Evaluation of Components of Sclerotinia Stem Rot (*Sclerotinia sclerotiorum*) Management in Canola: Seeding Rates, Avoidance Mechanisms, and Physiological Resistance Screening Methodologies

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Evaluation of Components of Sclerotinia Stem Rot (*Sclerotinia sclerotiorum*) Management in Canola: Seeding Rates, Avoidance Mechanisms, and Physiological Resistance Screening Methodologies

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE

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> "There is a difference between learning and acquiring knowledge. Learning ceases when there is only an accumulation of knowledge. There is learning only when there is no acquisition at all. When knowledge becomes all important learning ceases. The more I add to knowledge the more secure, the more assured the mind becomes, and, therefore it ceases to learn. Learning is never an additive process."
>
> J. Krishnamurti from "Freedom from the Known" p. 101.

Please enjoy reading up on my recent acquisitions of knowledge.

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ABSTRACT

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a serious disease of canola (*Brassica napus* L. and *B. rapa* (L.) Thell. Emend. Metzger). The most common method of control used by canola producers is the use of fungicides. The purpose of this series of experiments was to identify alternative methods of control: cultural and host resistance. The first study was an examination of the impact of seeding rate on the development of sclerotinia stem rot infection in four canola cultivars at seven locations in 2001 and 2002. It was found that seeding rates did affect sclerotinia stem rot disease incidence (DI). Seeding rates greater than twice the normal seeding rate (6.7 kg/ha) significantly increased DI, and rates less than normal did not affect DI. The second experiment was an examination of the plant architectural, crop structural, and phenological traits, which may act as escape or avoidance mechanisms to *S. sclerotiorum* infection. Eleven canola cultivars, which included two apetalous cultivars, were evaluated for 16 traits believed to aid in escape at seven locations in 2001 and 2002. The two apetalous lines had significantly less disease than the petalled lines. DI was significantly correlated to petal adhesion to leaves, lodging resistance, branch angle, and crop maturity. Plant breeders should be able to successfully select for these traits since their expression has stronger genotypic effects than environmental effects. The third experiment was the development of a growthroom screening methodology that accurately, reliably, and efficiently identified physiological resistance to *S. sclerotiorum*. Ten *B. napus* lines, seven with transgenes believed to confer physiological resistance to *S. sclerotiorum*, were screened using six different methods in growthroom studies and two years of field trials. The technique using canola petals infested with *S. sclerotiorum* mycelium resulted in the

best the accuracy, reliability, and efficiency of the six methods. The outcomes of these studies indicate that there are options available for producers and traits and techniques available for researchers to more effectively manage sclerotinia stem rot in canola.

FOREWORD

This thesis is written in a manuscript style. Therefore each chapter/manuscript has its own introduction, materials and methods, results, conclusion and acknowledgement sections. The style of these manuscripts and literature review is fashioned on the specifications of the Canadian Journal of Plant Pathology. This publication has been corrected for Canadian English spelling.

1.0 INTRODUCTION

Canola (*Brassica napus* L. and *B. rapa* (L.) Thell. Emend. Metzger), or oilseed rape, is an important oilseed crop in the world. Because of its physiology and unique nutritional qualities, it has become one of the largest crops in Canada, second only to wheat. As this crop has increased in acreage over the years, so have the occurrences of *Brassica* diseases. One of the major diseases of canola is sclerotinia stem rot. Currently, the only option available for canola producers to control this disease is the use of fungicides, which are costly to apply and are becoming less enticing because of environmental considerations. Therefore there is interest in exploring other options to manage sclerotinia stem rot, such as cultural controls and host resistance.

Canopy density in canola and other crops susceptible to *Sclerotinia sclerotiorum* (Lib.) de Bary has been found to have an effect on the development of this disease (Blad *et al.* 1978; Haas and Bolwyn 1972; Hu *et al.* 1999; Park 1993; Saindon *et al.* 1995). Seeding rates have been found to have some affect on canopy density in other crops but research on this in canola is lacking. The first objective of this research project is to determine whether seeding rate influences sclerotinia stem rot development in canola.

Sources for high levels of resistance to *S. sclerotiorum* infection are difficult to find since this fungus is a pathogen on many dicot species. There are reports of natural variation in disease reaction in soybeans (Arahana *et al.* 2001b; Boland and Hall 1987; Hartman *et al.* 2000; Kim and Diers 2000), beans (Kolkman and Kelly 2002; Miklas *et al.* 1992a; Miklas *et al.* 2001; Park *et al.* 2001; Schwartz *et al.* 1987), and canola (Brun *et al.* 1987; Li *et al.* 1999a; Liu 1991; Sedun *et al.* 1989). These effects are attributed to partial physiological resistance and escape or avoidance mechanisms. Jurke *et al.* (1998)

reported that an apetalous canola cultivar grown in Canada greatly reduced the incidence of sclerotinia stem rot. The second objective of this project was to quantify the effect of petals on infection and to determine other possible avoidance mechanisms that exist in canola cultivars.

Breeding novel sources of resistance into canola and other *S. sclerotiorum* susceptible crops is an effort being undertaken in many laboratories (Arahana *et al.* 2001a; Billings *et al.* 2003; Cober *et al.* 2003; Dickman *et al.* 2003; Dietz *et al.* 2003; Donaldson *et al.* 2001; Kesarwani *et al.* 2000; Thompson *et al.* 1995). *Sclerotinia sclerotiorum* can be a difficult pathogen to work with in the field because of its sensitivity to environmental conditions. For this reason, many researchers use laboratory or growthroom screening techniques to quantify physiological resistance, or resistance not due to avoidance mechanisms. However, there are no standardised procedures for these activities in any crop. The third objective of these experiments was to assess existing growthroom screening techniques on the basis of accuracy, reliability and efficiency for the measurement of physiological resistance in canola, and to develop new techniques if necessary.

2.0 LITERATURE REVIEW

2.1 The Host

2.1.1 History and Economic Importance

Canola, also known as oilseed rape, is grown on all continents of the world. The largest canola producing nations are Canada, Australia, and the United States of America, which produced a combined 5.0 million tonnes of canola in 2002. This represented 15.4% of the total world rapeseed and canola production (Table 2.1). Canola is also grown in India, UK, France, Germany, Poland, Sweden, Finland, Korea, China, Russia, Brazil, Iran, Egypt, and Mexico. The global canola acreage increased dramatically through the 1980s and 1990s but appears to have reached a plateau in recent years (Canola Council of Canada 2003).

Canola is an oilseed, which when crushed produces an edible oil low in saturated fats, which is considered to be a desirable and healthy oil. By definition, as given by the Canola Council of Canada (2003), canola is a rapeseed cultivar that has an oil profile with less than 2% erucic acid and has less than 30 μmols of glucosinolates per gram of oil-free solids (meal). This definition separates canola from rapeseed, the parent material from which the first canola cultivar was bred in 1974. Canola oil has less than 7.1% saturated fats, the lowest level of saturated fats of all major oils on the market (Canola Council of Canada 2003).

There are three species in the *Brassica* genus that have been modified to the canola definition: *B. napus*, *B. rapa*, and *Brassica juncea* (L.) Cosson. The majority of canola acres are planted to *B. napus* (Canola Council of Canada 2003). Within *B. napus*

Table 2.1 World supply of rapeseed and canola in millions of tonnes from major producing countries and regions 1999 to 2003.

Country	2002/03	2001/02	2000/01	1999/00
European Union	9.34	8.87	8.95	11.40
Central Europe	2.29	2.64	2.24	2.78
Canada	3.97	5.15	7.21	8.80
USA	0.71	0.91	0.92	0.62
China	10.53	11.32	11.38	10.13
India	3.40	4.85	3.75	5.10
Australia	0.73	1.77	1.78	2.43
Other countries	1.25	1.17	1.30	1.30

Note: Information is from (Oil World 2003).

and *B. rapa*, there are both spring-type and winter-type cultivars. Winter-type cultivars are usually more productive where conditions are favourable (Mendham and Salisbury 1995), but most canola acres are seeded to spring types.

2.1.2 Cropping Practices

Based on a review by Pouzet (1995), canola is a crop with a maturity range from 70 days for certain *B. rapa* cultivars up to 380 days for some Chinese winter *B. napus* cultivars. Canola must be seeded into a moist, well aerated, well-structured seedbed with minimal trash cover for maximum germination and growth. A soil temperature >5 C is needed for germination, limiting when spring type cultivars can be seeded. Seeding rates can vary and still result in similar yields, but generally rates are between 40 to 200 plants per square metre (1.2 kg/ha to 7.0 kg/ha).

Macronutrient requirements are 50 to 60 kg of N, 12 kg of P, 25 kg of K and 8 kg S per tonne of seed produced. Canola requires large amounts of water. Evapotranspiration in *B. rapa* has been recorded at 8 mm per day. Irrigation is not widely used in canola producing regions but can increase yields by 2.5 times (Pouzet 1995).

Grain loss because of shattering pods can be substantial if the crop is left to dry too much, especially in *B. napus* cultivars. Most producers swath or windrow their crops at growth stage 5.3 or 5.4 (Harper and Berkenkamp 1975) and harvest the windrows once grain moisture is less than 15%. Direct combining is a practice sometimes used if the crop matures evenly or if a desiccant chemical was used.

Weed management can be problematic in canola (Orson 1995). Weedy species include both annuals and perennials, and are found in all canola/rapeseed producing

regions of the world. Weed control is achieved with pre-seed tillage, pre-plant herbicides, and foliar applied herbicides, especially in herbicide tolerant cultivars.

There are many diseases that afflict canola crops. The more common diseases are blackleg (*Leptosphaeria maculans* (Desmaz.) Ces. & De Not.), sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary), alternaria leaf and pod spot or black spot (*Alternaria brassicae* (Berk.) Sacc., *A. brassicola*, (Schwein.) Wiltshire and *A. raphani* Groves & Skolko), light leaf spot (*Pyrenopeziza brassicae* Sutton & Rawlinson), white leaf spot (*Mycosphaerella capsellae* (Ellis & Everh.) Deighton), white rust (*Albugo candida* (Pers.) Kunze), downy mildew (*Pernospora parasitica* (Pers.:Fr.) Fr.), clubroot (*Plasmodiophora brassicae* Woronin), and soil-borne seedling diseases (*Fusarium* spp, *Pythium* spp, and *Rhizoctonia solani* Kuhn) (Rimmer and Buchwaldt 1995). Generally, control of most of these diseases is achieved through host resistance, with the notable exception of sclerotinia stem rot.

2.2 The Pathogen

2.2.1 Biology and Taxonomy

Sclerotinia stem rot in canola is caused by the fungus *S. sclerotiorum. Sclerotinia sclerotiorum* is a fungus belonging to the phylum Ascomycota, the class Discomycetes, the order Helotiales (Leotiales), and the family Sclerotiniaceae. Ascomycetes are fungi with a haploid mycelium possessing cross walls; they produce asexual conidia on conidiophores and sexual ascospores in asci. Discomycetes are ascomycetes that produce apothecia, i.e., cup-shaped or cushion-shaped ascocarps and discharge their ascospores forcibly. Helotiales are discomycetes producing cup-shaped apothecia and having septated ascospores. Members of the Sclerotiniaceae produce sclerotia (melanized, hardened vegetative survival structures) from which apothecial units emerge; all are necrotrophic parasites (Agrios 1997 and INRA 2003).

The genus *Sclerotinia* is delimited to those species possessing a free and discrete sclerotium, and an outer apothecial layer composed of globulose cells in chains oriented perpendicular to the receptacle surface (Kohn 1979). The genus *Sclerotinia* does not have an asexual state. *Sclerotinia sclerotiorum* has binucleate ascospores distinguishable from the tetranucleate ascospores of *S. trifoliorum* and *S. minor*.

2.2.2 Host Range and Distribution

Sclerotinia sclerotiorum has a broad host range. Boland and Hall (1994) found 408 species were reported in the scientific literature to be infected by this pathogen. Plant species infected were all in the sub-phylum Spermatophyta, both angiosperms and

gymnosperms. The majority of species infected were dicots. The family Brassicaceae was the third most reported host family for this fungus. *Sclerotinia sclerotiorum* has been reported to infect 25 species of monocots, among these the genera *Hordeum*, *Avena* and *Triticum*, but reports of infection in monocots are not very common. It appears that *S. sclerotiorum* prefers dicots.

Sclerotinia sclerotiorum is found world-wide, under all types of climatic conditions, and it is speculated that *Sclerotinia* can likely be found in every country in the world (Purdy 1979). Given its broad host range and the ease of sclerotia contaminating seed lots, this speculation is likely correct. It is found in all regions of Canada (Bardin and Huang 2001).

2.3 The Disease

2.3.1 Introduction

The disease caused by *S. sclerotiorum* in canola is commonly known as sclerotinia stem rot. This common name is also used for the diseases caused by the same pathogen in soybean, chickpea, lentil, cauliflower, and cucurbit crops. In other crops it has different common names. In bean, peas, pepper, potato, cabbage, broccoli, and tomato crops, it is known as white mold. In sunflowers, the disease caused by ascospores is called sclerotinia head rot, and root infection by fungal mycelium is known as sclerotinia wilt. Some other common names associated with *S. sclerotiorum* infection are: sclerotinia pink rot in celery, cottony rot in carrots, collar rot in avocado and tobacco, sclerotinia crown & root rot in beet, stem mold and rot in flax, drop or sclerotinia rot in lettuce, blossom blight in alfalfa, sclerotinia fruit rot in banana, hemp canker in hemp, sclerotinia blight in peanut, and soft watery rot in onion (American Phytopathological Society 2003).

Sclerotinia stem rot is a major disease of canola. It occurs sporadically in Canada and can cause large losses in yield in some years (Rimmer and Buchwaldt 1995). It is considered to be the most important disease of rapeseed in central China causing yield losses of up to 50% and an average loss of 20% annually (Hu *et al.* 1999). It is also a major cause of canola crop loss in France, Germany, other parts of Europe (Rimmer and Buchwaldt 1995) and more recently in Australia (Howlett *et al.* 1999).

2.3.2 Disease Cycle and Epidemiology

The impact of *S. sclerotiorum* depends on environmental conditions, host plant phenology, and the timing of ascospore release, all of which can vary greatly (Freeman *et*

al. 2002). Explained simply (Fig 2.1), the disease cycle in canola begins in its resting state, a sclerotium. The sclerotium germinates to produce apothecia, which release ascospores into the environment. The ascospores land on senescing petals, which fall onto leaves and into leaf axils. The ascospores germinate, colonise the petals to acquire enough energy to infect the leaf tissue. Once in the plant tissue, the fungus can grow into the stem, restricting nutrient flow, killing the plant, and can produce more sclerotia. The sclerotia return to the soil and the cycle is complete.

A sclerotium is a melanized, multicellular survival structure that can remain viable up to seven years (Gulya *et al.* 1997). A sclerotium germinates either myceliogenically to produce hyphae or carpogenically to produce apothecia and ascospores (Abawi and Grogan 1979). Myceliogenic germination is responsible for disease in some vegetable crops and for basal stem rot in sunflowers (Huang *et al.* 1998). In canola and most other field crops, carpogenic germination and the resulting apothecia and ascospores are considered to be the main mode of infection (Morrall and Dueck 1982). Only preconditioned (a minimum of four weeks of chilling temperatures) and functionally mature sclerotia are capable of carpogenic germination. The time and temperature required for preconditioning is isolate-dependent (Abawi and Grogan 1979). High soil moisture increases the frequency of carpogenic germination, but prolonged exposure to high soil moisture if oxygen supply is limited also encourages the rotting of sclerotia. Teo *et al.* (1989) found that after 2 years of high soil moisture, 65% of sclerotia in their study had rotted compared to 45% at low soil moisture. A closed canopy conducive to carpogenic germination due to reduced temperatures and increased water retention in the soil. In canola, most apothecia are not observed before the bud stage but rather are

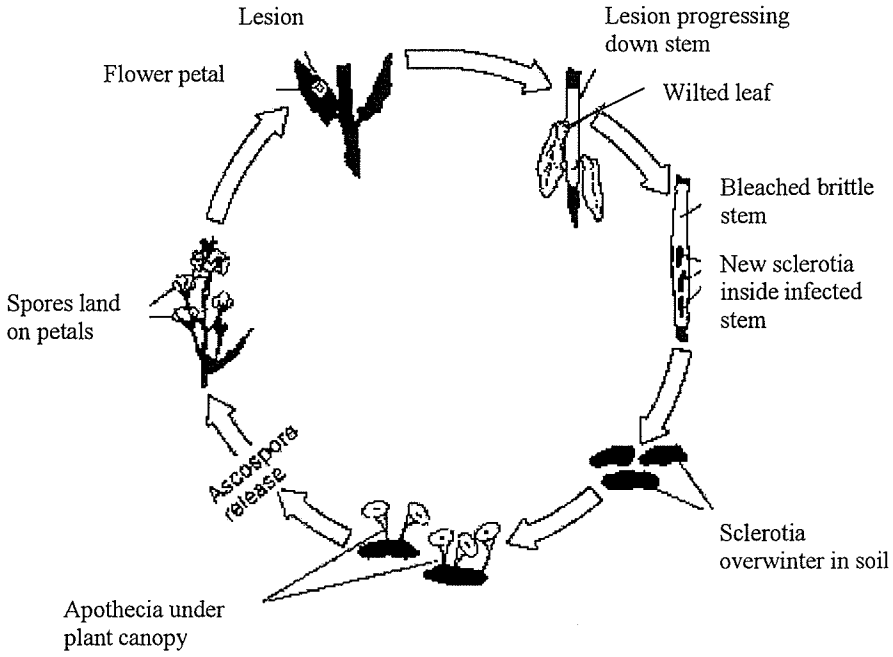

Figure 2.1. Disease cycle of sclerotinia stem rot in canola. Image courtesy of the Canola Council of Canada (2003).

observed in the middle of flowering (Morrall and Dueck 1982), which is an advantageous adaptation for the propagation of this fungus.

Ascospores are forcibly discharged from the apothecia by sudden changes in relative humidity (Rimmer and Buchwaldt 1995). This discharge lifts the ascospores about 1 cm above the apothecia, where they can be transported by wind (Abawi and Grogan 1979). One apothecium releases approximately 10^4 ascospores in a single discharge, usually during a two hour period around noon (Schwartz and Steadman 1978). Ascospores have been reported to travel distances greater than a kilometre (Abawi and Grogan 1979) but the majority of ascospores go no further than a 10 to 20 metres from the apothecia (Wegulo *et al.* 2000). No comprehensive study has been undertaken to observe the long-distance dispersal of ascospores. Ascospore dispersal has also been shown to be transported by bees (Stelfox *et al.* 1978) and infested pollen grains (Huang *et al.* 1998).

Ascospores are not very hardy structures. There is an increase in ascospore mortality as temperatures increase over 25 C and RH over 35% (Caesar and Pearson 1983). Ascospore survival in the field was increased by 21.5% when ascospores were within the shaded canopy compared to ascospores resting on leaves on the top of the soybean canopy; this was attributed to the deleterious effects of UV radiation. If conditions are not suitable for germination after ascospore release and dispersal, they may remain viable for a short time and germinate when conditions become favourable (Purdy 1979).

Ascospores require high moisture (Tu 1989) and senescing tissues to serve as a nutrient source for germination to infect above-ground plant tissues. Senescing tissues,

which can be utilised include: floral structures, cotyledons, leaves, seeds, seed capsules, pollen or injured plant tissue (Schwartz *et al.* 1978). In canola, the most important substrate or nutrient source, is believed to be petals (Rimmer and Buchwaldt 1995). Jamaux *et al.* (1995) also found that ascospores failed to adhere to and germinate on leaf surfaces due to the thick cuticle and epicutlicular wax, unlike their ready adhesion to and germination on petals. This understanding of petal usage coincides perfectly with canola phenology. Sclerotinia stem rot is not detected before mid to late flowering in canola (Morrall and Dueck 1982) when an abundance of senesced petals are available. Petals normally are infested with sclerotinia ascospores prior to abscission. After petals abscise, many lodge or adhere to lower plant tissues, and leaves in particular (Rimmer and Buchwaldt 1995). Lesions arise from the areas associated with *S. sclerotiorum* infested petals. Petal age also has an effect on *S. sclerotiorum* colonisation, and Heran *et al.* (1999) found that older petals produced significantly larger and less variable lesions compared to younger petals. Similarly, Seguin-Swartz and Lefol (2000) found that 7 day-old petals were entirely invaded by mycelium, while 1 day-old petals appeared to inhibit the growth of fungal germ-tubes. Lefol *et al.* (1997) found that extracts from petals younger than 6 days old decreased mycelial growth.

Outbreaks of sclerotinia stem rot are highly dependent on the weather (Morrall and Dueck 1982). Heran *et al.* (1999) studied the environmental effects on the infection process. Temperatures below 20 C and 25 C and relative humidity greater than 80% are required for optimal infection. Lower temperatures delayed the initiation of infection as did temperatures above 30 C. Relative humidity near 100% was required for infection to proceed; at an RH below 80%, infected petals dried up and fell off the leaf. Heran *et al.*

found that the presence of liquid water on the leaf was not necessary for infection if humidity was high, but larger lesions were formed when water was present. Water may aid the fungus in the utilisation of nutrients.

Inoculum load (density of ascospores) also has an effect on the infection process. Lesion size increased with increasing inoculum load up to a maximum of 80 ascospores/leaf, after which there was no increase in lesion size (Heran *et al.* 1999). Although Boland and Hall (1988) found a relationship between apothecia density and the resulting severity of sclerotinia disease in soybean, this has not yet been reported in canola.

Once leaf or stem tissue is infected, the fungus can grow unrestrained. *Sclerotinia* diseases can spread directly between diseased and healthy plants when these are in direct physical contact (Huang and Hoes 1980). However, temperatures exceeding 27 C will stop fungal growth (Phillips 1994) as will prolonged periods of leaf dryness.

It is possible that *S. sclerotiorum* can infect canola via substrates other than infested petals. Hudyncia *et al.* (2000) studied wounding effects in cabbage on infection *by S. sclerotiorum*. Cabbage (*B. oleracea*), a close relation to canola, was found to be infected directly by ascospores when plants were wounded by bruising, cutting, and non-lethal freezing. Freeze-treated plants showed the greatest severity of disease while cutting resulted in the least amount of infection among the wound treatments. The frequency of this occurrence has not been studied in canola.

Changes in microclimate caused by different canopy structures have been correlated with disease severity in dry bean, potato and peanut (Blad *et al.* 1978; Butzler *et al.* 1998). Turkington and Morrall (1993) reported that denser canola stands tended to

have slightly higher mean and minimum RH and longer periods of leaf wetness, those conditions under which sclerotinia stem rot develops quickly.

2.3.3 Pathogenesis

Sclerotinia sclerotiorum uses mechanical, chemical and enzymatic means to colonise host tissue. Jamaux *et al.* (1995) studied the initial stages of *B. napus* petal infection. Ascospores would adhere to petals but no appressorium was detected. Penetration of petal cells was direct and preceded by a cellular collapse in the immediate vicinity. Fungal development was then observed within the petal tissues. Thirty hours (h) after infection, extensive areas of the petal collapsed, and hyphae appeared on the under-surface of infected petals. If the infested petals were placed on leaf tissue, 20 h later hyphae would ramify on the leaf. By 70 h appressoria were observed in dense multicellular infection cushions and would mechanically penetrate the leaf tissue.

Once the cuticle is breached, *S. sclerotiorum* hyphae grow inter and intracellularly. The colonisation of tissues is mainly achieved enzymatically and chemically. The pathogen invades plant tissue by producing cell wall degrading enzymes such as polygalacturonases, galactonases, glucoamylase, α–L-arabinofuranosidase, cellulases, and pectinases (Lumsden 1979; Martel *et al.* 2002; Riou *et al.* 1991), and the toxin oxalic acid. The function of these enzymes is to kill cells in advance of growth since *S. sclerotiorum* is a necrotroph.

Oxalic acid appears to have the largest effect on the virulence of *S. sclerotiorum*. As early as 1886, oxalic acid was postulated to be involved in pathogenesis (de Bary 1886). Rowe (1993) quantified the effects of oxalic acid and the other fungal exudates and found that oxalic acid was responsible for two thirds of the effect of *S. sclerotiorum*

and *S. trifoliorum*'s pathogenicity. The function of oxalic acid in infection is believed to be three-fold: 1) reduced host viability as a result of acidification, which favours *S. sclerotiorum* growth; 2) occlusion of xylem vessels through the sequestration of Ca^{2+} and consequent formation of calcium oxalate crystals; and 3) suppression of host oxidative burst (Cessna *et al.* 2000; Ferrar and Walker 1993). The oxidative burst is a plant defence response to infection where controlled release of active oxygen species such as O_2^- and H_2O_2, limit fungal growth through a number of mechanisms.

