DEVELOPMENT AND GENETIC DIVERSITY OF *SCLEROTINIA SCLEROTIORUM* ON POTATO IN THE COLUMBIA BASIN

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of Zahi Kanaan-Atallah find it satisfactory and recommend that it be accepted

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Chair

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DEVELOPMENT OF WHITE MOLD AND GENETIC DIVERSITY OF *SCLEROTINIA SCLEROTIORUM* IN POTATO IN THE COLUMBIA BASIN

Abstract

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Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan, homothallic and necrotrophic ascomycetous fungus dispersed by airborne ascospores and soilborne sclerotia. It causes disease in over 400 host species. A significant correlation between potato yield losses and stem rot incidence has not been observed, while 1-3 fungicide applications are made to manage the disease. In the Pacific Northwest, iprodione, dichloran, quintozene and fluazinam are registered for stem rot control; labels recommend fungicide applications be made prior to row closure. Reduced control could be caused by mistimed applications, inadequate tissue coverage or a selection for fungicide resistant isolates.

Ascospores were captured and disease incidence was monitored in ten potato fields in the Columbia Basin to determine when infections occur. Peak ascospore release corresponded with initial full bloom (7 to 10 days after row closure) and disease onset occurred 10-14 days following row closure. Airborne *S. sclerotiorum* ascospores impacted potato blossoms attached to plants. Blossoms fell to the ground and onto stems, senesced and were colonized by the fungus. Potato vines became infected after dropping to the ground and coming in contact with the fungus. Flower removal and fungicide applications at initial full bloom reduced disease incidence, in comparison to untreated controls. These observations indicate that fungicide label recommendations need to be based on initial full bloom, instead of row closure, to provide

optimal protection at this critical time. Furthermore, no significant differences were found in efficacies of iprodione, dichloran or fluazinam in greenhouse and *in-vitro* trials; quintozene failed to provide effective protection on inoculated stems.

Canadian and southeastern US populations of *S. sclerotiorum* have been previously described as clonal, with a few genotypes composing the majority of populations. Analyses of isolates from the Columbia Basin and other U.S. areas using microsatellites revealed high genotypic variability, but genotypes were not segregated by host or geographic location. The discovery of a 25% rate of outcrossing in the Columbia Basin, in addition to gene flow explain in large part the high genotypic variability observed. Furthermore, no correlations were found between genotypes and mycelial compatibility groups, reduced sensitivity to fungicides, and response to various temperatures or aggressiveness.

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DEDICATION

My work is dedicated to the two people who sowed and grew the seeds of curiosity in me, my parents: Daad and Nabih. They believed in me and steered me toward safer shores.

I also dedicate my dissertation to my country: The land of Cedars, biblical Lebanon. Many forget that it gave the world its alphabet, Jesus' first miracle and more recently the mind behind "The Prophet", Jibran Khalil Jibran.

Say not, "I have found the truth," but rather, "I have found a truth."

Jibran on "Self-knowledge"

Preface

The two chapters included in this dissertation have been prepared for submission to professional journals. Chapter one will be submitted to Plant Disease and chapter two will be submitted to Phytopathology. Citations and references in those two chapters are formatted according to the guidelines of the respective journals and are listed at the end of each chapter before the accompanying tables and figures. Citations included in the general introduction section follow the guidelines of WSU graduate school and are found at the end of the dissertation.

Introduction

Cultivated potatoes (*Solanum tuberosum spp. tuberosum* L.) are plagued by a variety of fungal diseases of economic importance. Stem rot (aka white mold) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary occurs in temperate potato growing areas and was reported to have caused the destruction of entire crops in Ireland during the 19th century (Partyka and Mai, 1962). Symptoms of stem rot start as soaked/watery lesions on main and secondary stems that turn into severely rotted areas girdling infected stems and devouring the inner pith, thus causing the death of the canopy. Infected stems turn brownish, become soft and as they dry out acquire a bleached and papery appearance. A whitish mycelium is frequently observed on infected parts hence the name white mold is used to describe the symptoms on legume plants (chiefly bean and soybean). In potato the nature of the symptoms and their restriction to stems are in conformity with the name stem rot. Stems are hollowed by the fungus and are filled with black and hardened sclerotia, which allow the fungus to survive adverse conditions. With the degradation of stems on the soil surface or by plowing, sclerotia are liberated and after conditioning can either undergo a sexual cycle and produce apothecia, or germinate directly to infect plant tissues in the immediate vicinity.

S. sclerotiorum is a cosmopolitan inoperculate discomycete capable of generating cup-shaped apothecia from sclerotia. Those apothecia, usually 2-10mm in diameter, are lined with asci, which are filled with 8 hyaline ascospores (110-160µm x 6-10 µm) (Kohn, 1979). Those ascospores are forcibly ejected in puffs and are carried by wind currents. It is thought that because the sizes of apothecial stalks (stipes) are usually smaller than 5cm, sclerotia in the upper 5cm soil horizons are the only ones that germinate carpogenically (Steadman, 1983). Rainfall is known to increase disease incidence and following drought it induces carpogenic germination of

sclerotia in soybean (Natti, 1971; Boland and Hall, 1988). Decreases in relative humidity close to the apothecia are credited for the "puffing" of ascospores, while few ascospores were released at high relative humidity (McCartney and Lacey, 1992). Light was found to have little impact on ascospore releases. Ascospore releases usually peak around midday and plummet late in the afternoon and at night (McCartney and Lacey, 1992; Gutierrez and Shew, 1998).

The maximal extent of ascospore dissemination is still uncertain. While up to 90 percent of *S. sclerotiorum* ascospores are thought to deposit in a 100 meters radius from the source, the rest were assumed to travel up to 3-4 Km (Kohli *et al*., 1995; Cubeta *et al*., 1997). Furthermore, viable ascospores were captured at 6000m in altitude (Williams and Stelfox, 1979) indicating a possible dissemination over long distances. Depending on the crop growing in a field, apothecial production and ascospore releases may be staggered over a long period, especially given that soil shading and availability of moisture are required for a carpogenic germination. Epidemics occuring early in the season were usually traced back to ascopores produced by apothecia in neighboring fields cropped to winter crops (Abawi and Grogan, 1974; Abawi *et al*., 1975; Williams and Stelfox, 1979).

In potato the few available reports indicated that ascospores are responsible for disease and noted that outbreaks occur at row closure when the potato canopy has achieved row closure and the soil is totally shaded, thus providing a buffered microclimate where moisture is high with reduced air movement and solar radiation (Partika and Mai, 1962; Powelson, 2001). Thick, closed and drooping canopies of certain peanut, bean and soybean cultivars have been found to sustain more severe white mold outbreaks than cultivars with sparse or upright canopies (Schwartz *et al*., 1978; Boland and Hall, 1988; Butzler *et al*., 1998). Upright-growing and sparse canopy cultivars had minimal stem contact with *S. sclerotiorum* mycelium on the ground. Alternatively, other canopies provided a favorable microclimate for the carpogenic germination

of sclerotia, and stems came in contact with the inoculum on the soil surface as a result of the collapse under the weight of stems. High crop density, close row width and excess nitrogen fertilization were also linked with increased stem rot incidence in several crops (Natti, 1971; Grau and Radke, 1984).

Ascospores of *S. sclerotiorum* necessitate an external energy source to germinate. Flowers and senescing tissues, such as leaves on the soil surface, provide them with abundant and readily available nutrients, thus allowing them to build mycelial mats which could transfer the disease to healthy stems coming in contact (Keay, 1939; Abawi and Grogan, 1974, 1975; Abawi *et al*., 1975, Steadman, 1983; Powelson, 2001). In the absence of such accessible energy supplies, ascospores were unable to generate disease when ascospore suspensions were sprayed onto deflowered bean plants or intact green (unwounded) lettuce and clover leaves (Keay, 1939; Abawi and Grogan, 1974) even when in high humidity chambers. Flower contamination by *S. sclerotiorum* ascospores was associated with disease incidence in bean and canola (Abawi *et al*., 1975; Turkington and Morrall, 1993; Lefol and Morrall, 1996), where ascospores remained inactive until blossom senescence, which was followed by a rapid colonization of blossoms and subsequent disease where humidity was favorable (Steadman, 1983; Kohli *et al*., 1992; Turkington and Morrall, 1993). Moreover, fungicidal coverage of blossoms at full bloom delivered best control of white mold, as opposed to applications on stems and leave but not flowers, which had no effect on either disease incidence or severity (Natti, 1971; Abawi *et al*., 1975; Steadman, 1983; Morton and Hall 1989).

S. sclerotiorum is described as a homothallic fungus based on the ability of colonies produced by single ascospores or hyphal tips to produce fertile apothecia and viable ascospores without requiring the presence of a mate (Keay, 1939; Kohn, 1979). Furthermore, various attempts to force crossing between pairs of isolates in the Kohn lab (University of Toronto)

repeatedly failed to produce heterokaryons (Kohli *et al.*, 1992; Kohli and Kohn, 1998). However such findings were considered not to preclude the possibility of outcrossing, though the authors provided no specific details on such a phenomenon. No evidence of outcrossing was witnessed in the Canola production region of Canada over a decade and apothecia repeatedly yielded identical ascospore genotypes, indicating a lack of sexual recombination [sexual recombination is used here as a synonym to outcrossing, especially given that homothallic fungi can undergo outcrossing to produce ascospores] (Kohli *et al.*, 1992; Kohli *et al.*, 1995). In North Carolina (Cubeta *et al.*, 1997; Kohli and Kohn, 1998) and on wild *Ranunculus ficaria* (buttecup) plants in Norway (Kohn 1995), on the other hand, evidence for outcrossing under natural conditions was noted.

