INOCULATION TECHNIQUES, DEVELOPMENT OF *BRASSICA NAPUS* BREEDING LINES AND IDENTIFICATION OF MARKERS ASSOCIATED WITH RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY

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ABSTRACT

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is an economic disease affecting canola (*Brassica napus* L). Since expression of sclerotinia stem rot symptoms shows much variability and the trait is quantitative in nature, reliable phenotypic evaluation methods for characterization are needed. The three major objectives of this dissertation were to: i) evaluate eight different inoculation methods to discriminate between *S. sclerotiorum*-resistant and susceptible *B. napus* germplasm; ii) develop breeding lines with resistance to multiple diseases, and; iii) to identify QTL associated with resistance to sclerotinia stem rot using association mapping (AM). The eight methods evaluated were the detached leaves, detached stems, petiole inoculation (PIT), straw-inoculation, stem-piercing with toothpick, mycelial spray (MSI), petal inoculation and oxalic acid assay. MSI and PIT can better discriminate between the isolates and germplasm. Breeding lines resistance to *S. sclerotiorum, Leptosphaeria maculans*, and *Rhizoctonia solani* were developed from a cross between two moderately sclerotinia stem rot resistant plant introductions (PI). F_2 seedlings were screened for sclerotinia stem rot using PIT. Surviving plants were self pollinated and their progeny screened again. This process was repeated until the F_6 generation. In addition, F_5 seedlings were evaluated for their reaction to *R. solani* and F_5 and F_6 seedlings for their reaction to *L. maculans*. Eight lines were identified as moderately resistance to these three pathogens. The genomes of a group of 278 *B. napus* plant introductions were screened using Diversity Array Technology to detect QTL associated with resistance to sclerotinia stem rot. The population was classified into nine sub-populations and 32 significant markers each explaining between 1.5 and 4.6% of the variation were identified. Blastn search indicates

that similar nucleotide sequences are distributed throughout the genomes of *B. oleracea, B. rapa*, and *A. thaliana.*

Results of these studies suggest the PIT and MSI are reliable screening tools to evaluate materials for resistance to sclerotinia stem rot; materials identified as resistant to *S. sclerotiorum* were also moderately resistant against *R. solani* and *L. maculans* and could be valuable sources for canola improvement programs; and AM allowed us to identify QTL associated with resistance to sclerotinia stem rot.

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CHAPTER 1. INTRODUCTION

North Dakota (ND) ranked first in canola production in the United States with an estimated 663,400 tones harvested in 2007 and valued an estimated US$ 245 million (NASS, 2008). Canola production is affected by several factors but fungal diseases are the most important biotic constraints for canola production in ND. Among them, Sclerotinia stem rot, also known as white mold, is one of the most damaging diseases (del Rio et al., 2007). *Sclerotinia sclerotiorum* (Lib.) de Bary, an ascomycetous fungus, is the causal agent of sclerotinia stem rot (Lamey and Bradley, 2003). Sclerotinia stem rot is endemic in canola growing areas of ND with annual incidences ranging from 1 to 59%, and yield reductions of approximately 12.75 kg/ha for each incidence unit (del Rio et al., 2007).

Current sclerotinia stem rot management practices which emphasize the use of fungicides and 2-3 year crop rotations are not enough to prevent outbreaks and in some instances the economic returns from the former may not be consistent from one season to the next. Further, while growers usually rotate canola with cereals, the benefits of crop rotations for sclerotinia stem rot control are reduced by the ability of this pathogen to survive in the soil as sclerotia for longer than three years (Nelson, 1998). Foliar fungicides are applied at flowering time, however, rainy conditions which favor sclerotinia stem rot development also difficult the entrance of sprayers to fields resulting in some instances in applications being made pass the optimum time. Furthermore, the use of fungicides to manage sclerotinia stem rot increases the input costs of canola production.

The use of genetic resistance is the most effective means for disease management in most pathosystems; however, this option is not available at present time for many crops affected by *S. sclerotiorum* including canola (Bolton et al., 2006; Bradley et al., 2006; Zhao et

al., 2004). Resistance to this pathogen is polygenic in nature (Zhao et al., 2003a; Zhao et al., 2003b; Zhao et al., 2004), which makes identification of useful levels of resistance more difficult. Use of screening techniques that do not allow for consistent discrimination between resistant and moderately resistant or susceptible materials further compounds this situation. Because of this difficulty, very few lines with partial resistance have been identified (Khot, 2006; Zhao et al., 2003a; Zhao et al., 2003b).

While development of resistant lines is the ultimate goal of breeders and pathologists working on this area in many instances, only few quantitative trait loci (QTL)/markers that are closely associated to them have been identified. These QTL have been identified using the polymorphic characteristics of bi-parental populations and more recently, using association mapping. In a broad sense, the higher the number of markers detected, the easier it will be to verify the transfer of the genes of interest into breeding lines; in this sense, the use of association mapping seems to be more powerful than a biparental population (Collins and Morton, 1998; Flint-Garcia et al., 2003; Gupta et al., 2005). The recent discovery of additional sources of resistance among *B. napus* plant introductions (Khot, 2006) provides an opportunity to study the genetics of resistance associated with these disease.

The objectives of this study are: 1) evaluate eight inoculation methods for their ability to discriminate between *S. sclerotiorum*-resistant and *S. sclerotiorum*-susceptible materials; 2) develop *B. napus* breeding lines with resistance to multiple canola diseases; and 3) identify Diversity Array Technology (DArT) molecular markers associated with sclerotinia stem rot resistance in a collection of *B. napus* plant introduction materials using association mapping approach.

1.1. References Cited

Bolton, M. V., Thomma, B. P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-16.

Bradley, C. A., Henson, R. A., LeGare, D. G., del Rio, L. E., and Khot, S. D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Dis. 90:215-219.

Collins, A., and Morton, N. E. 1998. Mapping a disease locus by allelic association. Proc. Natl. Acad. Sci. 95:1741–1745.

del Rio, L. E., Bradley, C. A., Henson, R. A., Endres, G. J., Hanson, B. K., McKay, K., Halvoson, M., Porter, P. M., LeGare, D. G., and Lamey, H. A. 2007. Impact of Sclerotinia stem rot on yield of canola. Plant Dis. 91:191-194.

Flint-Garcia, S. A., Thornsberry, J. M., and Buckler, E. S. 2003. Structure of linkage disequilibrium in plants. Annu. Rev. Plant Biol. 54:357–374.

Gupta, P., Rustgi, S., and Kulwal, P. 2005 Linkage disequilibrium and association studies in higher plants: present status and future prospects. Plant Mol. Biol. 57:461.

Khot, S. D. 2006. Evaluation of *Brassica napus* accessions for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis. NDSU, ND, USA.

Lamey, A. and Bradley, C. A. 2003. *Sclerotinia* stem rot of canola biology and management. N. D. State Univ. Ext. Rep. No. PP-1201.

NASS. 2008. United States Department of Agriculture, 2008 Census of Agriculture. Washington, DC. [http://www.nass.usda.gov/index.asp (On November 1, 2010)].

Nelson, B. 1998. Biology of Sclerotinia. Pages 1-5 in: Proceedings of the 10th International *Sclerotinia* workshop, 21 Jan. 1998, Fargo, ND, USA. Department of Plant Pathology, NDSU, USA.

Zhao J., and Meng, J. 2003a. Detection of loci controlling seed glucosinolate content and their association with Sclerotinia resistance in *Brassica napus*. Plant Breed. 122:19–23.

Zhao, J., and Meng, J. 2003b. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 106:759–764.

Zhao, J., Peltier, A. J., Meng, J., Osborn, T. C., and Grau, C. R. 2004. Evaluation of Sclerotinia stem rot resistance in oilseed *Brassica napus* using a petiole inoculation technique under greenhouse conditions. Plant Dis. 88:1033-1039.

CHAPTER 2. LITERATURE REVIEW

2.1. *Brassica napus*

2.1.1. Taxonomy and nomenclature

Brassica napus L. belongs to the Brassicaceae family, which consists of approximately 3,200 species and is mainly grown in the northern hemisphere (Thomas, 2003). U (1935) described the cytogenetic relationships of major *Brassica* species that is currently in use (Figure 2.1). Each species has specific genome: for example, *B. rapa* is a diploid (AA, chromosome pair, *n* =10) and *B. napus* is an amphidiploid (AACC, *n* =19). *B. napus* is thought to have originated from a cross where the maternal donor was closely related to two diploid species, *B. rapa* (AA, *n*=10) and *B. oleracea* (CC, *n* =9).

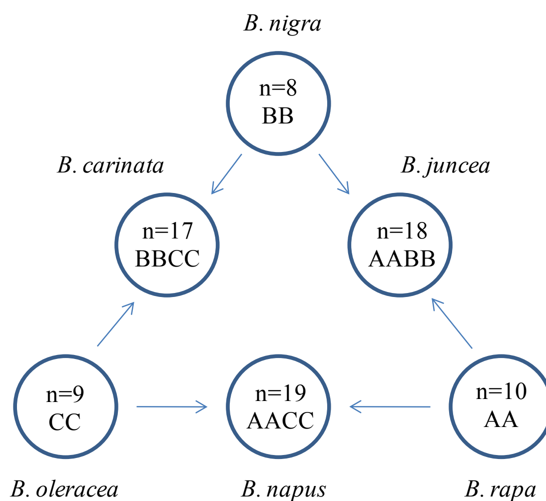

Figure 2.1. Cytogenetic relationships of *Brassica* species with specific genomes in each species; n: the number of chromosomes in each genome. (Adapted from U, 1935).

Rapeseed or oilseed (*Brassica* spp.) is a generic common name that encompasses plants from *B. napus*, *B. rapa* L. (syn. *B. campestris*), *B. juncea* (L.) Czern. (brown mustard) and *B. carinata* A. Braun. These species belong to the family Brassicaceae. Other common

names for these plants are oilseed rape, rapa, and rapeseed. A special group of rapeseed cultivars are called canola. The name "canola" was adopted in 1979 by Western Oilseed Crusher's Association (Canadian Oilseed Processors Association), Canada to identify *B. napus* and *B. rapa* varieties that produce oil with less than 2% erucic acid content and a defatted meal with less than 30 μmol/g of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free meal"; these lines are also known as "double low" or "double zero" varieties (Shahidi, 1990).

2.1.2. History of canola

Before 1970s, oil from *Brassica napus*, was used mostly in industrial applications due to its high eicosenoic and erucic acid contents. In 1968, the first rapeseed line with low eicosenoic and erucic acid contents was identified from an Argentina *B. napus* accession and released as cultivar 'Oro'; this cultivar also had low erucic acid content. Subsequently, the variety 'Bronowski', a *B. napus* line with low content of glucosinolates, was identified and used to generate the first double-low, low-erucic acid and low glucosinolates, cultivar 'Tower' in 1974 (Brown et al., 2008). Since then, many canola varieties have been bred via selection from wild *B. napus* accessions. These accessions are considered a precious gene pool for canola breeding. Now "canola" is officially defined as a commodity distinguished from common rapeseed by Canada's Food and Drugs Act, Feeds Act and Seeds Act. It has become the second largest oilseed crop in the world following soybean (FAS, 2009).

The term canola is broadly used to identify *B. napus*, *B. rapa* and *B. juncea* lines or their derivatives that have low erucic acid and low glucosinolate content (Thomas, 2003).

Canola-quality cultivars of *B. napus* and *B. rapa* are the predominantly grown species of rapeseed in the world which yields about 40% of their total dry weight as oil and meals containing about 38-43% protein. The canola meal contains essential amino acids which are absent in cereals i.e. lysine, methionine, cysteine, threonine, and tryptophan (Larsen and Sorensen, 1985). The canola oil of today's market contain 60-65% of monosaturated fats, 30-35% of polysaturated fats, 5-8% of saturated fats, and traces of erucic acid. Canola oil is good for human consumption purpose since it contain essential amino acids, and less erucic acid and lowest saturated fat content of all major edible vegetable oils (Sarwar, 1984).

2.1.3. Biology and morphology of *B. napus*

Canola can be classified as winter-type or spring-type, depending on whether they require vernalization or not. Winter types are planted in the fall while spring types are planted in the spring. Winter canola is planted in many European countries and in some parts of China, while spring rapeseed is produced in North Dakota (United States), Canada, and in some parts of Northern Europe and China. Spring rapeseed occupies a substantial portion of production in Canada, and is also grown in northern Europe, China, and India. Spring type *B. juncea* cultivars are dominant in India and are grown in limited areas in Canada and Europe to be used as a condiment (Shahidi, 1990). Spring type does not need vernalisation to flower, although vernalisation speeds up flowering.

B. napus has entomophilous flowers in a branching type inflorescence. Each flower contains four petals, four sepals, six anthers, one pistil with two functional nectarines at the base of the short stamens, and two non-functional nectarines at the base of the long stamens (Figure 2.2) (Downey et al., 1980).

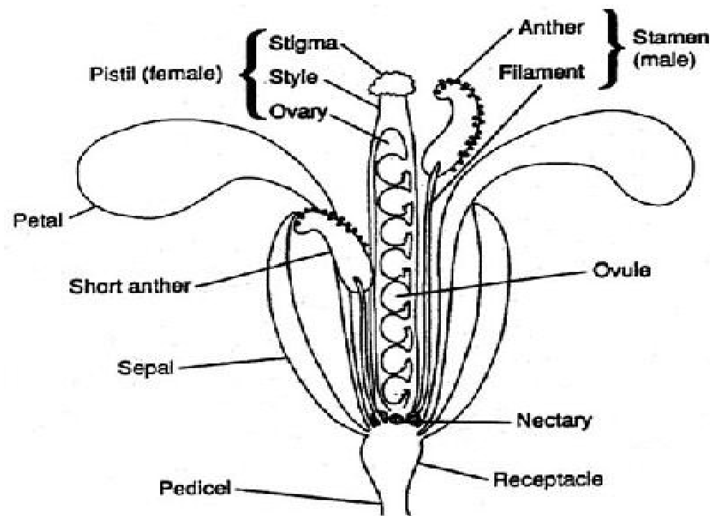

Figure 2.2. Cross-section of a canola flower (Source: http://www.ableweb.org/volumes/vol-15/12-ecker/12-ecker.htm)

B. napus has buds in the upper part of the branches. Fertilization of ovules usually takes place from self-pollination since the perfect flower can produce large amounts of pollen and out competes that from adjacent flowers. However, 12-47% of out-crossing occurs in the presence of physical contact, wind or pollinators (Becker et al., 1992; Williams et al., 1986). Pollen of *B. napus* is transferred from plant to plant by physical contact and over longer distances by wind and insects and the pollen viability gradually decreases over 4-5 days (Ranito-Lehtimaki, 1995). Temperature and relative humidity have large effects in pollen viability. Once fertilization takes place, siliques, which are commonly known as 'pods', are formed. Each pod contains about 15 to 25 seeds (1-2 mm in diameter). Seeds are primary means of reproduction. The average test weight for 1,000 seeds is 3.33 to 4.0 g (Buzza, 1991). *B. napus* growth stages include pre-emergence germination, seedling, rosette, bud, flowering and ripening. Days from seeding to harvest are an overall measure of the duration of canola growth stages. The length of each growth stage is greatly influenced by temperature, moisture, light (day length), fertilizers and types of varieties. Among them temperature is

considered the most important environmental factor regulating growth and development of canola in western Canada (Thomas, 2003).