The belief that oxalic acid is the prime infection factor is still being debated. Heller and Witt-Geiges (2003) presented information that oxalic acid was not detected in the early stages of infection (12 to 36 h post inoculation) but only detectable in the later stages (36 to 48 h) when sunflowers were inoculated with *S. sclerotiorum*. Calcium oxalate crystals accumulated in areas already digested by the fungus and not ahead of it, indicating that oxalic acid may not be the primary factor for infection. This report is challenging since there is a lot of evidence pointing to oxalic acid as being the primary factor. Godoy *et al.* (1990) generated oxalic acid minus *S. sclerotiorum* mutants and found that these mutant strains were non-pathogenic. Furthermore, soybean and canola lines have been transformed with genes that produce oxalic acid degrading enzymes and, subsequently, have greater levels of resistance to *S. sclerotiorum* (Billings *et al.* 2003; Cober *et al.* 2003; Donaldson *et al.* 2001; Thompson *et al.* 1995).

Polygalacturonases are common cell-wall degrading enzymes found in plants and in *S. sclerotiorum*. Four genes are believed to be responsible for producing polygalacturonases in *S. sclerotiorum* under differing conditions depending on whether the fungus is growing saprophytically or parasitically (Li *et al.* 2002a). Polygalacturonase

and pectinase activity are found in high concentrations at the advancing edges of lesions (Lumsden 1976) and require a low pH for optimal function. Cellulases may contribute to the nutritional status of the advancing hyphae.

In *Arabidopsis,* it was found that infection by *S. sclerotiorum* triggered an oxidative burst and the hypersensitive response (HR) in these plants (Govrin and Levine 2000). Govrin and Levine found that growth of *S. sclerotiorum* was suppressed on a HR deficient *Arabidopsis* mutant. The HR response is an efficient defence mechanism against biotrophic pathogens, but it facilitates colonisation by the necrotrophic pathogen, *S. sclerotiorum*.

2.3.4 Symptoms

Symptoms of sclerotinia stem rot in canola appear after flowering, growth stage 4.4 (Harper and Berkenkamp 1975). Rimmer and Buchwaldt (1995) describe sclerotinia stem rot symptoms succinctly:

> "Lesions on leaves are greyish, irregularly shaped and often associated with adhering petals. The lesions on stems are white or almost bleached, sometimes with darker rings showing stepwise growth of the fungus, often with a sharp line between infected and healthy tissue. Girdled stems are weaker and have a tendency to lodge at the point of infection. Stem-infected plants ripen prematurely and can be seen as brown patches in the field. At the end of the season, black sclerotia can be found inside the infected part of the stems, in infected pods, and under very humid conditions even on the outside of infected tissue."

2.4 Control Strategies

There are a number of strategies studied by researchers all over the world to control *S. sclerotiorum* infection in canola and other susceptible crops. Control strategies can be summarised into four types: chemical, biological, cultural, and host plant resistance.

2.4.1 Chemical Control

In canola, the most common and currently perhaps the most effective control against *S. sclerotiorum* infection is the use of fungicides. Foliar-applied fungicides are an effective control of sclerotinia stem rot in canola because this is essentially a monocyclic disease and only infects during the flowering stage. The fungicides azoxystrobin (Quadris), benomyl (Benlate) , iprodione (Rovral Flo), and vinclozolin (Ronilan DF) are all registered for use on canola in Canada (Saskatchewan Agriculture Food and Rural Revitalization 2003) and used to some degree in other parts of the world. Fungicide control is only effective when it is applied during early to full flower stages [growth stages 4.1 to 4.2 (Harper and Berkenkamp 1975)] prior to visual symptoms of the disease. Fungicide uses poses some difficulties. Recently, benomyl-resistant strains of *S. sclerotiorum* have been reported in canola in Western Canada (Gossen and Rimmer 2001). Also there is a predictive element for the efficient use of fungicides, since the development of an epidemic is largely influenced by environmental conditions. Indiscriminate fungicide application as a preventative measure is too costly for producers. Some people also have environmental concerns about fungicide use.

A number of disease forecasting models have been developed to avoid unnecessary fungicide treatment. The first was a checklist system based on risk assessment to determine the likelihood of a problem with sclerotinia stem rot in Canada and Scandinavia (Nordin *et al.* 1992; Thomas 1984; Twengstrom *et al.* 1998). Previously, the most widely used model used on the Canadian prairies was a petal-testing method which predicts the likelihood of a sclerotinia stem rot outbreak by evaluating the incidence of petals infested with ascospores with an agar plate assay (Gugel and Morrall 1986; Turkington *et al.* 1988; Turkington *et al.* 1991; Turkington and Morrall 1993). This assay gives an estimation of the inoculum load, and when used in conjunction with weather information, helps producers decide whether the disease risk warrants a fungicide application (Morrall and Thomson 1991). Since it takes several days to see fungal growth from these petals which can be a costly delay to producers, efforts have been made to make this model more efficient. Bom and Boland (2000) studied the use of polyclonal-antibody-based immunoassays to detect *S. sclerotiorum* on canola petals. Freeman *et al.* (2002) used PCR primers to detect ascospores on petals with success. Currently, neither of these assays is commercially available.

A more recent predictive model being used on the Canadian prairies analyses meteorological data to predict the development of a sclerotinia stem rot epidemic (ACE 2003). Surface soil moisture content over several days, relative humidity values, and temperature thresholds conducive to the germination of the sclerotinia spores are used to generate forecast maps. These maps show regions where the environmental conditions are favourable for the development of sclerotinia. This model assumes that the disease will occur if the environmental conditions are conducive.

There have been some suggestion that use of herbicides may aid in control of *S. sclerotiorum*. Nelson *et al.* (2002) applied a number of protoporphyrinogen oxidase-inhibiting herbicides on inoculated soybeans and found a decrease in the development of the disease. This protection is believed to be caused by an increase in phytoalexin production in the infected plant tissues which slows the disease development.

2.4.2 Biocontrol

Biocontrol, or the use of antagonist biological organisms, is a control strategy that has been examined for *S. sclerotiorum* since Wells *et al.* (1972) found that Trichoderma was antagonistic to sclerotia. Since then, *Coniothyrium minitans,* a mycoparastic fungus that is antagonistic to *S. sclerotiorum*, has become a commercial product named Contans, used in Europe and North America (Vrije *et al.* 2001). Other agents reported to have some efficacy in control of *S. sclerotiorum* are: *Spirodesmium sclerotivorum* attacks sclerotia (del Rio *et al.* 2002), *Serratia plumuthica* a soil bacterium attacks sclerotia (Thaning *et al.* 2001) and suppresses mycelial growth (Kalbe *et al.* 1996), *Erwinia herbicola* suppresses ascospore germination (Yuen *et al.* 1994), and *Pseudomonas* spp. suppress ascospore germination (Savchuk 2002). A novel biocontrol technique put forward by Dickman and Chet (1998) is to use biologicals to degrade the pathogen toxin, oxalic acid in this case. These researchers had identified two oxalic acid-degrading bacterial strains which effectively reduced sclerotinia disease incidence by more than 60% on beans inoculated with *S. sclerotiorum* and *S. rolfsii*.

2.4.3 Cultural Control

Cropping practices or cultural practices may have an impact on the development of sclerotinia stem rot in canola and in other crops. Understanding these effects can lead to control strategies. The benefit of this type of control strategy is that it often employs technologies already available to producers and often does not require additional inputs or costs. A literature review of the effects of cropping practices on the development of this disease reveals that there are a number of options available to producers. These options can be classified as such: plant density modification, physical plant canopy manipulation, crop rotation, planting date manipulation, soil fertility modification, irrigation manipulation and tillage regime manipulation. All these strategies operate on the basis of modifying the canopy microenvironment in order to produce an environment that does not encourage this disease.

Canopy density is believed to be very important to *S. sclerotiorum* infection. Blad *et al.* (1978) found that white mold severity in dry beans was greatly influenced by plant canopy. The densest canopies were the coolest and wettest and had the greatest severity of white mold. Open canopies had elevated temperatures, decreased leaf wetness and decreased white mold.

Researchers have been able to modify canopy density by varying seeding rates and row width. Park (1993) showed that decreasing plant row width in common bean increased the level of white mold. Similarly, Saindon *et al.* (1995) found that the number of *S. sclerotiorum* infected bean plants increased with increasing plant densities by using narrower row spacing. But there was a cultivar effect; some cultivars had larger increases in disease than others, suggested to be caused by cultivar growth habit. They concluded

that this increase in disease was not a significant concern for all cultivars given the benefit of increased yield that narrow row width provided. Irvine and Duncan (1992) studied the effects of seeding rates and row spacing on sclerotinia stem rot in spring canola. They found that increasing seeding rates and narrower row spacing resulted in increased lodging and increased incidence of sclerotinia stem rot, which resulted in a decrease in yield. They speculated that the effect of seeding rate and row spacing impacts the canopy microenvironment, which favours sclerotinia stem rot development. Unfortunately, these results came from one year of data. Contrary to this report, Hu *et al.* (1999) found that there was no difference in disease incidence under differing plant stands in winter rapeseed, although they cautioned that this finding was based on one year of data and with low levels of *S. sclerotiorum* infection.

Row orientation had an impact on sclerotinia stem rot in soybeans (Haas and Bolwyn 1972). Rows that ran parallel to the direction of the prevailing winds decreased disease incidence, likely caused by the increased drying action of the wind on the plant canopy and the soil.

Mechanical modification of the canopy structure has been found to change sclerotinia disease severity in at least two studies. Integrated management of *S. minor* in peanuts and pruning measurably increased soil temperature, which in turn reduced the disease (Butzler *et al.* 1998). Physically elevating the crop canopy of prostrate great northern bean cultivars with the use of wire tunnels and trellises was found to significantly reduce the amount of white mold infection (Fuller *et al.* 1984).

Crop rotation was not expected to have a large impact on sclerotinia diseases because of the longevity of sclerotia, the pathogen's capacity for long-distance dispersal

of ascospores and its wide host range (Krupinsky *et al.* 2002), and early data supported this hypothesis. Haas and Bolwyn (1972) found that rotation made no difference on the incidence of white mold in white bean in the late 60's early 70's. Steadman (1979) said that since sclerotia survive long periods in the soil and tillage operations generally assure the presence of sclerotia to be near the soil surface, sclerotia inoculum is likely present in most fields. However, recently some recent studies have found a rotation effect. Kurle *et al.* (2001) found that rotation had an effect on sclerotinia stem rot development in soybeans but found that rotation did not affect the sclerotial load in the soil nor apothecial density. They believed that this difference was because of better seedbed preparation for improved emergence and more readily available nitrogen that contribute to canopy structure differences creating a more favourable microenvironment for disease development. Gracia-Garza *et al.* (2002) studied the effects of crop rotation and tillage on apothecia production and found that crop rotation effected on apothecia production. Crops of corn and wheat in the rotation had lower mean apothecia numbers than a rotation of continuous soybean crops.

Planting date has been suggested as control strategy (Hu *et al.* 1999) but this would be difficult to implement. Since environmental conditions have a large effect on the development of an epidemic, changing the seeding date would require the producer to predict when those conditions favourable for the disease will occur.

Balanced fertility removes plant stress, improves physiological resistance, and decreases disease risk, at least in other pathosystems. Fertility deficiency can cause increased levels of disease in tan spot in wheat, alternaria in canola, leaf and head diseases in wheat, and root diseases in small grains (Krupinsky *et al.* 2002). However, the

effect of soil fertility and soil texture have been reported to have no effect on sclerotinia stem rot in white beans (Haas and Bolwyn 1972). Contrary to these findings, Hu *et al.* (1999) found that plants receiving a balanced fertiliser treatment had less disease than those that did not receive this treatment. The authors speculate that under optimum fertility the plants' defence systems operate more effectively.

Irrigation was found to have a large impact on white mold wilt in dry beans (Blad *et al.* 1978). A greater frequency of irrigation resulted in an increased severity of white mold.

Farm equipment, especially tillage implements, influence sclerotinia disease development. Kurle *et al.* (2001) found that tillage regime had an effect on inoculum density and on disease incidence in soybeans. A no-till system had reduced levels of disease compared to two tillage systems in this study. The authors believed that this reduction was due to a reduction in both the soil borne and airborne inoculum density. Gracia-Garza *et al.* (2002) reported that a no-tillage regime had lower apothecia production than systems using tillage. They also found a further reduction in apothecial numbers if the crop residue was chopped and left on the soil surface in the no-till system.

Trends towards crop diversification on the Canadian prairies also effected on sclerotinia stem rot in canola. Crop diversification has been reported to increase the risk of sclerotinia diseases by bringing more susceptible dicot crops into traditional cereal-growing regions (Krupinsky *et al.* 2002).

Kurle *et al.* (2001) examined several agronomic practices that influence sclerotinia stem rot in soybeans and found that differences in plant stand and canopy density are more important to the development of stem rot than differences in rotation,

tillage or inoculum density. But ultimately they state that planting a moderately resistant soybean cultivar was the single most effective management practice available for controlling sclerotinia stem rot.

2.4.4 Resistance

There are reports of resistance to *S. sclerotiorum* in many crops. Bean crops and soybeans have been studied the most thoroughly. Resistance to white mold in dry beans is quantitatively inherited (Fuller *et al.* 1984; Miklas and Grafton 1992) and consists of physiological resistance and avoidance mechanisms (Miklas *et al.* 2001). In *Brassica* crops, there are reports of both physiological resistance (Sedun *et al.* 1989) and avoidance mechanisms (Jurke *et al.* 1998) to sclerotinia stem rot.

2.4.4.1 Escape Mechanisms

Ascospores cannot infect plants directly; they require a nutrient source such as petals (Jamaux *et al.* 1995). In beans, the abundance of senescing blossoms and leaves, which accumulate within and around plants, indicates that many sites for potential colonisation by *S. sclerotiorum* exist. However, Schwartz *et al.* (1978) did not find a relationship between dry bean blossom production and white mold infection. This finding may be caused by a trial design flaw. They only tested five cultivars, each with different growth habits, therefore they would be unable to differentiate blossom effect from growth habit effect.

Apetalous (lack of petals) traits of *Brassica* species have been described in the literature since the 1940's (Ramanujam 1940), and it has been suggested as a possible solution to avoid sclerotinia stem rot (Kapoor *et al.* 1983; Mc Lean 1958). Jurke *et al.*

(1998) described field results showing that a cultivar with the apetalous trait did reduce disease incidence by 60% compared to the petalled cultivars. The authors suggest that this reduction was because of the apetalous characteristic, but with only one cultivar representing this trait, there were not enough degrees of freedom to verify this hypothesis. The reduction in disease incidence could have been attributed to other escape or physiological traits that were not measured. Furthermore, it is suggested that the apetalous trait may also be more efficient in photosynthesis and reallocation of assimilates because the yellow petal layer of *Brassica* crops reflect a large part of the photosynthetically active region of the light spectrum (Jiang and Becker 2003; Mendham *et al.* 1991). Plants with improved metabolic capabilities may be better able to mobilise defence responses to infection by *S. sclerotiorum* and other diseases.

The inheritance of the apetalous character depends on its source. In *B. rapa* it is the result of a single recessive gene (Singh 1961), and in *B. napus* it can be two recessive genes (Buzza 1983), four recessive genes [summarised by Jiang and Becker (2003)], an interaction of alleles at three loci (Kelly *et al.* 1995), or an interaction between cytoplasmic genes and two pair of recessive nuclear genes (Jiang and Becker 2003). There have been no reports of the apetalous trait being a single dominant gene.

In general, the escape mechanisms in bean and soybean crops are related to microenvironment modification. An open canopy and an upright growth habit promote the rapid drying of wet leaf and soil surfaces, facilitate air circulation and light penetration which reduce plant infection (Coyne *et al.* 1974). There have been no published reports of escape mechanisms that reduce the sites of infection available to the fungus in these crops (like the apetalous trait in canola). For example, Schwartz *et al.*

(1978) studied dry bean blossom production in relation to white mold disease and found that disease was not always correlated with the frequency of senescent blossoms. The canopy structure was found to have a greater influence on white mold infection.

Inherited escape mechanisms which have been found to be related to *S. sclerotiorum* infection are: canopy elevation by indeterminate upright growth habit and porous canopy in navy beans (Park 1993) and in dry beans (Kolkman and Kelly 2002), porous canopy in determinate beans (Schwartz *et al.* 1987), leaf area near the soil surface in dry bean (Schwartz *et al.* 1978), flowering duration in dry beans (Deshpande 1992), plant height in dry beans (Miklas *et al.* 2001) and soybeans (Boland and Hall 1987; Kim and Diers 2000), and lodging resistance and maturity in soybeans (Boland and Hall 1987; Kim and Diers 2000). This list is not universally accepted. Kim *et al.* (1999) found that canopy height and maturity in soybeans were not correlated with disease severity.

The variation in sclerotinia stem rot disease is usually attributed to environmental sensitivity of the fungus. Pennypacker and Risius (1999) found that the environmentally sensitivity of a soybean cultivar was partly responsible for the development of sclerotinia stem rot. Cultivars were divided into two groups: cultivars sensitive to changes in light intensity, and cultivars insensitive to changes in light intensity. As light intensity increased, disease ratings decreased for the sensitive cultivars and remained stable for the insensitive cultivars. Currently, no study such as this has been reported for canola.

2.4.4.2 Physiological Resistance

Physiological resistance has been detected in bean crops. Researchers have found partial physiological resistance in dry bean (Miklas *et al.* 1992a; Miklas *et al.* 1992b; Miklas and Grafton 1992), although they did not speculate what this mechanisms of

resistance might be. Quantitative trait loci (QTL) responsible for physiological resistance have been identified in soybeans (Arahana *et al.* 2001; Kim & Diers 2000) and in common and dry beans (Kolkman and Kelly 2003; Miklas *et al.* 2001). However, the effect of these physiological QTL was small, each explaining 10% of the variation in soybean, which will make the use of these QTL in breeding of these traits difficult. Not much is known about how these QTL affect resistance, except that one QTL was linked to resistance to oxalic acid (Kolkman and Kelly 2003).

In other crops, Kohler and Friedt (1999) found partial physiological resistance in wild sunflowers. Hahn (2002) found differences between head rot susceptibility between inbred sunflower lines, and these differences were moderately heritable. QTL for resistance for leaf resistance and head resistance were identified and these QTL explained 40% of the variation in *S. sclerotiorum* disease severity (Mestries *et al.* 1998).

In *Brassica* crops, Dickson and Petzold (1996) found partial and major resistance genes in *Brassica oleracea* germplasm. Sedun *et al.* (1989) inoculated five *Brassica* species including *B. napus* and *B. rapa* and observed that there were differences in lesion growth in stems, but heritability of this resistance was very low, indicating that these differences were likely environmental. The Chinese rapeseed cultivar, Zhongyou 821, generated by a wide cross of *B. napus* and *B. rapa*, is reported to have "good disease resistance" to *S. sclerotiorum* (Buchwaldt *et al.* 2003; Li *et al.* 1999c; Wu 1991). However, Zhongyou 821's resistance appears to be variable. This is explained by regional and environmental conditions. Li *et al.* (1999c) reported the mode of resistance prevents initial infection and slows mycelial growth in tissue suggesting the involvement of glucosinolates and may be related to oxalic acid tolerance. Seguin-Swartz and Lefol

(1999) have been studying the inheritance of resistance in dog mustard (*Erucastrum gallicum*) and found that they were able to select for increasing levels of resistance. They found that this resistance was polygenic.

Mullins *et al.* (1995) studied the effects of mutant *B. napus* lines on *S. sclerotiorum* infection. Partial resistance was found which was characterised by a reduction in the amount of mycelium in the inoculated stem. They believed that the resistance was caused by the plants producing fungitoxic phenolics.

An emerging field of study, which appears to have potential for controlling *S. sclerotiorum* is systemic acquired resistance. This is a physiological immune response triggered by pathogen infection or by synthetic or natural activators (Kessmann *et al.* 1994). Application of the herbicides Cobra (lactofen) and Action induced a systemic resistance response in soybeans (Yang and Lundeen 2001). Decreases in sclerotinia stem rot severity of 75% to 90% were observed over two years of trials in the field.

2.4.4.3 Novel Sources of Resistance to *S. sclerotiorum*

There are many species of plants that are not susceptible to *S. sclerotiorum* infection. Mechanisms of resistance can be identified and obtained from these species and moved into susceptible crops. Novel sources for resistance can be found in bacteria and other fungi as well. There are more possibilities for novel sources of resistance to *S. sclerotiorum* emerging every year. For example, there are many different proteins with antifungal and/or antibacterial activity detected in seeds such as: chitinases, β-1,3-glucanases, thionins, permatins, ribosome-inactivating proteins, and antifungal oligomeric proteins (Terras *et al.* 1992), to name a few. In this section we will review some of the

more popular novel sources of resistance which have been tested in canola or its close relatives.

Anti-apoptotic (anti-programmed cell death) genes are believed to be an effective method of controlling necrotrophic diseases. Researchers have transferred anti-apoptotic genes from both animals and plant species to tomato and tobacco lines (Dickman *et al.* 2001; Dickman *et al.* 2003). They have reported very high levels of resistance to *S. sclerotiorum* and other necrotrophic fungi in these lines.

Polygalacturonase inhibiting proteins (PGIP's) are believed to increase resistance to *S. sclerotiorum* and other polygalacturonase producing fungi. Li *et al.* (2002b) transformed *B. napus* with two cDNAs encoding PGIP's and they found that expression of these PGIP's in response to infection by *S. sclerotiorum* was dependent on the construct and the tissue being infected. One of the two constructs gave high levels of suppression of *S. sclerotiorum* growth in localised tissue, while the other construct was systemically produced and weakly effective against *S. sclerotiorum*. High levels of resistance to *S. sclerotiorum* in onions was achieved using PGIP's (Favaron *et al.* 1997). PGIP's are naturally found in all crops, even susceptible crops. PGIP's in *B. napus* are constituatively expressed in all organs of the plant and may be involved in regulating the polygalacturonases involved in the various aspects of plant development (Li *et al.* 2003). These genes seem to be inducible and concentrate in areas of plant damage or infection, so it becomes a matter of enhancing their expression.

If oxalic acid is the major pathogenicity factor of infection by *S. sclerotiorum,* then a method to control or neutralise oxalic acid could be effective to control infection. There are a couple of options. Oxalate oxidase genes have been identified in the true

cereals (barley, maize, oat, rice, rye, and wheat) and are believed to be a possible source of resistance (Lane 2002). The oxalate oxidase gene produces an enzyme (wheat germin) that catalyses the oxidation of oxalic acid by molecular oxygen to carbon dioxide and hydrogen peroxide ($H_2C_2O_4 \rightarrow 2CO_2 + H_2O_2$). The function of the oxalate oxidase gene appears to be four-fold (Lane 2002): 1) generation of H_2O_2 which has microcidal properties; 2) elicitation of the HR caused by low levels of H_2O_2; 3) H_2O_2 induced lignification of cell walls around an infection site; and 4) degradation of oxalic acid, which is an inhibitor of the HR.

Transgenic soybeans, sunflowers, and canola expressing the wheat oxalate oxidase gene have been created (Billings *et al.* 2003; Cober *et al.* 2003; Donaldson *et al.* 2001; Thompson *et al.* 1995). The most common source of this gene has been the wheat germin gene source. High levels of stem rot control in soybeans transformed with this gene have been found in the growthroom on inoculated leaves (Billings *et al.* 2003; Donaldson *et al.* 2001). But this finding has not been universal. Cober *et al.* (2003) found that the oxalate oxidase gene only gave partial levels of stem rot resistance in soybeans when tested in the field. This transgene has not been as effective in sunflowers, providing only partial resistance to *S. sclerotiorum* when tested in the growthroom (Billings *et al.* 2003). Transgenic canola, carrying a barley source of the oxalate oxidase gene, were found to have improved resistance in an oxalic acid assay (Thompson *et al.* 1995), but it was not tested against *S. sclerotiorum*.

As mentioned above, production of H_2O_2 is believed to have anti-microbial effects. This was found in potatoes transformed with a glucose oxidase gene, which increased the levels of H_2O_2 and resulted in improved resistance to non-oxalate secreting

fungi (Wu *et al.* 1995). However, there have not been reports of elevated H_2O_2 in transgenic soybeans, canola or sunflowers (Lane 2002).

