, radial growth was faster \((P \leq 0.05)\) on L-PDA than four other media on Day 3 (Table A2). A-PDA inhibited radial growth compared to the other media from Day 4 to Day 7. Colonies of \(B. \text{cinerea}\) reached the edge of the petri dishes by Day 4 on S-PDA and L-PDA, Day 5 on PDA, Day 6 on Na-PDA and after Day 7 on A-PDA.

Sporulation of \(B. \text{cinerea}\) tended to be initiated more quickly after the dark pretreatment than in the light, e.g. sporulation occurred on all six L-PDA dishes by Day 4 after dark pretreatment, but by Day 5 after the light pretreatment (Table A2). Similarly, a longer time was required for sporulation on other media after light pretreatment than after dark pretreatment. Alfalfa leaf amendment to PDA also tended to promote sporulation of \(B. \text{cinerea}\), e.g. following dark pretreatment, sporulation occurred by Day 4 on all six dishes of L-PDA, but by Day 6 on PDA and by Day 7 on A-PDA (Table A2).
Table A.1. The effect of amendments to potato dextrose agar on the radial growth of *Botrytis cinerea*, following pretreatment from Day 0 to Day 3 under a 14-hr day regime (Light) or continuous darkness (Dark) (n = 6).

<table>
<thead>
<tr>
<th>Media‡</th>
<th>Radial growth of <em>Botrytis cinerea</em> (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Light Pretreatment</td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>4.0 ab</td>
</tr>
<tr>
<td>A-PDA</td>
<td>3.6 b</td>
</tr>
<tr>
<td>Na-PDA</td>
<td>3.8 b</td>
</tr>
<tr>
<td>S-PDA</td>
<td>3.6 b</td>
</tr>
<tr>
<td>L-PDA</td>
<td>5.1 a</td>
</tr>
<tr>
<td>Mean</td>
<td>4.0</td>
</tr>
<tr>
<td>Dark Pretreatment</td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>6.3 C</td>
</tr>
<tr>
<td>A-PDA</td>
<td>4.4 C</td>
</tr>
<tr>
<td>Na-PDA</td>
<td>6.6 BC</td>
</tr>
<tr>
<td>S-PDA</td>
<td>6.9 BC</td>
</tr>
<tr>
<td>L-PDA</td>
<td>7.0 A</td>
</tr>
<tr>
<td>Mean</td>
<td>6.2</td>
</tr>
</tbody>
</table>

‡ PDA - potato dextrose agar, A - lactic acid, Na - NaCl, S - sucrose, L – fresh alfalfa leaves.
† Values in a column that are followed by the same letter and case did not differ based on Duncan’s Multiple Range Test at $P \leq 0.05$.
nd - not done. Colony size was not recorded because growth had completely filled the petri dish.
Analysis of variance of spore concentration demonstrated that spore production of *B. cinerea* was also affected by pretreatments and medium amendments (*P* ≤ 0.001), but not by their interactions. Dark pretreatment resulted in more spore production of *B. cinerea* (*P* ≤ 0.05) than light pretreatment (Table A3). Among the media, *B. cinerea* sporulated more abundantly (*P* ≤ 0.05) on L-PDA than on all of the other media (Table A3). Na-PDA also produced more (*P* ≤ 0.05) spores than A-PDA. No differences in sporulation occurred among A-PDA, PDA and S-PDA.

For *S. sclerotiorum*, radial growth was not affected by pretreatments. Medium amendments only influenced the radial growth at Day 3, in which L-PDA resulted in more (*P* ≤ 0.05) radial growth than A-PDA and Na-PDA (Table A4). Sclerotium formation of *S. sclerotiorum* tended to be initiated slightly more quickly after dark pretreatment than light pretreatment (Table A5), e.g. sclerotia were present on all of 6 dishes of L-PDA and A-PDA by Day 6 following dark pretreatment, but after Day 7 following light pretreatment. Nutrient amendments also slightly promoted sclerotiorum formation, e.g. sclerotia were formed on all of six dishes of L-PDA and A-PDA by Day 6, but by Day 7 on S-PDA and Na-PDA and after Day 7 on PDA.
Table A2. The effect of amendments to potato dextrose agar on sporulation of *Botrytis cinerea* after pretreatment for Day 0 to Day 3 under a 14-hr day regime (Light) or continuous darkness (Dark)*.