Clonality; i.e. the "repeated recovery of genotypes that can be identified with independent genetic markers and by the repeated recovery of such genotypes over a wide geographical area or from year to year" (Kohn, 1995); has characterized agricultural populations of *S. sclerotiorum* in Canada as illustrated by the recovery of one clone over a 2000km territory and during 4 successive seasons. Additionally, 9 clones were frequently recovered in Western Canada over 3 collection years (Kohn, 1995; Anderson and Kohn, 1995). From 213 isolates collected in Ontario and Quebec, 5 MCGs (mycelial compatibility group) represented 84% of isolates, while the other 16% was represented by 16 different MCGs (Hambleton *et al*., 2002). Mycelial incompatibility is described as "a failure of different strains to fuse and form one colony" (Kohn *et al*., 1990). In opposition, Ford *et al*. (1999) generated heterokaryons and observed that mycelial and vegetative compatibility (VC) loci were not linked. Vegetative incompatibility is described as "the inter-strain anastomosis and successful heterokaryon formation" (Glass and Kuldau, 1992; Ford *et al*., 1999). Ford *et al*. (1999) also concluded that isolates belonging to the same MCGs could belong to compatible VCGs. Such a finding could explain the disparity in the information regarding the

genotypic variability of *S. sclerotiorum* field populations, when using MCGs as markers (Kohli *et al.*, 1992; Kohli *et al.*, 1995; Kohn, 1995; Kohli and Kohn, 1998; Hambelton *et al.*, 2002).

Populations of *S. sclerotiorum* infecting *R. ficaria* in Norwegian forests were characterized by an elevated rate of diversity and a structured spatial distribution, while populations affecting potato and canola in neighboring agricultural areas exhibited a random distribution of genotypes and a genotypic uniformity of isolates (Kohn, 1995). Such genetic structuring and high genotypic variability indicate important rates of gene flow (allelic exchange), among studied fungal populations, which in turn affects the genetic composition of individual isolates (Balloux and Lugon-Moulin, 2002). On the other hand it is suspected that cultural practices select for certain genotypes, probably because of the reduction of susceptible taxa (Kohn, 1995).

The main objective of the following studies is to improve our ability to manage potato stem rot by investigating the reasons of the failure of current management practices. I try to shed light on the epidemiology of the pathogen and to determine the possible deficiencies in the current control practices. Conversely, I investigate the population biology of *S. sclerotiorum* and compare it with certain phenotypic characteristics to evaluate the diversity of the population in the Columbia Basin, and to identify potential phenomena driving the selection, which was described in other areas.

In addition to the ineffectual fungicide control strategies (Miller and Miller, 2001), to my knowledge conventional breeding methods are not likely to produce stem rot resistant potato cultivars. For those reasons I see it as crucial to answer a number of questions regarding the epidemiology of *S. sclerotiorum* to allow for the development of useful control approaches. Important aspects are inoculum sources (ascospores or mycelia) and dissemination; crop phenology; cultural practices; cultivar choice; watering and fertilization and disease etiology.

Sclerotinia sclerotiorum causes disease on a number of economically important crops in the US Pacific Northwest, such as potato, mint, vegetable seed crops and canola. There is no information on the population biology of the fungus in that region. I am also unaware of studies on the aggressiveness of "local" isolates (the quantity of disease induced by a pathogenic strain on a susceptible host), their response to fungicides or to temperature stimuli. Selection for isolates that are most aggressive, less sensitive to fungicides and/or highly adapted to environmental conditions could shape a clonal population in the Columbia Basin. To evaluate host specialization and geographic diversity of *S. sclerotiorum* isolates, I compared isolates from a variety of crops and regions in North America.

Chapter One

Development of Sclerotinia stem rot in potato fields in south central Washington

Abstract

Sclerotinia sclerotiorum (Lib.) de Bary is a discomycetous fungus dispersed by airborne ascospores and soilborne sclerotia. In the Columbia Basin of Washington, fungicide applications in potato are recommended prior to row closure and currently provide poor control. Studies in 10 potato circles demonstrated that in most cases ascospore release peaks corresponded with the initial full bloom (7 to 10 days after row closure) and disease onset occurred 10-14 days following row closure. Airborne *S. sclerotiorum* ascospores settled on potato blossoms that fell to the ground two days after blooming, senesced and were colonized by the fungus. Heavy potato vines became infected after dropping to the ground and coming in contact with the fungus. Flower removal and fungicide applications at initial full bloom reduced disease incidence by up to 82% in comparison to untreated controls. In opposition, fungicide applications at or prior to row closure failed to provide disease control. This implies that fungicide label recommendations need to be based on initial full bloom, as opposed to row closure, to provide adequate protection at this critical time.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan, homothallic and necrotrophic, ascomycetous fungus dispersed by airborne ascospores or soilborne sclerotia (16,25). *S. sclerotiorum* causes disease in over 400 host species including bean, canola, peanut, soybean, sunflower and potato (3,4,25). While the magnitude of this disease has long been recognized in many crops, reports on the economic impact on potatoes are scarce. Partyka and May (22)

reported that in Ireland stem rot (a.k.a. white mold) caused severe damages in the mid 19th century. In contrast, researchers in the U.S. Pacific Northwest (PNW) have not been able to relate significant potato yield losses with stem rot incidence (19). Nevertheless, one to three fungicide applications are made to manage *S. sclerotiorum* on potato in the Columbia Basin. Four fungicides are currently registered in the PNW for the control of stem rot, i.e. iprodione, dichloran, quintozene (syn. PCNB) and fluazinam. Label recommendations for all four fungicides advise starting applications prior to row closure (i.e. complete covering of furrow between rows). Current fungicide applications seek to cover stems for protection against stem rot, which compels growers to treat prior to row closure in order to obtain the greatest stem coverage. The potential contribution of blossoms to the disease is not taken into consideration. In the Columbia Basin, the cost per hectare per application was $112 ($45/acre) in 2000-2001 (A. Greenwalt, Quincy Farm Chemical, Quincy, WA) with control often deemed inadequate by growers and extension personel.

The Columbia Basin extends over 195 Km from north to south and 80 Km from east to west, in south central Washington and north central Oregon. On average of 65,000 ha of potatoes are grown annually in the Columbia Basin, making it the second largest potato producing area of the United States. Potatoes in the Columbia Basin are mostly grown under center pivot sprinkler irrigation at a density of 35 to 45 thousand plants per hectare. Determinate and indeterminate potato cultivars are grown. Indeterminate growth cultivars produce long viny stems that tend to lie on the ground soon after row closure, thus forming a closed canopy where humidity is often high; they also produce a number of bloom flushes. Determinate cultivars have limited bloom episodes and a smaller canopy volume. Air movement, solar radiation and fungicide coverage within canopies of determinate cultivars are generally greater than within closed canopies of indeterminate cultivars. Peanut and bean cultivars with upright growth habits and open canopies

had less severe stem rot compared with cultivars that developed thick and closed canopies (5,26). Also, closed soybean canopies provided a favorable microclimate for the carpogenic germination of sclerotia, while upright plant architectures reduced stem contact with mycelium on the ground (3). High crop density, close row width and excess nitrogen fertilization have also been linked with increased stem rot incidence in several crops (9,21).

Rainfall and irrigation were described as crucial factors in apothecial emergence and critical for ascospore germination. Rainfall increased disease incidence and when following drought it induced carpogenic germination of sclerotia in soybean (3,21). Between 48 and 72 hours of continuous wetness were essential for blossom colonization and subsequent disease (3).

Ascospores of *S. sclerotiorum* are a major source of inoculum in crops like bean, canola and soybean, where aerial parts are affected (2,3,27). In other crops, such as sunflowers, direct mycelial germination of soilborne sclerotia affects roots and crowns (11). In potato, intra-field apothecia were noted to supply most of the ascospore inoculum especially when their production coincides with row closure (23). Disease was also described to occur consequent to ascospore production, which coincided with the senescence of lower leaves following row closure (22). However, quantitative data was not presented in support of aerial ascospores as a major source of infection (22,23). Conversely, Pscheidt and Ocamb (24) noted that the disease appeared as a soft decay in the crown area, indicative of soilborne infection by direct mycelial germination of sclerotia.

The maximal distance of ascospore dispersal is uncertain but apothecia in neighboring fields are recognized as potential infection sources. Ascospores generated by apothecia outside bean and rapeseed fields were credited for initiating white mold epidemics in New York state and Canada (1,2,31). Up to 90 percent of *S. sclerotiorum* ascospores are thought to be deposited within a 100 m from the source (6). The remaining inoculum was assumed to travel farther than

150 m and up to 3-4 Km; viable ascospores of *S. sclerotiorum* were captured at 6000 m in altitude (15,31).

Ascospores are unable to readily infect green tissue, but they colonize flowers and senescing leaves that are then used as energy sources to infect green tissues (1,2,13,23,27). Attempts to inoculate lettuce and clover leaves with ascospore suspensions failed to initiate lesions in healthy tissues, but succeeded on declining tissues (13). Removing flowers from the crop canopy prevented stem rot occurrence in bean "even under ideal conditions" for infection (1). To our knowledge, the role of blossoms in the disease cycle and the exact timing of infection in potato have not been established. Both are crucial to developing a stem rot management strategy for this crop.

Limited information is available on the relative importance of ascospores and mycelium from sclerotia as inoculum sources on potato and on the spread of *S. sclerotiorum* in potato fields. Furthermore, little is known on the impact of cultural practices and crop phenology on disease establishment and development. Such knowledge is essential for developing disease control strategies, especially in the absence of stem rot resistant potato cultivars and the use of expensive fungicides. The objectives of this study were: i) to compare the relative roles of ascospores and direct mycelial germination from sclerotia as inoculum sources; ii) to identify potato growth stages and tissues essential for infection and disease development and iii) to assess the effects of vine growth habits, irrigation and chemical control practices on disease development.

Materials and Methods

Study area. Six commercial potato fields were selected for study in 2001 (fields 1 through 6) and four fields were selected in 2002 (fields 7 through 10) (Table 1). All ten fields were at least 53 ha in size, were planted with certified seed tubers from March through April of the respective years, and were irrigated by center pivot systems. All fields were not planted with potatoes for at

least three growing seasons preceeding the year of study. Rotational crops planted in these fields were corn, onions and wheat. Soils of all studied fields were sandy to sandy-loam deep soils and fields had been treated with Vapam® (metam sodium) or Telone C17® (1,3-dichloropropene + chloropicrin) prior to potato planting.