Another transgenic source to control oxalic acid is the oxalate decarboxylase gene. Products of oxalic acid decarboxylation are CO_2 and formic acid ($H_2C_2O_4 \rightarrow CH_2O_2 + CO_2$). The fungal species *Collybia velutipes* degrades oxalic acid directly to formate and CO_2 without any intermediate steps (Azam *et al.* 2001). This gene may have some benefit as a transgene even though it does not have the advantage of producing H_2O_2. Kesarwani *et al.* (2000) used this transgene in tobacco and tomato. In both an oxalic acid assay and an assay with *S. sclerotiorum*, they found a 60% reduction in lesion development.

Arahana *et al.* (2001a) used a variant oxalate degrading transgene: oxalyl Co-A decarboxylase in soybeans. They found that lines with this gene did not have increased levels of resistance to *S. sclerotiorum* compared to untransformed lines. This gene comes from the bacteria *Oxalobacter formigens*, and the oxalic acid breakdown is more complicated. Oxalic acid is activated to oxalyl Co-A, which is then degraded to oxalyl Co-A decarboxylase and requires ATP, coenzyme A (CoA), Mg^{2+}, thiamine pyrophosphate, and acetate (Azam *et al.* 2001).

Genes that have products with general fungitoxic properties have been tested in *B. napus*. Genes that encode for chitinase and glucanase genes (carbohydrates found in fungal cell walls) have been studied to this end. The mycoparasite *Coniothyrium minitans* produces a β-1,3-glucanase enzyme that enables it to digest the sclerotial cell walls of *S. sclerotiorum* (Giczey *et al.* 2001). This same enzyme was found to suppress *in vitro* growth of *S. sclerotiorum*. Chen *et al.* (1999) transformed Chinese rapeseed lines with both a chitinase and glucanase gene and observed that 43% of the transformed lines were

found to be resistant to *S. sclerotiorum*. Both the T_1 and T_2 generations showed resistance. Genes that metabolise the antifungal compound pyrrolnitrin were transformed in *Brassica* relative, *Arabidopsis,* by Dietz *et al.* (2003). Transformed plants where the enzymes were expressed in the chloroplast were highly resistant to *S. sclerotiorum*.

The use of transgenes have been the most popular methodology for introducing novel sources of resistance to *S. sclerotiorum* into crops. There are other methods, and Mullins *et al.* (1999) sought to improve resistance in canola using an induced mutagenesis technique. 1.7% to 5.1% of the canola lines treated with ethyl methane-sulphonate saw a significant increase in resistance in both the lab and the field. The authors concluded that the increase in resistance was caused by the additive effect of micromutations, which involve the mutation of dozens or possibly hundreds of minor genes, which largely control phenotypic characters. Some of these mutations may involve the deletion of susceptibility or resistance suppressor genes.

2.4.4.4 Screening for *S. sclerotiorum* Resistance

An effective screening methodology must be used to determine the level of resistance in a test line or a cultivar. Researchers have been studying resistance to *S. sclerotiorum* since the 1940's, and each researcher seems to have their own favoured screening methodology. Choice of a screening technique depends on the purposes of the experiment. All the techniques can be classified into two groups: field screening techniques and laboratory/growthroom screening techniques. The controlled environment of a growthroom gives researchers a greater ability to manipulate environmental variables, which increases reliability and repeatability. These techniques are enticing, but

ultimately, any new product, source of resistance, or control strategy must be tested in the field for a true measure of its efficacy to control *S. sclerotiorum* infection.

Field screening trials are perhaps more challenging to conduct than indoor trials since there is greater variability caused by environmental conditions. There are, however, a number of techniques that researchers employ to aid in infection success. One third of researchers conducting field trials ensure that sclerotia are present in the soil, either by spreading or sowing, while two thirds of researchers conducted experiments on land with a history of *S. sclerotiorum* infection. To ensure infection in the field, some researchers use field inoculation techniques. Kohler and Friedt (1999), Mestries *et al.* (1998) and Sedun and Brown (1989) used mycelial infested plugs to inoculate leaves or stems of sunflowers in the field. Although this technique is common in other crops, it has been reported infrequently on *Brassica* species (Dickson and Petzold 1996; Zhao and Meng 2003). Hahn (2002) used millet seed infested with *S. sclerotiorum* mycelia to inoculate sunflower heads in the field with good levels of success. Irrigation is sometimes used to ensure that soil moisture is adequate for carpogenic germination, and sprinkler or misting systems are used during flowering to ensure infection (Cober *et al.* 2003; Kim *et al.* 1999).

Screening for physiological resistance in the field is difficult because of the interaction of escape mechanisms, physiological resistance and environmental conditions. Growthroom techniques give a more accurate assessment of physiological resistance. These tests are usually done on individual plants with a technique that ensures infection

will be accomplished. Table 2.2 lists the most frequently used growthroom techniques to inoculate plants with *S. sclerotiorum*. This table is not comprehensive, but is representative of the majority of peer-review publications on *S. sclerotiorum* infection.

The most common or popular growthroom screening methodology for measuring physiological resistance is the mycelial plug technique. This technique involves growing a culture of *S. sclerotiorum* on an agar based medium, taking plugs or blocks of this infested agar, and placing them in contact with leaf tissue. This can be done on excised or intact leaves. Disease assessment is normally evaluated on lesion size, which can be recorded singly or multiple times. The allure of this technique is that it is easy to produce inoculum, simple to inoculate with, and fast to get results. This technique accounted for one third of all publications that described a growthroom technique. A variation of this technique is to use infested agar plugs on stems. The straw test is a further variation of this test but is only being used in publications on bean crops. This test involves placing an infested agar plug into a plastic straw and then placing this straw over the cut stem of a plant. The straw simply ensures that the plug remains in contact with the plant stem.

The second most utilised technique is a collection of methods used in bean and soybean crops. This technique is similar to the mycelial plug technique in that substrates bearing *S. sclerotiorum* mycelium are placed on plant stems or on leaves. The difference is that the substrate is autoclaved plant tissue such as wheat, oat, or barley seed, carrots, celery, or bean pods. Disease assessment is similar - measuring the lesion size.

Table 2.2. Number of publications of *S. sclerotiorum* inoculation techniques for evaluating resistance in the growthroom gleaned from peer-reviewed literature.

Growthroom Inoculation Technique	Initial Author	Number of Publications					
		Total	Crucifer Family	Soy-bean	Bean	Sun-flower	Other crops
Infested agar plug on leaf	Noyes and Hancock (1981)	21	10	5	2	2	3
Infested plant material	Hunter *et al.* (1981)	10	2	5	3		
Oxalate test	Marciano *et al.* (1983)	9	4	1	3		1
Ascospores on petals	Cline and Jacobsen (1983)	7	6	1			
Ascospore spray	Tu (1989)	4	1		1	1	1
Straw test	Park *et al.* (2001)	3			3		
Excised stem method	Chun *et al.* (1987)	3		2	1		
Matchstick test	Brun *et al.* (1987)	2	2				
Mycelium spray	Boland and Hall (1986)	2		2			
Direct inoculation with sclerotia	Cassells and Walsh (1995)	2	1				1
Infested agar plug on stem	Fang (1993)	2	2				
Soluble pigment assay	Wegulo *et al.* (1998)	1		1			

The oxalate test is a commonly used in experiments that examine the effect of oxalic acid in the infection process and thus has been used for the determination of resistance. Usually leaves or stems are excised and their base is placed in an oxalic acid solution for a period of time. Disease assessments are evaluated by measuring the progression of wilt symptoms up the stem or petiole.

The 'ascospores on petal technique' is used in crops where this is a common method of infection. This technique is more time consuming since it involves conditioning sclerotia, encouraging carpogenic germination, and harvesting and storing ascospores in such a way as to not lose viability. Petals are infested with ascospores and then placed in contact with leaf or stem tissue. Disease ratings are based on lesion size. Ascospores have also been directly applied to non-senescing plant tissue with varying degrees of success.

The excised stem ('tissue paper inoculum') method uses a 5 X 3 mm piece of tissue paper containing viable *S. sclerotiorum* mycelium. This infested paper is then wrapped around the apex of an excised stem and then incubated under high humidity. Disease assessments are made on lesion length.

The mycelium spray technique grows *S. sclerotiorum* in a liquid medium or on a solid medium. The mycelium and the medium are macerated together with water and applied as a foliar spray directly onto plants. This technique has the advantage that many plants can be inoculated at once and could possibly be used for field applications (this technique has not been published yet). Disease assessments are done on the percent of infected plants and/or on lesion size.

The matchstick method has only been published for use in canola. This method involves infesting matchsticks with *S. sclerotiorum* mycelium by placing wet autoclaved matchsticks on the advancing edge a *S. sclerotiorum* growing in culture. These infested matchsticks are then inserted into stems and lesion size is evaluated after a few days.

There have been two publications of techniques using sclerotia directly to infect plant tissue. To make it more effective, one author describes initiating myceliogenic germination before placing it in contact with artichoke plants. Lesion size is measured to assess disease severity.

The last technique to review has only been described in one publication. The soluble pigment technique was used in soybeans by Wegulo *et al.* (1998). This technique operates on the basis that a 'pink pigment' dissolved in oxalic acid from soybean stems was observed to be greater in more resistant cultivars than susceptible cultivars. Defoliated excised stems were placed in a solution of oxalic acid for 48 h. The oxalic acid was then analysed with a spectrophotometer to quantify soluble pigment levels.

Each of the above techniques has benefits and drawbacks. Some are more efficient, some produce less variable results, and some are more accurate in identifying resistance. There have been experiments where some of these techniques have been compared (Fang 1993; Kim *et al.* 2000; Steadman *et al.* 2001; Wegulo *et al.* 1998), and each has different conclusions. Fang's (1993) work is the only screening technique comparison study in canola to date.

2.5 Objectives

The objectives of this study into alternative controls for sclerotinia stem rot in canola are:

1. To examine the impact of seeding rate on sclerotinia stem rot development in canola.

2. To determine which characteristics of plant architecture, crop structure, and phenology influence the development of sclerotinia stem rot in canola.

3. To evaluate some growthroom screening techniques for the determination of physiological resistance to sclerotinia stem rot in canola based on their accuracy, reliability, and efficiency.

3.0 EFFECTS OF SEEDING RATE AND PLANT DENSITY ON SCLEROTINIA STEM ROT INCIDENCE IN CANOLA

3.1 Abstract

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, affects canola wherever it is grown. Seeding rates, which are believed to affect the microclimate beneath the canopy, were evaluated for their impact on sclerotinia stem rot incidence. A study with four seeding rates (2.2 kg/ha, 6.7 kg/ha, 13.3 kg/ha and 20.0 kg/ha) and four canola cultivars chosen for their variation in canopy structure was conducted in multiple locations in 2001 and 2002. A significant relationship between sclerotinia stem rot disease incidence (DI) and seeding rate was found. With an increase in seeding rate, the DI was increased in the lodging-prone cultivar AC Excel and in the mean of all four cultivars. Lodging significantly increased for all cultivars when seeding rates exceeded the standard 6.7 kg/ha. Multiple regression analysis revealed that both plant density and lodging explain the majority of the variation in DI, but plant density has a significantly larger impact on DI than lodging susceptibility. Our results indicate that increasing seeding rate does modify the microenvironment and increases the potential for lodging, which may be responsible for plant-to-plant spread of this disease.

3.2 Introduction

Sclerotinia stem rot of canola (*Brassica napus* L.) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, can be a devastating disease. Management of this disease consists mainly of the use of fungicides. Given the sporadic nature of this disease, chemical application can be costly, especially when applied as a preventative measure where risk may be low. A number of predictive models and testing protocols have been developed to determine the risk of disease development in order to make chemical application more cost effective (Bom and Boland 2000; Gugel and Morrall 1986; Turkington *et al.* 1991, 1993). Alternatives to chemical fungicides for managing sclerotinia stem rot, such as cultural control, are used but are not widely used for managing sclerotinia stem rot in canola.

One form of cultural control is the manipulation of plant density. A simple test of different seeding rates may indicate whether canola producers have another tool available to manage this disease. Many studies have suggested that crop density is related to sclerotinia stem rot incidence (Krupinsky *et al.* 2002; Nordin *et al.* 1992; Sigvald *et al.* 1991; Thomas 1984), but few have demonstrated this relationship in canola. Turkington *et al.* (1991) found that sclerotinia stem rot disease incidence increased as canopy density increased over a six-year study period sampling hundreds of farmers' fields. Some disease forecast models indicate that canopy density contributes to the development of sclerotinia stem rot. Sigvald *et al.* (1991) created a computer-based sclerotinia stem rot prediction model, which included crop density as one of the major factors. Turkington *et al.* (1993) reported that denser canola stands tended to have slightly higher mean and minimum relative humidity and longer periods of leaf wetness, the conditions under

which sclerotinia stem rot develops quickly. They found a significant relationship between disease incidence and crop height, which appeared to have an impact on canopy microenvironment. However, Nordin *et al.* (1992), studying sclerotinia stem rot of canola in Sweden, could not find a relationship between plant density and disease incidence.

The objective of our study was to examine specifically the impact of seeding rate and cultivar on sclerotinia stem rot development in canola. Jurke and Fernando (2002) had noted previously that there were consistent differences in sclerotinia stem rot infection between canola cultivars, which were attributed to cultivar architecture and morphology. Therefore, I examined the response of several different cultivars to a range of seeding rates and their impact on sclerotinia stem rot development and yield.

3.3 Materials and methods

3.3.1 Study Sites

Field trials in Manitoba, Minnesota, and North Dakota were conducted to obtain moderate-to-high levels of sclerotinia stem rot infection. Trials in 2001 were located in Carman, MB, Roseau, MN, and Valley City, ND, and trials in 2002 were located in Carman, MB, Minto, MB, Roseau, MN, and Langdon, ND. The sites in the USA were selected for a history of consistent sclerotinia stem rot infection, and the sites in Canada were selected for their ease of access and management. All trials were established on land using a rotation of at least three years between canola crops. The Carman, MB site was an irrigated sclerotinia stem rot nursery in a three-year rotation of soybeans-sunflowers-canola.

3.3.2 Experimental Design

A split-plot design was used at all sites in both years. In 2001, five seeding rates (main plots) were chosen: the normal seeding rate 'X' is 6.7 kg/ha; twice the normal seeding rate '2X' is 13.3 kg/ha; three times the normal seeding rate '3X' is 20 kg/ha; one half the normal rate '1/2X' is 3.3 kg/ha; and one third the normal rate '1/3X' is 2.2 kg/ha. In 2002, four rates were used: 1/3X, X, 2X, and 3X. Individual plot sizes were 1.5 m wide by 6 m long, with a 23 cm row spacing. The treatments were replicated three times in 2001 and five times in 2002.

Four canola cultivars (sub plots) were chosen on the basis of different canopy types. HyLite 225RR is a mid-height, mid-maturity, and moderately lodging resistant cultivar. AC Excel is a tall, mid-maturity, and lodging susceptible cultivar. Quantum is a mid-height lodging resistant cultivar. HyLite 201 is short, early maturing, apetalous canola cultivar. An apetalous canola cultivar was included in this trial to aid in understanding of the importance of petals and the relative impact of plant stand and plant-to-plant transmission of sclerotinia stem rot infection.

3.3.3 Inoculum

The trials in 2001 and 2002 were inoculated both by natural and artificial means. The 2001 Valley City site, the 2002 Langdon site, and the 2002 Roseau site relied solely upon natural inoculum. The 2001 Roseau site and the 2002 Minto site were artificially inoculated with a spray application of macerated *S. sclerotiorum* mycelium at mid-flower. The Carman sites, in both 2001 and 2002, had 100 conditioned *S. sclerotiorum* sclerotia/m^2 incorporated into the soil prior to planting.

3.3.4 Assessments

Disease assessments were made shortly before swathing [growth stage 5.3 (Harper and Berkenkamp1975)]. One square metre quadrats were marked out in the plots early in the growing season. These quadrats were placed specifically in a portion of the plot that had an even emergence and stand, which gave a representative sample of the plot and avoided bias in placement at the time of disease assessment. The total number of infected and non-infected plants were counted. A plant was considered to be infected if it showed any level of infection, whether it was a basal stalk infection or an upper canopy pod infection. A percentage of infected plants (disease incidence, DI) was calculated for each plot. Plant density was also calculated from the quadrat counts.

Ratings were taken on lodging resistance over the entire 1.5 X 6.0 m plot. These were visual ratings based on a 1 to 9 scale. A lodging resistance rating of 1 indicates that the whole plot is flat on the ground, while a 9 rating indicates there are no lodged stems at all.

Carman seed yield results were collected from the quadrats. The plants from these quadrats were removed by hand and put through a bundle-thresher harvester. The harvested seed was weighed at 7% moisture.

3.3.5 Data Analysis

Statistical analysis was carried out using Agrobase 20 (Agronomix Inc. Winnipeg, MB) and Statistical Analysis System 8.2 (SAS Institute Inc. Cary, NC) at $P = 0.05$. Analysis of variance was performed using a general linear model. Tukey's HSD (Honestly Significantly Different) test was used to compare treatment means. Multiple

regression analysis was performed on individual site years using the null hypothesis that the independent variables did not have an impact on DI.

3.4 Results

3.4.1 Site Success

The first year of trials had low levels of infection likely caused by high temperatures during flowering. The 2001 Carman site had moderate levels of patchy infection, which did not produce any significant differences in sclerotinia stem rot DI between seeding rates nor between cultivars. In 2002, consistent moderate levels of infection in Carman were found despite hot dry conditions during the flowering period. The remaining 2002 sites did not have any sclerotinia stem rot infection. Only lodging information was obtained from Minto.

3.4.2 Plant Density

Both the 2001 and 2002 Carman sites saw significant effects in the plant stand (number of plants/m^2) between the seeding rates and between cultivars (Table 3.1). The differences between the seeding rates were visually apparent. Plots with low seeding rates were noticeably sparse and their canopies closed only at mid flower resulting from a large amount of branching. The plots with high seeding rates (2X and 3X) had dense rows immediately after emergence and their canopies closed prior to bolting. At the time of harvest, the plots with high seeding rates had individual plants lodging and were short, un-branched, and thin-stalked.

Table 3.1. Levels of significance from analysis of variance on the effects of seeding rate and cultivar on plant density from Carman, MB in trials in 2001 and 2002.

	2001		2002	
Treatment/Interaction	F Value	Pr > F	F Value	Pr > F
Seed Rate	53.42	<0.0001	56.34	<0.0001
Cultivar	3.78	0.0016	5.64	0.0017
Seed Rate X Cultivar	1.64	0.0494	1.07	0.3938

3.4.3 Lodging

At the 2001 Carman site, the lodging results were too variable to draw any conclusions, because of low lying wet sections in the trial area, but at the 2002 Carman and Minto sites, the lodging data were better. At both Carman and Minto, there was an increase in lodging with an increase in seed rate (Figure 3.1). At Carman, the 3X rate was significantly more lodged than with the other seeding rates (P=0.0005), while at Minto, both the 2X and 3X rates were significantly more lodged than the 1/3X and X rates (P=0.0009). Similarly, examining the cultivar response, there was an increase in lodging for each cultivar as the seeding rate increased above the X rate. All cultivars were less lodged at the 1/3X rate at both sites except for Quantum, which was more lodged at the 1/3X rate in Carman.

Severely lodged plots had plants flat on the ground. Under these lodged canopies, moist conditions were evident even late into the growing season. In the most severely lodged plots, the ground was still damp and plant tissue nearest the ground was also wet. Lodged plots exhibited more plant-to plant contact than upright-standing plots. Under a lodged canopy it was not uncommon to find pockets of high levels of sclerotinia stem rot. Infected plants were covered with a visible layer of white *S. sclerotiorum* mycelium, which appeared to spread from plant to plant. AC Excel was the most lodging-prone cultivar, and at high seeding rates, plants were severely lodged in all five replications at Carman in 2002. Most plots had only low levels of lodging at lower seeding rates. These plots had upright plants with minimal plant-to-plant contact.

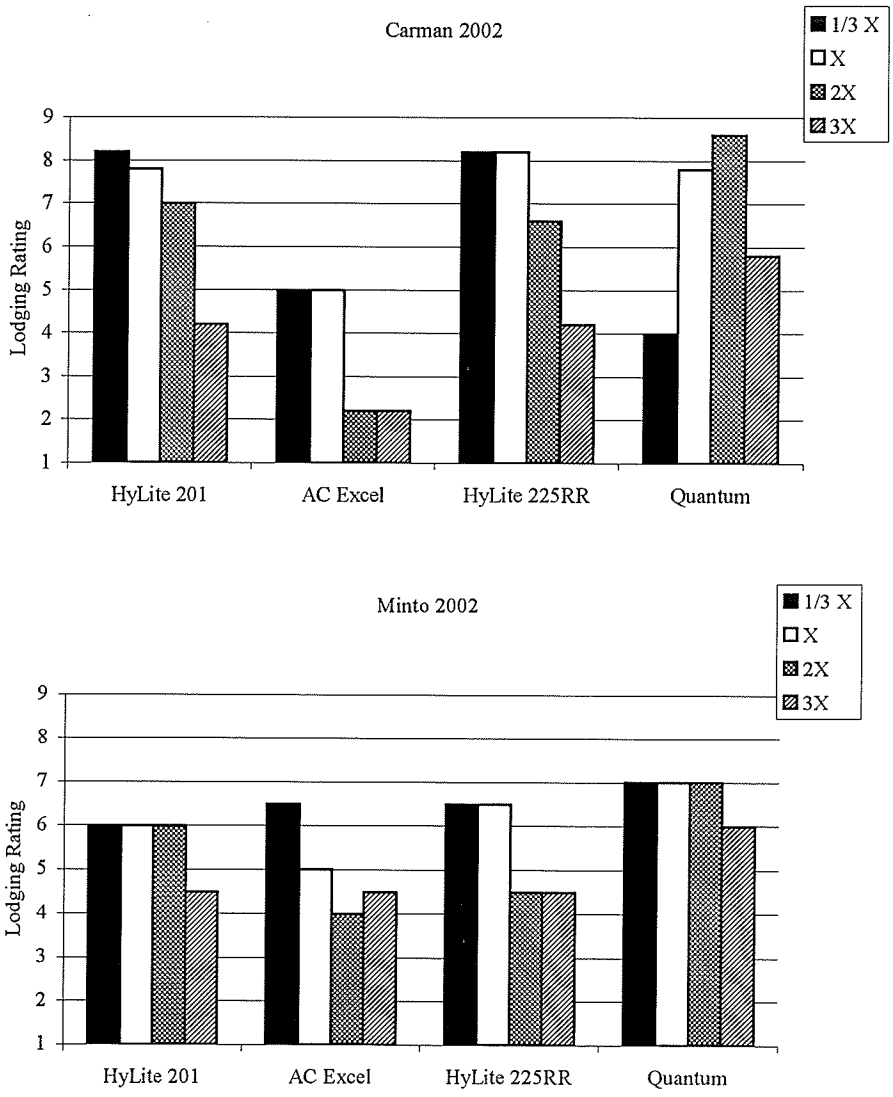

Figure. 3.1. Amount of lodging based on seeding rates and individual cultivars, in Carman and Minto, MB (2002). Lodging was evaluated using a 1-9 scale, where 1 is a severely lodged plot and 9 is with no lodged plants.

3.4.4 Disease Incidence

In Carman in 2001, a significant cultivar effect was found ($P<0.0001$) on DI, but the seeding rate effect was not significant ($P=0.1223$). In Carman in 2002, where the disease pressure was more consistent, there was a significant seeding rate effect ($P<0.0001$) and significant cultivar effect ($P<0.0001$). Figure 3.2 illustrates the seeding rate and cultivar effect at both sites. In 2002, the DI of the 3X rate was significantly greater than the 1/3X and X rates. The petalled cultivars in 2002 had more disease than HyLite 201 (apetalous), but this difference was significant only for AC Excel and HyLite 225RR (HSD=5.6%). There was no significant difference between HyLite 201 and the lodging resistant cultivar Quantum.

Individual cultivar response to seeding rates revealed significant differences only in the 2002 Carman site. Infection of each cultivar paralleled an increase in seeding rate (Figure 3.3). However, a significant increase (HSD= 19.2%) was found only in the more lodging-prone cultivar, AC Excel between the 1/3X and 3X rates. The apetalous cultivar, HyLite 201 and the lodging resistant cultivar, Quantum, did not have significant increases in infection.

A strong relationship between plant density for petalled cultivars and DI was observed (Figure 3.4). The cultivars were separated into two categories: petalled cultivars and apetalous HyLite 201, since a low disease incidence was observed in the apetalous cultivar. DI was significantly related to plant density for the petalled cultivars ($R^2=0.6697$, $P=0.0010$), but it was not significantly related to plant density in HyLite 201 ($R^2=0.6932$, $P=0.1670$), even though there was a similar R^2 coefficient.