<table>
<thead>
<tr>
<th>Media†</th>
<th>No. sporulating colonies of <em>Botrytis cinerea</em> (max. = 6)</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>A-PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Na-PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>S-PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>L-PDA</td>
<td></td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0</td>
<td>0.6</td>
<td>2.0</td>
<td>2.6</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Dark Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>A-PDA</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Na-PDA</td>
<td></td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>S-PDA</td>
<td></td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>L-PDA</td>
<td></td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.4</td>
<td>1.6</td>
<td>4.8</td>
<td>5.8</td>
<td>6.0</td>
</tr>
</tbody>
</table>

† PDA - potato dextrose agar, A - lactic acid, Na - NaCl, S - sucrose, L - fresh alfalfa leaves.

‡ No statistical analysis was possible because the trial was not repeated.
Table A3. The effect of amendments to potato dextrose agar on sporulation of *Botrytis cinerea* on Day 7 following pretreatment from Day 0 to Day 3 under a 14-hr day regime (Light) or continuous darkness (Dark) (n = 6)

<table>
<thead>
<tr>
<th>Pretreatment and Media</th>
<th>Mean conidia per plate ($\times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light pretreatment</td>
<td>178.1 b</td>
</tr>
<tr>
<td>Dark pretreatment</td>
<td>448.5 a</td>
</tr>
<tr>
<td>Media</td>
<td></td>
</tr>
<tr>
<td>A-PDA</td>
<td>55.1 C</td>
</tr>
<tr>
<td>PDA</td>
<td>197.4 BC</td>
</tr>
<tr>
<td>S-PDA</td>
<td>172.8 BC</td>
</tr>
<tr>
<td>Na-PDA</td>
<td>314.6 B</td>
</tr>
<tr>
<td>L-PDA</td>
<td>826.7 A</td>
</tr>
</tbody>
</table>

‡ PDA - potato dextrose agar, A - lactic acid, Na - NaCl, S - sucrose, L - fresh alfalfa leaves.
† Values in a column that are followed by the same letter and case did not differ based on Duncan’s Multiple Range Test at $P \leq 0.05$. 

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Table A4. The effect of amendments to potato dextrose agar on the radial growth of *Sclerotinia sclerotiorum* (n = 12) 

<table>
<thead>
<tr>
<th>Media*</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA</td>
<td>5.5 (\text{ab}^{\dagger})</td>
<td>6.5 (\text{a})</td>
<td>7.4 (\text{a})</td>
<td>7.8 (\text{a})</td>
<td>8.5 (\text{a})</td>
</tr>
<tr>
<td>A-PDA</td>
<td>4.4 (\text{b})</td>
<td>5.9 (\text{a})</td>
<td>7.5 (\text{a})</td>
<td>8.0 (\text{a})</td>
<td>8.4 (\text{a})</td>
</tr>
<tr>
<td>Na-PDA</td>
<td>4.6 (\text{b})</td>
<td>6.0 (\text{a})</td>
<td>7.4 (\text{a})</td>
<td>8.1 (\text{a})</td>
<td>8.5 (\text{a})</td>
</tr>
<tr>
<td>S-PDA</td>
<td>5.6 (\text{ab})</td>
<td>6.2 (\text{a})</td>
<td>7.6 (\text{a})</td>
<td>8.3 (\text{a})</td>
<td>8.5 (\text{a})</td>
</tr>
<tr>
<td>L-PDA</td>
<td>6.6 (\text{a})</td>
<td>6.9 (\text{a})</td>
<td>7.7 (\text{a})</td>
<td>8.3 (\text{a})</td>
<td>8.5 (\text{a})</td>
</tr>
<tr>
<td>Mean</td>
<td>5.4</td>
<td>6.3</td>
<td>7.5</td>
<td>8.1</td>
<td>8.5</td>
</tr>
</tbody>
</table>

\(\dagger\) Values are means of radial growth calculated across pretreatments (14-h photoperiod vs continuous dark).

\(\ddagger\) PDA - potato dextrose agar, A - lactic acid, Na - NaCl, S - sucrose, L - fresh alfalfa leaves.