In 2001, fields 2, 3, 4, 5 and 6 were treated with a mixture of iprodione and quintozene (Rovral 4F® at 1.7 l /ha and Blocker 4F® at 2.4 l /ha) prior to row closure. Field 1 was divided into six wedges, of which five were treated at row closure with one of the following: iprodione (Rovral 4F® at 2.4 l/ha), dichloran (Botran 5F® at 4.8 l /ha), iprodione + quintozene (Rovral 4F® at 1.7 l /ha and Blocker 4F® at 2.4 l /ha), dichloran + quintozene (Botran 5F® at 2.4 l /ha and Blocker 4F® at 2.4 l /ha) and fluazinam (Omega 500F® at 0.5 l/ha). A wedge was left untreated and served as a control. In 2002, fields 9 and 10 were treated with fluazinam (Omega 500F® at 0.5 l /ha) at initial full bloom. Field 7 (except for a 10 ha wedge left untreated) and field 8 were treated at row closure with iprodione + quintozene (Rovral 4F® at 1.7 l /ha and Blocker 4F® at 2.4 l /ha). Fungicides were applied through the irrigation system with approximately 0.875 cm water/ha.

Detection and quantification of inoculum. Petri dishes containing a semi-selective medium (10,12,28) were placed on top of plant canopies to capture airborne ascospores. Plates were exposed for approximately 30 minutes between 9AM and 2PM, coinciding with the diurnal peak in ascospore release as recorded in the literature (10,12,18). Eight to twelve plates were positioned along a randomly selected transect (sample transect) at 40-meter intervals from the edge of the field to the pivot center. Plates were also exposed on the top of the canopy of the last row at the edge of the field, as well as on top of the fertilizer tank in the center of the circle. The number of plates exposed depended on the radius of individual fields. Petri dishes were exposed

weekly starting 3-4 weeks after plant emergence in late April. Following the first appearance of stem rot symptoms the interval between exposures was increased to two weeks. Exposed plates were incubated at 25 °C for 3 to 4 days in the dark. Growing colonies were examined daily to identify *S. sclerotiorum*. Colonies of *S. sclerotiorum* were usually recognized within 3-4 days and were characterized by the absence of conidia, presence of whitish mycelium and a color shift from blue to yellow of the bromophenol blue dye of the culture medium, because of the production of oxalic acid (7, 28). Ascospore numbers were counted for fields 3, 7, 8, 9 and 10 and presence or absence of ascospores was recorded for fields 1, 2, 4, 5 and 6.

The number of ascospores captured was obtained by counting the number of *S. sclerotiorum* colonies obtained on the semi-selective medium with the assumptions that all viable ascospores captured germinated and that each colony resulted from a single ascospore. One-way analysis of variance (ANOVA) using PROC GLM in SAS (version 8.1, SAS Institute, Cary, NC) was used to analyze the number of captured ascospores (observational unit) among collection locations within a field on a given date and among collection dates for all fields. The latter two factors were analyzed separately.

At initial full bloom, five flower clusters were collected from all fields, in the same locations where Petri dishes were exposed. Two flowers per cluster were plated on the semi-selective medium to test for the presence of ascospores. Initial full bloom usually occurs 7 to 10 days after row closure depending on cultivar and growing conditions. One-way ANOVA using PROC GLM was used to analyze the number of captured ascospores (observational unit) among collection locations within a field on a given date and among collection dates for all fields.

Quantification of disease incidence. Potato plants along the sample transect were observed weekly for lesions. At the first appearance of stem rot symptoms, stems with lesions were

counted in two rows of 12 plants per row at the same sites where Petri dishes were exposed. Numbers of infected stems and not lesions per individual stem were recorded because lesions coalesce, potentially causing record-keeping difficulties. Two weeks after the first assessment, a second disease assessment was performed on a second set of two rows of 12 plants adjacent to the previously assessed rows. Different, but contiguous, rows were used because opening rows disrupted the canopy microclimate during the first disease assessment.

Sample transects in fields 1 and 7 were located in the untreated control wedges of both fields but disease assessments were carried out in all wedges. In field 1 five assessment sites were chosen in each of the six treatment wedges, and disease assessments were performed on 5 rows of 24 plants at disease onset. Disease assessments were made on 3 rows of 24 plants in the treated wedge of field 7 and at every ascospore trapping location along the sample transect in the control wedge. Fungicide treatments applied in field 1 were compared to the control by a chi-square goodness of fit test with the assumption that the only differences between the different wedges of the field were the fungicides applied (29). In the Poisson distribution, the mean and the variance are equal to the number of lesions per treatment, which allows us to compute a test of significance.

Monitoring of precipitation. Rain and irrigation water was collected with four rain gauges placed in each field to investigate the relationship between water management and disease incidence. Rain gauges (Tru-Check, Hummer Int., Earth City, MO) were placed at about 80 cm above soil level at 80-meter intervals along the sampling transect. Water amounts were recorded weekly. The relationship between disease incidence (dependent variable) and amount of irrigation water (independent variable) was investigated by linear regression using PROC REG in SAS.

Fungicide trials. To help determine the role of blossoms in disease development, blossoms were removed from plants or fungicides were applied to plant canopies in 3 rows by 10 m plots in field 7 in 2002. The test was conducted in the wedge-shaped section of the field (approximately 10 ha) not treated with fungicides. Flower clusters on plants were handpicked as they became apparent over a two-week period. Fungicides iprodione (Rovral 4F® at 2.4 l /ha) and fluazinam (Omega 500F® at 0.5 l /ha) were applied to the canopy at initial full bloom (i.e. first 100% bloom event; occurred following row closure) with a CO_2 pressurized sprayer at a rate of 281 l/ha at 210 kPa, using ConeJet® hollow cone nozzle TXVS-18 (R&D Sprayers Inc., Opelousas, LA). Blossoms were not picked and the canopy was not sprayed with fungicide in the non-treated control plots. The experiment was arranged in a randomized complete block design where treatments of fungicide applications or blossom removal were replicated four times. Disease incidence was assessed on 30 plants in the middle row by counting the number of stems with lesions 3 weeks after fungicide application. Disease incidence data was analyzed by one-way analysis of variance (ANOVA) using PROC GLM in SAS. Fungicides and flower removal served as treatments and lesion numbers were considered the observational unit. Fisher's protected least significant difference was used to compare treatment means.

Efficacies of registered fungicides were evaluated on stems of cv. Russet Burbank in the greenhouse to understand the nature of inefficacy observed in fields. Plants were propagated from certified seed pieces about 13 g in weight in a commercial potting soil mix (Soil Conditioners, Zillah, WA) and subjected to a 16-hour photoperiod. Stems of 1.5-month-old plants were treated with dichloran (Botran 5F® at 0.189 ml/plant and 0.095 ml/plant), iprodione (Rovral 4F® at 0.063 ml/plant and 0.031 ml/plant), dichloran+iprodione (Botran 4F® at 0.095 ml/plant and Rovral 4F® at 0.031 ml/plant) and fluazinam (Omega 500F® at 0.5 l /ha) (Table 4). Treated plants were left to dry for twelve hours, at 18±4°C, before being inoculated. Inoculations

were achieved by attaching colonized potato blossoms to potato stems, at 10 cm above soil line, and lightly wrapping inoculation points with gauze to hold the flower. Ten *S. sclerotiorum* isolates from the Columbia Basin were used in this experiment. Five isolates belonged to the most frequently collected mycelial compatibility group (MCG) while the other five isolates belonged to five different MCGs (Kanaan-Atallah and Johnson, unpublished data). Blossom colonization was achieved by placing autoclaved blossoms on top of a growing colony of *S. sclerotiorum* on potato dextrose agar for 3-4 days. Each isolate was replicated five times for each fungicide treatment. Inoculated but unsprayed plants served as controls. Plants were placed in mist chambers and subjected to an intermittent mist for 24hrs, which was stopped thereafter while the plants were kept at above 95% relative humidity in the mist chambers for 48hrs. Ambient temperature was 16 ± 2°C at night and 27 ± 2°C during the day. Lesions were counted on stems three days after inoculation. The experiment was repeated.

Results

Detection and quantification of inoculum. Ascospores were captured in 90% of the fields studied. The greatest ascospore numbers were captured beginning about a week before and continuing a few days after full bloom (Fig. 1). Ascospore numbers did not differ significantly among trapping sites within all fields ($P>0.05$). Significant differences in the numbers of captured ascospores were observed among collection dates in all fields ($P<0.05$).

One hundred percent of the flowers collected at initial full bloom from all fields except fields 9 and 10 were contaminated with *S. sclerotiorum*, as verified by mycelial growth from the blossoms plated on the semi-selective medium.

Quantification of disease incidence. In 60% of the fields sampled (fields 1, 3, 4, 5, 7 and 8) stem rot initially appeared on potato stems 14 to 20 days after row closure and 5 to 7 days following blossom fall. This coincided with stems touching the ground. Mycelial mats *of S.*

sclerotiorum colonized senesced blossoms on the ground and canopy following initial full bloom and blossom fall. Lesions developed on stems contacted by colonized blossoms. Healthy stems became infected in turn when contacting infected tissues, but rarely did whole plants die, unless infections occurred in the crown area at the base of plants. Stem rot lesions were detected in field 5 fourteen days before row closure and involved 5% of the plants. Lesions were located in the crown area, 1-2 cm below soil level and were associated with sclerotia directly germinating in the immediate vicinity of affect plants, which were killed as a result of such infections.