Maturity days vary considerably depending on location, cultivar, growing season and date of seeding. Generally speaking, *B. rapa* varieties mature seven to 28 days earlier than most of *B. napus* varieties (Table 2.1). The major differences between these two species are as follows:

Table 2.1. Differences between chief characteristics of *Brassica rapa* and *B. napus.*

Characteristics	***Brassica rapa***	***Brassica napus***
Cotyledons	Hairy and wrinkled on underside	Smooth
Rosette and Leaves	Small, 3 to 5 yellowish green leaves	Larger, up to 6 waxy, blue-green leaves
Branches	Up to 20 per plant	4 to 6 per plant on average
Flowers	Smaller, darker yellow, rely on cross pollination, compact bud clusters, buds held below uppermost open flowers	Self-pollinating, buds borne above open flowers
Leaves	Leaf blade clasps the stem completely	Leaf blade only partially clasps the stem
Height	15 to 125 cm	Taller, 75 to 175 cm
Pods	Smaller, shorter, long beak, smaller seeds, more pods	Large, medium length beak, fewer pods, larger seeds
Shattering	Resistant	Easily shattered

Source: (Thomas, 2003)

2.1.4. Canola production

The edible oil production of rapeseed comes mainly from *B. napus* and *B. rapa* and to a lesser extent from *B. juncea* and *Sinapis alba* L. (yellow mustard). Currently, more than 13% of the world's edible oil supply comes from *Brassicas* (rapeseed, canola, and mustard), making this group the second largest vegetable oil source after cotton and the world's second leading source of protein meal after soybean (FAS, 2009). Total world production of rapeseed has increased from 22.5 million metric tons in 1987 to 46.86 million metric tons in 2006-2007

(USDA, 2007). The United States ranks 10th worldwide in rapeseed production, and China, Canada, and India are in the top three positions, respectively. *B. napus* plants are taller, late maturing and liable to late spring frosts but they have higher yield potential and oil content compared to *B. rapa* (Thomas, 2003).

Canola hectarage has increased continuously in recent years in the United States because of continued strong demand for canola oil due to its healthy attributes and its favorable profile as a biodiesel and feedstock. Canola production in the United States is dominated by spring type *B. napus*. Major production areas in the country include the Northern Great Plains and Pacific Northwest with very limited production in other regions. Winter type *B. napus* can be grown in the Midwest, Great Plains, and Eastern regions of the United States (Raymer et al., 1990). The use of winter canola varieties expanded the production to the southern Great Plains centered in Oklahoma and Kansas. Canola planting also moved into the eastern portion of the southern Great Plains, with reports of a small hectarage being planted in Arkansas in 2007 (US Canola Association, 2008). Canola production increased dramatically from 60,000 ha. in 1995 to approximately 480,000 ha. in 2007 (USDA, 2007). Currently, canola is planted in approximately 450,000 ha. of which more than 90% are located in North Dakota (US Canola Association, 2008). North Dakota has been the leader of canola production in the United States since 1997 (NASS, 2008). In 2007, ND planted 91.8% of the nationwide production, and has a market value of US$ 245.5 million. The yield in the past ten years ranged from 1,237 to 1,618 kg/ha (USDA, 2007). One of the major impediments for economic production could be sclerotinia stem rot; a monocyclic disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary.

2.2. *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a facultative parasite, and one of the most nonspecific, and widespread, ascomycetous plant pathogens. It attacks more than 400 plant species which include 225 genera and 64 families primarily dicotyledonous and a few monocotyledonous species such as onion and tulip (Boland and Hall, 1994; Purdy, 1979). Between 1990 and 2008 approximately 101 plant species had been added to the host list of *S. sclerotiorum* (Saharan and Mehta, 2008). The diseases caused by *S. sclerotiorum* are known by more than 60 names in different hosts. *S. sclerotiorum* causes sclerotinia stem rot in canola, and soybean (*Glycine max*); white mold in dry bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), and pea (*Pisum sativum*); *Sclerotinia* wilt, middle stalk rot, and head rot in sunflower (Purdy, 1979).

Sclerotinia sclerotiorum causes significant reduction in yield and quality of several economic crops in North Dakota (ND) including canola. This fungus alone is responsible for more than US$ 200 million loss annually in the United States (USDA, 2005). Sclerotinia stem rots cause direct yield loss and indirect loss by degrading oilseed quality. In canola, yield is reduced by the shattering of prematurely ripened seed pods before and during harvesting. The loss of quality is due to the smaller, shrunken and chaffy seeds or mixture of sclerotia. The wide host range of *S. sclerotiorum* also leads to potential production loss due to abandonment of fields for growing preferred crops (Purdy, 1979). To combat *Sclerotinia* diseases in crops, the United States Congress approved the creation of the National Sclerotinia Initiative in 2004, which is managed by the Agricultural Research Service branch of the U.S. Department of Agriculture to conduct advance research on its biology, disease epidemics, host resistance and disease management (http://www.ars.usda.gov/Research/docs.htm?docid=20317).

2.2.1. Taxonomy and nomenclature

In 1837, Libert first described *S. sclerotiorum* as *Peziza sclerotiorum* (Libert, 1837; adapted from Bolton et al., 2006). After approximately 30 years of use, the species was re-classified to the new genus Sclerotinia (Fuckel, 1870 adapted from Bolton et al., 2006) and Fuckel named it as *Sclerotinia libertiana* Fuckel in honor of Libert (Purdy, 1979). Though plant pathologists accepted and used *S. libertiana* Fuckel for a long time, Wakefield (1924; adapted from Bolton et al., 2006) noticed the name was conflicting with the International Code of Botanical Nomenclature system. According to Purdy (1979), de Bary was the first to use the name *"S. sclerotiorum"* in 1884. Therefore, the proper naming and authority of this fungus was *S. sclerotiorum* (Lib.) de Bary. This name is currently in use.

Its taxonomical classification has changed over time, even after the name has been accepted. Based on its ascospore-producing nature, this fungus has been classified in the class Ascomycetes and belongs to Ascomycota division. Based on an old classification system, this fungus belongs to the family Sclerotiniaceae (group of fungi which produces sclerotia) in the order Helotiales. The new classification system moved this taxonomic family to the order Leotiales (group of fungi that produces ascospores in operculate asci), and class Discomycetes (group of fungi producing cup or disc shaped apothecia type of ascomata covered by hymenium layer until its maturity).

The modern taxonomical classification of *S. sclerotiorum* is as follows:

KINGDOM: FUNGI

DIVISION: EUMYCOTA

SUBDIVISION: ASCOMYCOTINA

CLASS: ASCOMYCETES

SUB CLASS: DISCOMYCETES

ORDER: LEOTIALES

FAMILY: SCLEROTINIACEAE

GENUS: *Sclerotinia*

SPECIES: *sclerotiorum*

Sclerotia of *S. sclerotiorum* are long-term survival structures (resting structures) that may remain viable up to eight years in soil (Adams and Ayers, 1979). Sclerotia are made of mycelial aggregates surrounded by a rind composed of several layers of cells with high melanin content; melanin is considered to play a vital role in protecting the sclerotia from adverse environmental conditions (Bell and Wheeler, 1986). Under favorable environmental condition, sclerotia germinate either carpogenically i.e. apothecia production or myceliogenically, i.e. somatic hyphae. Yellowish-brown apothecium (2-10 mm diameter) is supported by three to four cm long stem portion called stipe. One sclerotium can produce more than one apothecium. Apothecia are soft and have fleshy texture. Each apothecium has hundreds of rows of inoperculate asci that are cylindrical sac-like structures that produce 8-ascospores (4-6 x 9-14 µm) (Kohn, 1979). Length of ascospores are generally double its' width.

Two morphological characteristics facilitate differentiating between *S. sclerotiorum* and its two closely related species, *S. trifoliorum* and *S. minor*. *S. sclerotiorum* ascospores are uniform in size, binucleate, and have a length to width ratio of 2 (Saharan and Mehta, 2008). Ascospores of *S. trifoliorum* are tetranucleate, dimorphic in size and have a length to width ratio < 2, whereas ascospores of *S. minor* are binucleate, uniform in size and have a length to width ratio that could be smaller or bigger than 2.0. *S. trifoliorum* and *S. sclerotiorum* produce

2-20 mm long sclerotia at growing margins of culture plates forming concentric rings, radial lines and other patterns, whereas *S. minor* produces 0.5-2 mm long sclerotia throughout the colony.

2.2.2. Host-pathogen interaction

Studies on the interaction between host and necrotrophic pathogens are mainly concentrated on the hydrolytic enzyme activity and production of secondary metabolites e.g. toxins (Kars and van Kan, 2004; Thomas, 2003; Toth et al*.,* 2003). Cell wall degrading enzymes (CWDEs) and oxalic acid have been found to be the major pathogenicity factors of *S. sclerotiorum* (Bateman and Beer, 1965; Saharan and Mehta, 2008).

2.2.2.1. Cell wall degrading enzymes (CWDE)

S. sclerotiorum secretes CWDE to penetrate, macerate, and degrade plant cell wall components of host (Hancock, 1996; Lumsden, 1979; Riou et al*.,* 1992). Polygalacturonase produced by fungus dissolves the pectin present in cell walls and facilitates penetration and colonization of the host (Alghisi and Favaron, 1995). Polygalacturonases degrade unesterified pectate polymers, which are the structural polysaccharides found in middle lamella and primary cell wall of host. Polygalacturonases have been implicated as virulence factors in few other pathosystems i.e. *Mycosphaerella graminicola* and *Botrytis cinerea* (Garcia-Maceira et al*.,* 2001; Kars et al*.,* 2005; Shieh et al., 1997; ten Have et al*.,* 1998; Wagner et al*.,* 2000). Polygalacturonase activity in *S. sclerotiorum* is induced by pectins, such as galacturonic acid (Fraissinet-Tachet and Fevre, 1996; Riou et al., 1992). Polygalacturonase-inhibiting proteins (PGIP) are plant glycoproteins associated with resistant reaction to *S. sclerotiorum* found in soybean plants (DeLorenzo et al., 2001; Favaron et al., 1994; Favaron et al., 2004).

2.2.2.2. Oxalic acid

Oxalic acid (OA) has a major role in pathogenesis, which is supported by research findings that include the correlation between OA levels and disease severity (Bateman and Beer, 1965; Magro et al., 1984; Maxwell and Lumsden, 1970; Noyes and Hancock, 1981), the development of Sclerotinia disease-like symptoms after direct application of OA into plants e.g. soybean, canola (Bateman and Beer, 1965; Noyes and Hancock, 1981), and the recovery of OA from infected plant tissues (Ferrar and Walker, 1993; Godoy et al., 1990; Marciano et al., 1983).

OA plays an important role in pathogenicity and gives this fungus a competitive advantage over other fungal species for control of environmental nutrients (Dutton and Evans, 1996). When attacking plants, OA accumulates in colonized host cells (Bateman, 1964; Bateman and Beer, 1965). Increase in OA levels in the host tissue reduces the extracellular pH, which ultimately increases the activity of CWDE (Bateman and Beer, 1965; Marciano et al., 1983; Margo et al., 1984; Maxwell and Lumsden, 1970). Favaron et al. (2004) illustrated that due to its low pH, OA may also escape from the activity of PGIP produced by soybean plant defense (Favaron et al., 2004). OA inhibits the activity of polyphenol oxidases (Marciano et al., 1983; Magro et al., 1984) and chelates cell wall Ca^{+2}, allowing polygalacturonase to hydrolyze pectins, thus disrupting the cell integrity (Bateman and Beer, 1965; Kurian and Stelzig, 1979). OA suppresses the oxidative burst of infected host cells (Cessna et al., 2000), regulates stomatal opening, and inhibits abscisic acid-induced stomatal closure; as a consequence, wilting occurs (Guimaraes and Stotz, 2004). Transcriptional regulation of pH-regulated genes necessary for pathogenesis and developmental life cycle of *S. sclerotiorum* are also affected by lower pH (Rollins, 2003; Rollins and Dickman, 2001).

2.3. Sclerotinia Stem Rot in Canola

Sclerotinia stem rot is considered one of the most serious diseases affecting canola production in North Dakota (ND). During 1991 to 2002, sclerotinia stem rot incidence ranged from 7 to 19% in ND and 11 to 19% in Minnesota, resulting in direct economic losses estimated at US$ 94 million (Lamey et al., 2002; Lamey, 2003). One percent sclerotinia stem rot infection adds two percent yield loss in Canada (Thomas, 2003). In ND, every unit percentage of sclerotinia stem rot incidence equals a potential yield lost of 0.5% (equivalent to 12.75 kg/ha) (del Río et al., 2007). The collective annual losses due to sclerotinia stem rot are estimate at US$ 24 million (US Canola Association, 2008).

2.3.1. Symptoms

There is no unique symptom caused by this fungus in each plant. Sclerotinia stem rot symptoms are normally observed during the flowering stage. Dead tissues are easy sources of energy; therefore, in field conditions ascospores colonize casted petals or other senescent plant tissues more quickly than attached tissues (Jamaux et al., 1995). The pathogen then progresses towards leaf petioles producing water soaked lesion that expand rapidly. Water-soaked lesions later turn necrotic and develop patches of fluffy white mycelium, which are the most obvious sign of plants infected with *S. sclerotiorum*. Germination of sclerotia and development of mycelia is totally dependent on the level of host resistance, the pathogen's virulence, and environmental conditions (i.e. wavelength of light and relative humidity). Plant wilting occurs as the pathogen progresses into the stem and disrupts the movement of water and nutrient by destroying the cellular walls and causing cells to empty their contents into intercellular spaces. Sclerotia are usually formed in the stem pith, but in wet environments

sclerotia can also be formed on infected tissue's surfaces. Sclerotia thus formed will be black in color due to melanin content. The shape of sclerotia will be dependent on host and its location within or on host.