\(\dagger\) Values in a column that are followed by the same letter did not differ based on Duncan’s Multiple Range Test at \(P \leq 0.05\).
Table A5. The effect of amendments to potato dextrose agar on sclerotial formation of *Sclerotinia sclerotiorum* after pretreatment from Day 0 to Day 3 under a 14-hr day regime (Light) or continuous darkness (Dark).†

<table>
<thead>
<tr>
<th>Media</th>
<th>No. colonies of <em>Sclerotinia sclerotiorum</em> (max. = 6)</th>
<th>Light Pretreatment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 4</td>
<td>Day 5</td>
<td>Day 6</td>
<td>Day 7</td>
</tr>
<tr>
<td>PDA</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>A-PDA</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>S-PDA</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Na-PDA</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>L-PDA</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2</td>
<td>1.6</td>
<td>2.6</td>
<td>3.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

| Dark Pretreatment | | | | | |
|--------------------|---|---|---|---|
| PDA                | 0 | 2 | 3 | 3 | 5 |
| A-PDA              | 0 | 2 | 2 | 6 | 6 |
| S-PDA              | 0 | 1 | 2 | 3 | 6 |
| Na-PDA             | 0 | 1 | 2 | 4 | 6 |
| L-PDA              | 2 | 3 | 3 | 6 | 6 |
| Mean               | 0.4| 1.8| 2.4| 4.4| 5.8|

† PDA - potato dextrose agar, A - lactic acid, Na - NaCl, S - sucrose, L - fresh alfalfa leaves.

† No statistical analysis was possible on the data because the trial was not repeated.
2. Discussion

Different cultural methods and nutrient amendments were examined in order to identify combinations of factors that promoted sporulation of *B. cinerea* and sclerotial formation of *S. sclerotiorum*. The results in the trials in the laboratory showed that combining amendment of alfalfa leaves with a dark pretreatment induced the most rapid sporulation of *B. cinerea* (Table A3). This combination also slightly shortened the time requirement for sclerotial formation of *S. sclerotiorum* compared with commonly used methods (PDA or A-PDA with light pretreatment) (Table A5).

The effect of darkness on sporulation of *B. cinerea* is complicated. When incubated in continuous darkness for 2-3 days, the pathogen generally does not sporulate (Rubin, 1952; Stewart and Long, 1987; Tuller and Peterson, 1988). However, when incubation in darkness is followed by exposure to white or black light, *B. cinerea* sporulates abundantly and spore yields exceed production under continuous black or NUV light (Tan and Epton, 1973; Laha and Grewal, 1983). The results in the current study support the observation that a period of darkness increases subsequent sporulation (Table A3). Sporulation was also initiated earlier following a dark pretreatment than following a light pretreatment (Table A2).

Amendment with sucrose or NaCl did not induce sporulation of *B. cinerea* on PDA (Table A3), although the induction was observed on *B. fabae* (Leach et al., 1966). Induction of sporulation by salt and sucrose was probably due to high osmotic pressure and inhibition of fungal development (Leach and Moore, 1966). There was a tendency towards smaller colony size on Na-PDA compared to PDA, but the
differences were not significant (Table A1). Sporulation of *B. cinerea* may be promoted at higher concentrations of salt and sucrose. A trial to test the effect of higher concentration of salt and sucrose in PDA on sporulation of *B. cinerea* might be valuable.
Appendix B. Effect of anti-microbial amendments to potato dextrose agar on radial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum*

1. Introduction

Anti-microbial amendments such as antibiotics in PDA may help to inhibit contamination in field-collected samples. However, these amendments may also inhibit growth of the target pathogens. This preliminary trial was conducted to screen antibiotics which have no or little inhibitory effect on the radial growth of *B. cinerea* and *S. sclerotiorum*.

2. Materials and Method

Some commonly used anti-microbial agents were selected for test, including pimaricin, novobiocin, chloramphenicol, and Rose Bengal. All these agents are heat-stable except chloramphenicol. The herbicide paraquat was also selected for test. The concentrations of these chemicals in the test were chosen based on the dosage used for isolating *S. sclerotiorum* (Gugel and Morrall, 1986; Turkington, 1988), *B. cinerea* (Peng and Sutton, 1991) and other microorganisms (Dhingra and Sinclair, 1995) on other media.

Initially, pimaricin, novobiocin and Rose Bengal were assessed as amendments to PDA, to examine their effect on the radial growth of *B. cinerea* and *S. sclerotiorum*. The concentration of the chemicals per liter of solution of commercial PDA (39 g L\(^{-1}\)) was as follow: (i) PDA-P1: 1.0 mg pimaricin; (ii) PDA-P2: 5.0 mg pimaricin; (iii) PDA-N1: 50 mg novobiocin; (iv) PDA-P2: 100 mg
novobiocin; (v) PDA-RB1: 10 mg Rose Bengal; (vi) PDA-RB2: 50 mg Rose Bengal; (vii) PDA: no amendments.

