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Case Study: *Sclerotinia sclerotiorum*: Genetic Diversity and Disease Control

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1. Introduction

Mutation, selection, gene flow between populations, and genetic recombination within them are the primary factors that determine the genetic structure and dynamics of populations. Genetic isolation of populations and drift can lead to genetic divergence and, eventually, speciation. In the Ascomycetes, the capacity for sexual recombination often coexists with enormous asexual reproductive capacity, and may be complemented by parasexual recombination. Some populations within a species have completely lost sexual competence, and the fully asexual species within the Fungi Imperfecti may have originated as such populations diverged from their ancestors. The balance between sexual and asexual reproduction, the contribution of recombination to fitness and pathotypic diversity, the trends toward exclusive asexuality, and the genetic isolation are particularly important in pathogenic fungi, and can have telling consequences for disease management (Taylor et al., 1999).

Sclerotinia sclerotiorum, an important fungal pathogen, offers a pertinent case study of the multidisciplinary approach required to clarify the significance of recombination in agricultural pathosystems. This homothallic ascomycete has a wide host range, sometimes causing extensive necrotic lesions on aerial structures of approximately 400 species of plants worldwide, including important crops and numerous weeds (Boland and Hall, 1994). *S. sclerotiorum* poses a threat to dicotyledonous crops such as sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry pea, lentils, and various vegetables, as well as monocotyledonous species such as onion and tulip (Boland and Hall, 1994). Annual yield losses due to *Sclerotinia* diseases exceed millions of dollars each year all over the world. Extensive crop damage, lack of high levels of host resistance, and the general difficulty of managing diseases caused by *Sclerotinia* have been the impetus for sustainable research on this pathogen.

2. *Sclerotinia sclerotiorum* reproduction and disease cycle

Sclerotinia sclerotiorum (Lib.) de Bary: kingdom Fungi, phylum Ascomycota, class Discomycetes, order Helotiales, family Sclerotiniaceae, genus Sclerotinia. As a homothallic organism, *S. sclerotiorum* reproduces sexually by self-fertilization, resulting in the formation of apothecia and airborne ascospores, and asexually by formation of sclerotia.

The basic disease cycle of *Sclerotinia* diseases begins with the sclerotium, a pigmented, multi-hyphal structure that can remain viable over long periods of time under unfavorable conditions for growth. Sclerotia can germinate to produce mycelia (myceliogenic germination) or apothecia (carpogenic germination), depending on environmental conditions (Steadman, 1983).

Carpogenic germination usually requires the sclerotia to be in wet soil for one to two weeks prior to germination (Figure 1E). At soil depths of up to 2 cm, apothecia can extend from the sclerotia to reach the soil surface. One or several apothecia can emerge from a single sclerotium (Figure 1F). Apothecia are fleshy-colored discs measuring 2 mm to 10 mm in diameter, lined with asci, which are filled with eight hyaline ascospores (110-160 µm x 6-10 µm) (Kohn, 1979). Each apothecium may release up to 10 million ascospores over a period of several days into the environment. Most will fall on susceptible plants in a 100-meter radius from the apothecia, but some can travel up to 3-4 km by wind (Adams and Ayres, 1979; Kohli et al., 1995; Cubeta et al., 1997). Furthermore, viable ascospores were captured at altitudes up to 6000 m (Williams and Stelfox, 1979), indicating a possible dissemination over long distances. Prolonged periods of leaf wetness (16-48 h) with temperatures in the range of 12°C to 24°C promote ascospore germination and infection (Adams and Ayres, 1979; Steadman, 1983).

The pathogen produces oxalic acid and numerous enzymes that break down and degrade plant tissue (Marciano et al., 1983; Godoy et al., 1990), and highly susceptible host tissues, such as flower petals or senescing leaves, are typically the first ones to become infected. With adequate wetness, mycelium moves from the colonized tissue into the vigorous host tissues of stems, leaves, pods, among others (Figure 1B, 1C). Later in the disease process, sclerotia form, either on the plant surface or within stems and other plant parts (Figure 1A, 1B, 1D). As the plant or plant part dies, the sclerotia fall to the soil, where they can survive for multiple years (Figure 1E) (Natti, 1971; Adams and Ayres, 1979; Boland and Hall, 1988).

The other method of germination is myceliogenic, where the sclerotium produces mycelium. White hyphal strands extend from sclerotia, which have been stimulated to germinate by host plant exudates, infecting roots, crowns, and other plant parts that are touching the ground. Infection then spreads to aboveground plant parts (Steadman, 1983).

Ascospores of *S. sclerotiorum* are a major source of inoculum in crops like bean, canola, and soybean, where aerial parts are affected (Steadman, 1983; Boland and Hall, 1988). In other crops, such as sunflower, direct mycelial germination of soilborne sclerotia affects roots and crowns (Huang and Dueck, 1980).

The major adaptation strategies that contribute to the success of *S. sclerotiorum* in establishing a parasitic relationship with crops is the ability of the fungus to continue producing sclerotia on diseased foliar debris even after being detached from the living plant, thus increasing the amount of inoculum in the soil.

3. Genetic diversity, population structure and their implications for disease control

Estimating the genetic diversity of pathogen populations in a region is important to understand the epidemiology of the disease and to establish the strategies for the control,

Fig. 1. Soybean plants showing symptoms of *Sclerotinia sclerotiorum*. Mycelium colonizing tissue into the host tissues of stems (A-B), leaves, and pods (C). Newly produced sclerotia on stem (A-B), diseased pods (D), and soil surface (E). Apothecia from sclerotia on soil surface (F). (Source: Mônica C. Martins, Fundação BA, Barreiras, BA, Brazil, 2008/2009 growing season).

such as the use of fungicides or resistant cultivars. The analysis of *S. sclerotiorum* structure and population dynamics is an essential part of understanding how the underlying mechanisms are involved in the history of this pathogen and its distribution across different geographic areas and wide host range.