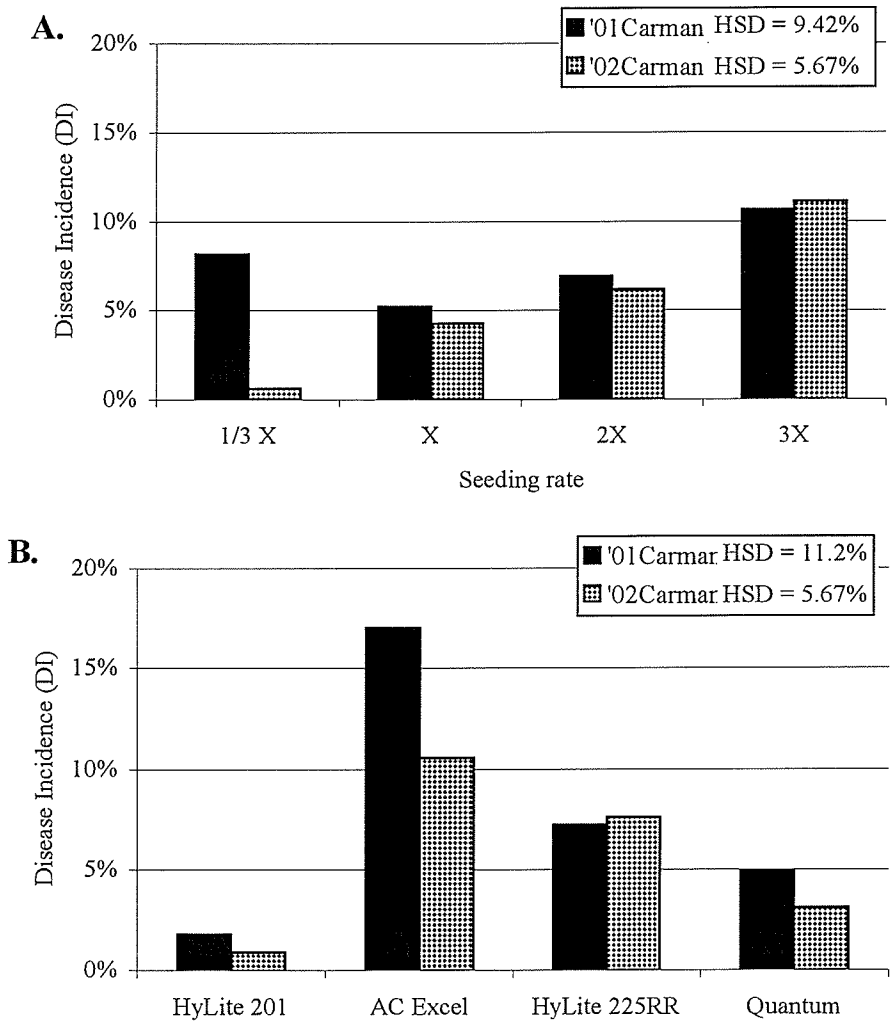

Figure. 3.2. Sclerotinia stem rot disease incidence (DI) based on seeding rate and individual cultivars in 2001 and 2002, Carman, MB site. **A.** DI based on the mean of all cultivars for each seeding rate. **B.** DI based on the mean of all seeding rates for each cultivar.

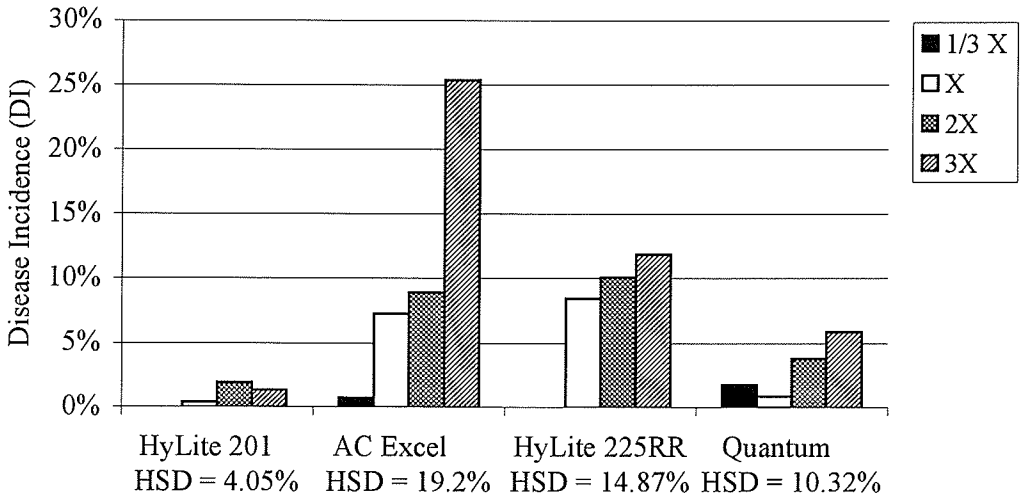

Figure. 3.3. Sclerotinia stem rot disease incidence (DI) based on seeding rates and individual cultivars in Carman, MB (2002). HSD values are calculated comparing DI means between seeding rates for each cultivar.

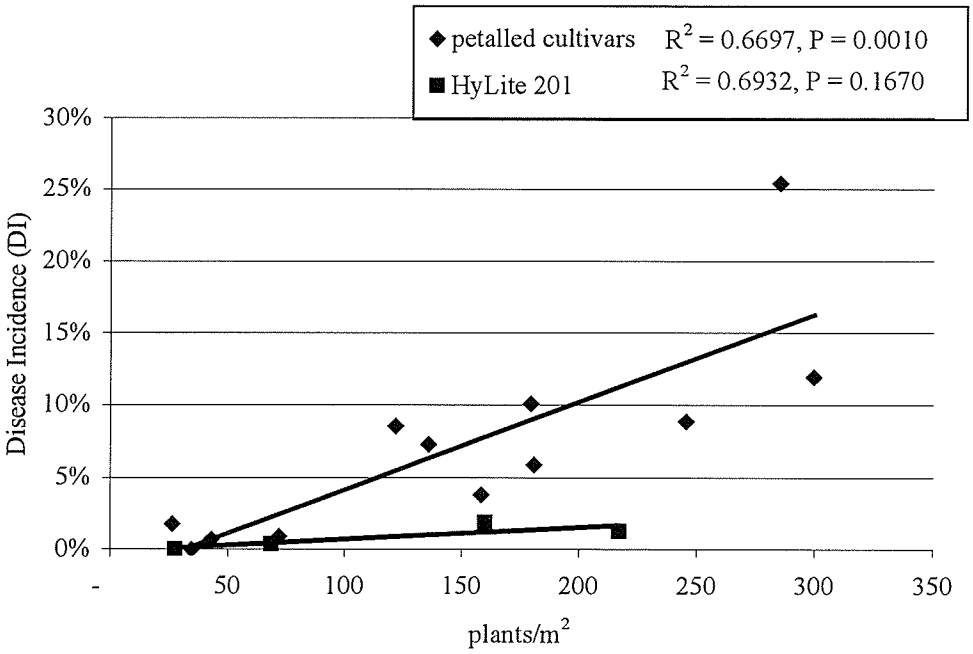

Fig. 3.4. Relationship between sclerotinia stem rot disease incidence (DI) and plant density in Carman, MB (2002). Petalled cultivars, AC Excel, HyLite 225RR and Quantum, are grouped together.

Figure 3.5 plots the impact of lodging on DI in Carman 2002. For each cultivar, with an increase in lodging there is an increase in sclerotinia stem rot infection. The R^2 values indicate that lodging explains only a fraction of the variation in DI, except in the case of the lodging-susceptible cultivar, AC Excel, which accounts for 52% of the variation.

Multiple regression analysis was performed on the 2002 Carman site data set to evaluate the combined effect of plant density and lodging on DI. The petalled cultivars data were combined, and a significant relationship (R^2=0.6952, P=0.0047) between the two independent variables and DI was found (Table 3.2). Lodging and plant density are not entirely independent variables (R^2=0.4107, P=0.0075). However, a partial correlation between DI and plant density to be 0.7231 and between DI and lodging to be –0.2825 was observed. Plant density explains more of the DI variation than lodging. The data for the individual cultivars showed no significant relationships between the independent variables and DI.

3.4.5 Yield

There were significant differences in yield between seeding rates at the 2001 Carman site but not at the 2002 Carman site (Figure 3.6). In 2001, the X rate was significantly lower yielding than both the 1/3X and 2X rates, and the 2X rate was significantly greater yielding than all but the 1/3X rate (P=0.0003). There were no significant differences in yield due to the differences between cultivars in 2002, and only AC Excel was significantly lower yielding in 2001 (P<0.0001).

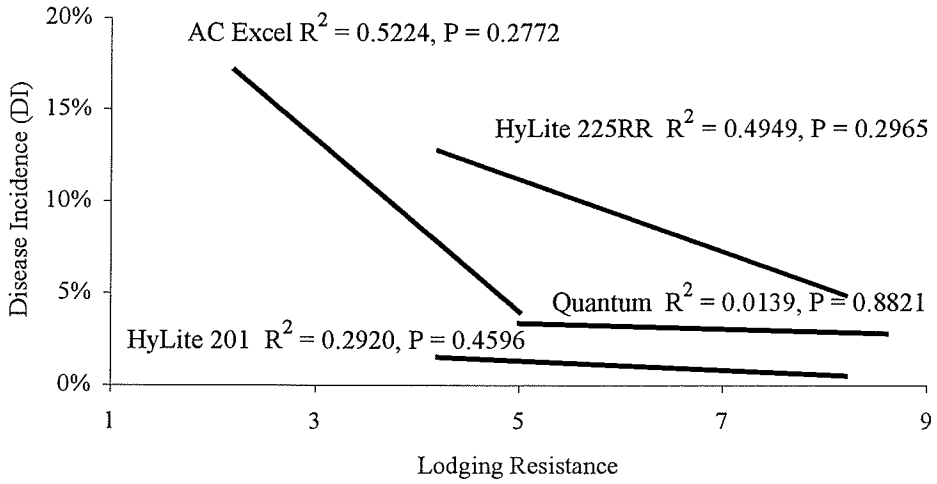

Fig. 3.5. Relationship between sclerotinia stem rot disease incidence (DI) and lodging resistance for each cultivar in Carman, MB (2002). Lodging resistance was evaluated using a 1-9 scale, where 1 is a severely lodged plot and 9 is no lodged plants.

Table 3.2. Multiple regression analysis of sclerotinia stem rot disease incidence on independent variables by cultivar or class of cultivars in 2002 Carman, MB.

Cultivar	Sample size	Multiple Regression Coefficient	Level of significance (P)	Independent Variables	Partial Correlation Coefficient	Level of significance (P)
All petalled cultivars	12	0.6962	0.0047	plant density	0.7231	0.0119
				lodging	-0.2825	0.4000
HyLite 201	4	0.9915	0.0923	plant density	0.9942	0.0685
				lodging	0.9873	0.1016
AC Excel	4	0.7596	0.4903	plant density	0.7046	0.5023
				lodging	0.3433	0.7769
HyLite 225RR	4	0.9803	0.1402	plant density	0.9803	0.1264
				lodging	0.9508	0.2006
Quantum	4	0.9819	0.1345	plant density	0.9908	0.0865
				lodging	-0.9590	0.1928

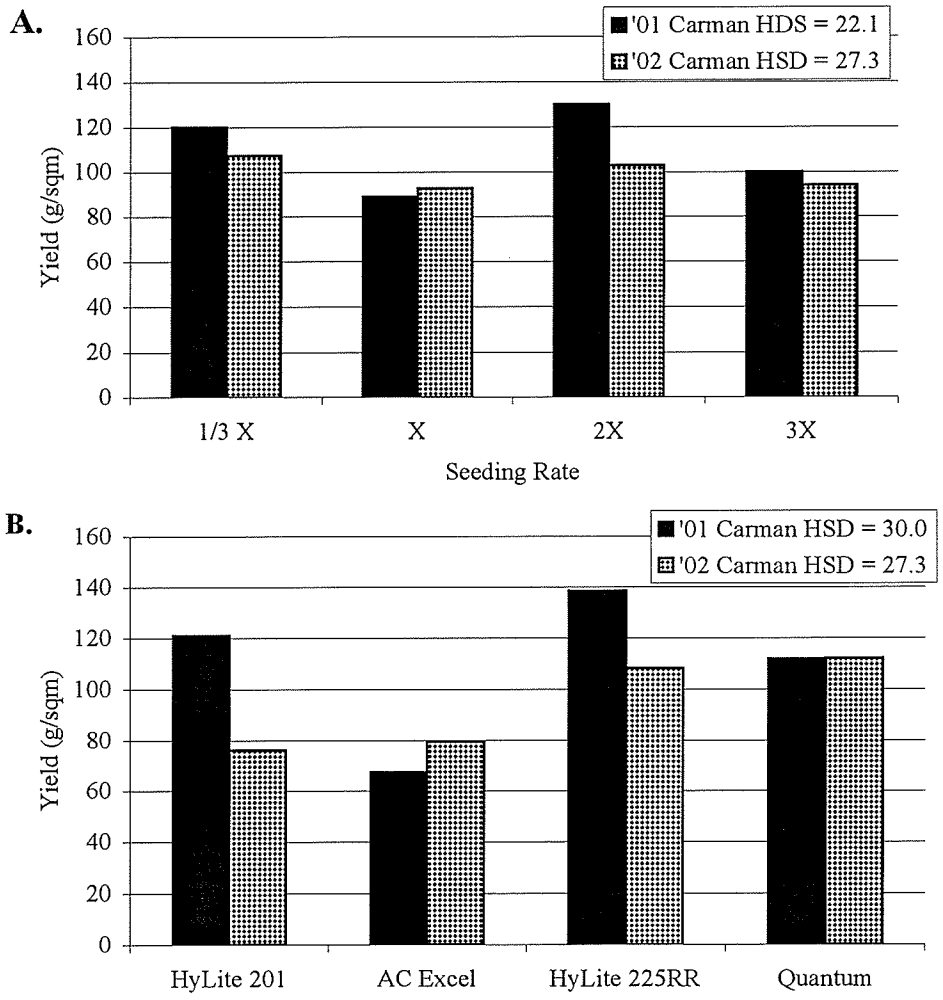

Figure. 3.6. Plot seed weights, harvested from a single metre square quadrat, based on seeding rates and individual cultivars, in Carman, MB (2001 and 2002). **A.** Seed weights based on the mean of all cultivars for each seeding rate. **B.** Seed weights based on the mean of each seeding rate for each cultivar.

3.5 Discussion

Seeding rate affects sclerotinia stem rot incidence in canola cultivars. There are only a few published studies that have looked at this relationship. This finding has been shown in canola (Irvine and Duncan 1992), in beans (Blad *et al.* 1978; Haas and Bolwyn 1972; Saindon *et al.* 1995; Steadman *et al.* 1973), and in sunflowers (Huang and Hoes 1980). Seeding rate, which is believed to affect plant density and the resulting canopy density, is not universally believed to have this effect on sclerotinia disease development. Nordin *et al.* (1992) found that there was no relationship between plant density and DI. Turkington and Morrall (1990) found a relationship between plant density and DI, but Turkington (1991) found that seeding rate's effect on DI is low and is mitigated by cultivar effects, environmental effects and soil fertility. Our study supports the conventional understanding that plant density plays a role in the development of sclerotinia stem rot and suggests that seeding rate is a strong component to the development of sclerotinia stem rot.

The 2002 Carman site best illustrates this relationship between seeding rate and DI. There was an increase in sclerotinia stem rot infection with an increase in seeding rate in AC Excel and in the mean of all the cultivars. Furthermore, there was a strong cultivar effect on disease incidence. Both AC Excel and HyLite 225RR had higher DI across all seeding rates than HyLite 201 and Quantum. This decrease in DI in HyLite 201 has been explained mostly by the lack of petals (Jurke and Fernando 2002), while Quantum's lower DI may be related to its lodging resistance. Regression analysis on plant density and DI showed that there was a significant relationship between these two factors in the petalled cultivars, but it was not significant in HyLite 201. Quantum showed the most

interesting DI results, with greater DI at low seeding rates. This effect can be explained since Quantum, being large-seeded, had the fewest plants at the low seeding rates, which caused profuse branching. Without adjacent plants for support, these isolated un-propped plants tended to lean and fall over. The lodging data did indicate that this variety had more lodging at low seeding rates than at higher seeding rates, which resulted in more disease.

Lodging in canola increases the development of sclerotinia stem rot. Lodged plots were found to have greater incidence of sclerotinia stem rot infection in this study. Lodged plots were associated with increased seeding rates, and the plants in these plots had very thin stems and were easily pushed over. Sections where disease incidence was highest were the sections where the greatest amount of plant-to-plant contact existed and the ground was wet for prolonged periods. Within these pockets of high levels of infection, the plants were visibly covered with white mycelium, which could be seen spreading from one plant to another. It appears that under these lodged conditions, not only is the microenvironment more conducive to sclerotinia stem rot development, but plant-to-plant contact is at its peak, permitting the physical spread of mycelium from one plant to another.

Seeding rate likely affects DI in two ways: 1) by modifying the microenvironment under the canopy, and 2) by decreasing the lodging resistance of the plant stand. This microenvironment effect has been explained in previous studies. Turkington (1991) found that plant stands with dense canopies have increased relative humidity and longer periods of leaf wetness than thin stands. Turkington *et al.* (1993) tried to establish the relationship between plant density and sclerotinia stem rot, finding that disease was inconsistently

related to their measures of canopy density. This inconsistent relationship was attributed to the difficulty in quantifying crop density. Although our study did not look at measures of canopy density, and therefore could not establish a relationship between seeding rate and measures of canopy density, visually, plots of higher seeding rates did appear to have a greater canopy density. There was a significant relationship between these visual density ratings and DI, but the value of the density ratings is not based on objectively measurable variables. The relationship between planting density and canopy density has been established in other crops. Blad *et al.* (1978), Haas and Bolwyn (1972) and Steadman *et al.* (1973) all found that row spacing and seeding rate affects disease risk by changing the proximity of individual plants and plant parts. Blad *et al.* (1978) found that a dense canopy in dry beans created temperature conditions that did not exceed 30 C and high moisture conditions that persisted up to 12 h, which are conditions that strongly favour sclerotinia stem rot development. There is a need to establish the relationship of seeding rate to crop density in canola.

Multiple regression analysis gives a deeper understanding of the relationship between DI and seeding rate. Analysis of all petalled cultivars indicates that 67% of the DI variation can be attributed to plant density and lodging. Since both plant density and lodging are largely affected by seeding rate, it is likely to be the main factor in explaining this variation. Of the two, plant density contributes more significantly to DI than lodging. This may be because each cultivar had different lodging characteristics, and combining these removes a noticeable effect.

Use of cultural methodologies to control disease can be difficult. Duczek *et al.* (1996), studying *Bipolaris sorokiniana* in multiple crops, found that reducing crop

density may encourage the growth of weeds which may aid in disease development by modifying the crop canopy, or may reduce the disease by trapping spores which might have landed on the susceptible crop. The influence of seeding rates on weed populations should be studied in order to determine whether this will impact on sclerotinia stem rot development in canola.

Reduction of seeding rate raises concerns about yield. Irvine and Duncan (1992) found that yield decreased as canola seeding rates increased, which they attributed to sclerotinia stem rot infection and lodging losses. However, our study indicated that yield was not affected by seeding rate in 2002 in Carman, which supports the finding that canola can compensate by branching readily to ensure that all gaps in the canopy are filled.

Decreasing the seeding rate may decrease the level of sclerotinia stem rot of canola in petalled cultivars. This reduction in disease can further be improved by the use of lodging resistant and apetalous canola cultivars.

3.6 Acknowledgments

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4.0 IDENTIFICATION OF AVOIDANCE TRAITS IN CANOLA TO SCLEROTINIA STEM ROT INFECTION

4.1 Abstract

There are no known forms of physiological resistance reported in canola (*Brassica napus* L. and *B. rapa* (L.) Thell. Emend. Metzger to fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal agent of sclerotinia stem rot, but there have been reports of escape or avoidance mechanisms in this crop. The objectives of this work were to identify plant architectural, crop structural, and phenological escape mechanisms in 11 Canadian canola cultivars. The objective of this study is to determine the relationship between sclerotinia stem rot disease incidence (DI) and 16 architectural/phenological traits (leaf number, leaf area, leaf litter, stem circumference, first branch height, branch angle, lodging resistance, days to flower, flowering duration, maturity, petals and sepal deposition on leaves and in leaf axils, maturity, crop height, and canopy density), collectively termed 'agronomic traits'. The canola cultivars used were: HyLite 201, an apetalous *B. napus* cultivar, an isogenic pair of *B. rapa* lines which differ for the apetalous trait, and 8 petalled *B. napus* cultivars which vary in these agronomic traits and in historical susceptibility to sclerotinia stem rot infection. The cultivars were evaluated for these agronomic traits at three locations in 2001 and 2002, and the results were compared to sclerotinia stem rot disease incidence (DI). Petals adhering to leaves and leaf axils was significantly correlated to DI. Principle component analysis revealed that the inclusion of apetalous biased the results and hindered the identification of other avoidance features. Removal of the *B. rapa* and HyLite 201 from analysis indicated that lodging resistance, branch angle and maturity

were also significantly correlated to DI. The apetalous trait gave the largest decrease in DI, and selection for lodging resistance and wider branch angles should aid in the development of cultivars with improved avoidance characteristics to sclerotinia stem rot.

4.2 Introduction

Canola, also known as oilseed rape, is a *Brassica* oilseed low in erucic acid (<2% of the oil) and low in glucosinolates (<30 μmol per gram of oil-free solids). The nutritional benefit of canola oil being low in saturated fats (<7.1%) has resulted in this crop growing remarkably in acreage in the past thirty years. There are three species from the *Brassica* genus which have cultivars possessing canola quality: *Brassica napus* L., *B. rapa* (L.) Thell. Emend Metzger and *B. juncea* (L.) Cosson. Sclerotinia stem rot of canola, caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is a major disease of canola. Annual yield losses in China related to sclerotinia stem rot average 20% and are as high as 50% in some years (Liu 1991). In temperate climates, infection rates and yield reduction vary from year to year. In Canada, the effect of sclerotinia stem rot varied from 0.5% incidence in Alberta to 12% in Manitoba in 2001 because of differences in environmental conditions (Benard *et al.* 2002; McLaren *et al.* 2002). The full impact of this disease depends on weather conditions and the synchronisation of host plant susceptibility with ascospore release, and thus can vary considerably from year to year (Freeman *et al.* 2002).

Sclerotinia sclerotiorum survives in the soil as sclerotia. In temperate climates, these sclerotia germinate carpogenically to produce apothecia which normally coincides with the flowering phase of the canola crop. Infested senescing petals, which fall through the canopy, act as the major nutrient source for the germinating ascospores. Petals adhere to lower canopy plant parts such as leaves and stems depending on their wetness (Heran *et al.* 1999). Lesions appear on leaves and infection proceeds into the stem, which can

result in stem girdling and plant death in severe cases. Sclerotia are produced in the stem of infected plants and are returned to the soil upon breakdown of the plant tissue.

There are reports of variation in susceptibility to *S. sclerotiorum* in many host crops. Resistance has been identified and is quantitatively inherited in bean crops (Fuller *et al.* 1984; Kolkman and Kelly 2002; Kolkman and Kelly 2003; Miklas *et al.* 1992a; Park *et al.* 2001) and in soybeans (Arahana *et al.* 2001b; Boland and Hall 1987; Kim and Diers 2000). The resistance observed consists of both physiological resistance and escape or avoidance mechanisms (Kolkman and Kelly 2003; Miklas *et al.* 2001). In *Brassica* crops, Sedun *et al.* (1989) reported that there may be physiological variation in resistance, but these results have not been expanded upon. Jurke *et al.* (1998) reported that an apetalous canola cultivar, HyLite 201, had significantly reduced levels of infection, and that this was attributable to the apetalous trait – an avoidance mechanism. But this hypothesis could not be verified since only one cultivar possessed this trait and no quantification of physiological resistance was made.

Disease escape can be the result of a genetically controlled avoidance trait or environmentally influenced conditions. There are a number of inherited avoidance traits reported in crops susceptible to *S. sclerotiorum*. Crop elevation in great northern bean and navy bean was found to reduce white mold infection (Fuller *et al.* 1984; Park 1993). Canopy structure was found to influence apothecia production in bean (Schwartz and Steadman 1978). Increases in plant height has been found to reduce white mold infection in dry bean (Miklas *et al.* 2001; Park 1993) but increase it in soybean (Kim and Diers 2000). Kim and Diers (2000) also found that white mold was influenced by lodging

resistance and maturity date. In contrast, Kim *et al.* (1999) reported that canopy height and maturity were not correlated with disease severity in soybeans.