2.3.2. Disease cycle and epidemiology

The disease cycle of sclerotinia stem rot begins with sclerotia, a melanized structure of mycelia that fall to the soil during harvest and can survive in or on the soil for several years. Due to its longevity and massive reproductive potential, sclerotia play a major role in the epidemiology of sclerotinia stem rot. The duration of sclerotial survival depends on soil types, crops planted after its production, initial population of sclerotia and environmental conditions (Duncan, 2003; Huang and Kozub, 1991; Willetts and Wong, 1980). Sclerotia may germinate carpogenically or myceliogenically depending on environmental condition (Willetts and Wong, 1980). Myceliogenic germination is triggered when sclerotia are exposed to high nutrient conditions or healthy plant tissues (Le Tourneau, 1979; Phillips, 1986) while carpogenic germination usually occurs when sclerotia are placed on substrate with low nutrients. Therefore, the numbers of sclerotia and apothecia are important primary factors for the disease epidemics. Ascospores are produced in asci in apothecia. Mature asci forcibly eject ascospores through a mechanism called puffing (Clarkson et al., 2003). Puffing has been associated with changes in relative humidity (≥90%) and temperature ≈15-16°C (Qandah and del Rio Mendoza, 2011). Ascospores are the primary source of inoculum for most of the diseases caused by *S. sclerotiorum* (Abawi and Grogan, 1979; Schwartz and Steadman, 1978; Steadman, 1979). In favorable environmental conditions in field, sclerotia need 10-30 days to produce apothecia; but mature apothecia can produce millions of ascospores (Schwartz and

Steadman, 1978). No asexual spore has been reported; however, microconidium has been reported on hyphae or on the apothecial hymenium (Kohn, 1979). The role of microconidia on sclerotinia stem rot epidemics is not known.

Apothecia are produced in the field when the plant canopy closes, which helps in maintaining high soil moisture (Clarkson et al., 2004). While high soil moisture and high relative humidity are favorable for apothecia production and ascospores release in the field, keeping soil moisture conditions at 25% of their moisture saturation capacity yields highest carpogenic germination; in contrast, soils kept at 100% moisture saturation are detrimental for germination (Nepal, 2009). When soil moisture fluctuates, highest carpogenic germination occurs in soils were moisture fluctuation ranges between 50-75% of saturation (Nepal, 2009). During canola flowering, the senesced petals that dropped onto lower portions of the plant, such as the plant leaf axils, provide *S. sclerotiorum* spores with an adequate seedbed and enough food for their germination and initial growth. These structures, sclerotia, play a major role in disease spread as they are the source for inoculum and are the primary long-term survival structures (Willetts and Wong, 1980).

Flowering is a critical stage for infection since senescent flower parts serve as the primary source of nutrient for germinating ascospores (Inglis and Boland, 1990; Turkington and Morrall, 1993). Ascospore germination and penetration is facilitated by long duration (36-40 h) of canopy wetness and temperature ranging between 20 and 25°C (Sharan and Mehta, 2008). Both senescent flower petals and wounded tissues serve as an excellent exogenous nutrient source for ascospore germination. Ascospores are covered by sticky mucilage, which helps in adhesion to the substrates. Upon landing, ascospores germinate producing a germ tube that penetrates the host tissue by mechanical force. When myceliogenic germination

takes place, hyphae penetrate host cuticle layer directly using enzymes or mechanical force via appressoria unless penetration occurs through stomata (Lumsden, 1979; Lumsden and Dow, 1973). Hyphal extension and secondary colonization of the host plant takes place through open stomata, as well as formation of sclerotia on the host surface. Hyphae colonize the tissues and use them to invade other areas of the plant (Abawi and Grogan, 1979).

After penetration and establishment in host, mycelium multiplies abundantly in infected host tissues and the tissues are degraded to the point that its nutritional status for the fungus is significantly diminished. Symptom development may vary among hosts (Bolton et al., 2006; Purdy, 1979). Wilting results mostly from destruction of vascular tissues, in addition oxalic acid also deregulates the stomata closure which leads more transpiration. Once the nutrients are depleted in the host cells, the fungus starts producing sclerotia in the stem pith of the infected host (Christias and Lockwood, 1973). Sclerotium development requires three stages: (i) initiation, aggregation of hyphae to form a white mass called sclerotial initials; (ii) development, increasing the size of sclerotial initials; and (iii) maturation, surface delimitation, melanin deposition in peripheral rind cells and internal consolidation, (Townsend and Willets, 1954). Sclerotia will remain attached to the host surface or fall down to the soil and remain viable for many years (Lamey and Bradley, 2003). After a period of conditioning, sclerotia may undergo carpogenic germination, which would usually occur during the growing season after the crop canopy has developed, and when periods of rainfall result in prolonged cool and humid conditions (Abawi and Grogan, 1979; Boland and Hall, 1987); otherwise, sclerotia may remain dormant for additional years (Purdy, 1979).

A sclerotium can produce several apothecia. Mature apothecia can release ascospores for 10 days or more at a rate of 1600 ascospores per hour (Clarkson et al., 2003). Viability of

ascospore is greatly influenced by environmental conditions before release and after landing on the host cell and can be blown away by general wind currents (Caesar and Pearson, 1983). However, ascospores can be exposed to direct sunlight for long enough to be airborne without losing viability (Clarkson et al., 2003).

2.3.3. Disease management

Sclerotinia stem rot is very difficult to control due to the wide host range of its causal agent and its ability to withstand adverse conditions (Purdy, 1979). The extent of damage also depends on the susceptibility of host, stage of host, infected part (i.e. main stem or branch) and environmental factors. Therefore, no single control measure is effective to manage this disease. Sclerotinia stem rot is a monocyclic disease, thus, reducing inoculum levels (number of sclerotia) in the field would be the approach that has the most significant impact on disease development, although an integrated disease management which includes use of cultural practices, chemical fungicides and biological control agents, and use of resistant cultivars would be most desirable (Lamey and Bradley, 2003).

2.3.3.1. Cultural practices

Several cultural practices have been suggested to manage diseases caused by *S. sclerotiorum*. Canola seeds infested with sclerotia help to increase the disease epidemics. Use of clean seed and crop rotation will reduce the amount of initial inoculum in the field. However, the impact of crop rotations in disease control is reduced because sclerotia can survive for longer than most commonly used rotation schemed. Other cultural practices, such as tillage and controlled irrigation are also effective (Lamey and Bradley, 2003) to manage disease in addition to use of proper fungicides (Bradley et al., 2006; Zhao et al., 2004).

2.3.3.2. Biological control

Coniothyrium minitans (Bremer et al., 2000; Chitrampalam et al., 2008; McQuilken et al., 1995; Subbarao, 1998) and *Sporidesmium sclerotivorum* (Adams and Fravel 1990; del Río et al., 2002) are effective biocontrol agents of *S. sclerotiorum*. However, to date only *C. minitans* has been commercially available. Results from the study by Chitrampalam et al. (2008) revealed that two applications of a commercial formulation of *C. minitans,* Contans, at manufacturer recommended rates (2.2 and 4.4 kg/ha) significantly reduced the incidence of lettuce drop caused by *S. sclerotiorum* and significantly increased the yield in desert lettuce experiments. In canola, application of 1 kg per ha produced better or comparable disease control than Ronilan 50% EG (Harikrishnan et al., 2005).

2.3.3.3. Chemical control

Fungicide application is the most practical and effective option to control sclerotinia stem rot. Several fungicides are registered for use in canola in North Dakota, i.e. Boscalid (Endura), Thiophanate Methyl (Topsin M), Prothioconazole (Proline) and Azoxystrobin (Quadris) (McMullen and Markell, 2011). Proper timing for fungicide application has a major impact on the effectiveness of fungicides. Quadris works best when applied at 10-25% bloom or 3-7 days after the first flower opens whereas Ronilan, Topsin or Endura can be applied at 20- 50 % bloom. The recommended dose for Quadris is 6.2 to 1.2 L/ha. However, 0.7 L may provide adequate control if applied before any petals begin to fall. In contrast, Topsin has been recommended at 1.12 to 2.24 kg/ha rate and Endura at the 440 mL/ha rate. Reports have shown that early application of Ronilan at 880 mL or 1.12 kg of Thiophanate Methyl ha^{-1} provides excellent sclerotinia suppression under severe disease pressure (McMullen and Markell, 2011).

2.3.3.4. Host resistance

Due to increasing environmental concerns related to pesticide applications, and the raising costs of chemicals and their application, selection of resistant cultivars to sclerotinia stem rot is of prime importance. While, there are no cultivars registered for sclerotinia stem rot resistance, several moderately resistance canola germplasm have been reported. In Germany, lines with less susceptibility to stem rot have been developed, but they are low yielding compared to susceptible cultivars grown in disease-free conditions (Kharbanda and Tewari, 1996). Sclerotinia stem rot incidence showed a positive correlation with earliness of flowering and negative correlation with cultivar height (Kharbanda and Tewari, 1996). Resistance to sclerotinia stem rot is multigenic and partial (Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2007), thus allowing heavy loss when weather conditions are favorable for disease development.

2.4. Breeding for Resistance Against Sclerotinia Stem Rot

Utilization of cultivars with resistance is always the first choice to manage a disease. However, research shows the majority of canola varieties are susceptible to sclerotinia stem rot (Lamey and Bradley, 2003). Therefore, proper selection of cultivar is wise. Use of canola cultivars with moderate sclerotinia stem rot resistance could be an economic and effective method of sclerotinia stem rot control.

2.4.1. Characteristics of resistance to sclerotinia stem rot

If genetic resistance to sclerotinia stem rot was available to canola growers, there would be less reliance on fungicides and canola production in Minnesota and North Dakota

would be more profitable (Lamey et al., 2002; del Rio et al., 2007). Hosts can escape from pathogen infection due to morphological or physiological characteristics or they could resist infection due to true genetic resistance. In some canola varieties, growth habit, morphological, or physiological traits play vital role to escape the infection of *S. sclerotiorum*. For example, tall varieties are more prone to lodging, which creates wounds, cool and humid condition; therefore will be more susceptible to sclerotinia stem rot than dwarf cultivars. Apetalous varieties tend to escape infection due to the absence of petals, which can serve as seedbed for the fungus (Rakow and Séguin-Swartz, 1999). The use of apetalous canola varieties started with the discovery of an apetalous *B. napus* mutant that showed less sclerotinia stem rot incidence than cultivar 'Westar' (Lü and Fu, 1990). A study later showed the inheritance of apetalous flowers resembled that of cytoplasmic male sterility in the sense that cytoplasm interacted with nuclear genes in determining phenotypes (Yang and Fu, 1990). Another important trait closely related to sclerotinia stem rot resistance is the seed glucosinolate content. Two QTL associated with aliphatic glucosinolate and 3-indolyl-methyl glucosinolate content in seeds were also associated with *Sclerotinia* resistance on the leaf at the seedling stage and on the stem of the mature plant, respectively (Zhao and Meng, 2003b). This finding may help explain why canola, low glucosinolate content species, is more susceptible than other *Brassica* species with higher glucosinolate content. Only few *B. napus* cultivars have relatively moderate level of sclerotinia stem rot resistance (Saharan and Mehta, 2008)**.**

Use of host genetic resistance or development of resistant cultivars is the most efficient and cost-effective means to manage plant diseases; however, no cultivar or source of complete resistance to *S. sclerotiorum* has been identified among the *Brassicaceae* (Bolton et al., 2006; Bradley et al., 2006; Zabala and del Rio, 2007; Zhao and Meng, 2003a; Zhao and

Meng, 2003b). Host responses to *S. sclerotiorum* are variable depending upon the environment in which the interaction takes place (Lamey and Bradley, 2003). However, differences in response to *S. sclerotiorum* among cultivars or breeding lines in some oilseed rape (Bradley et al., 2006; Zhao et al., 2004) have been reported, thus encouraging further efforts to screen germplasm for resistance to *S. sclerotiorum*. Some canola cultivars currently marketed in Canada (Kharbanda and Tewari, 1996) and the United States exhibit partial resistance to sclerotinia stem rot (Bradley et al., 2006; Zhao et al., 2004).

Several methods for phenotypic evaluation of sclerotinia stem rot, such as OA resistance test, stem inoculation, petiole and detached leaf inoculation have been used to identify resistance to *S. sclerotiorum* in canola (Bradley et al., 2006; Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2004). Phenotypic screenings of *B. napus* plant introductions in North Dakota identified several Korean materials with good level of resistance to sclerotinia stem rot (Khot, 2006). However, when screening materials, an important issue is repeatability of results. To reduce natural variability between plants evaluation of highly homozygous plants is desirable. Alternatively, doubled haploid lines or even clonal plants could be used. The ultimate goal for plant breeder to screen the plant populations phenotypically is to make progress in crop quality.

2.4.2. Tools used for QTL identification

2.4.2.1. Plant population

The objective of plant breeding is to improve various agronomic traits to sustain or increase crop productivity and quality. Crop improvement starts with the selection of desired traits from natural populations, followed by their introgression into more commercially

acceptable lines. Conventional plant breeding aimed at selecting superior individuals from segregating populations is laborious and time consuming. Crop improvement techniques are benefiting from recent advances in the use of molecular tools that speed up mapping genes. Once individuals with enhanced levels of resistance are identified, it is necessary to transfer the genes responsible for the trait into materials that can be marketed more easily. To do this, segregating populations (with individuals that differ in their response to disease) need to be produced. The two most commonly used approaches to develop such populations are the cross between a resistant and a susceptible parent (biparental population) and the development of doubled haploid populations from a heterozygous plant (that could have been produced by a cross of two lines as described above or that could be a plant from a synthetic population).

2.4.2.1.a. Biparental mapping populations

This has been the traditional approach to develop breeding populations; it has been used to move traits in a number of plant-pathogen systems (Chahal and Gosal, 2006). Use of biparental populations has been combined with molecular tools to detect QTL associated with disease resistance. Traditional biparental mapping approach is used mostly to identify and map QTL associated with sclerotinia stem rot resistance. In this approach, segregating mapping populations such as F_2 progenies are developed by crossing two contrasting genotypes (i.e. resistance and susceptible cultivars). Using this method, QTL associated with different levels of resistance to sclerotinia stem rot have been identified in Chinese winter-type cultivars (Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2004). Selection of promising materials can be made at different stages of development of biparental populations:

F_2 population

F_2 populations are produced by self pollinating individuals from an F_1 generation produced by crossing the selected parents. F_2 individuals are outcome of single meiotic cycles; therefore single recombination events are produced. The expected ratio for dominant markers is 3:1 and for co-dominant markers is 1:2:1. Developing F_2 populations requires short time; however, only one meiosis/recombination is available to generate maps for traits of interest and thus, use of F_2 populations carries low mapping resolution. Further, F_2 populations cannot be evaluated in replicated trials over locations and years because each F_1 seed gives a single plant. Thus, the effect of G x E interaction on the expression of quantitative traits cannot be precisely estimated.