3.1 Recombination *vs* clonal reproduction

There are two fundamental means by which fungi and other organisms transmit genes to the next generation – via clonal reproduction or via mating and recombination. Under clonality, each progeny would have one parent, its genome would be an exact mitotic copy of the parental one, and all parts of the genome would have the same evolutionary history. At the other extreme are genetically novel progenies formed by mating and meiotic recombination of genetically different parental nuclei, events that cause different regions of the genome to have different evolutionary histories. However, fungi do not fit neatly into these two categories. Recombination need not be meiotic or sexual because mitotic

recombination via parasexuality can mix parental genomes. Clonality need not be mitotic and asexual since self-fertilizing or homothallic fungi make meiospores with identical parental and progeny genomes. Furthermore, there is the complication that the same fungus may display different reproductive modes in different localities at different times (Taylor et al., 1999).

The methods currently available for studying microbial reproduction in nature can distinguish recombination from its absence, but they cannot reveal the mechanism nor estimate the amount of recombination (Taylor et al., 1999). Therefore, in this chapter, reproduction by recombination is defined as the production of progeny genomes that are mixtures of genetically different parental genomes, and reproduction by clonality is defined as the production of progeny genomes that are identical to the parental genomes.

S. sclerotiorum has a haploid somatic phase in which clonality is the result of both asexual reproduction by means of sclerotia and sexual reproduction by self-fertilization (Kohn, 1995) with the expectation that intraclonal variation is due to mutation (Carbone and Kohn, 2001a; Carbone et al., 1999). Predominant asexual or self-fertilizing modes of reproduction will determine a clonal population.

Prior researches on *S. sclerotiorum* population structure, in areas where canola, sunflower, cabbage, and other crops are grown, have shown that this pathogen populations are mainly clonal (Kohli et al., 1992, 1995; Anderson and Kohn, 1995; Cubeta et al., 1997; Kohli and Kohn, 1998; Carpenter et al., 1999; Hambleton et al., 2002), even though some recombination has been detected (Cubeta et al., 1997; Carbone and Kohn, 2001a), being more evident in subtropical populations than in temperate ones (Carbone et al. 1999; Carbone and Kohn, 2001b; Atallah et al., 2004; Sexton and Howlett, 2004; Sexton et al., 2006; Mert-Türk et al., 2007; Gomes et al., 2011).

Clonality of *S. sclerotiorum* in field populations has been evidenced in several ways: by detecting association of independent markers, such as mycelial compatibility groups (MCGs) and DNA fingerprints; by repeatedly recovering the same genotypes (over a wide geographic area or from year to year); or indirectly by statistical testing for lack of association of markers. Example of this was found by repeated recovery of MCGs in samples of *S. sclerotiorum* from canola made in Ontario in 1989 (Kohn et al., 1991), western Canada in 1990, 1991, and 1992 (Kohli et al., 1992, 1995; Kohli and Kohn, 1998), eastern Ontario and Quebec in 1999, and western Ontario in 2000 (Hambleton et al., 2002). Several clones were dispersed over large geographic areas, with clone 2 repeatedly isolated across 2000 km (recovered from Ontario, Manitoba, Saskatchewan, Alberta, and subsequently from cabbage in New York) over a 4-year period (Kohli et al., 1992; Anderson and Kohn, 1995).

Kohn et al. (1991) demonstrated that MCGs and DNA fingerprints, with a dispersed, repeated element pLK44.20, were linked in clonal populations of *S. sclerotiorum*. Members of different MCGs are incompatible, forming a reaction line where they meet; they also have DNA fingerprints that differ by five or more hybridizing fragments. In haploid organisms that reproduce asexually or by self-fertilization, genetically independent markers, such as MCGs and DNA fingerprints, will remain associated. In an outcrossing sexual fungus, independent markers will be shuffled and disassociated. Based on this concept, a group of isolates sharing the same DNA fingerprint and MCG is interpreted as a clone (Kohn et al. 1991; Kohli and Kohn, 1998; Carbone et al., 1999). Because a clone is a mitotic lineage, some

intraclonal variations due to mutations are expected. This is evident in variant fingerprints among isolates in an MCG (Carbone et al., 1999; Carbone and Kohn 2001b).

Characterizing the genotypic diversity in a population via MCG and fingerprinting constitutes a methodology that allows establishing the heterogeneity in the population and testing for clonality. Several works have deployed MCG typing in population studies of both *S. sclerotiorum* and *Sclerotinia minor* (Kohli et al., 1992, 1995; Cubeta et al., 1997; Carpenter et al., 1999; Carbone and Kohn, 2001b; Hambleton et al., 2002; Phillips et al., 2002; Durman et al., 2003; Hollowell et al., 2003; Kull et al., 2004; Atallah et al., 2004; Sexton and Howlett, 2004). Individual isolates are classified into clonal lineages by the use of two or more independent markers such as MCGs, DNA fingerprinting, or microsatellite markers (Kohn et al., 1991; Carbone et al., 1999; Sirjusingh and Khon, 2001; Hambleton et al., 2002; Auclair et al., 2004). DNA fingerprinting utilizes a probe for a multicopy transposon-like element in Southern blot analyses (Kohn et al., 1991). Microsatellite loci have high mutation rates and are multiallelic in nature, which makes them useful in phylogenetic inference (Sirjusingh and Kohn, 2001).

In an initial description of mycelial interactions in *S. sclerotiorum*, Kohn et al. (1990) reported relatively high mycelial incompatibility, with 21 strains incompatible with all other isolates and the remaining 10 strains tested forming four MCGs of two to three strains each. All self-self pairings were compatible. From each apothecium produced from four strains, all sibling ascospores were mycelially compatible with each other and with the parent homothallic isolate. This is consistent with selfed (homothallic), sexual reproduction, the results of which are indistinguishable from asexual reproduction in a haploid organism such as *S. sclerotiorum*.

Classification of isolates into MCGs is used routinely in many laboratories as a quick marker for genotyping *S. sclerotiorum* within populations. MCG determination is performed using a defined medium, originally developed for vegetative compatibility testing in *S. minor*, namely Patterson's medium (Patterson and Grogan, 1985), amended with red food coloring, and the current standard medium potato dextrose agar (PDA) (Schafer and Kohn, 2006). Compatible pairings formed one confluent colony, whereas incompatible pairings produced a visible reaction in the interaction zone, such as a red line visible on the colony reverse, or a line of fluffy, aerial mycelium or thin mycelium on the colony surface (Figure 2). Microscopically, challenging hyphae in compatible interactions did not necessarily anastomose.