The objective of this study was to determine which plant architectural, crop structural and phenological traits of canola influence the development of sclerotinia stem rot. Understanding what traits affect this disease may enable plant breeders to select and develop cultivars with improved avoidance mechanisms. The expression of these traits needs to be studied to determine if their phenotypic expression is largely the result of genotype or of environment.

4.3 Materials and Methods

4.3.1 Study Sites

Trials were conducted in 2001 and 2002 at multiple locations to obtain moderate to high levels of sclerotinia infection (Table 4.1). Sites in the USA were selected for a history of consistent sclerotinia infection, and the sites in Canada were selected for their ease of access and management. All trials were established on land using a rotation of at least 3 years between canola crops. The Carman, MB site was an irrigated sclerotinia nursery in a three-year rotation of soybeans-sunflowers-canola.

4.3.2 Experimental Design

Eleven canola cultivars chosen for their differing morphological characteristics were tested both years (Table 4.2). Trial design varied over years and over sites. A split plot trial design was used at all sites in 2001, with two main plots: an application of Rovral Flo (iprodione) fungicide (at a rate of 600 g ai/ha) and an unsprayed check; the sub plots were canola cultivars. These trials were replicated five times. Trial design in 2002 was an RCBD design with no fungicide application replicated 10 times in Carman, five times at Roseau and Langdon and three times at Minto. Individual plot sizes were 1.5 m wide by 6 m long, with a 23 cm row spacing.

The two *B. rapa* canola cultivars used were isogenic lines: one apetalous and the other petalled. These cultivars were used to gain a better understanding of the importance of petals in sclerotinia infection.

Table 4.1. Locations of sclerotinia stem rot avoidance trials, environmental conditions and resulting infection levels.

Year	Location	Success of Infection	DI (%)
2001	Carman, MB	hot and dry – low infection	3
	Roseau, MN	high levels of infection	52
	Valley City, ND	hot and dry – low infection	3
2002	Carman, MB	hot and dry – no infection	0
	Roseau, MN	flooding - no infection	0
	Langdon, ND	early frost – no plants	0
	Minto, ND	no infection	0

Note: DI is the sclerotinia stem rot disease incidence averaged over all cultivars at the site.

Table 4.2. General characteristics of canola cultivars used for avoidance trials under sclerotinia disease pressure.

Cultivar	Species	Petals	Plant Height	Maturity	Lodging Resistance
44A89	*B. napus*	mid size	short	early	mod. susceptible
46A65	*B. napus*	mid size	tall	mid	resistant
AC Excel	*B. napus*	mid size	mid	late	susceptible
CB9606	*B. rapa*	none	v short	v early	mod. susceptible
CB9607	*B. rapa*	small size	v short	v early	mod. susceptible
Ebony	*B. napus*	mid size	tall	mid	mod. resistant
HyLite 201	*B. napus*	none	short	early	mod. resistant
HyLite 225 RR	*B. napus*	mid size	mid	mid	mod. susceptible
Hyola 401	*B. napus*	mid size	mid	mid	resistant
Invigor 2153	*B. napus*	mid size	tall	late	susceptible
Quantum	*B. napus*	mid size	mid	mid	mod. resistant

4.3.3 Inoculum

The experiments in 2001 and 2002 were infected both by natural and artificial means. The 2001 Valley City site, the 2002 Langdon site and 2002 Roseau sites solely relied upon natural ascospore infection. The 2001 Roseau site and the 2002 Minto site were artificially inoculated with a single spray application of macerated *S. sclerotiorum* mycelium at mid flower. The Carman sites in both 2001 and 2002 had 100 conditioned sclerotia per square metre incorporated into the soil prior to planting. Also, the 2001 site was inoculated with the macerated sclerotinia mycelium spray. It was not deemed effective in 2001, so mycelial inoculation was not repeated in 2002.

The macerated sclerotinia mycelium spray was prepared by growing *S. sclerotiorum* mycelium in PDB (potato dextrose broth – Difco, Sparks, MD, USA) in 250 ml bottles for one week. This solution was then macerated using an electric hand-held blender. This macerated mash was applied with a back-pack sprayer at a concentration of 4 grams of mycelium per litre of water applied at a rate of 50 ml solution per square metre.

In 2002 at a location 5 km away from the Carman trials, rotorod impaction spore samplers (Model: GRIPS-99, Aerobiology Research Laboratories, Nepean, ON, Canada) and a Burkard 7-day recording volumetric spore sampler (Burkard Scientific Ltd., Uxbridge, U.K) were used to trap *S. sclerotiorum* ascospores through the growing season. Since these were not located on site, ascospores were not quantified, but rather, simply the days when ascospores were present in the atmosphere were noted.

4.3.4 Assessments

For simplicity sake, the term 'agronomic trait' refers to measurable phenotypic traits that are related to plant and crop structure and phenology that may contribute to a cultivar's escape of sclerotinia stem rot infection. Leaf litter is also in this classification. A total of 16 quantitative traits (agronomic traits) were scored for the 11 cultivars at three sites. Agronomic traits scored at Carman in 2001 and 2002 and Minto are listed in Table 4.3. Selection of traits was based on: 1) relation to the sclerotinia disease cycle in canola; 2) reports of important avoidance traits in other crops; and 3) ease of evaluation.

The duration of the flowering period ('flowering duration') and days to flower (DTF) were determined by recording when each plot was at 10% flower, 50% flower and 90% flower. Maturity ratings were a visual assessment done at 81 days after emergence on a 1 to 9 scale, where 1 = very mature and 9 = least mature.

Petals and sepals were evaluated by counting those found adhering to the surface of leaves and in leaf axils (the point where the leaf joins the stem) of five and three randomly sampled plants per replication in 2001 and 2002, respectively. Notes were taken on which plant parts contained the majority of flower debris and quantified as 1 = lower plant, 2 = whole plant, and 3 = upper plant.

'Leaf area' was measured in a number of ways. In 2001 leaf area was estimated by measuring the length and width of one lower large leaf blade from five randomly sampled plants. In 2002, a digital camera (Sony Cybershot DS75S) was used to photograph three leaves from 2 randomly sampled plants per plot. The leaves were sampled systematically: the lowest attached leaf, the largest mid-plant leaf, and one of the upper-most leaves on the plant. The digital image analysis software, Assess (APS Press, St. Paul, MN, USA),

was used to calculate the leaf area from each plant, which were averaged for each plot. The 'number of leaves per plant' trait was the average of three randomly sampled plants in 2001 and five in 2002.

'Canopy height' was measured using a yard-stick at one location in the centre of the plot. Five randomly sampled plants were pulled up from each plot to measure the distance from the soil surface to the first branch, which was referred to as 'height to first branch'. These same five plants were measured for the diameter of the base of the stem with a set of callipers for the 'stem diameter' evaluations, and the angle of the lowest branch from the stem was measured using a protractor for the 'branch angle' evaluations.

'Canopy density' was estimated differently each year. In 2000, a light meter (VWR Traceable, Model 62344-944, Texas USA) was used to measure the intensity of light above the canopy and beneath the canopy at the ground surface at two random points within a plot. In 2002, a metre-long Line Quantum Sensor, (Model LI-191SB, Li-Cor Inc., Lincoln, Nebraska, U.S.A.) was used to measure the light intensity above canopy and below at the ground. At the 2002 Carman site, readings were taken on 28-July (mid flower) and on 2-Aug (mid-late flower). The percentage of light intercepted ('% light extinction') was calculated by subtracting the quotient of below-canopy readings over the ambient readings from one. Visual canopy density assessments were made in 2001 using a 1 to 9 scale, where 1 is a very thin plot and 9 is a very dense plot.

'Lodging resistance' ratings were observed over the entire plot area. These were visual ratings based on a 1 to 9 scale. A lodging resistance rating of 1 indicates that the whole plot is lodged flat on the ground, while a 9 rating indicates there are no lodged stems in the plot.

'Leaf litter' was measured using digital images analysed with Assess software. These images were taken by separating the canopy and photographing the ground with a digital camera using a wide-angle view. The percentage of soil covered with leaf litter could then be determined based on colour spectrum of the digital image.

Sclerotinia stem rot disease evaluations were made shortly before swathing [growth stage 5.3 (Harper and Berkenkamp 1975)] with a one square metre quadrat placed randomly within the plot. The total number of infected and non-infected plants was counted. A plant was considered infected if it showed any level of infection, whether a basal stem lesion or an upper canopy pod lesion. A percentage of infected plants (disease incidence, DI) was calculated for each plot.

4.3.5 Statistical Analysis

Statistical analysis was carried out using Statistical Analysis System 8.2 (SAS Institute Inc. Cary, NC, USA). Analysis of variance was performed using a general linear model. Tukey's HSD (Honestly Significantly Difference) test was used to compare treatment means at P=0.05. Principle component analysis (PCA) was carried out using the SAS PRINCOMP procedure on the agronomic trait data to reduce and summarise the information. A PCA is used to explain the variance structure through a few linear combinations of the original variables (Johnson 1998). A scatterplot of the prime principle components illustrates the relationship between cultivars, since those in close proximity in the ordination space are similar with respect to gradients defined by PCA. The 34 original variables were reduced to seven, eight, or ten independent components depending on the number of cultivars included in analysis. Since two cultivars lacked petals, there was concern that analysis would result in petals playing a larger role in

analysis and interpretation than what is real. Therefore, the analysis was run in three ways: 1) the complete set of eleven cultivars; 2) *B. napus* cultivars only; and 3) petalled *B. napus* cultivars only. Values for the principle components were obtained for each experimental unit, and these new independent variables were correlated with sclerotinia stem rot DI. Pearson correlations coefficients (R) were calculated using PROC CORR to determine the relationship between DI and the agronomic traits and the principle components. Subsequently, the significant components were examined to assess which traits had the highest component loading to determine what these principle components may be representing.

4.4 Results

4.4.1 Site Success and Disease Incidence

Of the seven trials conducted, only the 2001 Roseau site had adequate levels of infection (Table 4.1). The mean DI for Roseau was 39% in the plots, treated with the fungicide iprodione and 65% in the unsprayed plots. The high level of infection in Roseau is a reflection of the cool and wet conditions throughout July; at the other 2001 locations it was significantly warmer and drier. The summer of 2002 was hot and dry at all locations through the flowering period at the time of ascospore release. In Carman that year, ascospores were trapped from July 3 to 13, July 20 to 23, and on July 26 during the flowering period (Fig. 4.1). This period coincided with daily maximum temperatures averaging 30 C and low minimum relative humidity, conditions that retard

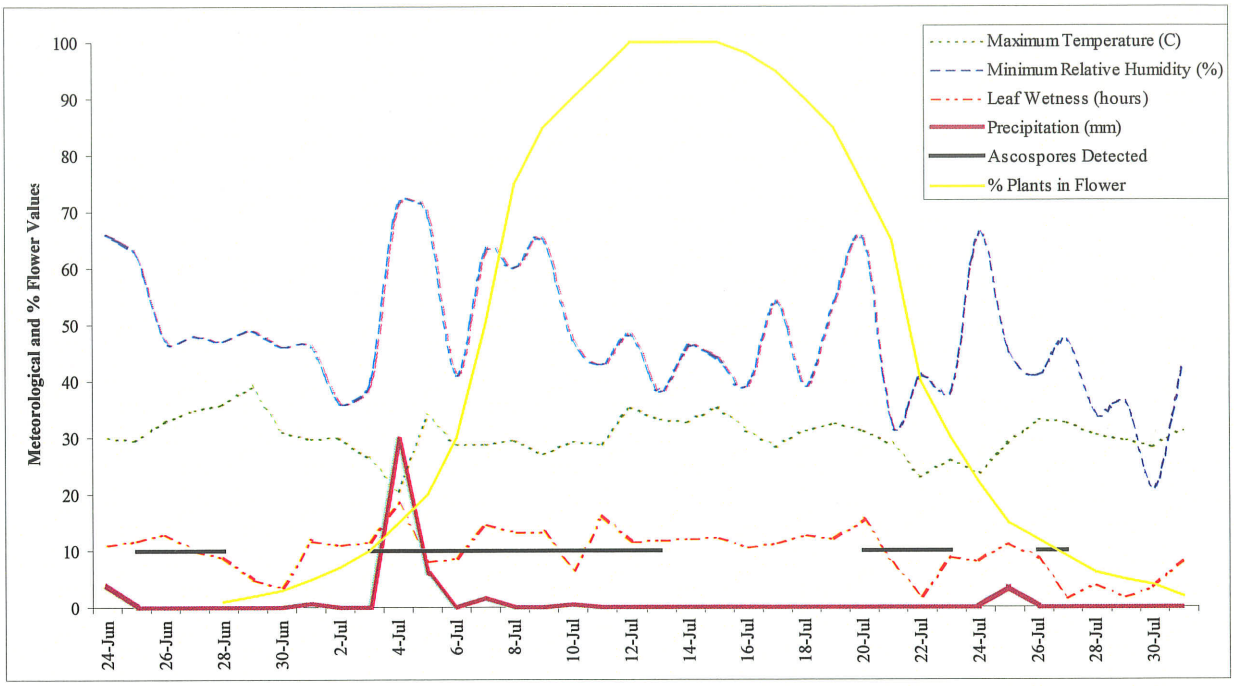

Figure 4.1. Meteorological, flowering, and ascospore presence data from Carman, MB in 2002. Ascospore data presented is not quantified, but the line denotes the presence or absence of ascospores for that day.

S. sclerotiorum growth. Forty-two mm of rain fell during the flowering period, but most of this occurred in the first few days.

The Roseau DI data showed that the apetalous lines had significantly lower DI than the petalled cultivars (HSD = 22.2% and LSD = 13.1%). The fungicide treatment significantly reduced DI by 42% for all cultivars, by 41% for the petalled cultivars, and by 45% for the apetalous cultivars. The cultivar 44A89 was the most infected cultivar, and DI was reduced only by 18% with the fungicide application, likely because of its early flowering time coinciding with more favourable conditions for sclerotinia stem rot development. The correlation between the sprayed and unsprayed cultivar's DI data was significantly high (R = 0.8548, P = 0.0033). The unsprayed data set (HSD = 29.11% and LSD = 17.16%) was used as the representative disease reaction data set for all future comparative methods.

The agronomic trait data was collected at Carman, the nearest study site. Even though there was little disease at this site in both years of study, information on the architectural, crop structural, and phenological traits of the canola cultivars was collected (Table 4.3). The remaining locations, other than the Minto site, were too remote to obtain agronomic trait data.

4.4.2 Analysis of Variance

To measure the environmental effect on the avoidance traits, analysis of variance was completed once the data sets were compiled. Significant genotypic and environmental (site) variance was found in the avoidance traits (Table 4.4). More significant variances were explained by environmental than by genotypic effects. Environment contributed significantly to leaf area, sepals on leaves, location of petal fall,

Table 4.3. Mean values for agronomic traits and disease incidence for 11 canola cultivars from Carman, MB in 2001 and 2002, Roseau, MN in 2001, and Minto, MB in 2002.

Traits	No. of replic-ations	Cultivar										
		44A89	46A65	CB9606	CB9607	Ebony	Excel	HyLite 201	HyLite 225 RR	Hyola 401	Invigor 2153	Quantum
Days to Flower												
2002 Carman	10	34.4	33.8	24.4	26.7	38.9	36.7	36.5	34.5	35.1	33.5	36.2
2002 Minto	3	36.7	36.7	26.0	27.3	39.7	37.7	35.0	36.0	36.3	35.0	38.7
Flowering Duration (days)												
2001 Carman	10	16.2	17.6	22.5	24.2	13.5	16.5	13.5	16.7	16.8	16.4	16.2
No. petals on leaves												
2001 Carman	10	22.5	21.3	5.4	20.0	15.2	16.7	10.9	19.3	27.8	29.0	18.2
2002 Carman	10	8.3	10.9	2.1	4.1	10.9	13.8	2.8	9.4	7.8	10.7	7.7
02 Minto	2	22.8	20.2	1.2	17.2	12.5	13.8	0.0	9.9	7.5	31.9	18.7
No. petals in leaf axils												
2001 Carman	10	3.3	3	0.7	3.7	2	2.2	1.0	2.5	3.1	4.3	1.8
Petal distribution on plant												
2001 Carman	10	1.2	1.5	1.4	1.5	1.1	1.4	1.1	1.3	1.0	1.2	1.1
2002 Carman	10	1.2	1.0	1.2	1.0	1.2	1.5	1.3	1.2	1.1	1.5	1.3
No. sepals on leaves												
2002 Carman	10	7.7	7.9	2.7	2.8	9.8	9.8	7.2	9.2	6.0	10.5	5.7
02 Minto	2	20.0	20.9	12.3	13.8	12.0	11.9	9.9	10.0	9.8	22.4	20.7
No. leaves/plant												
2001 Carman	10	7.0	7.3	6.9	8.7	6.4	7.4	6.6	6.9	7.9	7.6	7.9
2002 Carman	10	5.8	6.2	4.6	5.5	6.0	6.3	5.9	6.0	6.1	6.0	5.5
2002 Minto	2	8.5	11.4	11.0	10.5	7.7	8.1	11.2	6.9	15.1	9.4	8.5
Leaf Area (sq cm)												
2001 Carman	10	318	267	110	141	438	330	240	284	350	277	308
2002 Carman	10	503	442	424	427	697	521	517	586	653	688	487
2002 Minto	3	105	98	58	67	109	105	65	98	121	118	169
Leaf Litter (% ground cover)												
2002 Carman	4	39.9	43.9	8.6	19.3	42.4	48.1	48.4	45.5	51.4	46.3	39.9
Stem Diameter (mm)												
2001 Carman	10	7.59	6.6	6.2	7.62	7.47	7.59	7.01	6.86	7.37	6.38	7.6
2002 Carman	10	9.7	10.42	8.24	9.16	9.84	10.98	8.58	8.12	11.62	9.54	11.52
Branch angle (degrees)												
2001 Carman	10	28.4	29.4	29.6	33.7	31.7	28.6	32.5	27.9	31.7	23.7	28.2
2002 Carman	6	14.1	18.1	20.1	20.7	19.8	17.4	23.0	15.3	18.3	19.1	18.9
Distance from ground to first branch (cm)												
2001 Carman	10	23.7	17.0	13.3	6.9	35.4	22.8	20.0	23.2	12.4	19.5	28.0
2002 Carman	10	18.5	7.5	6.7	4.0	32.0	21.9	7.6	13.8	5.3	19.1	13.1
Lodging resistance (1-9)												
2001 Carman	10	3.6	5.8	4.4	4.8	7.0	4.2	4.6	5.4	7.2	4.4	4.8
2002 Minto	3	6.0	6.0	6.3	6.0	6.7	5.7	7.0	5.3	7.0	6.0	6.7
Canopy height (cm)												
2001 Carman	10	109	117	88	90	123	116	105	116	112	122	123
2002 Carman	10	97	106	82	79	115	110	90	92	98	109	105
2002 Minto	3	112	117	90	93	124	116	97	105	104	120	130
Canopy Density (% light extinction)												
2001 Carman	10	90.3	91.6	88.6	83.9	93.2	92.0	87.8	90.2	91.5	85.3	83.9
2002 Carman - 20-Jul	10	81.6	91.3	80.1	61.0	91.2	87.4	91.5	93.3	90.4	89.8	87.9
2002 Carman - 02-Aug	10	87.2	85.4	76.9	65.8	91.8	89.1	84.0	86.6	87.2	85.0	88.8
2002 Minto	2	88.5	88.9	86.3	77.5	88.3	92.6	87.0	91.5	86.7	93.8	86.4
Disease Incidence												
2001 Roseau - Fungicide	5	71.0	40.1	17.6	31.6	29.5	60.8	18.6	58.8	24.7	46.5	26.5
2001 Roseau - Unsprayed	5	86.9	72.1	33.9	69.2	60.0	75.6	32.6	81.5	60.2	83.8	62.2

Table 4.4. Mean square values from analysis of variance on agronomic traits in 11 canola cultivars across three site-years in Carman, MB in 2001 and 2002 and Minto, MB in 2002, and analysis of variance for sclerotinia stem rot from Roseau, MN in 2001.

		Mean Squares				
Source	df	Leaf area	No. leaves per plant	No. petals on leaf	Canopy density - July	Canopy height
Genotype (G)	10	13950.1	6.9 †	102.5 *	0.7	268.7 †
Environment (E)	2	247461.8 **	2.5	120.1 †	3.7 *	396.7 †
G X E	20	18153.6	6.0 †	89.8 **	1.6	89.5

Source	df	Sepals on leaf	Petal distrib-ution	Height to first branch	Stem diameter	Branch angle	Lodging resistance
Genotype (G)	10	21.0	0.2	205.9 **	1.8	40.7	1.46
Environment (E)	1	191.4 **	2.0 **	81.4	315.3 **	10.5	0.05
G X E	10	47.1	0.3	95.3	2.0	52.4	2.83 †

Source	df	Leaf litter	No. petals in leaf axils	Flower duration	Days to flower	Maturity rating	Canopy density-August	Disease incidence
Genotype (G)	10	1.6	11.1 **	7.0 *	2.8	0.4 †	0.9	31.0 **
Environment (E)	0							
G X E	10							

Note: An asterix indicates significance at $P < 0.05$, a double asterix indicates significance at $P < 0.01$, and the † symbol indicates significance at $P < 0.10$.

stem circumference, and canopy density in July. Genotype contributed significantly to the number of petals adhering to leaves and height to the first branch. Some traits were evaluated only at one site, and for these traits only a measure of the genotypic variation could be calculated. Of these traits, genotype significantly accounted for variation on the number of petals counted in leaf axils, flowering period duration and, at the Roseau site, disease incidence.

Petals on leaves and lodging resistance had a significant G X E interaction. The petals on leaves trait was influenced by cultivar at the Carman site in 2001 (MSE = 167.8, P = 0.0255), but not in 2002 (MSE = 23.7, P = 0.1518), which accounts for this G X E interaction. Lodging resistance was not significantly influenced by either genotype or by environment in the Carman 2001 and Minto sites. When the lodging data was compiled over both sites and analysis of variance completed on 13 reps of lodging resistance data, a significant cultivar effect was found for this trait (MSE = 8.65, P <0.0001). Seeding problems occurred at Carman in 2001, and the resulting plant stand was patchy, this influenced the lodging ratings.