F_2 derived F_3 ($F_{2:3}$) population

$F_{2:3}$ populations are obtained by selfing the F_2 individuals for a single generation. These populations are better suited for mapping purposes compared to F_2 populations when the intention is to map QTL for recessive genes. In this kind of population, selection or phenotyping is done in the third generation which allows researchers to have multiple data points for single F_2 lines. The $F_{2:3}$ populations can be used for reconstituting the genotype of respective F_2 plants, if needed, by pooling the DNA from plants in the family. These populations are basically similar to F_2 populations but have one additional recombination event and could be evaluated in replicated trials. Zhao and Meng (2003) used 128 $F_{2:3}$ populations to conduct genetic analyses of loci associated with partial resistance to *S. sclerotiorum* in *B. napus*.

Doubled haploid (DH) populations

Plant reproductive parts can be induced naturally or by using physical and chemical agents to produce haploids. Haploids can be obtained through either in vivo or in vitro methods (Palmer and Keller, 2005). Haploid plants carry gametic chromosome numbers i.e., in *Brassica napus,* the diploid state would have 2n=2X=38 chromosomes; the somatic cell of haploid canola plants would contain only 19 chromosomes (n=X=19). Microspore culture is the most commonly used procedure for DH production. Briefly, surface sterilized buds will be grinded in a sterile condition in 5 ml of B5-13 solution. The liquid mixer is pulled through a sieve and collected into a petri dish. The mixture is diluted with more B5-13 to make a 15 ml suspension that is then transferred with pipet into 50 ml centrifuge tubes and centrifuged at 800-1000 rpm @ 4° C for 3 min. Sedimented microspores are collected and their concentration estimated using haemacytometer. Microspores concentrations are adjusted to 10^{5} microspores/ml by adding NLN-13 medium to the suspension. Since both B5-13 and NLN-13 contain high percentage of sucrose and longer period is required to grow plantlets from microspores, each step is conducted under sterile condition. Fifteen milliliters of the suspension is placed in sterile petri plates and incubated for 24 hours at 32° C in a horizontal shaker. After that period, the suspension is transferred to an incubator set at 24° C for three to four weeks to allow for embryo growth. Once the embryos are formed, they are transferred to MS-basal medium for plantlet development and differentiation.

Use of DH populations with their two identical sets of chromosomes shortens the selection period and speeds up the breeding process to identify both recessive and dominant genes (Chase, 1974). In addition, DH populations are ideal for both applied research (such as breeding, mutation, and genetic transformation studies) as well as for basic research such as

genetic (Forster and Thomas, 2005), biochemical, cytogenetic (Sadasivaiah, 1974) and physiological studies including the study of embryogenesis and other biological processes i.e. mapping (Forster et al., 2007).

A DH population is homozygous (Bentolila et al., 1992; Murignerx et al., 1993). This excellent technique makes DH populations more appealing to researchers in order to have replicates for experiments such as, detection of multiple virus resistance in melon (Lotfi et al., 2003) and polyploidization studies in *B. napus* (Xu et al., 1999). Baenziger (1996) mentioned that use of DH populations is more valid and effective in line development from selected crosses than other traditional breeding approaches. Choo et al. (1985) also mentioned that production of DH lines in barley breeding programs could save four years compared to the use of the pedigree method in which selection occurs on F_6 lines. A genetic map of barley can be constructed within two to three years after the initial cross if DH populations are developed.

Use of DH becomes even more rewarding when it is used for detection of quantitative trait loci (QTL). Since the accuracy of QTL mapping depends on the robustness of the phenotyping procedure, having materials that are 100% homozygous as is the case of DH plants reduces the variability among individuals that is still observed in $F_{5:6}$ populations. Further, in situations where limited numbers of seed production by DH plants are available, production of clonal plants would also contribute to greatly reduce experimental error during phenotyping of reaction to disease. Some excellent results were already achieved in disease resistance QTL mapping in *B. napus* (Foisset et al., 1996; Liu et al., 2005; Yin et al., 2010).

2.4.2.1.b. Association mapping (AM) and linkage disequilibrium (LD)

AM is a population-based survey to identify relationships between phenotypic traits and markers based on LD which is the non-random association of alleles at two marker loci or two genes/QTL or between gene/QTL and a marker locus (Gupta et al., 2005). Use of AM or LD mapping has increased in recent years due to advances in high-throughput genotyping tools. AM has many advantages over traditional bi-parental approach. AM can use collections of germplasm of diverse origin, breeding lines, and cultivars, and therefore does not need to develop segregating populations as is the case of bi-parental mapping, which saves time and resources. AM provides a means for mapping disease resistance genes with considerably higher resolution compared to bi-parental approach (Collins and Morton, 1998). AM is an emerging powerful tool in genes/QTL mining since it can overcome these limitations (Flint-Garcia et al., 2003; Gupta et al., 2005). AM can be conducted either using genome wide scanning or candidate gene scanning techniques. If markers have been identified and mapped then candidate gene approach can be followed. If that is not the case, the genome-wide scanning process should be used.

LD measures the association between two alleles, which arises more often than can be accounted for by chance, because those alleles are physically close on a chromosome and infrequently separated from one another by recombination. The degree of LD in a population determines the resolution of the map. AM has been successfully used in mapping complex traits in humans (Lander and Schork, 1994). In recent years, it has been extended to plants to identify quantitative traits associated with diversified phenotypic traits such as flowering time of *Arabidopsis* (Aranzana et al., 2005), genetic AM and genome organization of maize (Yu et al., 2006), yield and its components in rice (Agrama et al., 2007), kernel size in wheat

(Breseghello and Sorrells, 2006), and phenological, morphological and quality traits in canola (Honsdorf et al., 2010). In addition to the identification of major genes or QTL linked to agronomical, morphological and physiological traits, AM have been used to identify QTL linked to disease resistance in several crops. Tomassini et al. (2007) reported that a 390-fold higher marker resolution can be achieved by AM of the *Stagonospora nodorum* blotch (SNB) resistance gene (*QSng.sfr-3BS*) in winter wheat using 44 varieties compared to QTL mapping using 240 RILs. They found LD decay in chromosome 3B was within 0.5 cM in 44 varieties compared to the 30 cM resolution produced by 240 RILs from a bi-parental cross. Kraakman et al. (2006) used AM to confirm *Rph3* as a major gene resistance to *Puccinia hordei* and the QTL *Rphq2* for prolonging latency to *P. hordei.* Their finding strongly suggested the existence of a barley yellow dwarf (BYD) resistance or tolerance gene on chromosome 2, linked to SSR marker HVM054. Steffenson et al. (2007) used whole genome AM to identify several candidate gene regions of leaf, stripe and stem rust resistance in barley and wheat.

Zhao et al., (2007) identified 13 associated markers linked to leaf traits and 11 markers were associated with flowering time of *B. rapa*. While AM has been successfully used to identify QTL in cereals for some fungal diseases, no reports have been made of its use to characterize the genetic resistance of canola/rapeseed to *S. sclerotiorum*, although several reports have been published on the identification of markers associated with QTL for resistance to this pathogen using bi-parental populations (Yin et al., 2010; Zhao et al., 2003a; Zhao et al., 2003b) and to resistance against blackleg using AM in *B. napus* (Jestin et al., 2010).

Once these populations are ready, phenotypic characterization of plant inoculations with the pathogen of interest and/or molecular marker analyses are needed to identify and

advance individuals that carry the genes of interest. QTL associated with disease resistance genes have been detected using a variety of molecular markers, like AFLP, RFLP, SNP, microsatellites, DArT etc.

2.4.2.2. Genetic and molecular markers used in QTL mapping

The genetic markers used to study resistance to plant pathogens can be grouped in two categories, morphological and molecular markers. Morphological markers, i.e. waxy grape berries (Mlikota-Gabler et al., 2003), and waxy genes for starch content in wheat (Briney et al., 1998) have been detected by plant breeders as they are the phenotypic expression of genes; as such, one of the main disadvantages of using these markers is that they are influenced by environmental conditions. Consequently, it is more difficult and/or time consuming to distinguish the heterozygous from homozygous genotypes using morphological markers. In contrast, molecular markers don't interact with the environment much and thus offer higher potential for use in plant breeding. Most molecular markers are the neutral site of DNA sequence polymorphism. Molecular markers can be used for several different applications including: germplasm characterization, genetic diagnosis, characterization of transformants, study of genome organization, phylogenic analysis, population diversity, marker-trait association etc. (Gupta et al., 1999). Numerous molecular marker types have been used to aid in the identification of QTL associated with disease resistance (Table 2.2).

Table 2.2. Genetic segregation ratio of marker locus in different marker–population combinations.

Marker	Nature	Genetic Segregation Ratio				
		F_2	RILs	DHs	Backcross population	
					B1	B2
RFLP	Co-dominant	1:2:1	1:1	1:1	1:1	1:1
RAPD	Dominant	3:1	1:1	1:1	1:0	1:1
AFLP	Dominant	3:1	1:1	1:1	1:0	1:1
Microsatellites	Co-dominant	1:2:1	1:1	1:1	1:1	1:1
DArT	Dominant	3:1	1:1	1:1	1:0	1:1

Reproducibility, cost, easy and cheap to detect, quantity of DNA required, nature of inheritance (co-dominant desirable), and random and frequent distribution throughout the genome are the basic requirements for suitable molecular marker systems. Various types of marker systems have been used to detect and amplify the naturally occurring polymorphisms in DNA.

a) Restriction fragment length polymorphism (RFLP): RFLPs were first developed to help map the human genome (Botsein et al., 1980); since then, RFLPs have been used extensively to develop genetic maps in plant species without the knowledge of DNA sequence data (Saghai-Maroof et al., 1994). The advantage of RFLP is that it is co-dominant marker and produces reliable and reproducible results between laboratories. However, the technique is expensive, labor intensive, time consuming, has higher quantity of DNA requirement and uses radio-isotope elements. RFLP was extensively used in mapping purpose in diversified crops i.e. maize, tomato (Helentjaris et al., 1986), canola (Yin et al., 2010).

b) Randomly amplified polymorphic DNA (RAPD): RAPD was the first PCR-based molecular marker technique used. It is considered as the simplest and cheapest molecular marker system. Short PCR primers (approximately 10 bases long) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome without the knowledge of DNA sequence data. RAPD shows a dominant relationship (show 3:1 ratio, unable to distinguish between homozyogotes and heterozygotes). The techniques required less quantity of DNA since it is a PCR based technique; however, this technique has reproducibility issues. Several linkage maps have been constructed on the basis of RAPD markers alone in pine and oil palm (Kubisiak et al., 1995; Moretzsohn et al., 2000) or in

combination with RFLP in rice (Huang et al., 1997; Mohan et al., 1994) or with SSR, RFLPs, AFLPs, and SNPs markers in canola (Wang et al., 2011).

c) Amplified Fragment Length Polymorphism (AFLP): AFLP is an advancement of RFLP and is based on selective amplification of restriction fragments (Vos et al., 1995). The restriction digestion of whole genome generates large numbers of fragments of genomic DNA whereas the selective amplification of these fragments helps detect polymorphisms. The system is PCR-based amplification of restriction enzyme fragments of genomic DNA. The high reproducibility, rapid generation and high frequency of detectable polymorphic bands made AFLP a suitable marker system for use in studies of many organisms including plant species. Several studies have been conducted in genetic map construction and population diversity study by using AFLP in several crops e.g. barley (Becker et al., 1995) and *B. napus* (Wang et al., 2011).

d) Simple Sequence Repeats (SSR)/Microsatellites: Simple sequence repeats, also known as microsatellites are present in the genomes of all eukaryotes and consists of several to more than one hundred repeats of a 1-4 nucleotide motif. These repeated motifs are denoted $(AAAC)_n$, where *n* is the number of tandem repeats. The sequences flanking these microsatellites are often conserved and can be used to design primers. These primers can be designed by constructing a novel genomic library and sequencing segments of the subject genome. Therefore, this system requires sequence data information. Already discovered sequence (i.e.: GENEBANK online database) can also be searched for SSR primer design. Polymorphism is based on the number of tandem repeats and therefore the length of the PCR products. SSR is a co-dominant marker. This marker system is the popular among

researchers these days due to its reproducibility, co-dominant nature, ease of use and moderate cost. Cheng et al. (2009) developed 627 new microsatellite markers for *B. napus.*

e) Single nucleotide polymorphisms (SNP): In recent years, nucleotide diversity has been widely used to understand the diversity of population and how it shapes the evolution of genome in different crops. This is the most popular, more robust and cheaper. Mutation or diversity in single nucleotide can be determined by this technique. SNPs are of more recent development, and are expensive to develop. Their use requires sequence information. SNP has been widely used in association mapping study of barley (Massman et al., 2010). More than 43,000 SNP markers have been developed for *B. napus* using Solexa transcriptome sequencing (Trick et al., 2009).

f) Diversity Array Technology (DArT): DArT does not use the sequence information to detect polymorphisms in a population. It has potential to be used in genes/QTL mapping, map based cloning and association genetics (Buckler and Thronsberry, 2002). Recently, the Diversity Array Technology group (DArT) developed 3072 DArT markers for genotyping of *B. napus* (Raman et al., 2011). Due to its whole genome system, cost, and reliability, the system is more appropriate for whole genome mapping. This technique requires less quantity of DNA and less time compared to other systems. Several linkage maps have been created in wheat and barley by using DArT marker system. DArT is also getting more popular and reliable for Linkage disequilibrium calculation in many crops including wheat and barley (A. Kinian, *Personal communication*).

Wang et al., (2011) used SSR, RFLPs, AFLPs, RAPDs and SNPs markers to publish a first genome-wide genetic map of different linkage groups (LG) of the three population-specific *B. napus* maps based on an automated implementation of a defined algorithm. This

study indicates that there is a good agreement over most of the LG of three population-specific consolidated maps and there is duplication of locus among *B. napus*, *B. rapa* and *Arabidopsis*. Marker assisted selection (MAS) has become more important today because traits conditioned by QTL are more difficult to introgress into desired cultivars using conventional plant breeding techniques. Furthermore, MAS has been used as a tool to enhance the selection efficiency of plant breeding. Molecular markers linked with phenotypic traits help breeder to select desired recombinants in the laboratory before they are actually evaluated in the field. For example, de la Pena et al. (1999) mapped Fusarium head blight resistance QTL in barley. Castro et al. (2003a and 2003b) used MAS to pyramid the stripe rust resistance QTL in barley.

2.5. References Cited

Abawi, G. S., and Grogan, R. G. 1979. Epidemiology of diseases caused by *Sclerotinia species*. Phytopathology 69:899–904.

Adams, P. B. and Fravel, D. R. 1990. Economical biological control of Sclerotinia lettuce drop by *Sporidesmium sclerotiorum*. Phytopathology 80:1120-1124.

Adams, P. B. and Ayers, W. A. 1979. Ecology of *Sclerotinia species*. Phytopathology 69:896–898.