Disease progress curves increased rapidly after the first appearance of symptoms in fields 1, 3, 4, 5, 7 and 8. In fields 1, 7 and 8 disease incidence significantly increased ($P<0.05$) between the first and second disease assessments (Fig. 2 and 3). Conversely, in fields 3, 4 and 5 disease incidence did not vary significantly between the first and second disease assessments ($P>0.05$; Fig. 3). Stem rot incidence reached a plateau between the first and second disease assessments in fields 3 and 4 planted with cvs. Shepody and Ranger Russet respectively. Initial full bloom in field 5, a late-season planting, occurred after ascospore numbers decreased drastically in Petri dishes.

Stem rot did not develop in fields 2, 6, 9 and 10. Considerable flower colonization occurred on the ground in fields 2 and 6 but stems did not become infected. Mycelium of *S. sclerotiorum* could not be detected on blossoms attached to plants and on the soil surface in fields 9 and 10.

Apothecia emergence coincided with row closure in fields 1, 2, 4, 5, 7 and 8, where 7 to 11 apothecia were counted in 80 m of row along the sample transect. No apothecia were observed in fields 6, 9 and 10.

Disease incidence in plots where flowers were removed in field 7 was significantly less than in control plots ($P<0.05$), but was not significantly different from disease incidence in plots treated with iprodione and fluazinam ($P>0.05$) (Table 2).

Monitoring of precipitation. Disease incidence in 2001 and 2002 were not related to irrigation water amounts applied (P>0.05) and coefficients of determination (r^2) were all □ 0.15.

Fungicide trials. Fungicide applications prior to row closure did not prevent the occurrence of stem rot in fields 1, 3, 4, 5, 7 and 8 (Fig. 3). Stem rot incidence was not different between treated and control areas in field 1 (Table 3) and field 7; applications at initial full bloom in field 7 resulted in significantly less disease in treated plots than in non-treated control (Table1). In field 1, chi-square results indicated no significant difference in the number of lesions observed between the control and wedges treated with dichloran, flazinam, iprodione and iprodione+quintozene; a significant difference was found with dichloran+quintozene that had a higher disease incidence (Table 3). The confidence interval for dichloran+quintozene was greater than the control mean estimate, indicating that the control had less disease than the treated wedge (Table 3).

Lesions did not develop on plant stems treated with iprodione, fluazinam or dichloran in greenhouse experiments. Lesions developed two days after inoculation on 100% of the untreated control stems (Table 4). The absence of disease in the fungicide treatments and 100% incidence in the untreated control precluded the need for statistical analyses.

Discussion

Epidemics of Sclerotinia stem rot of potato in six studied fields in the Columbia Basin were primarily initiated by ascospore production from May to mid-July. Studied fields were subjected to aerial inoculum early in the season prior to row closure and prior to apothecial emergence within the fields, suggesting that a substantial portion of ascospore inoculum originated outside studied fields. Apothecia emerged in studied fields soon after the plant canopy shaded the soil. Stem lesions initiated by ascospores became evident 14-20 days after row closure. The limited number of apothecia that emerged within studied potato fields and their relative short duration do

not account for the entire duration that ascospores were captured or the spatial pattern of those ascospores along the radius of fields. It is possible that if timed with full bloom, apothecia within a field could induce a substantial portion of the disease observed, but their relative significance as intra-field sources of inoculum is still unknown and necessitates further research. In field 10 where no apothecia were observed and no ascospores captured, no symptoms were observed, indicating the significant contribution of ascospores in the disease cycle.

Flowers proved to be a crucial link between the pathogen and host for development of Sclerotinia stem rot on potato. Flower blossoms became contaminated by airborne *S. sclerotiorum* ascospores when still attached to the plant canopy and acted as ascospore receptacles. This is similar to studies on canola where ascospore presence on flower petals was positively correlated with disease incidence (17,30) and on bean where blossoms were described as "essential intermediaries" between the fungus and the host (2). Ascospores evidently remained inactive until blossoms fell and senesced, after which they rapidly colonized blossom tissues (14,27,30) if within the humid microclimate of the crop canopy. Mycelium of *S. sclerotiorum* was then transferred from colonized blossoms to green stems as blossoms fell on foliage and when stems came in contact with colonized blossoms on the soil surface. Disease transmission occurred 14 to 20 days after row closure depending on cultivars and cultural practices. In fields 2 and 6, blossom colonization was observed on the soil surface following initial full bloom, but disease failed to occur as plants did not lay on the ground until a month after initial full bloom. This lag time allowed for the disintegration of the mycelial mats on the soil surface. Growers could employ such a disease escape strategy by delaying massive nitrogen inputs until after initial full bloom, in time for tuber setting and bulking.

All fungicides currently registered for the control of stem rot in potato are contact fungicides making them prone to wash off with daily irrigations or breakdown by sunlight. Furthermore, a

lag period of 14 to 20 days between fungicide applications at row closure and disease occurrence would allow protectants to be weathered and washed from stems, while applications at initial full bloom provide protection at a more appropriate timing.

Fungicide applications or removal of flowers at the onset of full bloom reduced the number of *Sclerotinia* stem rot lesions on potato stems (Table 2). Fungicide applications on potato, in field 9 and the experimental wedge in field 7, at initial full bloom reduced stem incidence because blossom contamination and subsequent colonization of blossoms by *S. sclerotiorum* ascospores was reduced at the time of peak ascospore release. Applications of iprodione, iprodione + quintozene and dichloran before row closure did not reduce incidence of Sclerotinia stem rot (Table 3 and Fig. 3). Blossoms had not developed and were not subsequently protected by fungicide applications. Similar observations were made when bean flowers were protected with benomyl at full bloom, which prevented white mold development even under optimal conditions (2,21,27). Applications of benomyl made after full bloom failed to provide effective stem rot control as flowers had already acquired the inoculum (20,21).

Similar to earlier studies (2,27) fungicides applied in all studied fields before full bloom had no impact on disease assessments performed in those fields. Differences in disease incidence between treated and untreated portions in fields 1 and 7 were found not significant, and disease incidence increased rapidly when effective inoculum in the form of contaminated blossoms became available.

There was minimal secondary stem rot spread from infected stems in studied fields. Secondary spread was restricted to stems coming in contact with infected plant parts. This indicates that if blossoms were protected with fungicides, application skips in a field would unlikely be a source of secondary increase in disease incidence.

A relationship was not found in any field between the amount of irrigation water applied and incidence of Sclerotinia stem rot. This indicates that amounts of water applied to fields are sufficient for disease development, similar to conclusions from soybean in the northern Great Plains (32). Furthermore, research on bean in Nebraska indicated that reduced watering frequencies decreased within-field apothecial production as well as white mold severity (27).

Planting dates and cultivar choices influence stem rot incidence, as fields planted earlier in the season are subjected to an extended ascospore-release period, while those planted later may escape ascospore releases that tend to decrease notably in July. Field 5 was planted late in the season and had a lower stem rot incidence compared to other fields (Fig. 3), as a result of a sharp reduction in the numbers of captured ascospores at initial full bloom. Field 5 was planted with the indeterminate cultivar Gem Russet and were it not for the delayed planting, it would have been expected to sustain a higher incidence of stem rot as a result of the extended growing season, repetitive blooming and constant vine growth.

Sanitation practices directed at reducing initial inoculum within a field, such as crop rotation and destroying sclerotia may not be effective control measures. This is because of the ubiquitous nature of *Sclerotinia sclerotiorum*, the survival of sclerotia in soil, and the large inoculum contribution of ascospores from neighboring and more distant fields. This conclusion agrees with reports from other crops where airborne ascospores contributed the majority of the inoculum and basal stem infections by direct mycelial germination being minimal to inexistent (8). An area-wide management scheme by neighboring growers could play a significant role in reducing the ascospore amounts generated in an area, or by restricting the period of ascospore release.

The epidemiology of stem rot in potato seems highly correlated with crop management decisions as a result of extended ascospore release periods and the relative inconsequentiality of direct mycelial germination. Even though no experiments were conducted to compare various

cultivars with regard to their growth habit it would seem that the choice of determinate or indeterminate cultivars plays a crucial role in the ability to manage the disease economically. Cultivars with a horizontal and indeterminate growth habit like Gem Russet are more likely to suffer higher stem rot levels, because of the high likelihood of contact with colonized blossoms on the ground and repeated blooms. If successfully bred, potato cultivars with an erect architecture would have a better inherent control through a reduced or delayed contact with the mycelial mats on the soil surface. Furthermore, the development of cultivars that do not bloom might offer economical means of stem rot control, especially since true seed is not consumed or used for commercial propagation of potato in developed countries.

Iprodione, dichloran and fluazinam effectively protected 100% of the treated plants from infection by *S. sclerotiorum* in greenhouse trials, when all surfaces of stems were thoroughly covered with fungicides to avoid skips. Reduced effectiveness in fields is best explained by improper application timing, inadequacy of blossom coverage, weatheribility (especially washing off by daily overhead irrigations) and reduced sensitivity to fungicides. Fungicide applications to bean blossoms at full bloom produced high levels of control, while applications made on stems and leaves, but not flowers, did not deliver stem rot control (2,27). Two fungicide applications may be warranted in indeterminate potato cultivars, with repeated blooming per season especially during ascospore production peaks.

In light of the recognition of a more appropriate timing for fungicide application, a re-evaluation of the efficacy of fungicides in reducing yield losses should be undertaken to elucidate the extent of protection provided. Similarly studies should be conducted to evaluate the contribution of apothecia emerging within potato fields around the time of initial full bloom in the disease cycle, as well as investigating the contribution of apothecia emerging in other fields.