MCG testing is a phenotypic, macroscopic assay of the self recognition system controlled by multiple loci common in fungi (Carbone et al., 1999). A distinction must be maintained between vegetative and heterokaryon compatibility, unless it is known that two strains not only anastomose but also form a stable heterokaryon (Schafer and Kohn, 2006).

In clonal populations, MCGs are transitive and each MCG is associated with one DNA fingerprint; additionally, no fingerprint is associated with more than one MCG. In clonal populations, a few MCGs are frequently sampled with many additional less frequent MCGs or singleton genotypes (Kohli et al., 1995; Hambleton et al., 2002). Even with some evidence of recombination, MCGs can still be identified and each one might be associated with a single fingerprint, or microsatellite, or RAPD genotype (Carpenter et al., 1999; Hambleton et al., 2002; Sexton and Howlett, 2004). If the population is recombining or presents genetic

exchange, markers, MCG, and fingerprint will not be associated. This has been observed in subtropical populations of *S. sclerotiorum* (Cubeta et al., 1997; Carbone and Kohn, 2001b; Meinhardt et al., 2002; Atallah et al., 2004; Sexton et al., 2006; Gomes et al., 2011; Litholdo Júnior et al., 2011). In a highly recombinant population, the expectation is that each isolate sampled, or most of them, is either incompatible with all other isolates or is part of an intransitive MCG, and each isolate either has a unique fingerprint or a fingerprint associated with more than one MCG. In temperate regions, clonal lineages persist over time, with new fingerprints and MCGs arising through mutation and infrequent outbreeding. Consistent with previous studies on *S. sclerotiorum*, there are more fingerprints than MCGs, an indication that transposition leading to new fingerprints occurs more frequently than mutational events resulting in new MCGs (Kohn et al., 1991; Carbone and Kohn, 2001b).

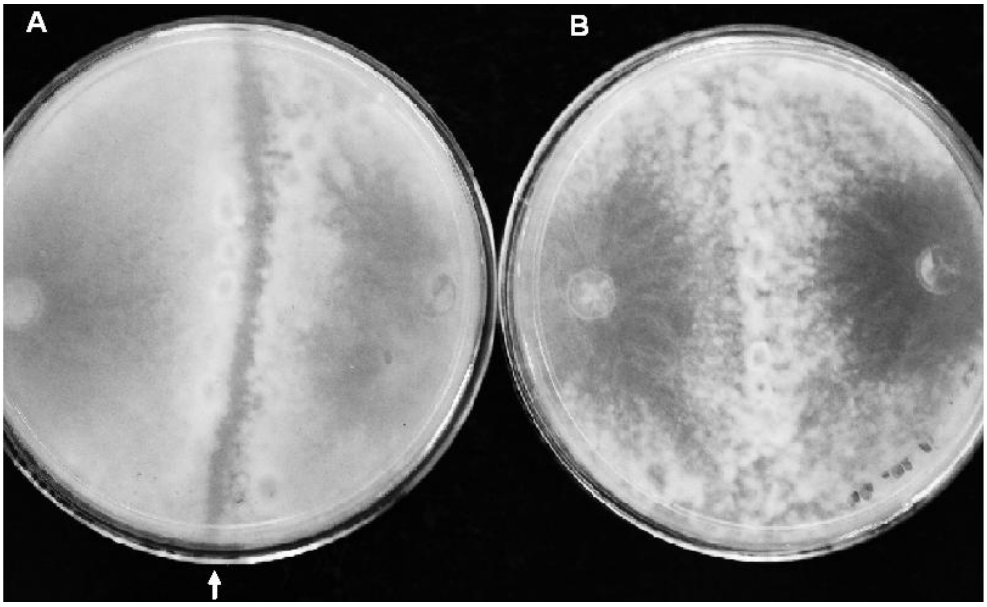

Fig. 2. Representative results from each mycelial compatibility group (MCG). (A) Incompatible reaction. (B) Compatible reaction. MCGs were determined using the method described by Schafer and Kohn (2006).

Factors influencing outcrossing and self-crossing are not well understood, but include weather and agricultural conditions affecting the timing and duration of various stages in the fungal life cycle. For example, if temperature and humidity are more favorable for ascospore production and survival (Clarkson et al., 2003), more simultaneous coinfection occurs, and subsequently more sclerotia might form after fusion of hyphae of two different genotypes, resulting in recombinant genotypes (Sexton et al., 2006).

3.2 Multiple genetic changes associated with gain of virulence

Although *S. sclerotiorum* is considered to exhibit little host specificity (Purdy, 1979), research is still in progress to determine whether genotypes of this fungus vary significantly in

aggressiveness or pathogenicity, or whether host cultivars show differential tolerance to pathogen genotypes. It is important to understand the diversity of this pathogen for the development of effective screening strategies to identify and deploy host resistance.

Pathogen aggressiveness is defined as the relative ability to colonize the host and cause damage, whereas virulence is considered the relative capacity to produce disease (Shurtleff and Averre III, 1997; Kull et al., 2004). Greenhouse inoculation studies on canola suggest that some *S. sclerotiorum* clones are more aggressive in forming lesions (Kohli et al., 1992, 1995).

Studies on *S. sclerotiorum* diversity and pathogenicity have been conducted for different crops. MCGs or microsatellite markers have not been associated with specific virulence characteristics or ecological adaptations of the pathogen, and lack of variation in virulence among isolates from defined geographic areas has been reported in a number of studies on agricultural populations (Kohn et al., 1991; Kohli et al., 1992; Cubeta et al., 1997; Atallah et al., 2004; Auclair et al., 2004; Kull et al., 2004; Sexton and Howlett, 2004; Sun et al., 2005; Malvárez et al., 2007; Litholdo Júnior et al., 2011).