Agrama, H. A., Eizenga G. C., and Yan. W. 2007. Association mapping of yield and its components in rice cultivars. Mol. Breed. 19:341–356.

Alghisi, P., and Favaron, F. 1995. Pectin-degrading enzymes and plant parasite interactions. Eur. J. Plant Pathol. 101:365–375.

Aranzana, M. J., Kim, S., Zhao, K. Y., Bakker, E., Horton, M., Jakob, K., Lister, C., Molitor, J., Shindo, C., Tang, C. L., Toomajian, C., Traw, B., Zheng, H. G., Bergelson, J., Dean, C., Marjoram, P., and Nordborg, M. 2005. Genome-wide association mapping in *Arabidopsis* identifies previously known flowering time and pathogen resistance genes. PLOS Genet. 1:531–539.

Baenziger, P. S. 1996. Reflections on doubled haploids in plant breeding. Pages 35–48 in: In vitro haploid production in higher plants. (Vol. 1) Mohan Jain, S., Sopory, S. K., and Veilleux, R. E. (eds.). Kluwer academic publishers, Dordrecht, The Netherlands.

Bateman, D. F. 1964. An induced mechanism of tissue resistance to polygalacturonase in Rhizoctonia-infected hypocotyls of bean. Phytopathology 54:438–445.

Bateman, D. F., and Beer, S. V. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis of *Sclerotium rolfsii*. Phytopathology 55:204–211.

Becker, H. C., Karle, R., and Han, S. S. 1992. Environmental variation for outcrossing rates in rapeseed (*Brassica napus*). Theor. Appl. Genet. 84:303-306.

Becker, J., Vos, P., Kuiper, M., Salamini, F., and Heun, M. 1995. Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genet. 249:69-73.

Bell, A. A., and Wheeler, M. H. 1986. Biosynthesis and functions of fungal melanins. Annu. Rev. Phytopathol. 24:411–451.

Bentolila, S. T., Hardy, T., Guitton, C., and Freyssinet, G. 1992. Comparative genetic analyses of F_2 plants and anther culture derived plants of maize. Genome 35:575–582.

Boland, G. J. and Hall, R. 1987. Evaluating soybean cultivars for resistance to *Sclerotinia sclerotiorum* under field conditions. Plant Dis. 71:934–936.

Boland, G. J., and Hall, R. 1988. Relationships between the spatial pattern and number of apothecia of *Sclerotinia sclerotiorum* and stem rot of soybean. Plant Pathol. 37:329-336.

Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 16:93-108.

Bolton, M. V., Thomma, B. P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-16.

Bostein, D., White, K. L., Skolnick, M., and Davis, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Bradley, C. A., Henson, R. A., LeGare, D. G., del Rio, L. E., and Khot, S. D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Dis. 90:215-219.

Bremer, E., Huang, H. C., Selinger, L. J., and Davis, J. S., 2000. Competence of *Coniothyrium minitans* in preventing infection of bean leaves by *Sclerotinia sclerotiorum*. Plant Pathol. Bull. 9:69–74.

Breseghello, F., and Sorrells, M. E. 2006. Association analysis as a strategy for improvement of quantitative traits in plants. Crop Sci. 46:1323–1330.

Briney, A., Wilson, R., Potter, R. H., Barclay, I., Crosbie, G., and Apples, R. 1998. A PCR-based marker for selection of starch and potential noodle quality in wheat. Mol. Breed. 4:427-433.

Brown, J., Davis, J. B., Lauver, M., and Wysocki, D. 2008. USCA canola growers' manual. Available from http://uscanola.com/index.asp?Type=B_BASIC&SEC={E0677795-D258-43C7-88A0-01E3358D89D6}: Accessed July 17, 2009.

Buckler, E. S., and Thornsberry, J. M. 2002. Plant molecular diversity and applications to genomics. Curr. Opin. Plant Biol. 5:107–111.

Buzza, G. 1991. Canola. Page 19 in: New crops: agronomy and potential of alternative crop species. Jessop, R. S., Wright, R. L. eds. Inkata Press, Sydney, Australia.

Caesar, A. J., and Pearson, R. C. 1983. Enviornmental factors affecting survival of ascospores of *Sclerotinia sclerotiorum*. Phytopathology 73:1024-1030.

Castro, A. J, Capettini, F., Corey, A. E., Fillichkin, T., Hayes, P. M., Kleinhofs, A., Kudrna, D., Richardson, K., Sandoval-Islas, S., Rossi, C., and Vivar, H. 2003a. Mapping and pyramiding of qualitative and quantitative resistance to stripe rust in barley. Theor. Appl. Genet. 107:922–930.

Castro, A. J., Chen, X. M., Hayes, P. M., and Johnston, M. 2003b. Pyramiding quantitative trait locus (QTL) alleles determining resistance to barley stripe rust: effects on resistance at seedling stage. Crop Sci. 43:651–659.

Cessna, S. G., Sears, V. E., Dickman, M. B., and Low, P. S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell 12:2191–2199.

Chahal, G. S., and Gosal, S. S. 2006. Principles and procedures of plant breeding: biotechnological and conventional approaches. Alpha science international ltd., Pangbourne, UK.

Chase, S. S. 1974. Utilization of haploids in plant breeding: breeding diploid species, pages 211–231 in: Haploids in higher plants: advances and potential. Kasha, K. J. (ed.). Proceedings of the first international symposium. Univ. of Guelph Press, Guelph, Canada.

Cheng, X., Xu, J., Xia, S., Gu, J., Yang, Y., Fu, J., Qian, X., Zhang, S., Wu, J., and Liu, K. 2009. Development and genetic mapping of microsatellite markers from genome survey sequences in *Brassica napus*. Theor. Appl. Genet. 118:1121-1131.

Chitrampalam, P., Figuli, P. J., Matheron, M. E., Subbarao, K. V., and Pryor, B. M. 2008. Biocontrol of lettuce drop caused by *Sclerotinia sclerotiorum* and *S. minor* in desert agroecosystems. Plant Dis. 92:1625-1634.

Choo, T. M., Reinbergs, E., and Kasha, K. J. 1985. Use of haploids in breeding barley. Plant Breed. Rev. 3:219–252.

Christias, C. and Lockwood, J. L. 1973. Conversion of mycelial constituents in four sclerotium-forming fungi in nutrient deprived conditions. Phytopathology 63:602–605.

Clarkson, J. P., Phelps, K., Whipps, J. M., Young, C. S., Smith, J. A. and Watling, M. 2004. Forecasting Sclerotinia disease on lettuce: toward developing a prediction model for carpogenic germination of sclerotia. Phytopathology 94:268–279.

Clarkson, J. P., Staveley, J., Phelps, K., Young, C. S., and Whipps, J. M. 2003. Ascospore release and survival in *Sclerotinia sclerotiorum*. Mycol. Res. 107:213–222.

Collins, A., and Morton, N. E. 1998. Mapping a disease locus by allelic association. Proc. Natl. Acad. Sci. 95:1741–1745.

de la Pena, R. C., Smith, K. P., Capettini, F., Rasmusson, D. C., GalloMeagher, M., Dill-Macky, R., Somers, D. A., and Muehlbauer, G. J. 1999. Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theor. Appl. Genet. 99:561-569.

De Lorenzo, G., D'Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopathol. 39:313–335.

del Río, L. E., Bradley, C. A., Henson, R. A., Endres, G. J., Hanson, B. K., McKay, K., Halvorson, M., Porter, P. M., Le Gare, D. G., and Lamey, H. A. 2007. Impact of Sclerotinia stem rot on yield of canola. Plant Dis. 91:191-194.

del Rio, L. E., Martinson, C. A., and Yang, X. B. 2002. Biological control of Sclerotinia stem rot of soybean with *Sporidesmium sclerotivorum*. Plant Dis. 86:999–1004.

Downey, R. K., Klassen, A. J., and Stringam, G. P. 1980. Rapeseed and mustard. Pages 495-509 in*:* Hybridization of crop plants. American Society of Crop Science, USA.

Duncan, R. W. 2003. Evaluation of host tolerance, biological, chemical, and cultural control of *Sclerotinia sclerotiorum* in sunflower (*Helianthus annuus* L.). M.S. Thesis, University of Manitoba, Manitoba, Canada.

Dutton, M. V. and Evans, C. S. 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42:881–895.

Favaron, F., D'Ovidio, R., Porceddu, E. and Alghisi, P. 1994. Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 195:80–87.

Favaron, F., Sella, L., and D'Ovidio, R. 2004. Relationships among endopolygalacturonase, oxalate, pH, and plant polygalacturonase inhibiting protein (PGIP) in the interaction between *Sclerotinia sclerotiorum* and soybean. Mol. Plant–Microbe Interact. 17:1402–1409.

Ferrar, P. H., and Walker, J. R. L. 1993. o–Diphenol oxidase inhibition – an additional role for oxalic acid in the phytopathogenic arsenal of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Physiol. Mol. Plant Pathol. 43:415–422.

Flint-Garcia, S. A, Thornsberry, J. M., and Buckler, E. S. 2003. Structure of linkage disequilibrium in plants. Annu. Rev. Plant. Biol. 54:357–374.

Foisset, N., Delourme, R., Barret, P., Hubert, N., Landry, B. S., and Renard, M. 1996. Molecular-mapping analysis in *Brassica napus* using isozyme, RAPD and RFLP markers on a doubled-haploid progeny. Theor. Appl. Genet. 93:1017-1025.

Foreign Agricultural Service (FAS), USDA. 2009. Oilseeds: Worlds market and trade. Available from http://www.fas.usda.gov/oilseeds_arc.asp: Accessed July 17, 2009.

Forster, B. P., and Thomas, W. T. B. 2005. Doubled haploids in genetics and plant breeding. Plant Breed. Rev. 25:57–88.

Forster, B. P., Heberle-Bors, E., Kasha, K. J., and Touraev, A. 2007. The resurgence of haploids in higher plants. Trends Plant Sci. 12:368–375.

Fraissinet-Tachet, L. and Fèvre, M. 1996. Regulation by galacturonic acid of pectinolytic enzyme production by *Sclerotinia sclerotiorum*. Curr. Microbiol. 33:49–53.

Garcia-Maceira, F. I., Di Pietro, A., Huertas-Gonzalez, M. D., Ruiz-Roldan, M. C. and Roncero, M. I. G. 2001. Molecular characterization of an endopolygalacturonase from *Fusarium oxysporum* expressed during early stages of infection. Appl. Environ. Microbiol. 67:2191–2196.

Godoy, G., Steadman, J. R., Dickman, M. B., and Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37:179–191.

Guimarães, R. L., and Stotz, H. U. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. Plant Physiol. 136:3703-3711.

Gupta, P., Rustgi, S., and Kulwal, P. 2005. Linkage disequilibrium and association studies in higher plants: present status and future prospects. Plant Mol. Biol. 57:461-485.

Gupta, P. K., Varshney, R. K., Sharma, P. C., and Ramesh, B. 1999. Molecular markers and their applications in wheat breeding. Plant Breed. 118:369–390.

Hancock, J. C. 1966. Degradation of pectic substances associated with pathogenesis by S*clerotinia sclerotiorum* in sunflower and tomato stems. Phytopathology 56:975–979.

Harikrishnan, R., Zabala, F., and del Rio, L. E. 2005. Evaluation of "Contans", a biological formulation for control of Sclerotinia stem rot (SSR) in canola, 2005. B&C Tests 21:FC043.

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A., and Nienhuis, J. 1986. Construction of linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet. 72:761-769.

Honsdorf N., Becker, H. C, and Ecke, W. 2010. Association mapping for phenological, morphological, and quality traits in canola quality winter rapeseed (*Brassica napus* L.). Genome 53:899-907.

Huang, H. C., and Kozub, G. C. 1991. Temperature requirements for carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* isolates of different geographic origin. Bot. Bull. Acad. Sin. 32:279–286.

Huang, N., Parco, A., Mew, T., Magpantay, G., and McCouch, S. R. 1997. RFLP mapping of isozymes, RAPD and QTLs for grain shape, brown plant hopper resistance in a double haploid rice population. Mol. Breed. 3:105-113.

Inglis, G. D., and Boland, G. J. 1990. The microflora of bean and rapeseed petals and the influence of the microflora of bean petals on white mold. Can. J. Plant Pathol. 12:129–134.

Jamaux, I., Gelie, B., and Lamarque, C. 1995. Early stages of infection of rapeseed petals and leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. Plant Pathol. 44**:**22–30.

Jestin, C., Lode, M., Vallee, P., Domin, C., Falentin, C., Horvais, R., Coedel, S., Manzanares-Daulex, M. J., and Delourme, R. 2010. Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Mol. Breed. 27:271–287

Kars, I., and van Kan, J. A. L. 2004. Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. Pages 99-118 in: Botrytis: biology, pathology and control. Elad, Y., Williamson, B., Tudzynski, P. and Delen, N., eds. Dordrecht: Kluwer Academic Publishers.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E. and van Kan, J. A. L. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43:213–225.

Kharbanda, P. D., and Tewari, J. P. 1996. Integrated management of canola diseases using cultural methods. Can. J. Plant Pathol. 18:168-175.

Khot, S. D. 2006. Evaluation of *Brassica napus* accessions for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis. NDSU, USA.

Kohn, L. M. 1979. A monographic revision of the genus *Sclerotinia*. Mycotaxon 9:365–444.

Kraakman, A. T. W., Martinez, F., Mussiralie, B., Eeuwijk, F. A. V, and Niks, R. E., 2006. Linkage disequilibrium mapping of morphological, resistance and other agronomically relevant traits in modern spring barley cultivars. Mol. Breed. 17:41-58.

Kubisiak, T. L., Nelson, C. D, Nance, W. L., Stine, M. 1995. RAPD linkage mapping in a longleaf pine x slash pine F_1 family. Theor. Appl. Genet. 90:1119-1127.

Kurian, P., and Stelzig, D. A. 1979. The synergistic role of oxalic acid and endopolygalacturonase in bean leaves infected by *Cristulariella pyramidalis*. Phytopathology 69:1301–1304.

Lamey, A., and Bradley, C. A. 2003. Sclerotinia stem rot of canola biology and management. N. D. State Univ. Ext. Rep. No. PP-1201.

Lamey, A., Knodel, J., Endres, G., Andol, K., Ashley, R., Beneda, R., Johnson, N., Knoke, S., Liane, M., Lykken, T., Miller, M., Nyegaard, C., and Peterson, N. 2002. 2002 Canola disease survey in Minnesota and North Dakota. N. D. State Univ. Ext. Rep. No. 80.

Lamey, H. A. 2003. The status of *Sclerotinia sclerotiorum* on canola in North America. *In*: Proceedings of 2003 Sclerotinia Initiative annual meeting, Bloomington, MN, USA.