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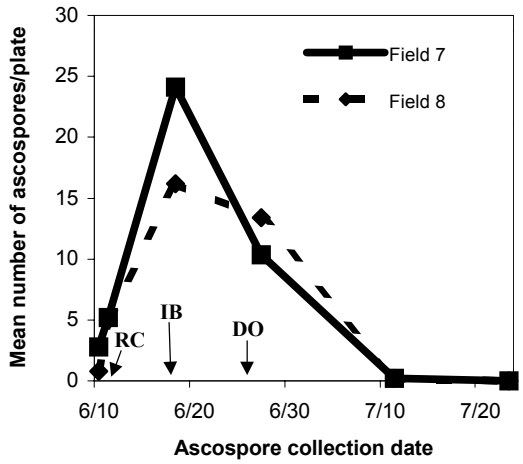

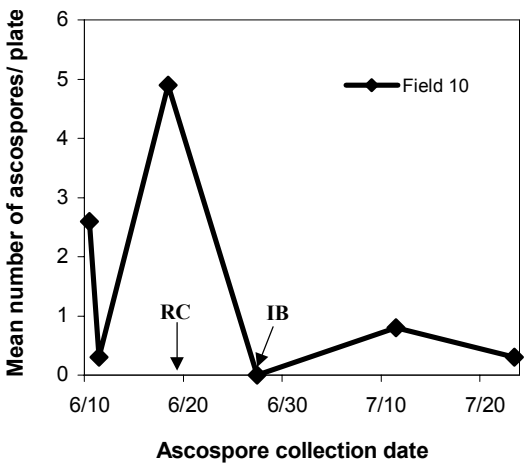

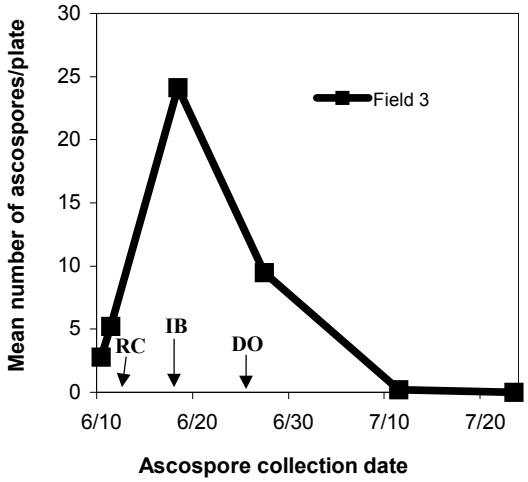

Fig. 1: Mean number of ascospores captured in north Pasco, WA (field 3) in 2001, north Pasco, in 2002 (fields 7 and 8) and west of Basin City, WA (field 10) in 2002. (RC: row closure, IB: initial full bloom, DO: disease onset)

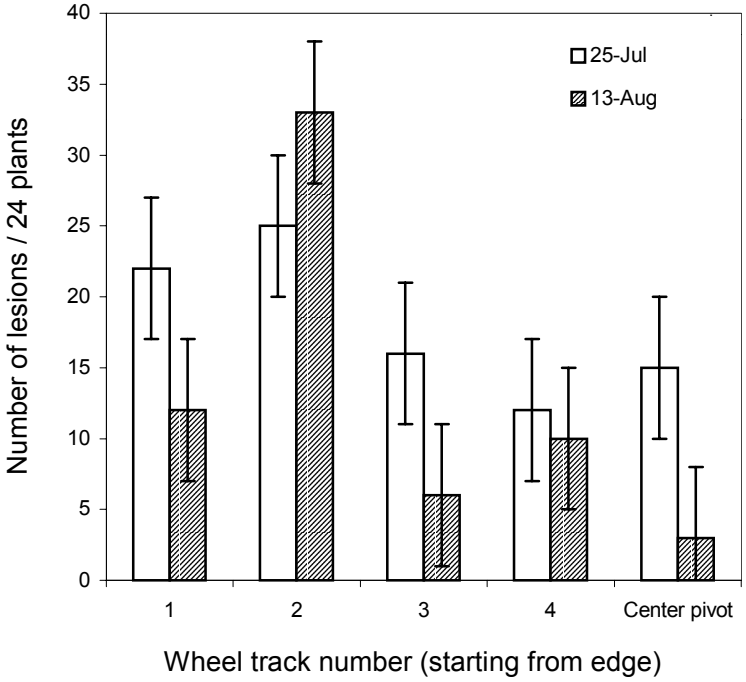

Fig. 2: Means of Sclerotinia stem rot incidence on two dates and at five locations along a transect from the edge to the center of circle of cv. Gem Russet, in 2001. No significant differences were found among disease assessment sites ($P > 0.05$), but counting dates are significantly different from each other ($P < 0.05$).

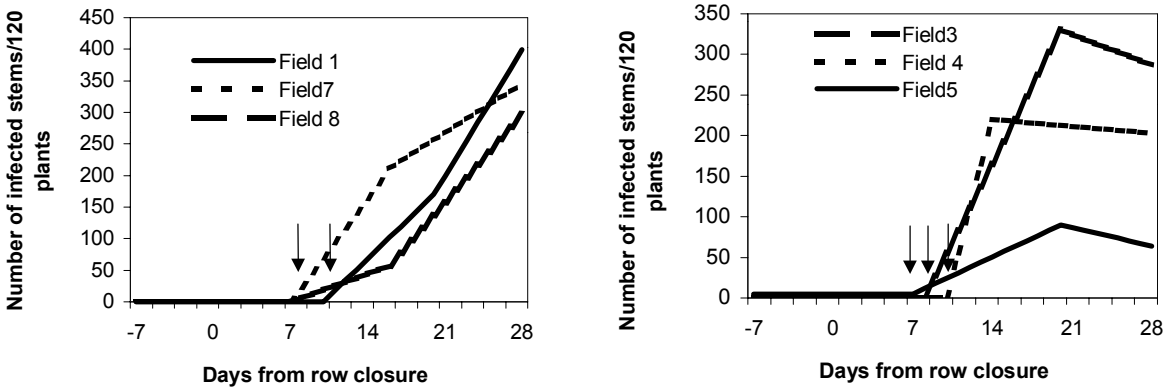

Fig. 3: Disease progress curves of Sclerotinia stem rot in commercial fields of cvs. Shepody (field 3), Ranger Russet (fields 4, 7 and 8) and Gem Russet (field 1 and 5) in the Columbia Basin. Arrows indicate date of initial full bloom.

Table 1: List of locations of studied potato fields. All fields are located in Washington State and the Columbia Basin Project.

	Field	Location	Cultivar
Southern Columbia Basin	1	50km Southwest Pasco	Gem Russet
	2	50km Southwest Pasco	Ranger Russet
	3	10km North Pasco	Shepody
	4	10km North Pasco	Ranger Russet
	7	10km North Pasco	Ranger Russet
	8	10km North Pasco	Ranger Russet
Northern Columbia Basin	5	10km West Warden	Gem Russet
	6	5km North Eltopia	Alturas
	9	15km West Basin City	Russet Burbank
	10	15km West Basin City	Umatilla

Table 2: Incidence of Sclerotinia stem rot when potato cv. Ranger Russet was treated with one of two fungicides or when blossoms were removed from the crop canopy

Treatment	Mean number of lesions [a]/ 10m of row
Iprodione	27
Fluazinam	16
Blossom removal	25
Control	87*

[a] Each value is a mean of four replicates. Values followed by an asterix is significantly different (P=0.05) from other values using the Fischer-LSD test

Table 3: Incidence of Sclerotinia stem rot in wedge-shaped section of a potato field of cv. Gem Russet treated with various fungicide and mixture of fungicides just before row closure.

Treatment	# Lesions/ 120 plants[a]	□[2]
CK	60	
Fluazinam	65	0.2
Dichloran	75	1.666
Iprodione	58	0.033
Dichloran + Quintozene	93	7.117**
Iprodione + Quintozene	53	0.433

[a] Each value represents the sum of five assessment sites per fungicide-treated wedge
** $P<0.01$

Table 4: Mean number of lesions when cv. Russet Burbank plants were treated with various fungicides or mixtures of fungicides and then inoculated with *S. sclerotiorum* in the greenhouse

Fungicide	Active ingredient	Rate[a] (l product/ha)	Amount/Plant[b] (ml product)	Lesion Number	Percent infected stems
Botran 5F	Dichloran	4.75	0.189	0	0
Botran 5F	Dichloran	2.35	0.095	0	0
Rovral 4F	Iprodione	2.37	0.063	0	0
Rovral 4F	Iprodione	1.66	0.032	0	0
Botran 5F + Rovral 4F	Dichloran + Iprodione	2.35 + 1.66	0.095 + 0.031	0	0
Omega 500F	Fluazinam	0.5	0.015	0	0
Control	-	-	-	50	100

[a]: the first rate is the label rate and the second is a half-rate
[b]: calculated amounts were dissolved in 10ml of distilled water and thoroughly sprayed onto individual stems

Chapter Two

High genetic variability, phenotypic uniformity and outcrossing in populations of *Sclerotinia sclerotiorum* in the Columbia Basin of Washington

Abstract

Sclerotinia sclerotiorum, the causal agent of stem rot, is widely distributed on various crops in Washington State and is not satisfactorily managed on potatoes. Because of the ubiquitous nature of the fungus and the size and crop diversity of this region, understanding population diversity of the pathogen could be helpful in developing management strategies. Canadian and southeastern US populations of *S. sclerotiorum* were previously described as clonal, with a few genotypes forming the majority of populations. Analyses using microsatellite markers in the present study revealed high genotypic variability. Population substructure analyses failed to differentiate between populations based on host or geographic location, while pairwise comparisons distinguished between some populations in the Columbia Basin. Out-crossing and a suspected high level of migration with vegetable and legume seed transport likely account for most of the high variability. In agreement with the discovery of a significant rate of outcrossing, no correlations were found between genotypes and mycelial compatibility groups, *in-vitro* response to fungicides and temperature stimuli or aggressiveness on potato stems.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an ascomycetous necrotroph reported to cause disease in about 400 host species (3, 4, 31). *S. sclerotiorum* is described as a homothallic fungus dispersed as airborne ascospores or soilborne sclerotia. Epidemics are initiated when ascospores land on open blossoms on the canopy. Contaminated flowers fall on stems and on the ground and fungal mycelia rapidly colonize the blooms. Stems or leaves contacting colonized blossoms acquire the disease. A mixture of ascospores generated within a potato field or blown in from neighboring fields causes the vast majority of observed lesions. Flower removal or fungicide applications at initial full bloom drastically reduced disease incidence (Kanaan-Atallah and Johnson, unpublished data). Infrequently, plants are infected at soil level by mycelia growing from sclerotia in close proximity of the plant crown.