Sexton and Howlett (2004) reported that among 44 isolates tested for virulence, the only two ones that caused relatively small leaf lesions were originally cultured from stem lesions rather than petals. One of these isolates had an identical microsatellite haplotype and MCG to three other stem isolates that caused large lesions, probably indicating that these isolates have genetic differences not reflected by microsatellite and mycelial compatibility markers. Lack of variation in virulence among *S. sclerotiorum* isolates has been noted in other studies. Atallah et al. (2004) found no significant differences in aggressiveness among 35 North American isolates from potato, and Auclair et al. (2004) tested isolates representing four Canadian clonal lineages and did not find any association between genotype and virulence in soybean. In contrast, Kull et al. (2004) reported variation in virulence among isolates from North America (Illinois) and South America (Argentina) collected from soybean. Variation in aggressiveness was highly significant for isolates in MCGs composed of members from different locations. MCGs do not appear to vary in aggressiveness. Although no isolate–cultivar interaction was detected, effective separation of resistant and susceptible cultivars was dependent on isolate selection (Kull et al., 2004).

Differences in virulence may be detected when comparing isolates from widely separate geographic regions. There has been no conclusive evidence to suggest host specialization among isolates of *S. sclerotiorum* (Kull et al., 2004). Diverse isolates of both *S. sclerotiorum* and *Sclerotinia trifoliorum* differed in virulence on alfalfa cultivars, and experiment–cultivar and experiment–isolate interactions were observed, but no isolate–cultivar interaction was detected (Pratt and Rowe, 1995). However, comparisons of *S. sclerotiorum* population on cultivated oilseed rape and on wild perennial host *Rannunculus ficaria* indicated major differences between agricultural and wild populations (Kohn et al., 1988). DNA fingerprint diversity is high in agricultural populations but low in wild populations and there is no evidence of outcrossing in agricultural populations, even though recombination occurs in wild populations.

While differences in pathogenicity or aggressiveness among *S. sclerotiorum* genotypes have been difficult to demonstrate, inconsistent correlations between field and greenhouse disease evaluations (Kim et al., 2000) may stem from incorrect assumptions of genetic

uniformity in pathogen populations. Greenhouse resistance screening programs may be conducted with one isolate of unknown relative aggressiveness and may not represent the range of pathogen variability existing in cultivated field populations. Designing field resistance evaluation experiments to eliminate or compensate for the variability in isolate aggressiveness and MCG profile may not be possible (Kull et al., 2004). It also makes sense to monitor pathogen genotypic diversity in performance trials; new crop varieties with partial resistance can be screened for durability against both established and emerging pathogen genotypes.

Primarily, the production of melanin in *S. sclerotiorum* is intended to protect sclerotia from adverse biological and environmental conditions. An association of melanin with pathogenicity has also been reported in other pathogens, such as *Magnaporthe grisea* and *Colletotrichum lagenarium* (Kubo et al., 2000). However, there are no reports accounting for a relationship between pigmentation and pathogenicity in *S. sclerotiorum.* Furthermore, Garg et al. (2010) found no correlation between pigmentation or colony diameter on PDA with the pathogenicity of different isolates of this pathogen, as measured by diameter of cotyledon lesion on the host genotypes.

Research on the molecular aspects of pathogenicity is mainly concentrated on the contribution of hydrolytic enzyme activity and production of certain metabolites that can act as toxins. This has been the major focus for research on *S. sclerotiorum* as well, with emphasis on the role of cell-wall-degrading enzymes (CWDEs) and oxalic acid. During the interaction with its host, *S. sclerotiorum* secretes a full complement of CWDEs, including pectinases, β-1,3-glucanases, glycosidases, cellulases, xylanases, and cutinases, which can facilitate penetration, macerate tissues, and degrade plant cell-wall components (Riou et al., 1991). Variation in pathogenicity has been associated with the production of pectolytic enzymes (Hancock, 1966; Lumsden, 1976, 1979; Errampalli and Kohn, 1995), cellulase (Lumsden, 1969), hemicellulase, phosphotidase (Maxwell and Lumsden, 1970), and oxalic acid (Noyes and Hancock, 1981; Marciano et al., 1983; Cessna et al., 2000). That oxalic acid is a necessary pathogenicity factor was shown by Godoy et al. (1990), who demonstrated that oxalic acid-deficient mutants were non-pathogenic. Also, at least part of the resistance to *S. sclerotiorum* infection among lines of the scarlet runner bean (*Phaseolus coccineus*) could be attributed to differences in oxalate sensitivity (Chipps et al., 2005). Nonetheless, variation in these factors does not appear to be the primary determinant of pathogenicity as correlations have not always been apparent (Morrall et al., 1972). As an example, in *S. trifoliorum*, a degenerative non-virulent isolate of the pathogen produces more proto-pectinases than a normal string, but only the normal isolate secretes a toxin and is considered virulent (Held, 1955).

In a recent study, our team (Petrofeza et al., data not published) evaluated the genetic interactions for disease response between cultivars of *Glycine max* and isolates of *S. sclerotiorum.* One of the hypotheses tested was the lack of interaction between production of CWDEs and aggressiveness of *S. sclerotiorum* isolates. Aggressiveness of field isolates varied, and this variation was highly significant for isolates in MCGs composed of members from different locations. Significant differences were detected in the activity level of the following CWDEs among the isolates: polygalacturonases, β-1,3-glucanases, cellulases, and xylanases. Furthermore, the level of enzyme activity does not appear to vary in aggressiveness. Nevertheless, the limited number of samples (80 isolates) may not be sufficient to accurately assess the relative contribution of CWDEs production in pathogen aggressiveness.

3.3 Global picture of genetic diversity in *Sclerotinia sclerotiorum* populations worldwide

The major focus of population genetics is to understand the evolutionary processes shaping and maintaining genetic variation within and among populations. Changes in genotype or allele frequencies in populations are considered evolutionary changes, although they often occur on microevolutionary time scales. The evolution of the species in response to selection by deployment of resistant host plants and the evolution of fungicide resistance in response to fungicide applications are perfect examples of population genetics problems in plant pathology. In this sense, plant pathology has provided many examples of how moving a fungus from its normal biogeographic range to a new environment across or between continents can change its reproductive mode and population structure.