Lander, E. S., and Schork, N. J. 1994. Genetic dissection of complex traits. Science 265:2037-2048.

Larsen, L. M., and Sorensen, H. 1985. The value of oilseed rape production in Denmark and the EEC. Pages: 1-18 in: Advances in production and utilization of cruciferous crops; Sorensen. H., Ed., Martinus Nijhof/Dr. Junk Publishers, Denmark.

Le Tourneau, D. 1979. Morphology, cytology and physiology of *Sclerotinia* species in culture. Phytopathology 69:887–890.

Liu, S., Wang, S., Zhang, J., Fitt, B., Xu, Z., Evans, N., Liu, Y., Yang, W., and Guo, X. 2005. *In vitro* mutation and selection of doubled-haploid *Brassica napus* lines with improved resistance to *Sclerotinia sclerotiorum*. Plant Cell Rep. 24:133–144.

Lotfi, M., Alan, A. R., Henning, M. J., Jahn, J., and Earle, E. D. 2003. Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. Plant Cell Rep. 21:1121–1128.

Lü, Z. J., and Fu, S. Z. 1990. Inheritance of apetalous character in rape (*Brassica napus* L.) and its implication in breeding. Jiangsu J. Agric. Sci. 6:30–36.

Lumsden, R. D. 1969. *Sclerotinia sclerotiorum* infection of bean and the production of cellulase. Phytopathology 59:653–657.

Lumsden, R. D., and Dow, R. L. 1973. Histopathology of *Sclerotinia sclerotiorum* infection of bean. Phytopathology 63:708–715.

Lumsden, R. D. 1979. Histology and physiology of pathogenesis in plant diseases caused by *Sclerotinia* species. Phytopathology 69:890–896.

Magro, P., Marciano, P., and Di Lenna, P. 1984. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. FEMS Microbiol. Lett. 24:9–12.

Marciano, P., Di Lenna, P., and Magro, P. 1983. Oxalic acid, cell wall degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. Physiol. Plant Pathol. 22:339–345.

Massman, J., Cooper, B., Horsley, R., Schwarz, P., Dong, Y., Neate, S., Dill-Macky, R., Franckowiak, J., Muehlbauer, G., Chao, S., and Smith, K. 2010. Association mapping of Fusarium head blight resistance QTL in contemporary barley breeding germplasm. Mol. Breed. 27:439-454.

Maxwell, D. P., and Lumsden, R. D. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60:1395–1398.

McMullen, M. P., and Markell, S. G. 2011. 2011 North Dakota field crop fungicide guide. N. D. State Univ. Ext. Rep. No. PP-622.

McQuilken, M. P., Mitchell, S. J., Budge, S. P., Whipps, J. M., Fenlon, J. S., and Archer, S. A. 1995. Effect of *Coniothyrium minitans* on sclerotial survival and apothecial production of *Sclerotinia sclerotiorum* in field-grown oilseed rape. Plant Pathol. 44:883–896.

Mlikota-Gabler, F., Smilanick, J. L., Mansour, M., Ramming, D. W., and Mackey, B. E. 2003. Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. Phytopathology 93:1263-1273.

Mohan, M., Nair, S., Bentur, J. S., Rao, U. P., Bennett, J. 1994. RFLP and RAPD mapping of the rice Gm 2 gene that confers resistance to biotype 1 of gall midge (*Orseolia oryzae*). Theor. Appl. Genet. 87:782-788.

Moretzsohn, M. C, Nunes, C. D. M., Ferreira, M. E, Grattapaglia, D. 2000. RAPD linkage mapping of the shell thickness locus in oil palm (*Elaeis guineensis* Jacq). Theor. Appl. Genet. 100:63–70.

Murignerx, A., Barloyz, D., Leroy, P., and Beckert, M. 1993. Molecular and morphological evaluation of doubled haploid lines in maize. 1. Homogeneity within DH lines. Theor. Appl. Genet. 86:837–842.

NASS. 2008. United States Department of Agriculture, 2008 Census of Agriculture. Washington, DC. [http://www.nass.usda.gov/index.asp (Accessed November 1, 2010)].

Nepal, A. 2009. Role of moisture and soil texture on apothecia formation by *Sclerotinia sclerotiorum* (Lib.) de Bary . M.S. Thesis, NDSU, USA.

Noyes, R. D., and Hancock, J. G. 1981. Role of oxalic acid in the Sclerotinia wilt of sunflower. Physiol. Plant Pathol. 18:123–132.

Palmer, C. E., and Keller, W. A. 2005. Overview of haploidy. Pages 1–10 in: Palmer, C. E., Keller, W. A., and Kasha, K. J. (eds.). Haploids in crop improvement II, Springer-Verlag, Berlin, Germany.

Phillips, A. J. L. 1986. Carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* after periods of conditioning in soil. J. Phytopathol. 116:247–258.

Purdy, L. H. 1979. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 69:875-880.

Qandah, I. S., and del Rio Mendoza, L. E. 2011. Temporal dispersal patterns of *Sclerotinia sclerotiorum* ascospores during canola flowering. Can. J. Plant Path. 33:159-167.

Rakow, G. and Séguin-Swartz, G. 1999. Development of apetalous *Brassica napus*. In: Proceedings of 10th International Rapeseed Congress. Canberra, Australia.

Raman, H., Raman, R., Nelson, M. N., Aslam, M. N., Rajasekaran, R., Wratten, N., Cowling, W. A., Kilian, A., Sharpe, A. G., and Schondelmaier, J. 2011. Diversity Array Technology markers: genetic diversity analyses and linkage map construction in rapeseed (*Brassica napus* L.). DNA research Pp:1-15. doi:10.1093/dnares/dsr041

Ranito-Lehtimäki, A. 1995. Aerobiology of pollen and pollen antigens. In Cox, C., and Wathes, C., (eds.) Bioaerosols Handbook. CRC Lewis.

Raymer, P. L., Auld, D. L., and Mahler, K. A. 1990. Agronomy of canola in the United States. Pages:25-36 in: Rapeseed/Canola: Production Chemistry, Nutrition and Processing Technology. Shahidi, F. ed. Van Nostrand Reinhold, New York, USA.

Riou, C., Freyssinet, G., and Fèvre, M. 1992. Purification and characterization of extracellular pectinolytic enzymes produced by *Sclerotinia sclerotiorum*. Appl. Environ. Microbiol. 58:578–583.

Rollins, J. A. 2003. The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. Mol. Plant–Microbe Interact. 16:785–795.

Rollins, J. A., and Dickman, M. B. 2001. pH signaling in *Sclerotinia sclerotiorum*: identification of a pacC/RIM1 homolog. Appl. Environ. Microbiol. 67:75–81.

Sadasivaiah, R. S. 1974. Haploids in genetic and cytological research. Pages 355–386 in: Haploids in Higher Plants: Advances and Potential. Kasha, K. J. (ed.). Proc. First International Symposium. Univ. of Guelph Press, Guelph, ON, Canada.

Saghai-Maroof, M. A., Biyashev, R. M., Yang, G. P., Zhang, Q., and Allard, R. W. 1994. Extra ordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations and population dynamics. Proc. Natl. Acad. Sci. 91:5466-5470.

Saharan, G. S. and Mehta, N. 2008. *Sclerotinia* diseases of crop plants: biology, ecology and disease management. Springer, Dordrecht, UK.

Sarwar, G. 1984 Available amino acid score for evaluating protein quality of foods. J. Assoc. Off. Anal. Chem. 67:623–626.

Schwartz, H. F., and Steadman, J. R. 1978. Factors affecting sclerotium populations of and apothecium production by *Sclerotinia sclerotiorum*. Phytopathology 68:383–388.

Shahidi, F. 1990. Canola and rapeseed: production, chemistry, nutrition, and processing technology. Van Nostrand Reinhold, New York., USA.

Shieh, M. T., Brown, R. L., Whitehead, M. P., Cary, J. W., Cotty, P. J., Cleveland, T. E., and Dean, R. A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, *P2c*, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63:3548–3552.

Steadman, J. R. 1979. Control of plant diseases caused by *Sclerotinia* species. Phytopathology 69:904–907.

Steffenson, B., J., Olivera, P., Roy, J., K., Jin, Y., Smith, K. P., and Muehlbauer, G. J. 2007. A walk on the wild side: mining wild wheat and barley collections for rust resistance genes. Aus. J. Agric. Res. 58:532-544.

Subbarao, K. V. 1998. Progress towards integrated management of lettuce drop. Plant Dis. 82:1068-1078.

ten Have, A., Mulder, W., Visser, J., and van Kan, J. A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant–Microbe Interact. 11:1009–1016.

Thomas, P. 2003. Canola growers manual. The canola council of Canada, Canada Available from http://www.canolacouncil.org/contents2.aspx: Accessed March 17, 2011.

Tomassini, L., Schnurbusch, T., Fossati, D., Mascher, F., and Keller, B. 2007. Association mapping of *Stagonospora nodorum* blotch resistance in modern European winter wheat varieties. Theor. Appl. Genet. 115:697-708.

Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. 2003. Soft rot erwiniae: from genes to genomes. Mol. Plant Pathol. 4:17–30.

Townsend, B. B., and Willetts, H. J. 1954. The development of sclerotia in certain fungi. Trans. Br. Mycol. Soc. 37:213–221.

Trick, M., Long, Y., Meng, J., Bancroft, I. 2009. Single nucleotide polymorphism (SNP) discovery in the polyploid *Brassica napus* using Solexa transcriptome sequencing. Plant Biotechnol. J. 7:334–346.

Turkington, T. K., and Morrall, R. A. A. 1993. Use of petal infestation to forecast Sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. Phytopathology 83:682–689.

U, N. 1935. Genome-analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jap. J. Bot. **7**:389–452.

U. S. Canola Association. 2008. U. S. canola production. Available from http://uscanola.com/index.asp?Type=B_BASIC&SEC={5CDA1D60-89B2-4F79-B96F-0B5AB2FBF781}: Accessed Jan 17, 2011.

USDA. 2005. Sclerotinia Initiative Brochure. Fargo, ND: United States Department of Agriculture, USA. Available at www.whitemoldresearch.com.

USDA, 2007. National Agricultural Statistics Service http://www.nass.usda.gov/Statistics_by_State/Ag_Overview/AgOverview_OK.pdf accessed Sept 20, 2010.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 11:4407–4414.

Wagner, F., Kusserow, H., and Schafer, W. 2000. Cloning and targeted disruption of two polygalacturonase genes in *Penicillium olsonii*. FEMS Microbiol. Lett. 186:293–299.

Wang, J., Lydiate, D. L., Parkin, I. A. P., Falentin, C., Delourme, R., Carion, P. W. C, and King, G. J. 2011. Integration of linkage maps for the amphidiploid *Brassica napus* and comparative mapping with *Arabidopsis* and *Brassica rapa.* BMC Genomics 12:101-121.

Willetts, H. J., and Wong, J. A. 1980. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum,* and *S. minor* with emphasis on specific nomenclature. Bot. Rev. 46:101–165.

Williams, I. H., Martin, A. P., and White, R. P. 1986. The pollination requirements of oil-seed rape (*Brassica napus* L.). J. Agric. Sc. 106:27-30.

Xu, H. X., Jing, H. C., Cheng, X. E., Iwanowska, A., Kieft, H., Bergervoet, J. H. W., Groot, S. P. C., Bino, R. J., and van Lammeren, A. A. M. 1999. Polyploidization in embryogenic microspore cultures of *Brassica napus* L. cv. Topas enables the generation of doubled haploid clones by somatic embryogenesis. Protoplasma 208:240–247.

Yang, G. S., and Fu, T. D. 1990. The inheritance of 'Polima' cytoplasmic male sterility in *Brassica napus.* Plant Breed. 104:121–124.

Yin, X., Yi, Ben, Chen, W., Zhang, W., Tu, J., Fernando, D., and Fu, T. 2010. Mapping of QTLs detected in a *Brassica napus* DH population for resistance to *Sclerotinia sclerotiorum* in multiple environments. Euphytica 173:25-35.

Yu, J., Pressoir, Briggs, W. H., Bi, I. V., Yamasaki, M., Doebley, J. F., McMullen, M. D., Gaut, B. G., Nielsen, D. M., Holland, J. B., Kresovich, S., and Buckler, E. S. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208.

Zabala, F., and del Rio, L. E. 2007. Screening the *Brassica rapa* NGRP collection for reaction to S. *sclerotiorum*. Phytopathology 97:S128.

Zhao J., and Meng, J. 2003a. Detection of loci controlling seed glucosinolate content and their association with Sclerotinia resistance in *Brassica napus*. Plant Breed. 122:19–23.

Zhao, J., and Meng, J. 2003b. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 106:759–764.

Zhao, J., Peltier, A. J., Meng, J., Osborn, T. C., and Grau, C. R. 2004. Evaluation of Sclerotinia stem rot resistance in oilseed *Brassica napus* using a petiole inoculation technique under greenhouse conditions. Plant Dis. 88:1033-1039.

Zhao, K., Aranzana, M. J., Kim, S., Lister, C., Shindo, C., Tang, C. Toomajian, C., Zheng, H., Dean, C., Marjoram, P., and Magnus, N. 2007. An Arabidopsis example of association mapping in structured samples. PLoS Genet. 3:71–82.

CHAPTER 3. COMPARISON OF EIGHT INOCULATION TECHNIQUES TO SCREEN FOR SCLEROTINIA STEM ROT RESISTANCE IN CANOLA.

3.1. Abstract

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is an economic fungal disease affecting rapeseed (*Brassica napus* L.) worldwide. Since expression of sclerotinia stem rot symptoms shows much variability and the trait is quantitative in nature, reliable phenotypic evaluation methods for characterization of SSR resistance are needed. Eight different inoculation techniques were compared for their ability to discriminate between *S. sclerotiorum*-resistant and susceptible germplasms using two isolates that differ in virulence. These techniques were evaluated using two *S. sclerotiorum* isolates (WM031 and WM192) collected in North Dakota and clonal plants derived from doubled haploid resistant (PI458940 x PI 649136) and susceptible (Westar) *B. napus* materials. The inoculation techniques evaluated were detached leaves (DLA) and stems (DSA) assays, petiole inoculation (PIT), straw inoculation method (STR), stem-piercing with toothpick (SPT), mycelium spray (MS), petal inoculation (PET), and oxalic acid assay (OAA). The experiment was conducted using a randomized complete block design with four replications and was repeated once. Inoculated materials were incubated at 16 h light and 22±2 °C daily. PET and SPT were highly correlated to each other (rho = 0.98) ($P < 0.001$) whereas, MSI and PIT methods can better discriminate between the isolates and germplasm compared to other methods. Therefore, PIT and MSI are considered more reliable methods for breeders to evaluate materials with for their reaction to sclerotinia stem rot.