A clonal population of *S. sclerotiorum* was identified in the "canola belt" of Canada with a restricted number of clones accounting for a large part of the population; a number of other genotypes were recovered infrequently (6, 21, 22). Clonality is described as the repetitive recovery of the same genotypes over an extended period of time and a large geographic area (24). A single clone was repeatedly sampled over 4 years, across 2000Km (1, 24). While outcrossing was reported in North Carolina (6, 20), studies conducted in Canada produced no indication of such events (21, 22). Comparisons between wild and agricultural populations of *S. sclerotiorum* in Norway exhibited tendencies for genotypic uniformity in isolates from potato and canola, and a higher diversity was found in isolates from the wild *Ranunculus ficaria* (buttercup, lesser

celandine) (24). Wild populations of *S. sclerotiorum* were characterized by spatial structuring while agricultural populations showed a random distribution of genotypes.

Mycelial compatibility groupings (MCGs) and DNA fingerprinting have been used to assess populations of *S. sclerotiorum*. Mycelial incompatibility is described as "a failure of different strains to fuse and form one colony" and is characterized by the formation of a barrage of dead cells between two incompatible colonies (25, 26). This technique allows for easy genotype identifications since several loci are believed to control mycelial compatibility, but does not identify heterokaryon incompatibilities (11, 26). Attempts to cross different clones of *S. sclerotiorum*, without forcing markers, have failed to produce heterokaryons; nevertheless, naturally-occurring "cryptic sex" events have not been ruled out as a potential source of genetic variability (20, 21). The occurrence of some level of outcrossing (sexual recombination) or heterokaryosis could explain the high variability of genotypes collected in Canada, but would be hard to distinguish from high levels of mutation (20). Heterokaryon generation by Ford *et al.* (11) and vegetative compatibility grouping (VCG) assignments did not correlate with MCG, indicating that the two phenomena are governed by different genetic determinants.

Simple sequence repeats (SSR) or microsatellite loci have been successfully used for intraspecific population studies to investigate genetic structuring of populations (3), especially since they are widely dispersed and evenly distributed in the genome of eukaryotes (12, 39). Microsatellites are characterized by high allelic variation in the numbers of 2 to 5 base pair (bp) repeated units due to elevated rates of mutation (18, 39). Mutation rates in microsatellites are best described by the stepwise mutation model (SMM) where the accumulation of polymorphisms is by replication slippage or by loss of

repeats (3, 10, 12, 18, 33, 39), rather than substitution like in minisatellite markers (12). The advantages of microsatellites over other markers, such as RAPDs, are their high specifity, reproducibility, polymorphism, co-dominance, easy handling and scorability (17, 38). Null alleles, which might be induced by reaction conditions, are rare in microsatellites and are treated as missing data, while they are scored in RAPD (38). Due to their inherent high variability of microsatellite loci, underestimations of population differences by as much as one order of magnitude in comparison to traditional markers (e.g. allozymes) have been reported (2, 17). Such underestimations are exacerbated when gene flow is restricted and with constraints on the number of repeats and back mutations. Also, statistical analyses based on comparisons of homozygosity indices between subpopulations and the total population may loose some of their discriminatory powers. Nevertheless, the high variability of microsatellite markers would improve the differentiation among individuals of a clonal organism such as *S. sclerotiorum*.

There is no published information on the population biology and sexual recombination of *S. sclerotiorum* in the US Pacific Northwest, where the fungus causes disease on a number of economically important crops such as potato, mint, vegetable seed crops, canola, etc. Also, no studies have been completed on the aggressiveness of available isolates (the quantity of disease induced by a pathogenic strain on a susceptible host) and response to fungicides. Clonality could be influenced by a selection for isolates that are highly aggressive, least in sensitivity to fungicides and highly adapted to environmental conditions.

The objectives of this study were to: (i) elucidate the population structure of *S. sclerotiorum* on potato in the Columbia Basin of Washington; (ii) compare isolates

collected from potato to isolates collected from other crops in Washington and other North American regions; and (iii) evaluate the aggressiveness of the various collected genotypes as well as their response to fungicides and temperatures.

Materials and methods

Isolates. Isolates of *S. sclerotiorum* from potato and other hosts were obtained from fields in the Columbia Basin and from collaborators in Idaho, North Carolina, Nebraska, and Ontario, Canada (Tables 1 and 2). In 2000, isolates from potato were isolated from stem lesions in four fields near Othello, WA (fields OA, OB, OC and OD); isolates were collected from a randomly selected 10x10m plot at initial disease appearance (Table 1). In 2001, potato isolates were collected from two fields southwest and north of Pasco, WA (Fields SP and NP, respectively) and one near Warden, WA (Field WD). Three sites per field were sampled along a randomly selected transect running from the edge of the field to the center of the irrigation pivot. Fields were separated by more than 50Km. Isolates were collected in three ways. (i) Prior to initial full bloom, plates of a semi-selective medium (15, 37) were exposed on top of plant canopies for 30 minutes between 9:00 AM and 2:00 PM, to capture ascospores (15). (ii) At initial full bloom, flowers were collected from the same ascospore trapping sites and plated on the semi-selective medium. (iii) At first disease appearance, about 14-20 days following row closure, potato stems with lesions were collected and infected tissue was plated on potato dextrose agar (PDA). All isolates were purified by transferring single hyphal tips to Petri plates containing PDA and generated sclerotia were stored at -20°C until used.

Genetic variation: mycelial compatibility groups. All 167 potato isolates were subjected to MCG (mycelial compatibility group) testing. Three 2mm mycelial plugs

were paired in 6.5cm diameter Petri plates containing potato dextrose agar (PDA) amended with 175µl per liter of McCormack's red food coloring (25, 30). Agar plugs were obtained from the edge of colonies growing on non-amended PDA. Plates were incubated in the dark at 25°C for 7 days. Pairings of the 167 potato isolates were performed in a pyramid design, where groups of 10 isolates were paired in all-pairwise combinations; only non-compatible isolates were paired subsequently. Compatible isolates were distinguished by the fusion of mycelia. Incompatible reactions produced a barrage recognized by an obvious red line on the bottom side of Petri dishes, or by the formation of aerial mycelia along the barrage line.

Genetic variation: analysis of microsatellite loci. Agar plugs from the growing edge of colonies were transferred to potato dextrose broth in 250ml flasks and allowed to grow for 5 days under constant shaking to increase biomass for DNA extraction. Mycelia were filtered on miracloth (Calbiotech; San Diego, CA) and freeze dried. Fifteen milligrams of dried mycelium were then subjected to DNA extraction with Fast-DNA extraction kit (Qbiogene; Carlsbad, CA) according to the manufacturer's indications.

Twenty-five microsatellite primer pairs developed by Sirjusingh and Kohn (2001) were used to identify polymorphisms among 223 *S. sclerotiorum* isolates. Amplifications were performed following the procedures described by Sirjusingh and Kohn (35). PCR products were run on 5% polyacrylamide denaturing gels and stained with silver nitrate. Bands of differing sizes were scored accordingly, in increasing size order.

All isolates that failed to produce bands on polyacrylamide gels were amplified up to three times. Isolates that failed to produce any products after three amplifications with the specific primer pair(s) in question were considered to have missing data for those loci.

Relationships between populations and gene-flow patterns are mostly estimated using Wright's fixation index (F_{ST}) and/or Slatkin's R_{ST} (3,12, 27, 33, 36). F_{ST} is a function of migration and mutation, while R_{ST} is based on allele frequencies and independent of the mutation rate. F_{ST} and R_{ST} comparisons were performed in the FSTAT version 2.9.3.2 software (14).

The genetic structure of the population of *S. sclerotiorum* in the Columbia Basin was analyzed with the analysis of molecular variance (AMOVA) that uses an analysis of variance framework (9, 28). Computations were made in the Arlequin ver. 2000 software (34). AMOVA, initially developed to estimate population genetic structures from haplotype frequencies, was shown to compute an analogue to Slatkin's R_{ST} (8, 28). To adjust for the increased chances of type I-error due to the large number of comparisons performed, the applicable significance level for pairwise comparisons of populations was computed to be 0.00019 ($P<0.00019$ significant). Subsequently, the significance of P-values was based on the sequential Bonferroni corrections method (32).

Detection of outcrossing. Twelve apothecia were collected in a mustard field north of Pasco, WA, in October 2002, and inverted over water agar to collect ascospores. Twenty individual ascospores were transferred to PDA plates and incubated for 2 days at 25°C. Colonies from individual ascospores from each individual apothecium were paired in all combinations on PDA amended with red food coloring to test for difference in MCGs, which are indicative of outcrossing.

Response to temperature and fungicides. One hundred potato isolates, representing all MCGs were tested for *in-vitro* growth at a range of temperatures and fungicide concentrations. Isolates were grown on non-colored PDA and 2mm in diameter agar

plugs from actively growing colonies were transferred to new PDA plates and subjected to 10, 18, 25 and 33°C in the darkness for 3 days. Plates were arranged in a randomized complete block design (RCB) with three replicates. Agar plugs of colonies were also transferred to PDA plates amended with iprodione, dichloran, quintozene and fluazinam. Label rates were converted to µg a.i./ml culture medium on the basis of Petri dish areas (Table 3). Cultures were incubated in a RCB design of three replicated cultures in the dark at 25°C for 3 days and compared with the same isolate on non-amended PDA. Two colony diameter measurements were made at a 90° angle using Vernier calipers, and the average was retained. The experiments were repeated.

Data were analyzed by one-way analyses of variance (ANOVA) using PROC GLM in SAS (version 8.1, SAS Institute, Cary, NC). Fungicides and temperatures served as treatments while colony diameter functioned as observational units. Because data were not normally distributed, a rank transformation was implemented and the ANOVA was computed on the ranks.