The world population of *S. sclerotiorum* is composed of subpopulations that may be associated with phenotypic variability, such as MCGs and aggressiveness, some of which are older or isolated and endemic, whereas others are younger or highly dispersed. And certain crop samples have shown the occurrence of more recombination in subpopulations in subtropical climates than in temperate zones. Unveiling the structure and dynamics of *S. sclerotiorum* populations represents an essential part of understanding the underlying mechanisms involved in the pathogen history and distribution along geographic areas and different hosts. In a brief report, we describe the current knowledge about genetic diversity in *S. sclerotiorum* populations worldwide (Table 1).

Origin	Population/host	Analysis/method	Conclusions	Reference
Canada	Canola (*Brassica napus* L. or *B. rapa*)	MCG DNA fingerprinting Mitochondrial SSrDNA RFLP in Southern probe with mitochondrial 24S RNA from *N. crassa*	Population structure predominantly clonal. Each field infected by several clones. Individuals of some clones can be recovered from different geographic areas and over several years.	Carbone et al. (1999); Kohn et al. (1991); Kohli et al. (1992, 1995)
Norway	*Ranunculus ficaria*	MCG DNA fingerprinting	Genetic markers decoupled. Fingerprinting diversity low and localized. Evidence of some recombination. Spatial substructuring in the distribution of the fingerprints.	Kohn (1995)
USA (North Carolina/ Lousiana)	84 from four cabbage production fields in North Carolina and 16 isolates from an experimental cabbage field plot in Louisiana (*Brassica oleracea* L. var. *capitata*)	MCG DNA fingerprinting	Clonal, but deviated from one-to-one association of markers. Six clones recovered from fields 75 km apart. MCG was associated with more than one DNA fingerprint.	Cubeta et al. (1997)

Canada and USA	Canola (*Brassica napus* L. or *B. rapa*) Cabbage (*B. oleracea* L. var. *capitata*)	DNA fingerprinting	Clonality supported by statistical tests. Clonal fraction higher in canola samples. Some genetic exchange and recombination in cabbage samples.	Kohli and Kohn (1998)
Canada	213 isolates from 10 fields and three performance trials of soybean (*Glycine max*)	MCG DNA fingerprinting	Population structure predominantly clonal. Clones recovered from previous studies. Soybean infected by genotypes residual from other crop or weed hosts.	Hambleton et al. (2002)
USA and Argentina	Diverse hosts/locations, 24 isolates; Argentine set, 21 isolates; soybean set, 254 isolates.	MCG	Within widely dispersed MCGs, isolate aggressiveness varied.	Kull et al. (2004)
USA (Columbia Basin of Washington)	167 isolates from potato	MCG Microsatellite	High haplotypic diversity. Recombinant ascospores in apothecia examined. Microsatellite haplotypes were not correlated with MCGs. Potential for outcrossing.	Atallah et al. (2004)
USA (Alaska)	Vegetable crops	DNA markers	Populations predominantly, if not exclusively, comprised of non-recombining clonal lineages.	Winton et al. (2006)
USA and Canada	294 isolates, three populations: California (CA, lettuce), Washington (WA, pea/lentil), and Ontario (ON, lettuce)	MCG DNA fingerprinting Multilocus DNA sequence	Genetic exchange and recombination could explain the high diversity in California.	Malvárez et al. (2007)
USA (Salinas, San Joaquin, and Santa Maria Valleys in California)	Lettuce drop incidence and structure of *S. minor* and *S. sclerotiorum* populations	MCG	Populations of *S. sclerotiorum* exhibited greater diversity.	Wu and Subbarao (2006)
USA	69 isolates from four natural populations on lentils from eastern Washington	MCG Microsatellite	Clonal lineages dominate each population. 46 MCGs and 53 hyplotypes. Populations differentiated by both location and collection year.	Wang and Chen (2007)
Iran	38 isolates from canola in Iran	rep-PCR primers	High level of genetic diversity. Most isolates from the same regions grouped in the same cluster or in a close cluster.	Karimi et al. (2011)

Iran	48 samples isolated from 29 rapeseed fields in northern Iran	MCG Morphological characteristics	31 MCGs identified. High rates of out crossings as well as evolutionary potentials found within the population.	Barari et al. (2011)
Iran	65 isolates from infected rapeseed, lettuce, bean, tomato, cucumber and wild synapses plants in various fields of northern provinces in Iran	Microsatellite MCG	High level of genetic diversity. 39 MCGs and 26 hyplotypes. High rates of outcrossing as well as evolutionary potential within population of the pathogen in different collecting locations.	Barari et al. (2010)
Iran	276 isolates representing 37 field populations from four provinces in northern Iran	Microsatellite	Moderate levels of gene diversity. Occurrence of outcrossing. Populations randomly mate and have a number of shared haplotypes among regional populations.	Hemmati et al. (2009)
Iran	12 isolates from different provinces of Iran	RAPD	RAPD profiles markedly differed between isolates.	Colagar et al. (2010)
Jordan	25 isolates from vegetable crops in the Jordan valley	MCG RAPD Specific gene amplified	30 MCGs. Occurrence of genetic diversity.	Osofee et al. (2005)
Pakistan	16 isolates associated with the stem rot of chickpea from various localities of Pakistan	MCG Morphological characteristics	Differences in all morphological characteristics observed.	Akram et al. (2008)
Turkey	36 isolates from oilseed rape (*Brassica napus* L.) fields	MCG Microsatellite	High level of genetic diversity within and among population. 19 MCGs identified. Data suggest the possibility of a high rate of outcrossing as well as evolutionary potential within the population of the pathogen in oilseed rape fields.	Mert-Türk et al. (2007)
Turkey	119 isolates from cucumber plants in Kumluca, Finike, and Demre districts of Antalya	MCG Pathogenicity	70 MCGs. Significant differences determined in virulence of isolates within MCGs.	Yanar and Onaran (2011)
China, Canada and the United Kingdom	205 isolates: 5 from England, 33 from Canada, and 167 from China	MCG Pathogenicity	39 MCGs – 64% represented by a single isolate. Isolates from different countries were incompatible. Significant differences were found in radial growth, aggressiveness, production of oxalic acid, and total acids within and among MCGs.	Li et al. (2008)