3.2. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary causes Sclerotinia stem rot in canola/rapeseed (*Brassica napus* L.) (Purdy, 1979). SSR epidemics on dry bean, sunflower, and canola have been responsible for US$ 24.5 million losses per year in North Dakota and Minnesota during the year 2000 (Lamey et al., 2001), although the pathogen also affects numerous other crops (Boland and Hall, 1994). Canola growers have a menu of management practices to use against sclerotinia stem rot that includes use of chemical fungicides guided by a forecasting model Sclerotinia Risk Map (Northern Canola Growers Association, 2009); use of biofungicides that antagonize the pathogen's mycelial growth, parasitize its sclerotia or enhance plant defense mechanisms; and cultural practices, like tillage, crop rotation with non-host crops, and controlled irrigation (Kharbanda and Tiwari, 1996; Lamey and Bradley, 2003; Sharan and Mehta, 2008). However, the best and most effective way of managing a disease, use of resistant cultivars, is not included. Commercial canola or rapeseed cultivars with acceptable levels of resistance to sclerotinia stem rot are not available.

Resistance to sclerotinia stem rot is polygenic and additive (Zhao and Meng, 2003; Zhao et al., 2006), which makes the identification and selection of resistant genotypes difficult. The use of molecular tools has helped to identify quantitative trait loci (QTL) associated with resistance to *S. sclerotiorum* in a number of crops (Ender et al., 2008; Kelly et al., 2003; Miklas, 2007); however, the discriminating power of these tools depends on the quality of the data collected while assessing the plants' reaction to the pathogen. Therefore, use of an inoculation method that provides consistent results is of critical importance. Numerous screening techniques have been developed to evaluate the phenotypic reaction of plants to *S. sclerotiorum* under controlled conditions. Some of these methodologies are:

excised or detached stem (DSA) and detached leaf (DLA) assays in bean and soybean (Chun et al., 1987; Kull et al., 2003), cut-stem inoculation in dry bean, soybean and sunflower (Vuong et al., 2004), petiole inoculation technique (PIT) in soybean and canola (Bradley et al., 2006; del Rio et al., 2001; Zhao et al., 2004), straw test (STR) in dry bean (Petzoldt and Dickson, 1996), stem-piercing with toothpick (SPT) in canola (Zhao and Meng, 2003), mycelium spray inoculation (MSI) method in soybean (Chen and Wang, 2005) and oxalic acid assay (OAA) in soybean (Peltier and Grau, 2006).

Not all inoculation methods work similarly well in all crops. In canola, Bradley et al. (2006) compared the reaction of commercial canola cultivars to *S. sclerotiorum* using the PIT, OAA and DLA in controlled conditions and compared it to field evaluations using *S. sclerotiorum* ascospores. Results demonstrated that PIT and OAA were capable of discriminating between cultivars but DLA was not. On dry beans the PIT did not work well because the plants tend to drop the petioles before the fungus enters the stems (del Rio, *personal communication*); instead the preferred inoculation method is STR in which the main stem rather than a petiole is inoculated with a piece of agar loaded with *S. sclerotiorum* mycelium (Petzoldt and Dickson, 1996). While many more methods have been developed, most seem to have a common disadvantage, their limited ability to consistently predict the reaction of cultivars in the field. These inconsistencies could be due in part to heterozygous condition of the populations evaluated, to the differential effect of environments, or simply to the inoculation method itself. Comparison of several inoculation techniques using fully homozygous materials could help identify the best methods. This study intends to compare eight different inoculation techniques for their ability to discriminate between moderately resistant and susceptible *B. napus* materials using two different *S. sclerotiorum* isolates.

3.3. Materials and Methods

Two highly homozygous plant materials, one considered moderately resistant and the other susceptible to *S. sclerotiorum*, were used to compare the ability of eight inoculation techniques to discriminate between them. The methods evaluated were DLA, DSA, MSI, PIT, petal inoculation (PET), SPT, STR, and OAA.

3.3.1. Doubled haploid and clones preparation

The moderately resistant DH line (DHP) was produced from a cross between *B. napus* plant introduction materials 458940 and 649136, using a protocol developed by Su (2009), which are considered to be resistant to *S. sclerotiorum* (Khot, 2006). The susceptible DH line (WDH) was derived from cultivar 'Westar'. In brief, an F_1 seed from the PIs 458940 X 649136 cross, and 'Westar' were planted under greenhouse conditions. Immature buds from corresponding plants were aseptically homogenized in B5 media and re-suspended in NLN media. Microspores were cultured in NLN media for 4-6 weeks. Once the embryos were formed, they were transferred to MS basal media for rooting and shooting. Due to the limited number of DH progenies formed, a decision was made to clone the plants. Briefly, 3.8 cm long pieces containing a node were cut from one to two week old branches from each DH line and deposited in sterile distilled water to prevent them from dehydrating. In the laboratory, the pieces were surface disinfested by immersing them in a 10% aqueous solution of commercial bleach (0.525% NaOCl) for one minute before rinsing them in sterile water. Four to six pieces were placed in plastic containers (Caisson laboratories, North Logan, UT, USA) containing ~100 ml of MS medium (Murashige and Skoog, 1962) supplemented with NAA at 1 µM (MS1N) to induce root production. The pieces were incubated under 16 h light at 26±2

°C for 15-20 days. Clones were transferred to 10 x 10 cm plastic pots filled with Sunshine Mix # 1 (Fison Horticulture, Vancouver, B. C.) approximately 10 days after root initiation. Plants were kept in mist chamber for 48 hr. after transplanting. A total of 288 media-free clones from each line were prepared.

3.3.2. Inoculum preparation

S. sclerotiorum isolates WM031 and WM192 with different level of virulence, collected in North Dakota, were used for all inoculations. These isolates are well characterized and had been used in screening canola germplasm before (del Rio, *personal communication*). Cultures of both isolates were obtained from sclerotia produced from single ascospores. Colonies of both isolates were grown at 21 $\pm$ 2°C (room temperature) on 4-mm-thick layer of half-strength potato dextrose agar (HPDA) medium. At maturity, sclerotia were harvested and stored in a refrigerator at 4°C until use. When needed, sclerotia were incubated on HPDA for 4 days at room temperature with 10 h of light daily to produce a mycelial colony. For the methods that required hyphal tips, DLA, DSA, PIT, and STR, colonies were grown for two to three days. Agar plugs containing hyphal tips were harvested using a 5 mm diameter cork-borer before the colonies could reach the edge of the dishes. For the SPT method, round wooden toothpicks (Diamond toothpicks, Jarden Homebrands, IN, USA) were autoclaved at 121°C with 103 kPa for 20 min. Sterilized toothpicks were soaked on sterile distilled water and laid flat on the surface of petri dishes containing HPDA medium; then the medium was seeded with an agar plug containing *S. sclerotiorum* hyphal tips. The colonies were incubated for three days to allow the fungus to grow on the toothpicks and immediately used for inoculation. For PET, freshly opened flowers from cv. Westar were surface

disinfested with 10% NaOCl solution for 30 sec and rinsed twice in sterile distilled water. Rinsed flowers were placed in contact with actively growing mycelium of *S. sclerotiorum* in HPDA medium. The dishes were incubated for at least six hours to allow the fungus to contact and grow over the flowers. After incubation, the flowers were used for inoculations.

3.3.3. Experimental design

The experiment was conducted using a randomized complete block design with four replications. A factorial arrangement of treatments was used; each factor, isolate and genotype, had two levels. Each experimental unit had three samples (i.e. stems or leaves or plants). The experiment was repeated once.

3.3.4. Inoculation techniques

The trial was conducted simultaneously in greenhouse and in laboratory facilities of the Canola Pathology program in the Department of Plant Pathology at NDSU. DLA, DSA, and OAA were evaluated in laboratory; whereas MSI, PIT, PET, SPT, and STR were evaluated in greenhouse.

For OAA, a preliminary test was conducted using six concentrations of oxalic acid (Sigma-Aldrich, USA). Leaves of *B. napus* cv. Westar were excised from plants using a sharp razor blade and immediately the tip of the petioles were immersed in water and transported to the laboratory. For the experiment, 50 ml of oxalic acid solutions ranging from 25 to 45 mM at 5 mM intervals were prepared and placed in different containers. Each concentration was replicated four times and in each replication three leaves were used. The tip of the proximal end of each petiole was cut off to expose fresh tissue and the tip was immediately immersed

with upright position in the oxalic acid suspension for 48 h at 21±2°C. The OA concentration used was selected on the basis of discoloration for further study. Once a single concentration was selected, leaves of each genotypes (i.e. DHP and WDH) were collected as described and brought to the lab. The incubation was as described for the preliminary tests. Image software ASSESS was used to scan the percentage of infected leaf area. This inoculation was considered one of the three treatments evaluated simultaneously with DLA and DSA.

For DLA, the fourth leaf of clonal plants was collected from canola seedlings that were in greenhouse for 20 days. Leaves from each genotype were excised from plants using a sharp razor blade and immediately the tip of the petioles were immersed in distilled water and transported to the laboratory. In the lab, Kull et al. (2003) protocol was followed. Briefly, sterile paper towels moistened with sterile distilled water were placed at the bottom of plastic containers (25 x 15 x 7.6 cm SC Johnson & Son Inc., Racine, Wisconsin), then a plastic mesh screen was placed over the paper towels, and the excised leaves were placed on the plastic mesh screen with their adaxial side up. A single agar plug containing hyphal tips from an actively growing *S. sclerotiorum* colony was placed upside down on the middle of each leaf. After inoculation, the containers were closed to prevent desiccation of the materials, and the leaves were incubated on a laboratory bench for 72 h at 21 ± 2°C.

For DSA, straight 8 cm long plant stems ≈ 6 mm diameter were cut approximately 3.5 cm above ground from plant clones 25 days after they had been transferred to greenhouse. Stem collections were conducted at the same time that leaves were collected for DLA and OAA. Detached stems were placed in plastic containers similar to those used for DLA and inoculated using agar plugs containing hyphal tips of actively growing colonies similar to those used for DLA. Agar plugs containing hyphal tips of actively growing *S. sclerotiorum*

colonies were kept in contact with the wounded side of basal part of stem. After inoculation, the containers were sealed and incubated as described for DLA. Lesion development was measured on each detached leaf or stem segment 24, 48, and 72 h after inoculation.

For MSI, *S. sclerotiorum* colonies were grown in potato dextrose agar in 90 mm diameter plastic petri dishes (VWR International, Inc., Bridgeport, NJ) for three days as described in inoculum preparation. After incubation, colonies from ten dishes were mixed with 500 ml of sterilized distilled water and blended for 30 seconds using a Hamilton Beach blender (Hamilton Beach/Proctor-Silex, Inc.). Approximately five ml of inoculum was sprayed with pressure sprayer on each plant. Three plants per replications were sprayed on 20 days after transferring to greenhouse. This method and the PIT, STR, SPT, and PET were conducted simultaneously in the same greenhouse room. Treatments were arranged in a RCB design with four replications. Plants were incubated in a mist chamber for 24 h at 22±2°C. After incubation, plants were brought to a greenhouse room at 23±2°C under 16 h light daily for 31 days after inoculation. Additional fluorescent light was supplied during the experiment for consistent growth of mycelia on host. Plain HPDA plug was used as a control.

For PIT, we follow the methodology described by del Rio et al. (2001). Briefly, hyphal tips of 3 to 4 day-old culture of *S. sclerotiorum* growing on HPDA were loaded on 100 µL sterile pipette tips by cutting the mycelium with the broad base of the tips. The petiole of the fourth fully-expanded canola leaf on each plant was cut with a razor blade approximately 2 cm away from the main stem. Then the petiole tip was capped with an inoculum-loaded pipette. Care was taken to ensure the petiole tip was flush with the side of the agar that contained fungal growth. Plants were incubated in room as aforementioned.

For STR, the inoculum was produced as described for PIT but was loaded into 1000 μL pipette tips. The main stem of approximately one month plants was cut with a razor blade approximately 3.5 cm below the tip of plant. Then the stem tip was capped with a pre-loaded pipette and rated similar to PIT. Inoculated plants were incubated as described for PIT.

For SPT, the main stem (about 6.5 mm in diameter) of approximately one month old plants was pierced approximately 15 cm below the stem tip using the mycelia covered tooth-pick, prepared as described in inoculum preparation. Inoculated plants were moved to mist chamber for 24 h and to greenhouse as mentioned before for PIT.

For PET, one complete flower with *S. sclerotiorum* mycelia growing over petal was gently placed on the axil of the 6th fully expanded leaf and main stem. Inoculated plants were kept in mist chamber for 48 h after inoculation at 22±2°C. After incubation, plants were brought to a greenhouse room at 23±2°C under 16 h light daily for 31 days after inoculation.

3.3.5. Disease assessment and data analyses

The numbers of wilted or dead plants were counted over time for plants inoculated with PIT and MSI whereas lesion length was measured for other techniques i.e. DLA, DSA, PET, SPT, STR and percentage of discolored area was measure for OAA. For OAA, leaves were observed at 12 h time intervals to detect symptoms such as discoloration, wilting, and drying of leaf tissues. To obtain a more accurate estimation of the leaf area affected by oxalic acid, leaves were scanned 48 hours after incubation using software ACCESS (Lamari, 2002). Water-soaked, bleached and shrunken area were considered as lesion for disease assessment. Plants were observed daily during first 10 days after inoculation to record time for first wilting symptoms or time to first water-soaked lesion development and after 10 days of

inoculation, plants were observed until 31 days after inoculation with three day intervals for PIT, MSI, PET, STR and SPT. A plant was considered to be wilted when 100% leaves and tip lost its rigidity and considered dead when plants wilted permanently and toppled down.

Standardized area under mortality curve (SAUMC) were calculated for PIT and MSI methods using plant mortality percentage data whereas standardized area under disease progress curve (SAUDPC) was calculated for DSA, DLA, STR, PET and SPT. While standardized percentage of leaf area infected was calculated for OAA. Since, response to different inoculation techniques were measured in different units, AUDPC, AUMC and standardized percentage of leaf infected were converted to percentile based on susceptible line for further analysis to determine the significant inoculation techniques. Standardized disease reaction of virulent isolate on susceptible line was considered as 100 % of disease for percentile conversion. Standard error was calculated for each reaction based on their replicate observations. Spearman's rank correlation coefficients were calculated using SAS PROC CORR (SAS 9.1.3) among inoculation techniques to see whether the cultivars response to the different methods in similar fashion. A test for homogeneity of variances was conducted to determine whether the two runs of each study could be combined for analysis. Homogeneity of variances was evaluated using the Brown-Forsythe option in PROC ANOVA of SAS (SAS Version 9.1.3) for each inoculation methods. Analysis of variance was performed independently for greenhouse conducted (i.e. MSI, PIT, PET, STP, and STR) and laboratory conducted (i.e. DLA, DSA and OAA) studies using SAS (SAS Version 9.1.3) after verifying homogeneity of variances. Least significant difference (LSD) was used to compare the means among different inoculation techniques for two isolates in both moderately resistant and susceptible clones in laboratory and greenhouse conditions independently.