Agressiveness. Potato plants used for aggressiveness tests were propagated from certified seed tubers. Seed pieces, about 13 g in weight, were planted in a commercial potting soil mix (Soil Conditioners, Zillah, WA) and subjected to a 16-hour photoperiod in the greenhouse at 25±5ºC. Inoculations were performed on 60cm long stems, by attaching potato blossoms colonized with one of 36 *S. sclerotiorum* isolates collected from the Columbia Basin to stems. Ten isolates representing MCG 1, five isolates representing MCG 6, five isolates representing MCG 10, and 15 isolates randomly chosen from the population in the Columbia Basin were used in the aggressiveness study. Blossom colonization was achieved by placing autoclaved blossoms on top of a growing colony of

S. sclerotiorum on PDA for 3 to 4 days. Each isolate was represented five times. Each colonized blossom was lightly wrapped with gauze to hold the flower on the stem and to preserve moisture. Plants were subjected to an intermittent mist for 24 hours in mist chambers. Misting was stopped after 24 hours but the plants were kept at above 95% relative humidity in the mist chambers for 48 hours. Ambient temperature was 16 ± 2°C at night and 27 ± 2°C during the day. On the third day post-inoculation, plants were transported to the greenhouse and kept at approximately 25°C during the day and 18°C at night for 3 additional days, and lesion lengths were measured using Vernier calipers. The experiment was repeated.

Data was analyzed by one-way ANOVA using PROC GLM in SAS. Isolate was the treatment and lesion length was the observational units. The data was not normally distributed and a rank transformation was used for the ANOVA test.

Results

Genetic variation: mycelial compatibility. Eighty-two MCGs were identified from 167 potato isolates. The largest MCGs were MCG 1, MCG 10 and MCG 6 representing 30%, 6.5% and 6% of the potato isolates, respectively (Fig. 2). Most of the other MCGs were collected only once, 16 other groups were collected twice to five times. All self-pairings were compatible. Isolates belonging to the same MCG had similar to highly variable microsatellite haplotypes.

MCG 1 was collected from all seven fields across the Columbia Basin. MCG 1 was collected in the 4 fields in 2000, only from lesions in the field in Warden and from ascospores only southwest and north Pasco in 2001. MCG 10 present only southwest and

north of Pasco, in the southern Columbia Basin. MCG 6 was collected in Warden in the northern Basin and included 4 isolates from stem lesions and one from an ascospore.

Genetic variation: analysis of microsatellite loci. Eleven of the 25 microsatellite primer pairs exhibited polymorphic alleles (Table 4). AMOVA analyses failed to separate isolates at any level and indicated a lack of population structuring. In all analyses more than 90% of variability was attributed to within-population variability, making segregation based on date, crop or geographic location impractical. Similarly, analyses of dimorphic loci using AMOVA yielded similar responses. Allele frequencies corresponding to the various collection dates in 2001 were not significantly different from each other, indicating no grouping by collection date in the 3 potato fields.

Pairwise comparisons between fields using F_{ST} and R_{ST} tests (Table 5), failed to detect differences between crops, dates and North American regions, but identified differences between potato fields, especially in comparisons between 2000 and 2001 isolates. Isolates from fields OA and OC were different from isolates from field OD and fields in the southern (fields SP and NP) and northern (field WD) Columbia Basin. Isolates from fields OA, OB and OC had similar allele frequencies and were separated from each other by 10 miles.

F_{ST}, R_{ST} and AMOVA tests yielded no significant differences among non-potato isolates in the Columbia Basin, regardless of the crop. Populations from potato, carrot, pea, mint or other crops in Washington State were not significantly differentiated from each other (Table 5). Comparisons among isolates from the other areas of North America produced similar results. Population comparisons using F_{ST}, R_{ST} and AMOVA tests failed

to separate between populations of *S. sclerotiorum* in the Columbia Basin and other North American regions.

Detection of outcrossing. Three out of twelve apothecia collected from the Columbia Basin in October 2002 contained ascospores belonging to different MCGs. This can be considered evidence of sexual recombination.

Response to temperature and fungicides. Fungal growth did not occur *in-vitro* at labeled rates of iprodione, dichloran and fluazinam. Mycelial plugs transferred from amended PDA plates to non-amended plates failed to grow when transferred from iprodione-amended plates, demonstrating a fungicidal activity. In contrast, plugs transferred from dichloran and fluazinam-amended plates grew normally after transfer to non-amended PDA thus demonstrating a fungistatic activity. All isolates grew at 0.145µg dichloran/ml and at $2.7x10^{-4}$µg iprodione/ml, while fluazinam prevented mycelial growth at $1.26x10^{-4}$µg fluazinam/ml. No differences in colony diameters were observed among isolates at all fungicide concentrations at which growth was not inhibited. Quintozene failed to prevent the growth of *S. sclerotiorum* colonies at the highest field rate (at 10.67 l Blocker 5F®/ha); growth reduction was 20% compared to non-amended controls.

S. sclerotiorum grew normally at 10, 18 and 25°C, but failed to grow at 33°C. Colony growth was delayed by 3 days at 10°C, but picked up rapidly thereafter. Colonies filled the 9cm diameter Petri dishes in 3 days at 18 and 25°C and 8 days at 10°C. Colony diameters did not differ at any temperature used.

Aggressiveness. Lesions were apparent on stems three days post-inoculation. All isolates produced lesions on potato stems, but lesion size and number of lesions did not differ significantly among isolates ($P > 0.05$).

Discussion

Ascospores were abundant at the time of initial full bloom in the various fields, and were regularly captured in Petri dishes on top of plant canopies between May and mid-July. Colonies produced by germinating ascospores usually belonged to different MCGs (Fig. 1). In comparison to previous reports of high clonality (6, 21, 22), *S. sclerotiorum* in the Columbia Basin showed high rates of genotypic variability. This disparity could be generated by the crop diversity in the Columbia Basin compared to other areas, where monoculture over large areas could be the selection factor shaping populations. The lack of differentiation between isolates of *S. sclerotiorum* from potato and other crops in the Columbia Basin could be viewed as indicative of a lack of host specialization of the pathogen. Fears by growers of having separate "potato" populations seem unsubstantiated by our analyses. This implies that control of *S. sclerotiorum* should be based on a global management regardless of the crop grown within a locality. Such a finding also indicates that fungicide alternations should be implemented across grown crops, in order to avoid building resistance to fungicides, especially that those registered to control potato stem rot are used to control *S. sclerotiorum* in other crops.

The observed differences in pairwise comparisons between *S. sclerotiorum* isolates collected in certain potato fields in 2000 and others collected in 2001 could be exacerbated by a geographic concentration of fields in Othello, WA, in 2000, as opposed to collections from a number of localities performed in 2001. But the lack of differentiation with isolates from other crops in the Columbia Basin could indicate new migrations in 2000 that were subsequently diffused in 2001. Such phenomena necessitate more careful studying to identify sources of variability in the Columbia Basin.

The observed outcrossing in the Columbia Basin could partially account for the greater number of clones in this area compared with the Canadian plains (6). Three out of twelve apothecia from the Columbia Basin had mycelially incompatible ascospores. Environmental or cultural conditions could prevent outcrossing in the Canadian plains and enforce a strict homothallism. The high diversity and proximity of host crops in the Columbia Basin might offer the fungus with better conditions for outcrossing. Microconidia described on hyphae, apothecia and sclerotial surfaces of *S. sclerotiorum* and other Discomycetes (7, 19, 23) were found essential to the fertility of apothecia in genera related to *Sclerotinia* (7), but were considered non-essential in the homothallic *S. sclerotiorum* (23). It is probable that higher levels of outcrossing occur in the Columbia Basin as a result of more favorable conditions for microconidia to fertilize receptive individuals. Another reason for outcrossing could lie in the high diversity of microsatellite haplotypes forming one MCG; hyphae of isolates belonging to same MCG could fuse and produce heterokaryons. Even though apothecia were readily produced *in-vitro* by several isolates used in this study, this does not preclude homothallic fungi like *S. sclerotiorum* from outcrossing if conditions are propitious. Further studies are required to quantify the extent of outcrossing because of its potential impact on white mold management in various crops.

Outcrossing mitigates the effects of selection by host, cultural practices and the environment on the genotypic selection. It also reshuffles the genome thus disassocating between markers that are physically unlinked (29). This disassociation of unlinked markers means that using genotypes from neutral molecular markers to predict certain phenotypic characters is rendered unachievable because of the lack of association

between markers. Even though a high correlation was found between DNA fingerprints and MCGs in previous studies (22), such a correspondence was not observed between microsatellite haplotypes and MCG groups in this study. MCGs encompassed isolates with varying microsatellite haplotypes, while fairly similar haplotypes were grouped in different MCGs. Looking at MCGs alone would have indicated a moderate level of clonality, especially since one group encompassed 30% of the potato isolates (Fig. 2). Nevertheless, no signs of clonality emerged from microsatellite haplotypes and only a handful of isolates shared identical haplotypes.

Isolates from potato collected in the Columbia Basin did not differ in aggressiveness on potato plants, sensitivity to fungicides or *in-vitro* response to temperature. The rationale behind such tests was that clonality of *S. sclerotiorum* populations could be greatly influenced by selection by any of those three factors. Previously, differences in isolate aggressiveness were reported from canola populations in Canada, but selection based on such a phenomenon was ruled out (22). The lack of differentiation in the current study could be induced by the nature of the test itself. A test based on the production of oxalic acid, reported to be the pathogenicity determinant in *S. sclerotiorum* (13), might provide additional information on possible differences.