There were significant differences between canola cultivars for many of the avoidance traits. The most obvious difference was in the production of petals and the resulting petal adhesion to leaves (Fig. 4.2). The two apetalous lines had significantly less petals adhering to leaves than most petalled cultivars at the three sites (HSD = 31.5%). HyLite 201 was a true apetalous line producing no petals during the flowering season. Petals adhering to leaves in the plot are due to contamination from adjacent plots containing petalled cultivars. Line CB9606 produced some petals during the flowering

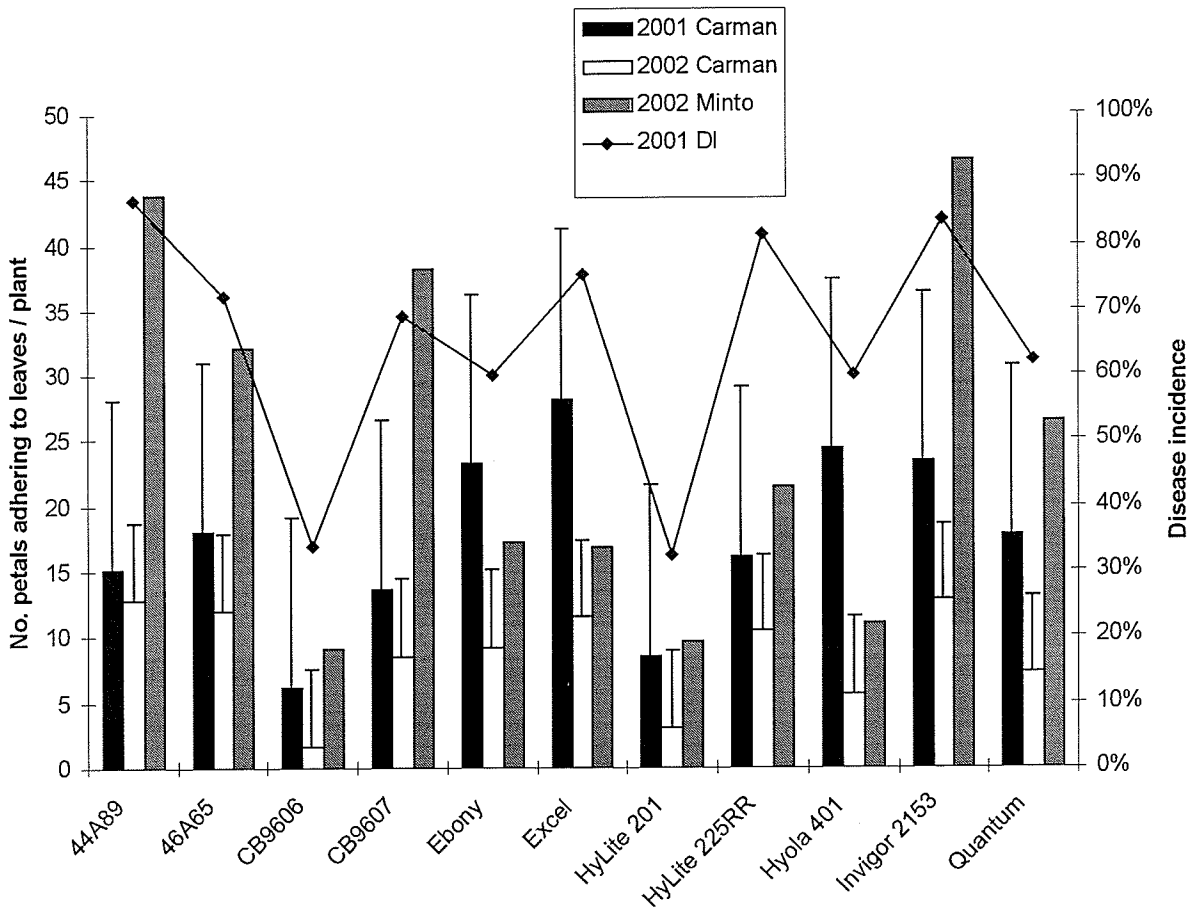

Figure 4.2. Number of petals adhering to leaves of 11 canola cultivars at Carman, MB 2001 and 2002 and in Minto 2002, and the sclerotinia stem rot disease incidence (DI) recorded in Roseau, MN in 2001. Error bars indicate Tukey's HSD at P = 0.05 to compare cultivars at each site. Error bars for 2002 Minto could not be calculated since only two replications of data were available.

period. An estimated 15% of the flowers contained petals although these were reduced in size. Combining the data from all three sites, HyLite 201 had an average of 5.3 petals on leaves/plant, CB9606 3.4, and CB9607 12.6 petals, the lowest number for a petalled variety.

4.4.3 Principle Components Analysis

Principle component analysis on all eleven canola cultivars indicated that the first two principle components explained 60.3% of the sampled variance (Fig. 4.3). The second component was significantly correlated to DI and it accounted for 14.8% of the total variation and was composed primarily of petal and sepal adhesions to leaves and leaf axils, flowering duration, and leaves per plant variables (Table 4.5). A biplot of principle components 1 and 2 (Fig. 4.3) illustrated that the two *B. rapa* cultivars (CB9606 and CB9607) were noticeably different from the *B. napus* cultivars. The apetalous *B. napus* cultivar HyLite 201, also was spatially separated from petalled cultivars. Therefore PCA was also calculated excluding the *B. rapa* cultivars and excluding both HyLite 201 and the *B. rapa* cultivars.

PCA excluding the *B. rapa* cultivars indicated that the first two principle components accounted for only 48.2% of the total variation (Fig. 4.4). The second principle component was significantly correlated to DI and was composed primarily of branch angle, days to flower, and leaf area variables. Both HyLite 201 and Ebony are outliers.

PCA excluding HyLite 201 and the *B. rapa* cultivars indicated that the first two principle components accounting for 50.0% of the total variation. The first principle

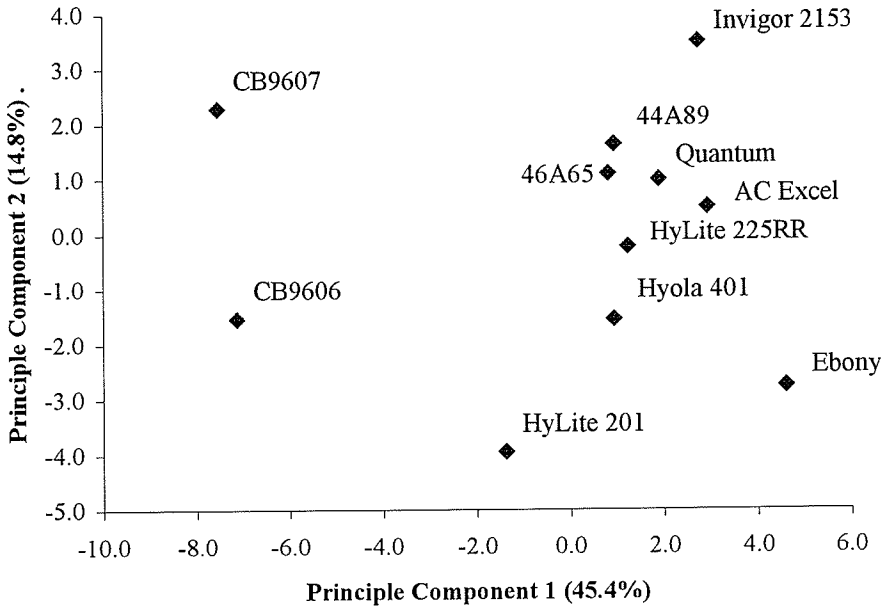

Fig. 4.3. Principle components analysis biplot for all canola cultivars tested in relation to agronomic traits.

Table 4.5. Agronomic traits measured at Carman, MB in 2001 and 2002 and in Minto, MB in 2002 of three groupings of the canola cultivars used in principle component analysis, the contribution of the first two components, and the correlation of the components to sclerotinia stem rot disease incidence in Roseau, MN.

	All Cultivars		*B. napus* Cultivars		Petalled *B. napus* Cultivars	
	Principle Component		Principle Component		Principle Component	
	1	2	1	2	1	2
Eigenvalue	15.45	5.04	8.37	8.01	10.18	6.79
Total Variance (%)	45.4	14.8	24.6	23.6	29.9	20.0
Characteristic Loading						
Days to Flower						
2002 Carman	0.243 *	-0.043	0.249 *	0.213	0.296 *	-0.010
2002 Minto	0.231 *	-0.114	0.097	0.323 *	0.307 *	0.017
Flowering Duration (days)						
2001 Carman	-0.217	0.170	0.029	-0.260	-0.264	-0.097
Maturity rating (1-9)						
2002 Minto	0.228 *	-0.102	0.127	0.191	0.213 *	-0.099
No. petals on leaves						
2001 Carman	0.110	0.270 *	0.037	-0.237	-0.230	-0.113
2002 Carman	0.210 *	0.151	0.263 *	-0.093	0.044	0.244
2002 Minto	0.096	0.389 *	0.208	-0.249	-0.146	0.203
No. petals in leaf axils						
2001 Carman	0.047	0.343 *	0.083	-0.270	-0.241	0.041
Petal distribution on plant						
2001 Carman	-0.130	0.156	0.079	-0.127	-0.063	0.184
2002 Carman	0.140	0.030	0.117	-0.070	0.003	0.247 *
No. sepals on leaves						
2002 Carman	0.224 *	0.028	0.165 *	-0.078	0.010	0.326 *
2002 Minto	0.054	0.330 *	0.137	-0.218	-0.124	0.106
No. leaves/plant						
2001 Carman	-0.085	0.279 *	0.027	-0.170	-0.142	-0.177
2002 Carman	0.181	0.035	-0.033	-0.040	-0.046	0.020
2002 Minto	-0.092	-0.095	-0.215	0.001	-0.092	-0.308
Leaf Area (sq cm)						
2001 Carman	0.234 *	-0.077	0.209	0.214 *	0.275 *	-0.049
2002 Carman	0.175	-0.062	0.080	0.054	0.073	0.024
2002 Minto	0.177 *	0.136	0.196 *	-0.030	0.064	-0.107
Leaf Litter (% ground cover)						
2002 Carman	0.215 *	-0.042	-0.189	0.000	-0.078	-0.117
Stem diameter (mm)						
2001 Carman	0.058	0.000	0.088	0.208	0.193	-0.113
2002 Carman	0.125	0.082	0.106	0.030	0.068	-0.223
Branch angle (degrees)						
2001 Carman	-0.113	-0.257	-0.151	0.296 *	0.196 *	-0.242
2002 Carman	-0.098	-0.189	-0.107	0.156	0.137	-0.021
Distance from ground to first branch (cm)						
2001 Carman	0.196 *	-0.104	0.234 *	0.185	0.247 *	0.180
2002 Carman	0.184	-0.004	0.278 *	0.094	0.199	0.261 *
Lodging resistance (1-9)						
2001 Carman	0.074	-0.192	-0.004	0.184	0.139	-0.218
2002 Minto	-0.003	-0.264	-0.115	0.211	0.140	-0.255
Canopy height (cm)						
2001 Carman	0.245 *	0.050	0.292 *	-0.014	0.144	0.158
2002 Carman	0.234 *	0.044	0.293 *	0.027	0.171	0.154
2002 Minto	0.213 *	0.129	0.305 *	-0.014	0.144	0.145
Canopy Density (% light extinction)						
2001 Carman	0.114	-0.199	0.018	0.129	0.100	-0.054
2002 Carman - 20-Jul	0.195 *	-0.167	-0.087	0.076	0.035	-0.022
2002 Carman - 02-Aug	0.234 *	-0.111	0.257 *	0.210	0.304 *	0.011
2002 Minto	0.187 *	0.025	0.124	-0.215	-0.123	0.299
Correlation to DI						
R	0.460	0.763	0.535	-0.692	-0.686	0.590
P	0.154	0.006	0.137	0.039	0.060	0.124

Note: An asterix indicates the strong partial contribution of the architectural traits to the principle components.

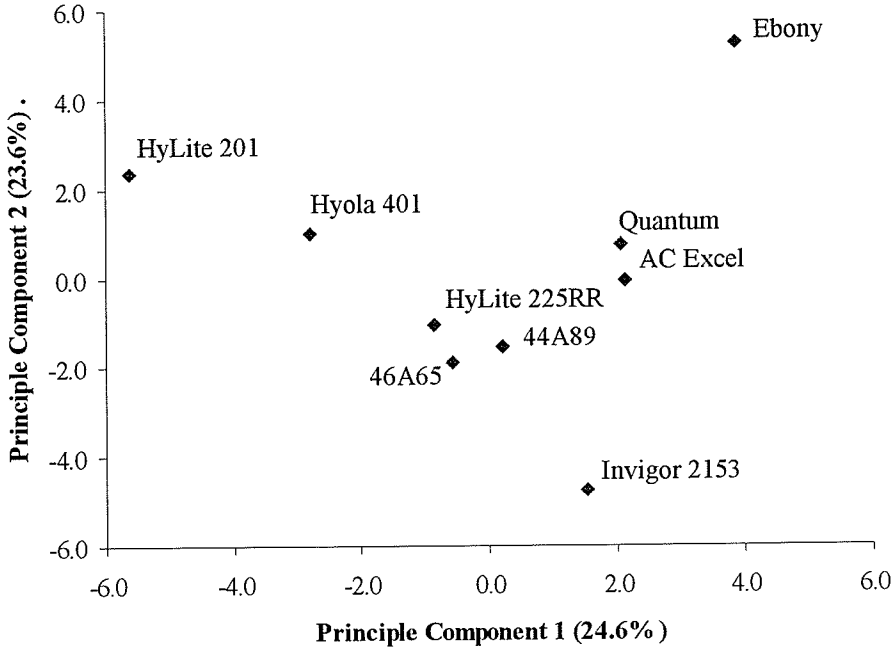

Figure 4.4. Principle component analysis biplot for *B. napus* canola cultivar in relation to agronomic traits.

component was most tightly but not significantly correlated to DI and was composed primarily of days to flower, maturity, leaf area, branch angle, distance from ground to first branch and canopy density variables. A biplot (Fig. 4.5) of the first two principle components now shows that the petalled cultivars are more evenly dispersed than the previous biplots, indicating a more homogeneous data set.

4.4.4 Correlations

Sclerotinia disease incidence was significantly correlated to a number of avoidance traits (Table 4.6). As was done for PCA, the data was compared in three ways: 1) using all 11 cultivars; 2) using only the *B. napus* cultivars; and 3) using only the petalled *B. napus* cultivars. The number of petals adhering to leaves from all three sites were significantly correlated to DI in the first two groups of cultivars (Fig. 4.6 A and B) but not in the third (Fig. 4.6 C). The same relationship existed for petals observed in leaf axils. Branch angle was significantly correlated with DI across all three cultivar groups. Lodging resistance was significantly negatively correlated for all three groupings of cultivars only at the Minto site, but it was significantly correlated to the petalled cultivar grouping at the 2001 Carman site. If the two apetalous cultivars are removed from this relationship, then the correlation between lodging resistance in Carman in 2001 increases from –0.133 to –0.7292 ($P = 0.258$). There was a significant negative correlation between the maturity ratings and DI, but only in the petalled cultivar grouping. The remaining traits did not correlate significantly with DI.

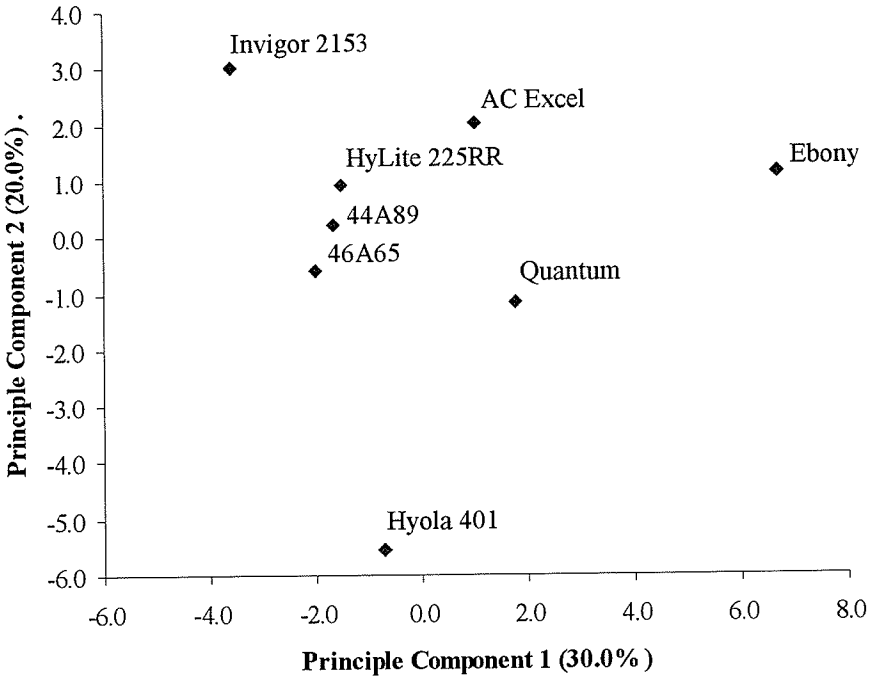

Figure 4.5. Principle component analysis biplot for petalled *B. napus* canola cultivars in relation to agronomic traits.

Table 4.6. Pearson correlation coefficients for agronomic traits with sclerotinia stem rot disease incidence on three groupings of canola cultivars.

Agronomic Trait	All cultivars	*B. napus* cultivars	Petalled *B. napus* cultivars
Days to Flower			
2002 Carman	0.376	-0.012	-0.663 †
2002 Minto	0.235	-0.549	-0.636 †
Flowering Duration (days)			
2001 Carman	-0.071	0.675	0.366
Maturity rating (1-9)			
2002 Minto	0.231	-0.212	-0.822 *
No. petals on leaves			
2001 Carman	0.744 **	0.600 †	0.262
2002 Carman	0.715 *	0.686 *	0.186
2002 Minto	0.806 **	0.748 *	0.546
No. petals in leaf axils			
2001 Carman	0.803 **	0.762 *	0.575
Petal distribution on plant			
2001 Carman	0.520 †	0.454	0.468
2002 Carman	0.483	0.481	0.332
No. sepals on leaves			
2002 Carman	0.060	0.351	0.342
2002 Minto	0.162	0.143	0.285
No. leaves/plant			
2001 Carman	0.280	0.254	-0.164
2002 Carman	0.524 †	0.122	0.077
2002 Minto	-0.427	-0.413	-0.393
Leaf Area (sq cm)			
2001 Carman	0.360	0.049	-0.598
2002 Carman	0.245	0.050	-0.178
2002 Minto	0.425	0.260	-0.469
Leaf Litter (% ground cover)			
2002 Carman	0.359	-0.319	-0.121
Stem diameter (mm)			
2001 Carman	0.238	-0.139	-0.392
2002 Carman	0.237	0.004	-0.644 †
Branch angle (degrees)			
2001 Carman	-0.541 †	-0.759 *	-0.714 *
2002 Carman	-0.767 **	-0.856 **	-0.693 †
Distance from ground to first branch (cm)			
2001 Carman	0.154	-0.005	-0.194
2002 Carman	0.364	0.302	0.049
Lodging resistance (1-9)			
2001 Carman	-0.133	-0.310	-0.768 *
2002 Minto	-0.687 *	-0.805 **	-0.810 *
Canopy height (cm)			
2001 Carman	0.474	0.345	-0.365
2002 Carman	0.398	0.256	-0.364
2002 Minto	0.476	0.334	-0.296
Canopy Density (% light extinction)			
2001 Carman	0.069	0.079	-0.140
2002 Carman - 20-Jul	-0.002	-0.393	-0.360
2002 Carman - 02-Aug	0.205	0.093	-0.579
2002 Minto	0.357	0.644 †	0.682 †

Note: An asterix indicates significance at $P < 0.05$, a double asterix indicates significance at $P < 0.01$, and the † symbol indicates significance at $P < 0.10$.

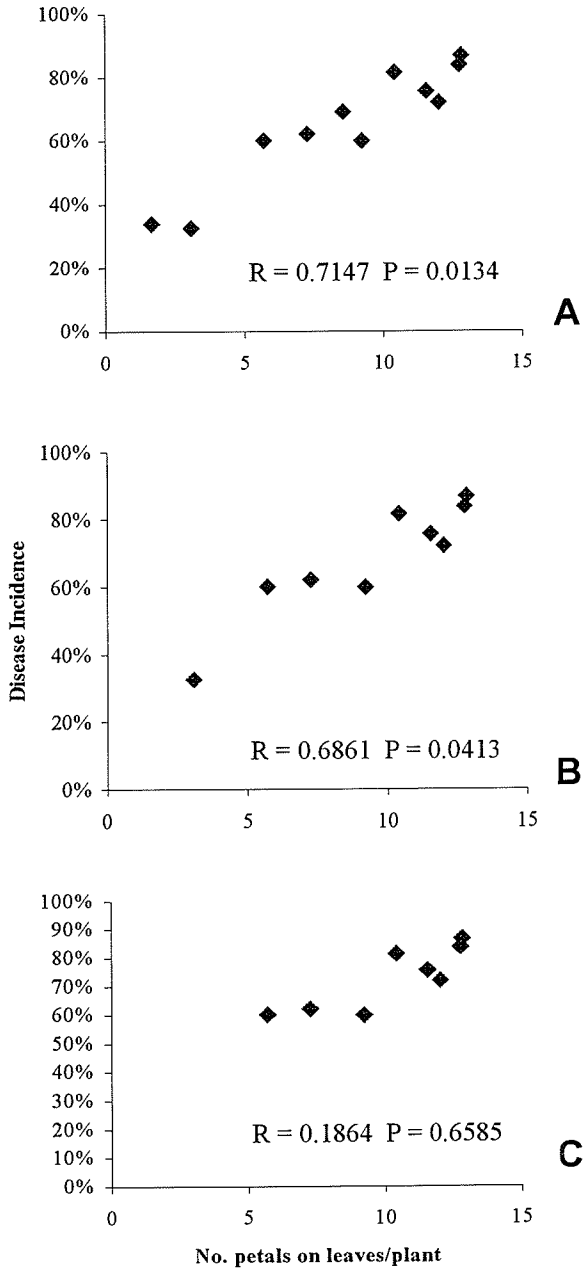

Figure 4.6. The relationship of number of petals adhering to leaves per plant of three groupings of canola cultivars counted in Carman, MB in 2002 with sclerotinia stem rot disease incidence from Roseau, MN in 2001. **A.** all eleven canola cultivars; **B.** only the *B. napus* cultivars; **C.** only the petalled *B. napus* cultivars.

4.5 Discussion

Often many trials need to be planted over a wide geographical area to obtain one site of land with sufficient levels of sclerotinia infection for comparative purposes. Temperatures greater than 27 C will arrest *S. sclerotiorum* growth and development (Phillips 1994). Despite the use of a misting system at the Carman site, infection was low because of high ambient temperatures and low RH values throughout the day. For this reason, it was not possible to collect both sclerotinia stem rot infection and plant avoidance trait data from the same location. Regardless, since the Roseau site had very high levels of infection, and these results match data on infection from previous trials (Jurke *et al.* 1998, unpublished data), the DI data set is likely accurate. Also, the ability to obtain multiple site-years information for many of the agronomic traits allows an understanding of the influence of genotype and environment on the expression of these traits. This understanding is critical for plant breeders to effectively select those traits that aid in sclerotinia avoidance.

Principle components analysis is useful in understanding the dimensionality of the data set and the inter-relationships between the experimental units (cultivars). PCA revealed that there was a high amount of inter-relationship between the agronomic traits. Biplots of the first and second principle components (PC1 and PC2) revealed that *B. rapa* cultivars were clustered separately from the bulk of *B. napus*, cultivars indicating considerable differences in many of the agronomic traits. This observation justified the decision to also run the analyses without the *B. rapa* lines. PCA confirmed that the presence of the apetalous lines masked the importance and contribution of other

avoidance traits. Therefore the additional analysis without HyLite 201 and the *B. rapa* cultivars also was justified.

Petals adhering to leaves and leaf axils were found to be very important to the development of sclerotinia stem rot. This finding was only possible by including apetalous cultivars in this trial. When the apetalous lines were removed from the analysis, the full impact of petals over obscured since all petalled cultivars produced about the same number of petals adhering to leaves. It is only by having apetalous cultivars in this study that the full effect of petals on the infection process could be observed. However, there were some differences between petalled cultivars for this trait, but the trait was largely influenced by environmental effects and, possibly, by other effects which were not measured. These include leaf wax composition and structure, leaf angle, and leaf shape.

The importance of petals in the infection process appears to be dominant over all other agronomic traits. This finding supports the hypothesis that petals are the major nutrient source for germinating ascospores (Kapoor *et al.* 1983; Mc Lean 1958). The hypothesis that there may be variation in petal adhesion in the petalled cultivars was found to be true. But such variation in the petalled cultivars was not correlated to DI and these differences were environmentally influenced. This suggests that if petals are being produced, then there is no value in selecting for petal adhesion to leaves. The value lies in true apetally, and this is a key avoidance trait.

The importance of petals may not be applicable to other *S. sclerotiorum* susceptible crops. Schwartz *et al.* (1978) did not find a relationship between dry bean blossom production and white mold infection, which is what was observed in the petalled

B. napus cultivar, However, this finding may be because apetalous bean lines are not available.

Sepal adhesion to leaves was essentially the same for all canola cultivars. The environmental effect of this trait was high. This was similar to petal adhesion in the petalled lines, i.e., adhesion was influenced more by environmental variables than by genetics. Sepals have a role in the infection process, but a much smaller one than petals. Leaf lesions around infested sepals were observed in other experiments, but less frequently than lesions developing from infested petals.

Lodging resistance is another trait that appears to have a significant impact on sclerotinia stem rot development in canola, which agrees with findings in soybeans (Boland and Hall 1987; Kim and Diers 2000) and beans (Kolkman and Kelly 2002; Schwartz *et al.* 1987). Fuller *et al.* (1984) found that elevating dry bean canopies from a prone position with artificial supports decreased white mold infection significantly. Blad *et al.* (1978) and Kolkman and Kelly (2002) reported that the microclimate of a lodged canopy is more conducive to white mold development and spread of the disease. In this study, lodging resistance was significantly related to DI in all the cultivar groupings and was an important component of PC2 in the *B. napus* group of cultivars. There was neither a strong genotypic effect nor environmental effect on the expression of lodging resistance at both sites where this trait was evaluated, but the G X E was significant indicating a possible problem at one of the sites. Patchy emergence at the Carman site in 2001 resulted in uneven stands within the plots. When the data from these two sites was compiled, a strong cultivar effect was present and the lodging resistance data correlated well with

previous observations on these cultivars. Lodging resistance likely has a strong genotypic influence and can be easily selected by plant breeders.