China, Canada, and the United Kingdom	96 isolates from sunflower	SRAP markers	The five populations isolated from the three countries showed various levels of genetic variability. Population significantly differentiated from others.	Li et al. (2009)
China, Canada, Poland, and Slovakia	Populations from carrot (*Daucus carota* var. *sativa* DC), oilseed rape (*Brassica napus* L.), and soybean plants	RAPD	Highly significant genetic variation among and within populations. Isolates from East Mongolia, in China, and Manitoba, in Canada, were clustered in the same lineage without differentiation.	Sun et al. (2005)
China	76 isolates from different regions and hosts	SRAP markers	Little genetic difference identified among isolates from different host species. Variation among populations differed according to geographic and ecological region.	Chen et al. (2010)
New Zealand	75 isolates from four populations	MCG DNA fingerprinting	High level of variation both within and between populations.	Carpenter et al. (1999)
Australia	154 isolates from four Australian canola fields	MCG Microsatellite	Moderate to high levels of differentiation between populations. Both clonal and sexual reproduction contributed to population structure.	Sexton et al. (2006)
Australia	105 isolates from four Australian canola fields	MCG Microsatellite	Higher genotypic diversity. Occurrence of both clonal reproduction and outcrossing.	Sexton et al. (2006)
Australia	8 isolates from Mount Barker and Walkway regions of Western Australia	Multilocus DNA sequence Pathogenicity Colony characteristics	Significant differences between isolates in relation to pathogenicity. No correlation between pigmentation or colony diameter on PDA with the pathogenicity of different isolates.	Garg et al. (2010)
Australia	250 isolates from sunflower in Queensland and New South Wales	Multicopy Restriction Fragment length polymorphisms MCG RAPD	Individual sclerotia comprised of only one genotype. Temporal studies revealed genetic uniformity was maintained across years.	Ekins et al. (2011)
Brazil	21 isolates from bean (*Phaseolus vulgaris*), one from potato and one from bell peppers	MCG PCR/RFLP of the ITS1-5.8S-ITS2 ribosomal subunit regions Chromosomal telomere sequence Microsatellite	Genetic polymorphisms among isolates within the same MCG.	Meinhardt et al. (2002)

Brazil	isolates from bean (*Phaseolus vulgaris*)	Microsatellite ITS1-5.8S-ITS2 ribosomal subunit regions		Gomes et al. (2011)
Brazil	40 isolates from various fields widely distributed throughout Brazil	MCG RAPD	High level variability. Genetic differences between individuals. The host culture did not have a significant effect.	Litholdo Júnior et al. (2011)
Argentina **(Buenos Aires Province)**	140 isolates: bean (60 isolates), lettuce (59 isolates), and sunflower (21 isolates) fields	MCG	50 different MCGs. Populations from field crops are made up of various and different MCGs. No differences in aggressiveness between MCGs.	Durman et al. (2003)

Table 1. A list of reported *Sclerotinia sclerotiorum* genetic diversity.

3.3.1 *Sclerotinia sclerotiorum* populations in North America

Over a period of 20 years, population studies on *S. sclerotiorum* carried out in the Unites States and Canada have revealed a predominantly clonal mode of reproduction (Kohn et al., 1991; Kohli et al., 1995; Cubeta et al., 1997; Kohli and Kohn, 1998; Winton et al., 2006), with some evidence of outcrossing contributing to the population structure in a few temperate regions of North America (Atallah et al., 2004; Malvárez et al., 2007), as previously described in item 3.1.

In Canadian oilseed rape (*Brassica rapa* or *B. napus*) fields, clonality dominated in *S. sclerotiorum*, evidenced by the association of each of a relatively large number of DNA fingerprints with MCG. Nevertheless, no evidence of sexual recombination was found within a large population composed of a small number of clones, with a single clone repeatedly isolated across 2000 km over a 4-year period (Kohli et al., 1992; Anderson and Kohn, 1995).

3.3.2 *Sclerotinia sclerotiorum* populations in Asia, Europe and Oceania

A series of intensive analyses of population structure from fields in Australia have been performed. A total of 250 isolates, collected hierarchically from sunflower in Queensland and New South Wales, were proven to belong to one large genetic population of *S. sclerotiorum.* MCGs and molecular markers analyses showed that the majority of sunflower plants were infected by only one genotype and the temporal studies revealed that genetic uniformity had been maintained over the years (Ekins et al., 2011). This corroborates another research that indicated the existence of large populations of *S. sclerotiorum* in canola fields in Australia (Sexton and Howlett, 2004). *S. sclerotiorum* from canola fields in New South Wales were shown to be genetically divergent but with no corresponding difference in virulence from canola-derived isolates collected in Victoria. Genotypic disequilibrium measures were consistent with the occurrence of both clonal reproduction and outcrossing (Sexton et al., 2006).

Several reports concerning the population structure of *S. sclerotiorum* in Asia are available: Iran (Hemmati et al., 2009; Barari et al., 2010, 2011; Colagar et al., 2010; Karimi et al., 2011), Jordan (Colagar et al., 2010); Pakistan (Akram et al., 2008), Turkey (Mert-Türk et al., 2007; Yanar and Onaran, 2011) and China (Sun et al., 2005; Li et al., 2008, 2009; Chen et al., 2010). Different vegetable crop fields were analyzed in these studies and most authors came to the general conclusion that populations exist somewhere in the spectrum between sexual outcrossing and clonal.

In Norway, located in Northern Europe, comparisons between wild and agricultural populations of *S. sclerotiorum* exhibited tendencies for genotypic uniformity of isolates from potato and canola. Also, great genetic diversity was found among isolates from wild perennial host *Ranunculus ficaria,* evidenced by very low fingerprint and MCG diversity accompanied by disassociation of fingerprint with MCG on a very local spatial scale (Kohn, 1995).