Migration could also impact the genotypic variability of populations. Migration of isolates into the Columbia Basin was not investigated, but is expected to be relatively high since the transport of vegetative material is high and diverse. Seeds of a variety of crops (vegetables, legumes, grain crops, etc.) are imported for growing in the Columbia Basin; seed batches could easily carry sclerotia of *S. sclerotiorum*. Genotype migration (3), high mutation rates in microsatellite loci (2, 17) and the wide host range of *S.*

sclerotiorum (5) could induce the lack of differentiation between populations of the Columbia Basin and other regions in North America. Gene flow could be achieved by dispersal of sclerotia in vegetative material or by ascospore transport in air currents. Even though the extent of ascospore dissemination is deemed not to exceed 3 to 4Km (6), the capture of viable ascospores at high altitudes (up to 6000m) raises the possibility of long distance dissemination by air currents over natural barriers, such as the Rocky Mountains (40). Substantial gaps remain in our understanding of population differences between the various regions of North America, of the mechanisms controlling homothallism and outcrossing in *S. sclerotiorum* and dispersal of ascospores.

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Table 1: List of potato isolates of *S. sclerotiorum* collected from the Columbia Basin of Washington.

Location	Field code	Collection dates	# Isolates
2000			
Othello	OA	6/28	15
	OB	7/14	3
	OC	8/14	14
	OD	8/22	16
2001			
Warden	WD	6/18, 6/26, 7/25	41
North Pasco	NP	6/05, 6/18, 6/22, 7/13	42
Southwest Pasco	SP	5/30, 6/05, 6/10, 6/22. 6/29	36
Total			167

Table 2: List of non-potato *S. sclerotiorum* isolates from the Columbia Basin and other regions in North America

Region	Host	Contributor	Code	# Isolates
Columbia Basin, WA				
Pasco	Pea	G. Pelter	PePa	10
Othello	Pea	G. Pelter	PeOt	10
Grant Co.	Carrot	L. Dutoit	Cagr	7
Klickitat Co.	Carrot	L. Dutoit	Cakl	3
Royal City	Mint	D. Johnson	Mint	3
Grant and Klickitat Co.	Squash, Brussels sprout, etc.	G. Pelter and L. Dutoit	Miswa	7
Other regions				
Kimberley, ID	Bean	T. Miller	Id	5
Nebraska and North Carolina	Bean, various	J. Steadman and M. Cubeta	East	7
Ontario	Canola	L. Kohn	Lmk	4

Table 3: List of fungicides and rates used to identify sensitivity of *S. sclerotiorum* isolates from the Columbia Basin

Fungicide	Active Ingredient	Label rate (l/ha)	Rates used (µg a.i./ml culture medium)
Botran 5F	Dichloran	4.75	1.45, 0.145, $1.45x10^{-2}$, $2.9x10^{-3}$
Blocker 5F	Quintozene	10.67	$6.3x10^{-2}$
Omega 500F	Fluazinam	0.591	$6.32x10^{-2}$, $6.32x10^{-3}$, $6.32x10^{-4}$, $1.26x10^{-4}$
Rovral 4F	Iprodione	2.375	$2.7x10^{-2}$, $2.7x10^{-3}$, $2.7x10^{-4}$, $5.5x10^{-5}$

Table 4: List of microsatellite primers showing polymorphism with *S. sclerotiorum* isolates. Primers were developed by Sirjusingh and Kohn (2001).

Locus	Repeat Motif	Primer sequence (5' - 3')	Size Range (bp)	No of Alleles observed
5	$(GT)_8$	GTAACACCGAAATGACGGC	318 - 325	5
		GATCACATGTTTATCCCTGGC		
7	$(GA)_{14}$	TTTGCGTATTATGGTGGGC	160 - 172	3
		ATGGCGCAACTCTCAATAGG		
12	$(CA)_9$	CGATAATTTCCCCTCACTTGC	215 - 225	2
		GGAAGTCCTGATATCGTTGAGG		
13	$(GTGGT)_6$	TCTACCCAAGCTTCAGTATTCC	284 - 304	2
		GAACTGGTTAATTGTCTCGG		
17	$(TTA)_9$	TCATAGTGAGTGCATGATGCC	345 - 390	3
		CAGGGATGACTTTGGAATGG		
55	$(TACA)_{10}$	GTTTTCGGTTGTGTGCTGG	173 - 221	4
		GCTCGTTCAAGCTCAGCAAG		
92	$(CT)_{12}$	TCGCCTCAGAAGAATGTGC	374 - 378	2
		AGCGGGTTACAAGGAGATGG		
106	$(CATA)_{25}$	TGCATCTCGATGCTTGAATC	491 - 571	3
		CCTGCAGGGAGAAACATCAC		
110	$(TATG)_9$	ATCCCTAACATCCCTAACGC	362 - 378	3
		GGAGAATTGAAGAATTGAATGC		
114	$(AGAT)_{14}(AAGC)_4$	GCTCCTGTATACCATGTCTTG	351-391	4
A7	$(GT)_{10}$	GGACTTTCGGACATGATGAT CCTGATATCGTTGAGGTCG ATTTCCCCTCACTTGCTCC	202-212	2

Table 5: P-values of pairwise comparisons using F_{ST} statistics among *S. sclerotiorum* potato isolates (columns SP through WD) and other crops (columns Cagr throught PePa) from the Columbia Basin in Washington and isolates from other regions in North America (Columns East, Id and Lmk).

	SP	NP	OA	OB	OC	OD	WD	Cagr	Cakl	Miswa	Mint	PeOt	PePa	East	Id	Lmk
SP		0.06946	0.00016*	0.03121	0.00004*	0.09675	0.06916	0.03676	0.08251	0.00204	0.01906	0.00948	0.00425	0.00463	0.05418	0.03693
NP			0.00003*	0.02927	0.00001*	0.1025	0.00184	0.0599	0.22171	0.00639	0.00486	0.00608	0.00689	0.00072	0.13843	0.04283
OA				0.01203	0.00135	0.00011*	0.00008*	0.01203	0.0116	0.00213	0.03613	0.00033	0.00217	0.01183	0.03668	0.03483
OB					0.00465	0.07419	0.37547	0.39889	0.79967	0.10753	0.0993	0.07258	0.12243	0.59959	0.19907	0.39924
OC						0.00006*	0.00001*	0.00455	0.00485	0.00056	0.01883	0.0006	0.00033	0.00455	0.01838	0.01814
OD							0.0263	0.00825	0.37429	0.00645	0.01283	0.01294	0.02109	0.00562	0.05125	0.06444
WD								0.10083	0.39368	0.18144	0.00943	0.0002	0.00998	0.00184	0.12334	0.29487
Cagr									0.89872	0.01749	0.0998	0.29178	0.25003	0.1508	0.1009	0.40152
Cakl										0.10736	0.39793	0.3785	0.78458	0.19828	0.2983	0.49937
Miswa											0.04851	0.00143	0.01192	0.01708	0.0961	0.14188
Mint												0.02258	0.04736	0.10017	0.1675	0.16703
PeOt													0.28875	0.42768	0.15503	0.02262
PePa														0.01816	0.09452	0.28653
East															0.30103	0.10067
Id																0.33376
Lmk																

* $P<0.00019$

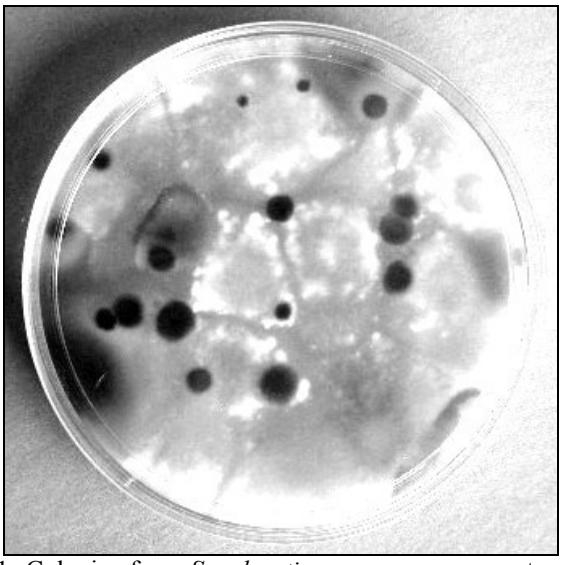

Fig. 1: Colonies from *S. sclerotiorum* ascospores captured on top of plant canopy. Colonies belong to different MCGs as evident by the formation of barrages between mycelia.

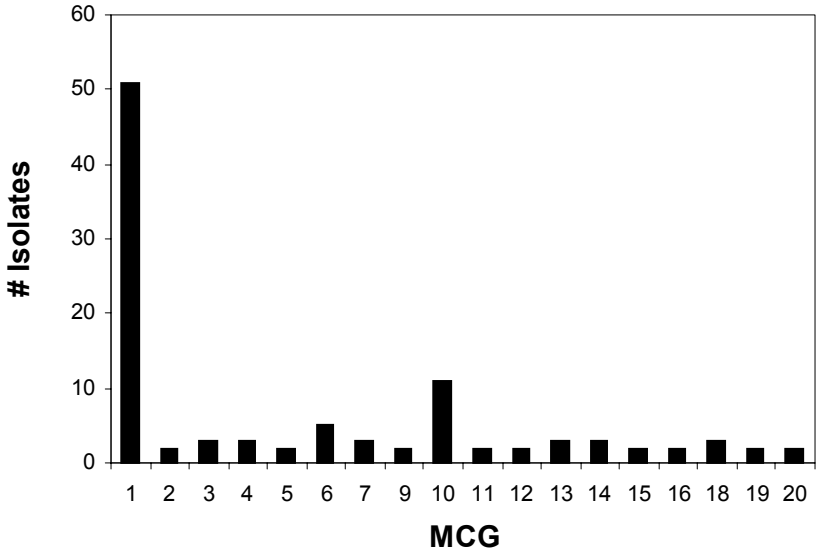

Fig. 2: Histogram of frequencies of the 20 MCGs including more than one individual out of a population of 167 potato isolates of *S. sclerotiorum*. Nine MCGs included 2 isolates, 6 included 3 isolates and another 62 MCGs were formed by individual isolates. MCG 1 comprised 51 isolates and was the largest group, MCG 10 contained 11 isolates and MCG 6 consisted of 5 isolates

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