The amendments were added to PDA prior to autoclaving. Six 9-cm dia. petri dishes were prepared for each combination of medium and pathogen. Each dish was inoculated with a 5-mm mycelial plug taken from the edge of an actively growing 3- to 4-day-old colony of *B. cinerea* or *S. sclerotiorum*. These dishes were incubated on a lab bench with a 14-h photoperiod. The radial growth of each colony (mean of widest diameter and right angle) was assessed each day after 2 or 3 days of incubation.

In the second trial, the impact of chloramphenicol, paraquat and fresh lettuce leaf amendments to PDA on the growth of *B. cinerea* and *S. sclerotiorum* from inoculated alfalfa flowers were assessed. The concentration of the amendments in commercial PDA (39 g L⁻¹) was as follow: (i) CL-PDA: 200 mg chloramphenicol and 20 g lettuce leaves; (ii) CLP-PDA: 200 mg chloramphenicol, 5 mg paraquat and 20 g lettuce leaves; (iii) PDA: no amendment.

Five 9-cm petri dishes were prepared for each combination of medium and pathogen. Alfalfa plants were inoculated as described in Section 3.2.1, and incubation and colony measurement were conducted as described in Appendix A. Analysis of variance of colonial sizes was performed using SAS. Differences among means were examined using Duncan’s Multiple Range Test at *P* ≤ 0.05.

2. Results

Amendments of 5 or 10 mg L⁻¹ of pimaricin to PDA did not affect radial growth of *B. cinerea* or *S. sclerotiorum* compared to PDA (Fig. B1 and B2). The
radial growth of *B. cinerea* reached the edge of dishes by Day 5 on PDA + P1, PDA + P2 and PDA, by Day 6 on PDA + N1, by Day 8 on PDA + N2, and after Day 8 on PDA + RB1 and PDA + RB2 (Fig. B1). For *S. sclerotiorum*, the size of colonies on PDA + P1 and PDA + P2 were equal to those on PDA, and the radial growth reached the dish edge by Day 2. By comparison, the colony reached the edge of dishes by Day 5 on PDA + N1 and after Day 6 on PDA + N2, PDA + RB1 and PDA + RB2 (Fig. B2).

In the second trial, CL-PDA promoted radial growth of *S. sclerotiorum* compared to PDA (*P* ≤ 0.05), but had no effect on growth of *B. cinerea* (Table B1). Addition of paraquat to the CL-PDA media had no effect on growth of *S. sclerotiorum*, but inhibited the growth of *B. cinerea* (*P* ≤ 0.05).

3. Discussion

Adding pimaricin to PDA did not affect the radial growth of the two pathogens, while Rose Bengal inhibited their growth (Fig. B1, B2). Amendment of novobiocin also inhibited the growth of the two pathogens, its effect was less pronounced than Rose Bengal. Based on the results, pimaricin at 5 mg l⁻¹ and novobiocin at 50 mg l⁻¹ were selected for further testing in field trials.

CL-PDA did not inhibit either pathogen, but paraquat inhibited *B. cinerea*. Therefore, chloramphenicol was also selected in combination with lettuce leaves. Because alfalfa is the host of blossom blight caused by the two pathogens, lettuce leaves were replaced by alfalfa leaves in the field trials.
Figure B1. The effect of amendments to potato dextrose agar on the radial growth of *Botrytis cinerea* incubated on the lab bench at room temperature with 14-h photoperiod (n = 6). P1 - 1.0 mg pimaricin, P2 - 5.0 mg pimaricin, N1 - 50 mg novobiocin, N2 - 100 mg novobiocin, RB1 - 10 mg Rose Bengal, RB2 - 50 mg Rose Bengal.
Figure B2. The effect of amendments to potato dextrose agar on the radial growth of *Sclerotinia sclerotiorum* incubated on the lab bench at room temperature with 14-h photoperiod (n = 6). P1 - 1.0 mg pimaricin, P2 - 5.0 mg pimaricin, N1 - 50 mg novobiocin, N2 - 100 mg novobiocin, RB1 - 10 mg Rose Bengal, RB2 - 50 mg Rose Bengal. Values for PDA + P1 and PDA + P2 are equal to those for PDA.
Table B1. The effect of amendments to potato dextrose agar on radial growth (colony dia. in cm) of *Botrytis cinerea* and *Sclerotinia sclerotiorum* incubated on the lab bench for five days (n = 6).