Shared haplotypes of *S. sclerotiorum* have been found between countries in different studies. Sun et al. (2005) reported that the relationship between Canadian and Polish populations of *S. sclerotiorum* was very close, although the geographic distance between them was large. Also, Li et al. (2009) analyzed five populations of *S. sclerotiorum* isolated from sunflower in three countries, Mongolia, in China, Canada, and the United Kingdom. Despite the fact that various levels of genetic variability were found, the four populations from West, Middle, and East Mongolia and Canada were clustered into one subgroup, in which the isolates from West and Middle Mongolia belonged to one population and those from East Mongolia and Canada essentially belonged to another. Both East Mongolia and Manitoba, in Canada, are at a high latitude north (over 52°) and have similar climates, which supports the hypothesis that similar ecotypes in two sunflower production areas might produce genetically similar populations.

Comparison of allelic patterns of 22 Australian isolates (Sexton et al., 2006) with Iranian populations (Hemmati et al., 2009) revealed that 16 out of the 30 alleles are common between Australian and Iranian isolates, although no shared haplotypes were found (Hemmati et al., 2009). Furthermore, Australia appears to harbor populations of the fungus that are distinct from North American populations. In a study of southern Australian isolates, Sexton and Howlett (2004) found distinct alleles and allele frequencies from those detected in North America by Atallah et al. (2004). Also, in a study of northern Australian *S. sclerotiorum* isolates from sunflower, using DNA fingerprinting multicopy probe, all Australian genotypes were distinct from Canadian and American genotypes (Ekins et al., 2005).

3.3.3 *Sclerotinia sclerotiorum* populations in South America

In South America, information relating to the population biology of *S. sclerotiorum* is still emerging, with few reports coming from Argentina and Brazil.

In Argentina, *S. sclerotiorum* is one of the most important fungal pathogens of some economically important crops, causing diseases such as *Sclerotinia* stem rot of soybean, head rot of sunflower, and stalk rot of sunflower. In a study designed to analyze MCGs of 140 isolates of *S. sclerotiorum* obtained from soybean (60 isolates), lettuce (59 isolates), and sunflower (21 isolates) fields in a 6500-km^2 area of the Buenos Aires Province, the partition of total diversity showed that 98.4% corresponded to variations of diversity within populations. These populations were made up of 50 different MCGs (Durman et al., 2003).

In Brazil, white mold caused by the fungus *S. sclerotiorum* occurs primarily in many economically important crops, such as common bean, soybean, cotton, several vegetables, among others, and can cause serious yield losses. This pathogen was firstly reported in the country in the 1920s and spread to the main agricultural regions probably due to infected seeds. White mold is one of the major problems of highly mechanized irrigated winter production of common beans (*Phaseolus vulgaris*) and other vegetable crops in tropical regions in the states of São Paulo, Minas Gerais, and Goiás. During the dry winter months in these states, crops must be grown under center-pivot irrigation, which along with lower ambient temperatures create a microclimate that is suitable for infection and growth of *S. sclerotiorum*. In an initial work, 21 isolates of *S. sclerotiorum* were obtained from a center-pivot-irrigated field and MCG analyses revealed the presence of only two MCGs. PCR/RFLP analyses of the ITS1-5.8S-ITS2 ribosomal subunit regions of these field isolates failed to show any genetic differences between MCGs. DNA amplification with a chromosomal telomere sequence-based primer and one microsatellite primer revealed genetic polymorphisms among isolates within the same MCGs. Isolates taken from beans and two other crops from another region of Brazil showed the same two MCGs and had identical banding patterns for the telomere and microsatellite primers (Meinhardt et al., 2002). In a subsequent work, Gomes et al. (2011) analyzed 79 isolates of *S. sclerotiorum* collected from four common bean center-pivot-irrigated fields and using microsatellite markers observed high genetic diversity within and among populations. The authors suggested that the occurrence of exclusive alleles in the populations analyzed may indicate the introduction of new genotypes in these areas at different moments through contaminated seeds or agricultural implements, in addition to the occurrence of sexual outcrossing and clonal reproduction.

Some specific questions that should be addressed with respect to genetic diversity in *S. sclerotiorum* are:

1. Is *S. sclerotiorum* clonal and/or recombining in nature?
2. What is the relative contribution of both modes of reproduction to genetic diversity, in different locations, and at different times?
3. How variable are virulence factors among populations within the putative recombination belt in different regions?
4. What are the environmental limits to outcrossing sexual reproduction? Do agricultural practices in different regions affect the population biology of the pathogen?

4. Phylogenetics: Inferring evolutionary development

Plant pathologists have led the way in *S. sclerotiorum* population genetics, making fungi in genera such as *Sclerotinia* among the best understood in terms of how they reproduce in nature, the limits of their genetically differentiated or isolated groups, and their spread through space and time. However, a question that has driven much of the recent studies on population genetics of this pathogen has been, "*S. sclerotiorum is a pathogen of virtually all dicots worldwide. Is there a single population with a common origin or are there several populations associated with different geographic areas? Or associated with different hosts?*".

Phylogeography is the study of spatial distributions of genealogical lineages (phylogenies), especially lineages within species and among closely related species

(Taylor et al., 1999). Phylogenetic inference from molecular sequences (loci) is becoming an increasingly popular tool to trace the patterns of pathogen dispersal. Reconstructing both the evolutionary history and spatial process from these sequences provides fundamental understanding of the evolutionary dynamics. Single or multi-locus gene genealogies derived by phylogenetic, coalescent, or Bayesian approaches can be explored to estimate the contribution of the key drivers to evolution in populations: mutations, selection, changes in population size, gene flow, genetic exchange, and recombination (Carbone and Kohn, 2001a, b).