Branch angle was correlated to DI irrespective of the cultivar grouping. A 'tighter' or more acute branch angle was related to an increase in DI. This relationship appears logical since an acute branch angle leads to a more compact plant canopy and can retain more plant debris in the branch joint, which can lead to increased opportunities for infection. These evaluations were highly variable, and had no significant genetic or environmental effect, which would make selection of this trait a difficult one. Park (1993) reported that more "porous" bean canopies had less white mold infection. Likely a wider branch angle does create a porous canopy.

Canopy density had no relationship to DI. This result was not surprising since canopy density is strongly influenced by environmental conditions. Canopy density is difficult to evaluate and variability in results was high. There was very little correlation among density assessments among sites and even between evaluations done weeks apart at the same site. Turkington and Morrall (1993) had similar difficulty in finding a relationship between canopy density and sclerotinia stem rot incidence in canola. They found that DI was inconsistently related to their measures of canopy density. Turkington (1991) summarised that canopy density is the result of fertility status, meteorological conditions, seeding rate and cultivar effects. Since this study is interested in identifying traits with moderate to high heritability, selections based on canopy density evaluations would not be effective for escaping sclerotinia stem rot infection.

Canopy height has been found to be an avoidance trait in beans (Park 1993). But this trait was not correlated to DI in this study. Neither canopy height nor branch height

evaluations were correlated with DI, and both of these traits had strong weighting in the PC variables that did not correlate to DI. For this reason it is reasonable to deduce that these traits have very little impact on the ability of a cultivar to escape sclerotinia stem rot.

Measurements of leaf area and the number of leaves were expected to have a relationship to DI since these affect the surface area for petal adhesion, which in turn affects the number of possible infection sites. For example, leaf removal was suggested to be an important method of reducing sclerotinia blight in peanuts (Butzler *et al.* 1998). This was not the case in canola. Leaf area is another trait strongly influenced by environmental conditions. Measurement of leaf litter, an indirect phenotypic trait, was significantly related to number of leaves at the same site and was highly variable. The hypothesis that leaf litter may influence below-canopy inoculum levels was rejected, since there was no relationship between leaf litter and DI.

The phenological traits, days to flower, flowering duration and maturity, were not evaluated consistently across all sites, and therefore only limited comments can be made on the genetic and environmental influences on these traits. None of these traits were significantly correlated to DI except maturity in the petalled *B. napus* cultivar grouping. Phenological traits such as days to flower are normally highly heritable, so cultivar maturity should have an impact on DI (Thurling and Kaveeta 1992). For example, the early maturing cultivar 44A89 had the highest DI score and the lowest reduction in DI following fungicide treatment. This result is likely due to timing; 44A89 happened to be in full flower when conditions were ideal for infection. This also illustrates the problem with using one site for our DI results: it is only one site and timing at this site is very

important. Studies in other crops have reported significant relationships between DI and crop phenology (Boland and Hall 1987; Deshpande 1992), but Kim *et al.* (1999) found no such relationship. Phenology had a variable impact on white mold of beans and was dependent on the environment (Kolkman and Kelly 2002). These phenological traits are not likely to be used successful in reducing sclerotinia stem rot outbreaks in canola. The timing of flowering cannot be adjusted to avoid prime conditions for sclerotinia development, unless one can predict the weather accurately. Modifying planting date could have a greater chance of success. Kolkman and Kelly (2002) found a similar result - that days to flower and maturity effects of dry bean varied across environments and populations.

The objective of this study was to identify heritable traits in canola that influence sclerotinia stem rot development. Kolkman and Kelly (2002) completed a similar study in dry bean and found that the ideal dry bean cultivars that avoid sclerotinia stem rot should possess some physiological resistance, be indeterminate, have a Type II growth habit, be resistant to lodging, have a medium canopy width and branching pattern, and have a medium height. For canola, the list may be smaller. Petal production and ultimately adhesion to leaves is the most significant factor relating to sclerotinia stem rot, but selection for this trait in petalled cultivars may not be effective. Use of apetalous lines significantly reduces, but does not eliminate disease incidence. Lodging resistance and branch angle are two additional avoidance traits which plant breeders may be able to use successfully for sclerotinia stem rot avoidance.

4.6 Acknowledgements.

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5.0 COMPARISON OF GROWTHROOM SCREENING TECHNIQUES FOR THE DETERMINATION OF PHYSIOLOGICAL RESISTANCE TO SCLEROTINIA STEM ROT IN *BRASSICA NAPUS*.

5.1 Abstract

Screening *Brassica napus* L. lines for resistance to sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary in the field is usually unreliable. Six growthroom screening methodologies were evaluated and compared to accurately, reliably, and efficiently measure 10 *B. napus* lines for resistance to sclerotinia stem rot. These lines were planted in 2001 and 2002 in a nursery infested with *S. sclerotiorum* sclerotia and evaluated for disease incidence and tested in four growthroom experiments. The growthroom methodologies consisted of: 1) leaves of the *B. napus* lines inoculated with fragments of *S. sclerotiorum* mycelium suspended in a potato dextrose broth (Myspray technique); 2) *B. napus* petals bearing *S. sclerotiorum* mycelium (Mypetal technique); 3) *B. napus* petals bearing *S. sclerotiorum* ascospores (Ascpetal technique); 4) leaf petioles dipped in a solution of oxalic acid (Oxalate technique); and 5,6) both leaves and stems inoculated with agar plugs with *S. sclerotiorum* mycelium (Plug Leaf and Plug Stem techniques). Infection was low and highly variable in the field trials with CV's of 205.9% and 133.6%, respectively, and with low correlation between them ($R = 0.6164$, $P = 0.1405$). The Plug Leaf technique had low correlations among growthroom trials and resulted in inconsistencies among cultivar rankings between the trials. The Plug Stem technique had high levels of variation and was not considered to be accurate. The Ascpetal technique, although regarded as more accurate, was found to be highly variable

and was cumbersome to use. The Myspray technique had the greatest variability of all the techniques with a CV of 57.3% and low correlation between the growthroom trials. Myspray technique was significantly correlated with field data ($R = 0.9150$, $p = 0.0039$) and with the Ascpetal technique ($R = 0.7945$, $p = 0.0060$). The Oxalate test was the most efficient of all techniques but did not correlate with any other technique. The Mypetal technique was chosen to best assess physiological resistance since it had the least variation in its results and cultivar ranks between growthroom trials. It was accurate in determining resistant lines observed in the field, and it was efficient to employ.

5.2 Introduction

Sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary) is a difficult disease to manage in canola (*Brassica napus* L. and *Brassica rapa* L.). Fungicides are the only effective method of control available for producers, but these are costly and can have environmental concerns. The ideal control strategy is host resistance, but as *Sclerotinia sclerotiorum* infects more than 400 plant species (Boland and Hall 1994), finding and incorporating genetic resistance by conventional breeding practices has proved to be challenging. Field resistance to sclerotinia stem rot is the result of physiological resistance and escape mechanisms. Escape mechanisms in canola include lodging resistance, short open canopies, apetally (lack of flower petals), and short flowering duration (Jurke and Fernando 2002). Many of these escape mechanisms are polygenic and difficult to breed into elite germplasm. The use of a monogenic source of strong resistance has an appeal to plant breeders selecting for resistance for sclerotinia stem rot.

Transgenic sources of resistance for sclerotinia stem rot are available. Transgenes encoding for enzymes such as oxalate oxidase have been found to have some efficacy in canola, soybeans, and sunflowers (Billings *et al.* 2003; Donaldson *et al.* 2001; Lefol 1998; Thompson *et al.* 1995). To successfully determine whether a source of resistance is adequate at controlling sclerotinia stem rot infection, an effective screening methodology must be available.

Ideally, an indoor screening technique, which correlates well with field-level performance, is the most desirable method for screening for *S. sclerotiorum* resistance. However, disease nurseries in the field have their limitations. The development of sclerotinia stem rot influenced strongly by environmental conditions, which are not

always conducive for disease development. Furthermore, since field-level resistance in many species is influenced by the growth-habit of the crop, which includes avoidance and escape mechanisms, these factors can hamper the measurement of the physiological resistance. This can be better accomplished with a growthroom inoculation technique.

Researchers working in different crops have used a number of screening techniques for the determination of resistance to *S. sclerotiorum* (Table 5.1). These techniques have tested various plant organs and used many different nutrient substrates, depending on the goals of the experiment. Some researchers have conducted studies comparing these inoculation methodologies to assess which might be most appropriate for their respective crop. Wegulo *et al.* (1998) compared five techniques for the determination of resistance in soybean and found that the use of soluble pigment determination and oxalic acid assays were more reliable methodologies than using mycelial disks to identify resistant lines. Kim *et al.* (2000) also compared inoculation techniques in soybeans, but found that mycelial disks could be used reliably to obtain preliminary information on white mold resistance. Fang (1993) examined four inoculation techniques in *B. napus* and found that the foliar applied mycelial disk method produced the most uniform results for the determination of sclerotinia stem rot resistance.

Our objective was to evaluate six growthroom *S. sclerotiorum* inoculation techniques on canola for their efficacy in measuring physiological resistance. Accuracy, reliability, and efficiency were the determinants used for selecting the best technique(s).

Table 5.1. Inoculum sources, target tissue, and references for growthroom techniques for resistance screening to *S. sclerotiorum* as described in the scientific literature.

Fungal structure/toxin	Nutrient substrate	Target tissue	Host crop	Author(s)
Mycelium	*PDA[a] plugs*	*Leaf*	*B. napus*	Fang (1993), Mullins *et al.* (1995), Zhao and Meng (2003)
			sunflower	Mestries *et al.* (1998)
			soybean	Kim *et al.* (2000) and Wegulo *et al.* (1998)
Mycelium	*Autoclaved carrot pieces*	*Leaf*	G. max	Cline and Jacobsen (1983)
Mycelium	*PDA plugs*	Stem	*B. napus*	Brun *et al.* (1987) and Buchwaldt *et al.* (2003)
			cabbage	Dickson and Petzold (1996)
			soybean	Arahana *et al.* (2001b) and Vuong and Hartman (2003)
			sunflower	Kohler and Friedt (1999)
Mycelium	Autoclaved barley or oat grains	Stem	*B. napus*	Scott (1984) and Thomson and Kondra (1983)
			soybean	Cober *et al.* (2003) and Kim *et al.* (2000)
Mycelium LTI[b]	Autoclaved celery stick pieces	Stem	bean	Hunter *et al.* (1981)
	Autoclaved celery sticks or green bean pods	Stem	soybean	Boland and Hall (1986) and Cline and Jacobsen (1983)
Mycelium	Autoclaved carrot pieces	Stem	soybean	Wegulo *et al.* (1998)
Mycelium	Autoclaved matchsticks	Stem	*B. napus*	Brun *et al.* (1987)
Mycelium	Blended carrot and water	Whole plant	soybean	Wegulo *et al.* (1998)
Crumbled sclerotia	None	Stem base	*B. napus*	Frencel *et al.* (1987)
Ascospores	Petals	Leaf	*B. napus*	Brun *et al.* (1987) and Lefol and Seguin-Swartz (1998)
		Leaf axils	bean	Hunter *et al.* (1981)
Ascospores	None	Leaf	*B. napus*	Thomson and Kondra (1983)
			soybean	Cline and Jacobsen (1983)
Oxalate		Whole plant	soybean	Kolkman and Kelly (2000)

[a]Potato Dextrose Agar.
[b]Limited Term Inoculation

5.3 Materials and methods

5.3.1 Trial Design

Several trials were undertaken to evaluate *S. sclerotiorum* inoculation techniques on up to 10 *B. napus* lines (Table 5.2). This included two field experiments conducted in an irrigated sclerotinia nursery in Carman in 2001 and 2002 and four indoor growth-room trials (GR trials) to evaluate the six inoculation techniques.

The *S. sclerotiorum* isolate FR1 was used in all cases. It was isolated from a soybean crop in Homewood, MB in 1998 and maintained on potato dextrose agar (PDA). This isolate was chosen for its aggressiveness, its ease of culturing, and its consistency of infection. A number of preliminary trials were done to develop much of the technical aspects of each inoculation technique. Preliminary trials on age of inoculum, isolate, inoculum concentration, inoculum storage, plant age, and environmental conditions for infection were all completed before the test techniques were compared.

Three *B. napus* plants were seeded per pot - this represented the experimental unit for all growthroom tests. The growthroom diurnal cycle was 22 C for 16 h days and 18 C for 8 h nights. Leaves were the target tissues for five of the test techniques (Table 5.3). For these techniques, the plants were inoculated at the pre bolting stage [growth stages 2.6 to 2.8 (Harper and Berkenkamp 1975)] when leaf size was at a maximum prior to flowering. Three to seven leaves were inoculated per plant. Lesion size was determined by measuring the lesion diameter with a set of callipers. If the lesion was not circular, the mean of the length and width of the lesion was used.

Table 5.2. List of sclerotinia stem rot inoculation techniques used in comparative trials and notes on trial performance.

		Growthroom inoculation techniques[a]									
Trial name	Field	Plug Leaf	Plug Stem	My-petal	My-spray	Asc-petal	Ox-alate	No. replic-ations	CV (%)	No. *B. napus* lines	Note[c]
2001 Field	X							3	205.9	7	no Oxox lines and no ZhongYou 821
2002 Field	X							5	133.6	10	
GR Trial[b] A		X		X	X	X		2	35.0	9	no ZhongYou 821
GR Trial B		X		X	X			3	81.7	9	no ZhongYou 821
GR Trial C		X		X	X	X		3	23.0	10	
GR Trail D			X	X			X	3	31.4	10	

Note: An 'X' denotes that this techniques was tested in the trial.

[a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid.

[b] Growthroom trial.

[c] Note explains what *B. napus* lines were not included in each experiment

Table 5.3. Inoculation techniques, life stage of *S. sclerotiorum*, nutrient substrate used, host target tissue, and reference source of technique used in evaluation trials to determine physiological resistance in *B. napus* to sclerotinia stem rot.

Technique	Fungal structure	Nutrient substrate	Target tissue	Reference
Plug Leaf	mycelium	PDA[a] plug	Leaf	Fang (1993)
Mypetal	mycelium	*B. napus* petal	Leaf	
Myspray	mycelium	PDB[b]	Leaf	Boland and Hall (1986)
Ascpetal	ascospores	*B. napus* petal	Leaf	Lefol and Seguin-Swartz (1998)
Oxalate	none	none	Leaf	Kolkman and Kelly (2000)
Plug Stem	mycelium	PDA plug	Stem	Buchwaldt *et al.* (2003)

[a]Potato Dextrose Agar.
[b]Potato Dextrose Broth.

A humidity chamber was constructed to ensure consistent high humidity for the leaf inoculation techniques. Two residential ultrasonic cool mist humidifiers were attached on top of the chamber, continually delivering mist evenly and heavily to all plants.

5.3.2 B. napus Lines

Brassica napus cultivars and lines were chosen on the basis of potential susceptibility and resistance to sclerotinia stem rot (Table 5.4). Six of these lines were transformed with genes believed to confer some level of physiological resistance to *S. sclerotiorum* infection. The parent canola cultivar, Drakkar, was transformed using an Agrobacterium-mediated technique to produce these transgenic events. The specific transgenic lines used in this study were selected from a screening program completed prior to this experiment, which identified potential physiological resistance. Zhongyou 821 is a Chinese rapeseed cultivar reported to have moderate levels of sclerotinia stem rot resistance (Buchwaldt *et al.* 2003; Li *et al.* 1999c). HyLite 201 is an apetalous canola cultivar that has been shown to effectively escape sclerotinia stem rot infection (Jurke *et al.* 1998; Jurke and Fernando 2002). HyLite 225RR is a canola cultivar with no physiological resistance or escape mechanisms to sclerotinia stem rot.

Table 5.4. *Brassica napus* lines used to evaluate sclerotinia stem rot inoculation techniques and notes on their resistance.

B. napus line	Level of resistance[a]	Resistance gene	Source of resistance
Drakkar	none	none	
HyLite 225RR	none	none	
HyLite 201	very good	apetally	*B. napus*
678-22	minor	Rs-AFP2 (Anti-fungal protein)*	*Raphanus sativus*
479-84	minor	Ox-ox (Oxalate-oxidase)*	*Triticum aestivum*
783-182	minor	Chi/glu (Chitinase and β-1,3-glucanase)*	*Nicotiana tabacum*
375-144	minor	Ox-dec (Oxalate decarboxylase)*	*Collybia velutipes*
375-112	moderate	Ox-dec*	*C. velutipes*
375-53	good	Ox-dec*	*C. velutipes*
Zhong You 821	good	unknown	

[a]Level of resistance determined from previous field and growthroom screening trials.
*Transgenic genes

5.3.3 Mycelium-Infested Agar Plug Technique (Plug Leaf)

Sclerotinia sclerotiorum mycelium was grown on PDA (Becton Dickson Co., Sparks, MD, USA) plates. Mycelium-bearing agar plugs, 6 mm in diameter, were cut with a cork borer. Since the age of the culture was found to be important, plugs were cut only within one cm of the advancing margin of a new culture (about 2 days old). If the mycelium was allowed to reach the margins of the plate (older cultures), its infection ability was found to decline substantially. The plugs were then inverted, mycelium side down, onto a large canola leaf and pressed with the fingers to keep it in place. If these plugs were not pressed onto the leaf they often would roll or slide off the leaf before leaf infection would occur. Plants were inoculated at the pre-bolting stage [growth stages 2.6 to 2.8 (Harper and Berkenkamp 1975)] prior to flowering when leaf size was at a maximum. Three to seven leaves were inoculated per plant. Infected plants were placed in the humidity chamber for 48 to 72 h. Severity was rated on the diameter of the lesion upon removal from the chamber. A mean score of all lesions was calculated for each pot.

5.3.4 Stem Inoculation Technique (Plug Stem)

This technique was modified from Buchwaldt *et al.* (2003). Four mm diameter plugs from *S. sclerotiorum* mycelium growing on PDA were placed mycelium side down against canola stems and gently wrapped with parafilm to prevent desiccation. Plants were not placed in the humidity chamber. Plants were at the bolting to early flower stage and were inoculated up the length of the stem every 10 cm. Seven days after inoculation, the parafilm was removed and lesion length was measured. A qualitative description of

the lesion also was assessed at the same time: C for collapsed lesion, S for soft lesion, F for a firm lesion, and B for a firm lesion with a black outline.

5.3.5 Mycelium-Infested Petal Technique (Mypetal)

A potato dextrose agar plug containing mycelium of *S. sclerotiorum* was transferred to potato dextrose broth (PDB, Sigma-Aldrich, St. Louis, MO, USA), and allowed to grow for one week at room temperature with 16 h days and 8 h nights (when the broth appeared to be thick with mycelium). The mycelium was strained from the PDB, weighed and added to sterile distilled water at a concentration of 0.15g of mycelium per ml of water. It was then macerated using a hand-held blender/food processor. Canola petals of a similar age, which were previously collected and stored at –15 C, were submerged in this slurry for 10 minutes. A single petal, infested with *S. sclerotiorum* mycelium, was placed on a leaf, and minimum of three leaves per plant were infected in this way. The inoculated plants were placed in the humidity chamber for at least 72 to 96 h when lesions were large enough to differentiate between *B. napus* lines. Infection was evaluated by measuring the diameter of the lesion. A mean score of all lesions was calculated for each pot. Figure 5.1 illustrates a typical lesion resulting from this technique.

5.3.6 Macerated Mycelium Spray Technique (Myspray)

Sclerotinia sclerotiorum mycelium was grown in PDB for about one week at room temperature with 16 h days and 8 h nights. The solution of mycelium and PDB was macerated using a hand-held blender and diluted to a concentration of 0.15g mycelium per ml PDB. The mycelial solution was then sprayed on to the plants using a hand-held

Figure 5.1. Lesion produced by the Mypetal inoculation technique on a *B. napus* leaf in the growthroom.

misting bottle until the leaves were wet with the solution. An effort was made to ensure that all leaves of the plants were evenly covered. The inoculated plants were then placed in the humidity chamber for five to six days. Disease severity was scored in two ways: 1) percent leaf area covered with lesions; and 2) diameter of the largest lesion on a single leaf. A mean score for each scoring method was calculated for each pot. Figure 5.2 illustrates the types of lesions resulting from this spray technique.

5.3.7 Ascospore-Infested Petal Technique (Ascpetal)

Ascospores were collected from sclerotia using a modified protocol described by Lefol (1998). Sclerotia harvested from PDA plates were placed on moist sand and incubated in 24 h darkness at 15 C for 1 to 2 months. When stipes were produced, the sclerotia were transferred to a 1% agar medium with 16 h of daylight at room temperature. Apothecia were soon formed, and ascospores were collected with a vacuum filter (Model MF 75, Nalgene, Rochester, NY, USA). Ascospores were collected on filter paper with a pore size of 0.2μm and stored at –15 C. Canola petals, which were previously detached and stored at –15 C, were dipped for 10 minutes in a solution of ascospores ($8.0X10^5$ spores/ml sterile distilled water). Single infested petals were placed on a minimum of three leaves per plant, and the plants kept in the humidity chamber for five to six days. Disease severity was rated by the diameter of the lesion. A mean lesion diameter was calculated for each pot.

Figure 5.2. Lesions produced by the Myspray inoculation technique on a *B. napus* leaf in the growthroom.

5.3.8 Oxalic Acid Technique (Oxalate)

Inoculation of excised canola leaves was done in a large tub containing 4 litres of a 20mMol oxalic acid solution. Two leaves, with a minimum leaf blade length of 10 cm, were detached at the base of the petiole, labelled, and suspended above this solution on inverted Petri dishes with the petioles submerged. Progression of wilt symptoms up the petiole and along the main vein of the leaf was measured 24 h following immersion.

5.3.9 Plant Age

Two experiments were conducted examining the effect of plant age on growthroom inoculation techniques. The first experiment was a split-split plot design used the Mypetal and the Myspray techniques as main plots, the 6 leaf and flowering developmental stages as sub plots, and six *B. napus* lines as sub-sub plots. The second experiment was a split plot design. The Mypetal technique was used to inoculate the four main plot 4 growth stages (cotyledon, 3 leaf, 6 leaf, and flowering) of the six *B. napus* sub plots.

5.3.10 Field Trials

For comparative purposes, field trials with the same lines were conducted in 2001 and 2002 at Carman, MB. The transgenic lines used in this experiment restricted experimentation to only one location per year. A randomised complete block design with three replicates of single row plots 3 m in length was used. Trials were seeded in late May into soil that had 100 sclerotia/m^2 incorporated into the soil. Tents were erected over the site and a misting system was installed to ensure high humidity. Disease assessments

were made shortly before swathing [Harper and Berkenkamp (1975) growth stage 5.3]. The total number of infected and non-infected plants were counted. A plant was considered to be infected if it showed any level of infection, whether it was a basal stalk infection or an upper canopy infection. The percentage of infected plants (disease incidence, DI) was calculated for each plot.

5.3.11 Data Analysis

Statistical analysis was carried out using Agrobase 20 (Agronomix Inc. Winnipeg, MB) and Statistical Analysis System 8.2 (SAS Institute Inc. Cary, NC) at $P = 0.05$. Analysis of variance was performed using a general linear model. Tukey's HSD (Honestly Significantly Different) test was used to compare treatment means. Mean values for each *B. napus* line for each inoculation technique were calculated by averaging all of the individual replications from the six experiments involved in this study. Additionally, the values of each *B. napus* line were normalised to the untransformed parent line, Drakkar for each replication. These normalised values were averaged from all six experiments. The means and normalised values were used in correlation analysis and rank correlation analysis. Pearson correlation coefficients among inoculation techniques were calculated on the mean values and the normalised mean values of the *B. napus* lines. Spearman's coefficients of rank correlation were used to measure the relationship among the ranks of cultivars among inoculation techniques.

Choice of the most effective inoculation technique was based on accuracy, reliability, and efficiency criteria. Accuracy was defined as the ability of an inoculation technique to correctly identify physiological resistance in a *B. napus* line to *S. sclerotiorum*. It was examined in two ways: 1) correlation of inoculation technique with

field trial data; and 2) a qualitative assessment of the representation of an inoculation technique to the natural infection processes. Reliability was defined as the repeatability of an inoculation technique to consistently identify resistance or susceptibility of a *B. napus* line. This criterion was quantified by an examination of the correlation of results and ranks between growthroom trials, the variance of each technique, and frequency of failed inoculations. Efficiency was determined by the amount of plant material needed, the basis of length of time to obtain results, and the simplicity in the application the inoculation technique. For each criterion, quantification by rank of these techniques was used to determine the most effective inoculation technique.