<table>
<thead>
<tr>
<th>Media(^\d)</th>
<th>Radial growth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. cinerea</em></td>
</tr>
<tr>
<td>PDA</td>
<td>6.5 a(^\d)</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>6.9 a</td>
</tr>
<tr>
<td>CLP-PDA</td>
<td>1.7 b</td>
</tr>
</tbody>
</table>

\(\d\) PDA - potato dextrose agar, C - chloramphenicol, L - lettuce leaves, P - paraquat.

\(\d\) Values in a column, followed by the same letter, did not differ based on Duncan's Multiple Range Test at \(P \leq 0.05\).
Appendix C. Recovery of *Botrytis cinerea* and *Sclerotinia sclerotiorum* isolated from alfalfa flowers on PDA with various amendments in repetition C

**Table C1.** Recovery of *Botrytis cinerea* and *Sclerotinia sclerotiorum* isolated from alfalfa flowers on PDA with various amendments in an inoculated trial at the AAFC research farm at Saskatoon in 1997 (in repetition C, n = 5)

<table>
<thead>
<tr>
<th>Media</th>
<th><em>Botrytis cinerea</em></th>
<th><em>Sclerotinia sclerotiorum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA†</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PL-PDA</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>NL-PDA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CL-PDA</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PNL-PDA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCL-PDA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A-PDA</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>2.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

† PDA - potato dextrose agar, A - lactic acid, C - chloramphenicol, L - alfalfa leaves, N - novobiocin, P - pimarin
‡ Incidence was assessed on Day 16.
Appendix D. Recovery of *Botrytis cinerea* isolated from alfalfa flowers on PDA with various amendments in repetition G

**Fig. D1.** Effect of medium amendments on recovery of *Botrytis cinerea* from alfalfa flowers collected in an inoculated field trial at Saskatoon, SK in 1998 (repetition G, n = 5). Amendments are described in Section 3.2.2.
Fig. D2. Effect of medium amendments on contaminant colonies isolated from alfalfa flowers collected in an inoculated field trial at Saskatoon, SK in 1998 (repetition G, n = 5). Amendments are described in Section 3.2.2.
Fig. D3. Effect of medium amendments on the number of contaminant colonies isolated from alfalfa flowers collected in an inoculated field trial at Saskatoon in 1998 (repetition H, n = 5). Amendments are described as Section 3.2.2.
Appendix E. Composition of soilless mix

(1) 1 × 113 litre bale sphagnum peat moss
(2) 2 × 110 litre bags medium grade vermiculite
(3) 3.2 kg fine ground calcium carbonate
(4) 3.5 kg “Osmocote” fertilizer
(5) 700 g 20% Superphosphate
(6) 15 g “Fritted” Trace Elements #555
(7) 15 g 13% Chelated Iron
Appendix F. Surface sterilization

1. Materials and methods

Two trials were conducted to develop an effective procedure for surface sterilization of alfalfa leaves. In the first trial, 40 florets per treatment were inoculated with *B. cinerea*, then surface disinfested by immersion in 70% ethanol for 5 vs. 10 sec., followed by 0.6% NaOCl for 45 vs. 60 sec., and then three rinses in sterile distilled water. The florets were then plated onto PDA (four florets per plate) and incubated on a laboratory bench for 10 to 14 days. Based on this result, together with several other preliminary tests, a second test was designed. In the second trial, 40 florets per treatment were immersed in 70% ethanol for 10 vs. 30 sec., followed by 90 sec. in 0.6% NaOCl and 10 rinses in sterile distilled water. The immersion in ethanol and disinfestation in NaOCl were performed with continuous stirring. Each treatment was replicated three times in the second trial.

2. Results and Discussion

The recovery of the pathogen in the four surface sterilization treatments in the first trial ranged from 30% to 43%. This was considered to be unacceptably high because the flowers were sterilized immediately after inoculation. Therefore, the high frequency of recovery indicated that the surface-sterilization method was not sufficiently effective. A longer duration of disinfestation was used in the second trial. Recovery of the pathogen was 6% in the treatment with 30 sec. in ethanol vs. 13% in
the treatment with 10 sec. in ethanol. Based on this result, the combination of 30 sec. in 70% ethanol, 90 sec. in 0.6% NaOCl and 10 rinses in sterile distilled water was selected as a standard procedure in all subsequent tests in this study.