The evolutionary history of population haplotypes indicates that isolates of *S. sclerotiorum* may be grouped into subtropical, temperate, wild, and two relatively recently evolved temperate subtropical populations. Carbone and Kohn (2001b) reconstructed the evolutionary history of haplotypes, on both population and species scales, using a combination of parsimony, maximum likelihood, and coalescent methods. For the population level, samples from Canada, the United States, Norway, and New Zealand isolated from different hosts were analyzed. Samples from extensive crops, like canola (*Brassica napus* or *B. rapa*), sunflower (*Helianthus annuus*), tobacco (*Nicotiana tabacum*), as well as from vegetable crops, like cabbage (*Brassica oleracea*), and smaller samples from other species (*Ranunculus ficaria*, *Apios americana*, *Geranium* sp, *Cannabis sativa*, *Raphanus* sp) provided a broad base for the population analysis. Haplotyping was based on DNA sequence data for seven loci in 385 isolates: the intergenic spacer (IGS) region of the nuclear ribosomal RNA gene repeat unit (IGS; 4,000 bp), an anonymous nuclear region (44.11; 700 bp), portions of the genes encoding translation elongation factor 1α (EF-1α; 350 bp), calmodulin (CAL; 500 bp), chitin synthase (CHS; 300 bp), actin (ACT; 300 bp), and ras protein (RAS; 350 bp). In their study, Carbone and Kohn (2001b) identified five populations from samples of diverse crops and hosts. The most recently diverged populations, 3-1 and 3-2, are dispersed in both temperate and subtropical areas, and diverge via dispersal/isolation by distance. Population 3-5 was limited to temperate areas, whereas 3-4 was a metapopulation restricted to wild buttercup (*Ranunculus ficaria*) in Norway (Carbone and Kohn, 2001a, b). The oldest population, numbered 3-3, appears to be endemic in the subtropical region of the southeastern United States (Carbone and Kohn, 2001a; Phillips et al., 2002).

In a subsequent study, Malvárez et al. (2007) applied this standard to extend a multiple gene genealogical approach aiming to detect geographic differentiation and identify putative biological species among different populations of *S. sclerotiorum*. Isolates of *S. sclerotiorum* were determined to represent three genetically differentiated populations: California (CA, lettuce), Washington (WA, pea/lentil), and Ontario (ON, lettuce). The California population from lettuce was highly diverse, highly divergent from the other populations, and old compared with Washington pea/lentil and Ontario lettuce samples, representing two other populations. The California sample does not represent a new species, despite genetic isolation relative to other populations. As demonstrated in the multilocus phylogeny, the California population was recently derived from a common ancestor in *S. sclerotiorum*, along with representatives of populations 3-3 (southeastern United States), 3-2 (north-central United States and adjacent Canada), and 3-4 (Norwegian wild buttercup), as well as isolates from Uruguay and Argentina, while the representatives of other named and cryptic *Sclerotinia* species are derived from a more distant, more ancient, common ancestor.

This geographic clustering infers that ecological conditions may contribute to adaptations associated with growth temperature range, light intensity, or sclerotial vernalization requirements (Taylor et al., 1999; Kull et al., 2004; Wu and Subbarao, 2006). Nonetheless, outcrossing appears to occur in agricultural populations of *S. sclerotiorum* at a rate that is important in generating new genotypes, which makes it likely that long-term evolution of asexual lineages does not contribute significantly to population genetic structure.

Although pathogens from all hosts are now considered to be one species (Carbone et al., 1999; Carbone and Kohn, 2001b), the extent of gene flow among pathogens of different hosts remains unclear.

5. Disease management and future perspectives

Management practices such as tillage, crop sequence, variety selection, seed treatment, plant population, row width, fertilizer, lime, water (in irrigated crops), herbicide, and fungicide application should be used as integrated management strategies in order to minimize crop losses all over the world.

Management of white mold in any crop requires adoption of an integrated package of practices for disease control. Management practices interact with the white mold pathogen at every stage of the fungal life cycle. No single management practice or variety can be relied on to control white mold. Instead, control of white mold requires application of a combination of management practices to be most effective. The effectiveness of combinations of variety selection and management practices has been tested in Brazil in research plots and on-farm studies examining crop rotation, tillage, variety, and irrigation system selection as tactics for limiting losses with success and substantially reducing white mold incidence.

Establishment and spread of *S. sclerotiorum* among geographic areas is achieved by several mechanisms and disseminative propagules, such as wind dispersal of airborne ascospores and transportation of sclerotia through farming operations, including tillage and irrigation, which are important means for long distance distribution of the pathogen. Sclerotia formed on diseased plant tissues may be dislodged and persist in soil for several years. Sclerotia-contaminated seeds and seeds infected with mycelium are also potential forms of pathogen introduction. Factors such as the different dispersal types for *S. sclerotiorum*, wide host range, genotypic structure with outcrossing potential, and absence of pathogenic races constitute a great challenge for producing resistant crops in the near future.

However, based on the rapid growth of knowledge of molecular genetics and *S. sclerotiorum* genome sequencing, project genome is in progress (Broad Institute through a project sponsored by the USDA Microbial Genomics Program) and advances in the following areas are expected to implement the control of this pathogen:

- Development of methods to assess the amount of recombination as part of larger integrated studies of their epidemiology, evolution, and population biology.
- Development of phylogeography studies, perhaps with multilocus sequence analyses to assess worldwide movement and diversification of genotypes. A better understanding of the evolutionary potential of the pathogen will provide insight into the most suitable breeding strategies for durable resistance.

- Detailed studies are required to understand the mechanism of plant-pathogen interactions, such as virulence factors and aggressiveness, which vary across populations.
- Development of biological control products should be very effective, environmentally safe, and economically acceptable.
- Development of transgenic crops with superior resistance to *Sclerotinia.*

6. Acknowledgments

I am grateful to Dr. M. F. Grossi de Sá for critically reviewing earlier versions of this manuscript.

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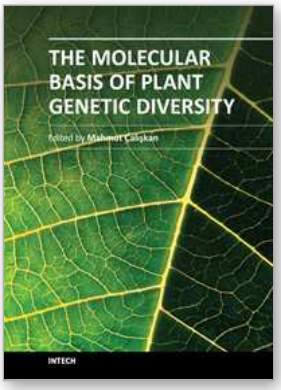

The Molecular Basis of Plant Genetic Diversity

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ISBN 978-953-51-0157-4
Hard cover, 374 pages
Publisher InTech
Published online 30, March, 2012
Published in print edition March, 2012

The Molecular Basis of Plant Genetic Diversity presents chapters revealing the magnitude of genetic variations existing in plant populations. Natural populations contain a considerable genetic variability which provides a genomic flexibility that can be used as a raw material for adaptation to changing environmental conditions. The analysis of genetic diversity provides information about allelic variation at a given locus. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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