5.4 Results

5.4.1 Field trials

Infection levels were low in the field trials in both 2001 and 2002. Sclerotinia stem rot symptoms were present but only occurred in random patches. In both years, the flowering period coincided with three weeks of maximum temperatures averaging 30 C and low relative humidity. As a result, the coefficient of variation (CV) for the 2001 and 2002 trials were 206% and 134% respectively (Table 5.2) and the mean DI of the check line, Drakkar, was only 5.2% and 8.8% respectively (Table 5.5). Combining and analysing the two field trials together increased the CV to 155% and decreased Drakkar's mean DI to 7.4%. Given the high variability in these trials and the overall low infection rates, their reliability and usefulness is questioned.

Table 5.5. Sclerotinia stem rot infection evaluations in *B. napus* lines from field trials at Carman, Manitoba and growthroom inoculation trials.

	Field Trials				Growthroom inoculation techniques[a]											
	2001		2002		Plug Leaf		Plug Stem		Mypetal		Myspray		Ascpetal		Oxalate	
B. napus Lines	DI[b]	Rank	DI	Rank	Lesion Size[c]	Rank	Lesion Size	Rank	Lesion Size	Rank	Lesion Size	Rank	Lesion Size	Rank	Wilt Length	Rank
Drakkar	5.2%	6	8.8%	8	37.3	10	19.0	3	21.2	10	14.6	8	18.6	3	76.9	2
HyLite 225 RR	7.3%	7	15.6%	9	27.2	5	31.8	10	15.1	4	15.6	10	25.3	10	87.8	9
HyLite 201	2.1%	5	0.0%	1	29.1	4	31.6	9	19.8	8	11.3	5	19.3	6	76.1	4
678-22	3.4%	4	0.0%	1	36.2	9	31.4	8	20.3	9	9.6	4	19.8	8	93.3	8
479-84			5.9%	5	32.7	7	20.8	4	14.4	3	13.3	6	20.5	9	75.6	3
783-182			2.0%	4	24.0	3	30.2	7	13.8	2	12.5	7	19.4	7	60.4	1
375-144	2.1%	3	11.6%	10	31.5	8	26.9	6	15.2	5	8.9	2	18.3	2	91.9	10
375-112	0.0%	2	6.3%	6	22.8	2	22.8	5	18.5	7	8.7	3	18.8	4	92.2	6
375-53	0.0%	1	0.0%	1	22.2	1	16.4	2	10.6	1	5.7	1	9.0	1	89.8	7
Zhong You 821			6.4%	7	31.1	6	11.7	1	16.2	6	11.0	9	18.9	5	87.2	5
No. replications	4		6		8		3		11		8		4		3	
Mean	2.9%		6%		29.4		24.3		16.5		11.1		18.8		83.1	
HSD[d]	5.1%		27.8%		15.08		21.59		10.00		12.86		27.52		74.07	

[a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid.
[b]Disease Incidence (percentage of infected plants).
[c]Lesion size and wilt length measured in millimetres.
[d]Tukey's Honestly Significantly Different test

5.4.2 Plug Leaf technique

The Plug Leaf technique produced the largest lesions in the shortest amount of time. Small lesions were already apparent 36 h after inoculation and lesions were large enough for rating by 72 h. Significant differences in lesion size were found between *B. napus* lines (Table 5.5). There was no significant growthroom trial effect, nor was there a significant line by trial interaction. The correlations of line performance between trials were low (R = 0.134 to 0.563). The ranks of the lines varied between GR trials, but lines 375-53 and 375-112 had consistently less disease than the other lines, and these two lines were only marginally different from one another.

5.4.3 Plug Stem technique

The appearance of lesions occurred after 4 days after inoculation as incipient brown necrosis. After seven days, the lesions had progressed up and down the stems from the inoculation point. There were no significant differences among the lines, nor was there a replication or interaction effect with this technique (Table 5.5). Ranks from this technique did not reflect the ranks from the previous techniques, although line 375-53 had the second smallest amount of lesion development. Zhongyou 821 had the smallest lesions and ranked first, which is contrary to its ranking with all other inoculation techniques.

5.4.4 Mypetal technique

Lesions formed more slowly on *B. napus* lines inoculated with the Mypetal (Figure 5.1) than the Plug Leaf technique. Small lesions were observed 48 h after inoculation and by 96 h these were large enough for rating. Significant differences in lesion size were found between *B. napus* lines (Table 5.5). There was no significant growthroom trial effect, nor was there a significant line by trial interaction. Correlations of line performance between trials were high for most trials (Table 5.6), indicating the repeatability of results was good with this technique. Like the Plug Leaf method, ranks of lines varied from one GR trial to another. Only line 375-53 was consistent in its ranking having the smallest lesion size using this technique.

5.4.5 Myspray technique

Lesions on plants inoculated with this mycelial mist appeared as small black speckles 96 h after inoculation. These spot-like lesions often covered the margins of the leaf and would grow into larger distinguishable lesions after 5 to 6 days in the humidity chamber (Figure 5.2). Significant differences between lines were not demonstrated using this technique (Table 5.5). Correlations of lines between trials was low (R = 0.248 to 0.546). Line ranks between trials varied. Line 375-53 had the smallest lesions in two of three trials but was 6^{th} in the third, and line 375-112 ranked 2^{nd} in two trials and was 4^{th} in the third. There were significant GR trial and replication effects using this technique (data not shown).

Table 5.6. Matrix of Pearson correlation coefficients for sclerotinia stem rot disease evaluations for the Mypetal inoculation technique between the growthroom trials.

Growthroom Trial	GR Trial A	GR Trial B	GR Trial C
GR Trial B	0.5006		
GR Trial C	0.7815 *	0.6453 †	
GR Trial D	0.4224	0.7451 †	0.6102

Note: An asterix denotes significance at P<0.05 and the † symbol indicates significance at P <0.10.

5.4.6 Ascpetal technique

Lesions first appeared 96 h after inoculation and were large enough to rate by 5 to 6 days. There were no significant differences in lesion size among lines, nor were there any significant trial, replication, or interaction effects (Table 5.5). Correlation of line performance between the two GR trials was low (R = -0.01794). Ranks between the two trials varied significantly. Line 375-53 had the lowest lesion size in GR trial C and ranked 3rd in GR trial A. HyLite 201 ranked first in GR trial A but consistently ranked low with all other inoculation techniques.

5.4.7 Oxalate technique

The appearance of wilt symptoms progressing up the petiole was visually different from the lesions of the other indoor techniques used. The wilting progressed with time from the base of the severed petiole, and after 24 h was evident along the veins in the leaf blade. There were no significant differences between lines using this technique, even when employing a less conservative measurement of difference such as Fisher's LSD (Table 5.5). There was a significant replication effect. The ranks obtained with this technique did not reflect the trends observed with the previous techniques; line 375-53 ranked 7th.

5.4.8 Comparison of inoculation techniques

To compare inoculation techniques, mean scores across all growthroom trials were tabulated (Table 5.5) and Pearson's correlation coefficients (R) were calculated (Table 5.7). Significant relationships were found between the Myspray and the Ascpetal

Table 5.7. Matrix of Pearson correlation coefficients of sclerotinia stem rot disease evaluations for various inoculation techniques from the growthroom and the field trials at Carman, MB.

Inoculation technique	2001 field	2002 field	Plug Leaf[a]	Plug Stem	Mypetal	Myspray	Ascpetal
2002 field	0.6164						
Plug Leaf	0.5287	0.1477					
Plug Stem	0.4113	-0.0073	-0.0056				
Mypetal	0.2666	-0.0204	0.6077†	0.2448			
Myspray	0.9150**	0.5147	0.4002	0.2371	0.2938		
Ascpetal	0.7384†	0.5594†	0.3332	0.5037	0.4033	0.7945**	
Oxalate	-0.2986	0.2244	0.0543	-0.1693	0.0739	-0.4996	-0.1482

Note: An asterix indicates significance at $P < 0.05$, a double asterix indicates significance at $P < 0.01$, and the † symbol indicates significance at $P < 0.10$.

[a]'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems.

techniques and between Myspray and the 2001 field trial. Spearman's rank correlation analysis found only a significant relationship between the Myspray technique and the 2002 field trial. Using the data set normalised to the untransformed parent line, Drakkar, the correlations remained the same. Correlations were also tabulated without the data from the apetalous cultivar, HyLite 201, and the relationships remained the same. Of the leaf inoculation techniques, the Plug Leaf technique consistently produced the largest lesions and had the least number of misses (Table 5.8) across all GR trials. Although, the Mypetal technique had a greater overall CV in lesion size and had a greater frequency of misses than the Plug Leaf technique, its repeatability between GR trials was the highest for all growthroom techniques. The Oxalate technique was the quickest to perform and required the fewest number of plants.

Quantification of the accuracy, reliability, and efficiency of each technique was done (Table 5.9). The techniques were ranked according to these criteria and summarised. The Plug Leaf and Mypetal techniques had the lowest i.e., best scores.

5.4.9 Plant age

An experiment examining the effect that plant age might have on these growthroom techniques was carried out using the Mypetal technique. Plant age was found to have little effect with the inoculation technique (Table 5.10). Lesion size increased significantly with plant age. The *B. napus* line rankings were similar in the cotyledon, 6 leaf, and flowering plant stages but were significantly different at the 3 leaf stage. An earlier experiment using the Mypetal and Myspray techniques at the 6 leaf and flowering plant stages had the same rankings for both techniques (data not presented).

Table 5.8. Comparisons of aspects on the efficiency and reliability of the inoculation techniques used in this experiment.

Technique[a]	Number of plants required	Number of days from planting to rating	Mean Lesion Size (mm)	Frequency of misses (%)	Coefficient of Variation (%)
Plug Leaf	12	31	29.2 b	10	31.2
Plug Stem	12	80	24.3 bc	1	27.5
Mypetal	12	33	17.7 d	16	36.4
Myspray	12	35	11.1 e	43	57.3
Ascpetal	12	35	19.0 cd	42	37.0
Oxalate	6	29	83.1 a	0	27.6
Field	400	110	n/a	?	155.0

Note: Means followed by a letter (a, b, c, etc.) differ significantly using P = 0.05 with Tukey's HSD, which controls Type 1 experiment-wise error.

[a] 'Plug Leaf' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Plug Stem' PDA plugs bearing *S. sclerotiorum* mycelium on *B. napus* stems; 'Mypetal' *B. napus* petal bearing *S. sclerotiorum* mycelium on *B. napus* leaves; 'Myspray' solution of PDB and *S. sclerotiorum* mycelium fragments sprayed on *B. napus* leaves; 'Ascpetal' *B. napus* petal bearing *S. sclerotiorum* ascospores on *B. napus* leaves; 'Oxalate' *B. napus* leaf petiole submerged in oxalic acid.

Table 5.9. Comparison by rank of growthroom inoculation techniques on the basis of their efficiency, reliability and accuracy.

Technique	Accuracy	Reliability	Efficiency	Summary
Plug Leaf	3	2	2	7
Plug Stem	3	4	6	13
Mypetal	2	1	4	7
Myspray	3	6	3	12
Ascpetal	1	5	5	11
Oxalate	6	3	1	10

Note: Lower number indicates higher rank.

Table 5.10. Effects of plant age on relative lesion sizes in *B. napus* lines caused by *S. sclerotiorum* using the Mypetal technique (infesting *B. napus* petals with *S. sclerotiorum* mycelium).

	B. napus growth stage							
	Cotyledon		3 leaf		6 leaf		Flowering	
B. napus line	Lesion mean	Rank	Lesion mean	Rank	Lesion mean	Rank	Lesion mean	Rank
Drakkar	14.4	5	16.8	4	28.9	3	43.4	4
HyLite 225RR	13.8	4	15.2	5	38.1	6	48.7	6
479-08	8.8	2	20.1	6	33.5	4	42.3	3
783-182	15.7	6	14.6	2	33.9	5	46.2	5
375-144	9.8	3	14.8	3	22.4	2	24.7	2
375-53	7.9	1	10.5	1	17.6	1	20.5	1

Note: Lesion means are recorded in mm.

5.5 Discussion

Sclerotinia stem rot is a difficult disease to investigate in the field. The ability to achieve consistently high levels of infection is hampered by this fungus' sensitivity to environmental conditions (in a disease nursery). Since measurements of physiological resistance in the field can be confounded by escape mechanisms, the use of a growthroom technique for sclerotinia stem rot screening is preferred for the sake of accuracy. Reliability and efficiency also are important in a breeding program. For this reason the Mypetal technique appears to be the best overall choice for accuracy, reliability and efficiency of selecting lines for sclerotinia stem rot resistance in *B. napus.*

The question of accuracy needs elaboration since a technique is not useful unless it is representative of resistance that can be observed in the field. Since the field trials had very low levels of infection and very high levels of variation, good correlations among growthroom and field results were not expected. Furthermore, since escape mechanisms due to the plant's morphology may be involved in field results, even with consistent levels of infection, field results would not correlate well with a growthroom technique. For example, HyLite 201, lacking petals, had no infection in the field trials but was quite susceptible in all growthroom trials. Nevertheless, field trials are still useful to verify susceptibility and to verify the accuracy of an inoculation technique. As an example, the Plug Stem technique is not considered to be very accurate since Zhongyou 821 was identified as the most resistant line, but in the field trials it ranked 7th, being one of the more susceptible lines tested. All foliar growthroom techniques found that the *B. napus* line 375-53 had the smallest lesions, and no infection was seen in this line in the field.

The Mypetal technique proved to be the most repeatable and therefore the most reliable of the inoculation techniques judging by the correlation results and the ranks among GR trials. The Mypetal technique was relatively simple to employ and efficient. However, production of inoculum require the extra step of growing *S. sclerotiorum* in liquid medium compared to the agar medium only for the Plug Leaf technique. In preliminary trials, individual petals were removed from *B. napus* flowers for storage, which was quite tedious. Later flowering racemes were cut and stored, which took considerably less time. The Mypetal technique appears to be an accurate system. The Ascpetal technique is believed to best represents the actual mode of infection in the field. The Mypetal technique uses the same substrate, or the same nutrient source as what is seen in the field. It differs in it by-passing the ascospore germination phase.

The Plug Leaf technique also appeared useful and was a close second. It has been used successfully in canola by Fang (1993), Li *et al.* (1999b), Liu *et al.* (2001), and Mullins *et al.* (1999). It has also been used in soybeans (Arahana *et al.* 2001b; Hoffman *et al.* 2002; Kim *et al.* 2000; Vuong *et al.* 2001), in sunflowers (Bert *et al.* 2002; Noyes and Hancock 1981) and in beans (Zhou and Boland 1999). The reason for ultimately selecting the Mypetal technique over the Plug Leaf technique is reliability. The Plug Leaf technique had the lowest CVs among the foliar techniques, but the correlations between GR trials were low. The Plug Leaf technique was more efficient than the Mypetal technique because of its ease in inoculum production, ease in application, and fewer days from planting to rating. This technique uses PDA as a nutrient source, which may give the fungus a nutritional boost to facilitate infection compared to the nutrients available in petals. This speculation is reflected by the fact that it initiated infection the quickest and

produced the largest lesions of the foliar techniques. Sensitivity with this technique is also a concern. In preliminary trials, barley leaves were successfully infected with the Plug Leaf technique. This raises the concern that this technique might be overly aggressive and unrepresentative.

The Plug Stem technique was largely rejected on the basis of the difficulty in inoculation and the length of time to retrieve data. Concern about the variation of this technique has been raised by other researchers. Fang (1993) found that the variability with this technique was too high to be of any use in *B. napus* screening suggesting that this might because of plant age. Cline and Jacobsen (1983) suggested plant age as well in soybeans might be responsible for this variation. But it has been indicated that this variability might be caused by variation in stem size, which can be accounted for by selecting appropriate stems (Buchwaldt *et al.* 2003). However, accuracy of this technique, like the Agar Leaf technique could not be assessed fully (Kim *et al.* 2000).

The Ascpetal technique can be regarded as the most accurate measure of physiological resistance since it uses the same propagules and nutrient substrate as the pathogen occurs naturally in the field. But it was rejected as a useful growthroom technique for high-throughput disease screening because of its low efficiency and high variability. This technique was cumbersome, requiring a constant and viable source of ascospores, which can take up to four months to produce in the laboratory. This technique also had a longer lag period between inoculation and the time lesions were large enough for disease assessment, compared to the Plug Leaf or Mypetal techniques. This could cause logistical problems for a large-scale screening program. Also, there was high

variability between replications and GR trials, which might be because of variability in viability caused by the storage of ascospores for longer periods of time.

The Myspray technique correlated well with the Ascpetal technique, but the variability between replications and GR trials was very high. This technique had the greatest frequency of misses, questioning its reliability. It could not be determined if a plant with no lesions was truly resistant or whether it was an escape. This suggests that this technique might measure some form of foliar avoidance mechanisms such as leaf angle, leaf orientation, or cuticular wax structure and composition.

The Oxalate technique was the most efficient of the growthroom techniques. Oxalic acid tests have been done on detached leaves with success in soybeans (Kolkman and Kelly 2000), but in canola, this technique does not appear to be effective. As it did not correlate with any other technique, its results were deemed not to accurately represent physiological resistance. Furthermore, since many of the sources of resistance in the test lines used mechanisms other than oxalate-degrading enzymes, the usefulness of this test may not go beyond those lines with an oxalate-resistant gene.

These findings are in agreement with comparative studies conducted by Fang (1993) and Kim *et al.* (2000) and that foliar applied mycelium-bearing substrates are effective for determining physiological resistance. Fang found that the Plug Leaf technique was the best assessment method. However, the scope of Fang's experiments was smaller and did not include any other foliar techniques. By using four foliar techniques, and comparing these, the Plug Leaf technique, while it was efficient and relatively reliable, was found to be of questionable accuracy. As well, Fang found that the Plug Leaf technique did not correlate well with field results. Kim *et al.*'s (2000) findings

were that the Plug Leaf technique was not superior to other foliar techniques in soybeans, and that any of these would be useful in screening for white mold resistance.

Critical to development of a screening methodology is the availability of susceptible and resistant plant material. The difficulty with sclerotinia stem rot is that most *B. napus* material is largely susceptible. This study included two cultivars reported to have resistance in the field and lines with four transgenes that were believed to confer resistance. HyLite 201 possesses an avoidance mechanism but has no physiological resistance (Jurke *et al.* 1998; Jurke and Fernando 2002). Zhongyou 821 from China is reported to have field tolerance to sclerotinia stem rot (Li *et al.* 1999c). The transgenic canola lines contained the Ox-ox gene from wheat, Ox-dec from the basidiomycete *Collybia velutipes*, Rs AFP2 (anti-fungal protein) encoding gene from *Raphanus sativus* and chitinase and beta-1,3-glucanase genes from tobacco (Table 5.4). The enzyme oxalate oxidase (Ox-ox) oxidises oxalic acid and has been shown to provide some level of resistance in transformed soybean (Billings *et al.* 2003; Donaldson *et al.* 2001). Mehta and Datta (1991) have shown that the oxalate decarboxylase enzyme from *C. velutipes*, which is inducible by the presence of oxalic acid, is able to decarboxylize oxalic acid effectively. Kesarwani *et al.* (2000) using tobacco and tomato transformed with the oxalate decarboxylase gene found good levels of control against *S. sclerotiorum*. Anti-fungal proteins have been found to have an effect on a number of filamentous fungi, including *S. sclerotiorum* (Terras *et al.* 1992). Chitinase and β-1,3-glucanase, have been found to be involved in sclerotial degradation (Gigzey *et al.* 2001) and found to provide some control of *S. minor* (El-Tarabily *et al.* 2000).

This study would have been more productive if better levels of resistance were available. Of the transgenic lines, only 375-53 had a moderate level of physiological resistance, while the remainder had similar susceptible reactions. Since these lines did not differ significantly from each other or from the untransformed parent, the correlations between GR trials, and between techniques were not particularly strong. Rank correlation analysis was less useful in this regard, since a small difference between lines changed the ranks to a larger degree.

The experiments on plant age were done to confirm that these techniques could be used at any plant developmental stage. The general agreement in results indicates that the differences detected between lines are not age-mediated and that the resistance genes are expressed throughout the plant's life. This provides a large window for inoculation and enhances the value of the foliar inoculation techniques.

Nelson *et al.* (1991) found that growthroom evaluations had limited value in a breeding program for soybeans. But like Kim *et al.* (2000), it is believed that growthroom inoculation techniques can be employed to obtain useful preliminary data on the physiological resistance of genotypes to *S. sclerotiorum*. The Mypetal technique was assessed to be the best growthroom inoculation technique for determining physiological resistance to *S. sclerotiorum* in *B. napus*. Lines identified to have resistance in the growthroom will need to have this verified in field trials. Nevertheless, susceptible lines could be identified and discarded in growthroom tests. Ultimately field data is what will be most important for growers.

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6.0 GENERAL CONCLUSIONS

The purpose of this research was to evaluate two alternative management strategies, cultural control and avoidance traits, to fungicide control of sclerotinia stem rot in canola and to identify a methodology to enable a third alternative, physiological resistance, to be evaluated.

Prior to development and registration of fungicides for controlling sclerotinia stem rot in rapeseed, the only methods of control available to producers were cultural ones such as long rotations. Since the development of canola in 1974 and the increase study of canola and rapeseed diseases, much is now known about the epidemiology and the infection process by *S. sclerotiorum*. A common theme discussed in the scientific literature is canopy density and canopy microenvironment when speaking about cultural controls and escape/avoidance mechanisms in *S. sclerotiorum* diseases.

Seeding rate manipulation is a cultural or agronomic option that canola producers currently have available and has been found to be effective against *S. sclerotiorum* disease development (Blad *et al.* 1978; Haas and Bolwyn 1972; Huang and Hoes 1980; Irvine and Duncan 1992; Steadman *et al.* 1973). The findings on seeding rate effects reported here support the results of the previous studies. Seeding rate affected plant density which subsequently affected both lodging and sclerotinia stem rot development. Seeding rates greater than the currently recommended rate in Canada (6.7 kg/ha) resulted in significantly increased disease incidence. Seeding rates lower than normal did not significantly affect disease incidence. Lodging increased the incidence of sclerotinia stem rot, and the most severely infected areas were those most severely lodged. The lodging

susceptible canola cultivar, AC Excel, had the most lodged plants and the highest levels of disease. Not increasing seeding rates and choosing lodging resistant cultivars should reduce the severity of sclerotinia stem rot in the field. Seeding rates and DI did not have a demonstrable effect on canola yields in these experiments.

There have been a number of escape or avoidance mechanisms identified in physiologically susceptible crops that reduce *S. sclerotiorum* disease levels in the field. Traits such as crop height, growth-habit, leaf area, lodging resistance, and maturity, to name a few, been found to affect disease expression in soybeans and other bean crops. However, there have been no such studies done with canola. Evaluating these reports from other crops and understanding the disease cycle in canola, traits suspected to be avoidance mechanisms were measured. Canola cultivars that lack petals (apetalous cultivars) were found to have large significant decreases in disease incidence. The number of petals adhering to leaves and leaf axils was significantly correlated to disease incidence suggesting that petal production is the largest contributor to sclerotinia stem rot in canola. Petalled cultivars varied in disease incidence but this disease variation was not attributed to petal adhesion. Other avoidance traits significantly related to disease incidence included lodging resistance, branch angle and crop maturity. Since these traits are strongly heritable, plant breeders should be able to select for them and develop cultivars with improved avoidance characteristics.

Avoidance traits and physiological resistance both affect field resistance. Measuring physiological resistance in the field is challenging because of the environmental sensitivity of *S. sclerotiorum* and the confounding effects of avoidance traits. To effectively study physiological resistance, researchers require a reliable,

standardised growthroom screening or inoculation procedure. This has not been available for canola. Building on the research of others, most notably Fang (1993), existing screening methodologies were compared and a new methodology was developed that was more effective in identifying resistance. The mycelium infested petal technique, referred to as the Mypetal technique, proved to be the most reliable, one of the most accurate, and one of the most efficient of techniques tested. It was therefore selected as the most desirable technique for identifying physiological sclerotinia stem rot resistance under growthroom conditions. The mycelial disk (Plug Leaf) technique, while comparable to the Mypetal technique, was found to be more variable and likely too aggressive for studies requiring sensitivity and specificity.

Based on the findings of this research, more effective management of sclerotinia stem rot is achievable and data presented here should be useful to plant breeders, plant pathologists and ultimately to canola producers.

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