3.4. Results

Both isolates, inoculated using seven different techniques, produced typical sclerotinia stem rot symptoms in the susceptible as well as the moderately resistant lines. Initially, water-soaked lesions developed, then white fluffy mycelia were visible on leaves and stems; wilting occurred in the final stage of infection of live plants. In detached plant tissue assays (DSA, DLA and OAA) (Figure 3.1), the first symptoms appeared 24 h after inoculation. White mycelium but not stem discoloration were visible on stem pieces inoculated using DSA. No aerial mycelial growth was observed on leaves inoculated using DLA; however, in all cases inoculated leaf tissues rot completely within three days after inoculation. Stem water-soaked lesions appeared three days after inoculation when using the SPT and after six days when using the PIT, MSI, and STR and PET (Figure 3.1). Lesions expanded from the point of

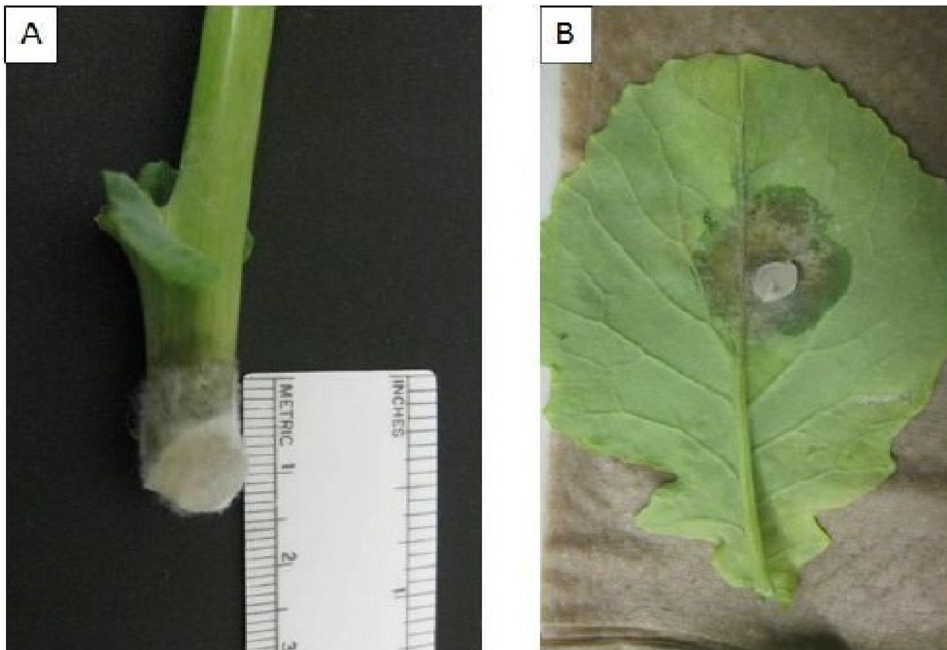

Figure 3.1. Pictorial view of symptomatic stem, leaves or plants using different inoculation methods; A) Detached stem assay (DSA); B) Detached leaf assay (DLA); C) Straw test (STR); D) Mycelium spray inoculation (MSI); E) Petiole inoculation technique (PIT), F) Stem-piercing with toothpick (SPT); G) Petal inoculation technique (PET); and H) Oxalic acid assay (OAA).

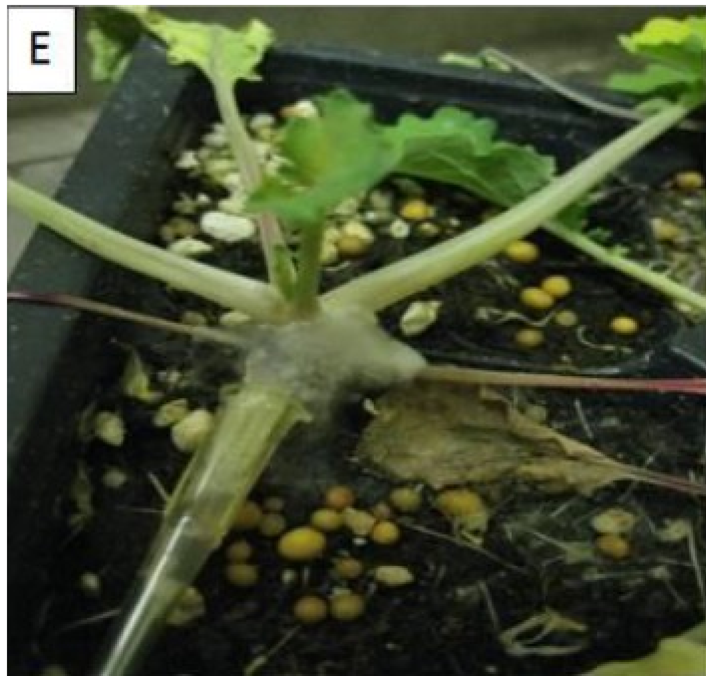

Figure 3.1. Pictorial view of symptomatic stem, leaves or plants using different inoculation methods. (Continued)

Figure 3.1. Pictorial view of symptomatic stem, leaves or plants using different inoculation methods. (Continued)

inoculations and reached to the main stem in plants inoculated with PIT, MSI, PET and STR. Plants started wilting once the vascular tissue of main stem was girdled. All three laboratory inoculation assays evaluated discriminated between moderately resistant and susceptible lines but, their ability to discriminate between isolates was very poor (Figure 3.2). Under laboratory conditions, DLA can better separate the virulence level of isolates compared to DSA.

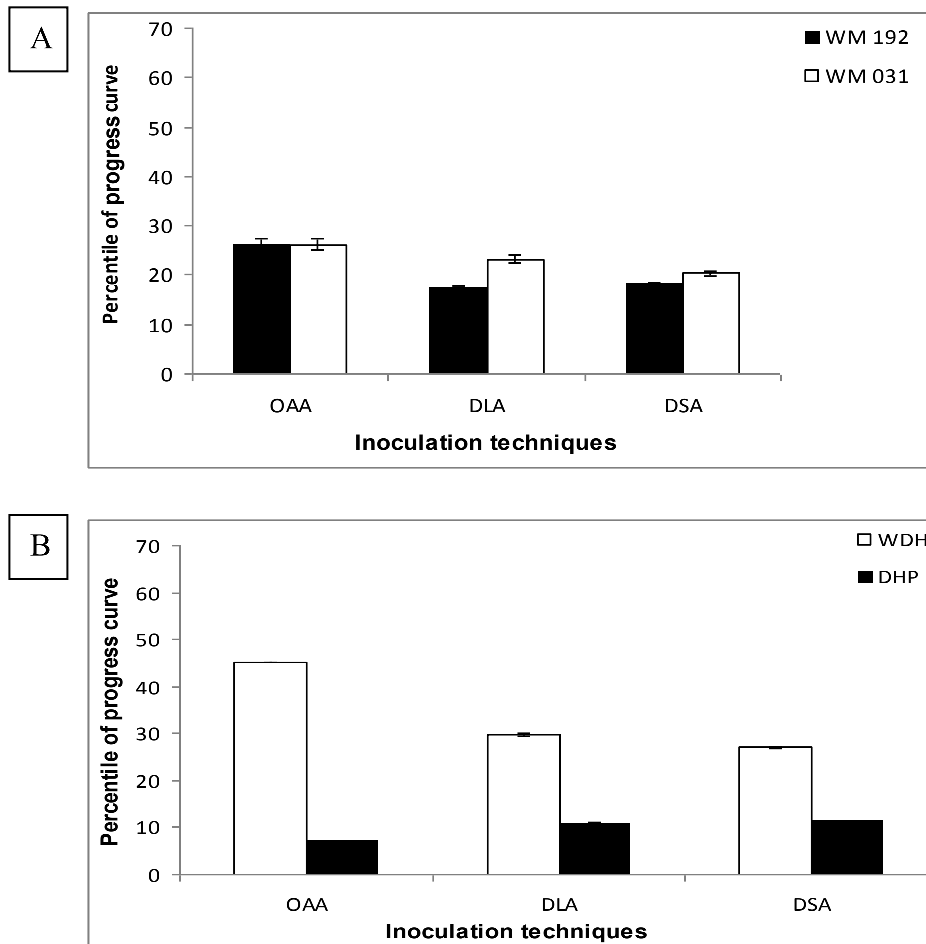

Figure 3.2. Percentile of standardized area under the disease progress curve and standardized area under mortality curve produced by: A) inoculation of two *S. sclerotiorum* isolates, WM031 and WM192, on *B. napus* doubled haploid genotypes one moderately resistant, derived from the cross between PI 458940 and PI 649136 (DHP), and one susceptible, derived from cv. Westar (WDH), and B) inoculation of *S. sclerotiorum* on two *B. napus* doubled haploid genotypes in laboratory condition. In both instances, the pathogen was delivered using two inoculation methods: detached leaf assay (DLA), detached stem assay (DSA), and one organic compound (oxalic acid) in OAA. Least significant difference (P=0.05) for isolates, lines and for disease progress curve were 1.59, 1.59, and 2.51, respectively. Suspension with similar concentration of OA was duplicated to compare different isolates for OAA. Each bar represents the percentile of progress curve with standard error.

Among the five different methods used to inoculate whole plants, MSI and PIT caused variable plant mortality, whereas PET, STP, and STR produced lesions in the stem but did not kill the plants. Therefore, AUMC was calculated for the first two methods and AUDPC was calculated for the rest of them. All greenhouse methods clearly separated susceptible lines

from moderately resistant lines, whereas only the MSI and PIT separated the virulence level of isolates clearly (Figure 3.3a). In addition, MSI and PIT were better to discriminate between susceptible and moderately resistant plants compared to other methods (Figure 3.3b). Moderately resistant plants i.e. DHP inoculated using the PIT or MSI produced an SAUMC

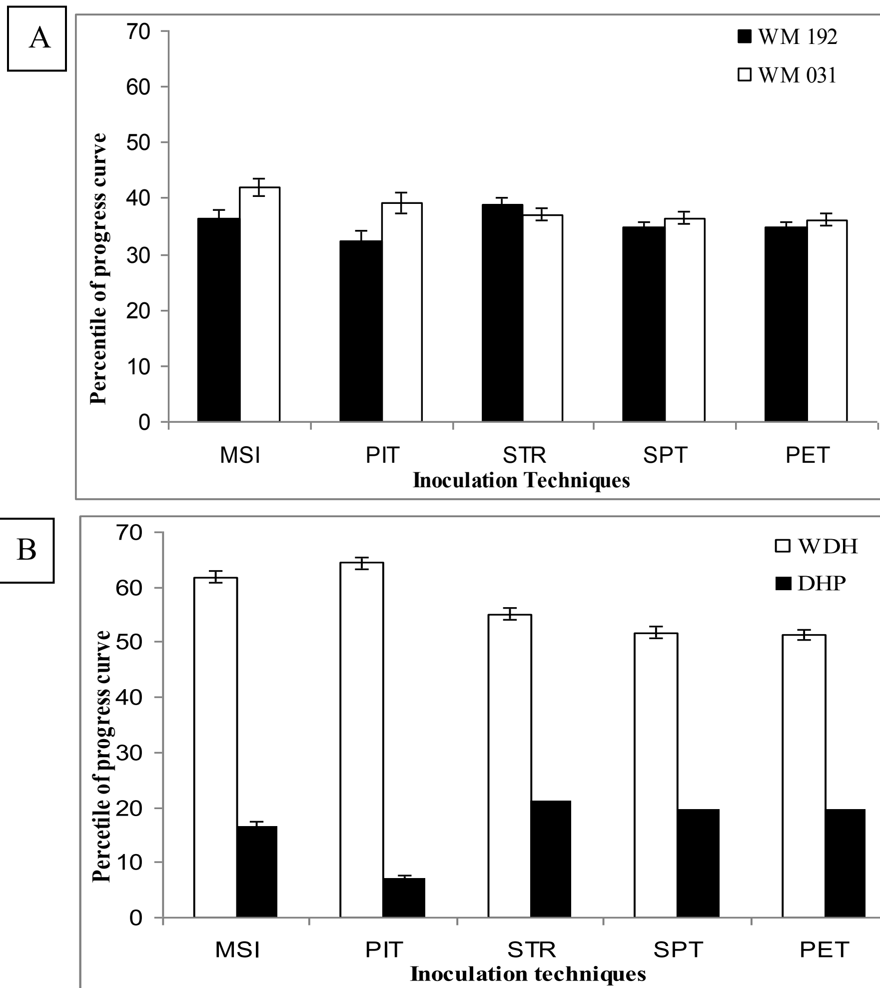

Figure 3.3. Percentile of standardized area under the disease progress curve and standardized area under mortality curve produced by: A) inoculation of two *S. sclerotiorum* isolates, WM031 and WM192, on *B. napus* doubled haploid genotypes one moderately resistant, derived from the cross between PI 458940 and PI 649136 (DHP), and one susceptible, derived from cv. Westar (WDH), and B) inoculation of *S. sclerotiorum* on two *B. napus* doubled haploid genotypes in greenhouse conditions. In both instances, the pathogen was delivered using five inoculation methods: mycelium spray inoculation (MSI); petiole inoculation (PIT); petal inoculation (PET); stem-piercing with toothpick (SPT); and straw test (STR). Least significant difference (P=0.05) for isolates is 0.74; for lines is 0.74; and for progress curve is 0.91). Bars represent the percentile of progress curve with standard error.

that was 5.0 times smaller than that of the susceptible line. However, when inoculated using the SPT, STR, PET, or DLA, the moderately resistant line had an SAUDPC that was on average 2.6 times smaller than that of the susceptible line. Similarly, PIT or MSI were better to discriminate between the reactions to both isolates. Moreover, all of these inoculation techniques consistently differentiate the difference between lines and isolates. For disease severity, highly significant correlations were obtained among all inoculation techniques. Rank correlation coefficients between the SAUMC or SAUDPC or standardized area affected with oxalic acid obtained with the eight different inoculation methods ranged between 0.6 and 1.00 (P<0.0001) (Table 3.1). PET and SPT were highly correlated to each other (rho = 0.98) (*<0.0001*). DHP had consistently smaller lesions, SAUMC and SAUDPC than Westar no

Table 3.1. Rank correlation coefficients1 for disease severity caused by *Sclerotinia sclerotiorum* inoculated on *B. napus* double haploid lines using eight different inoculation techniques.

	SPT	STR	DLA	DSA	MSI	PIT	PET	OAA
Stem Piercing (SPT)	1							
Straw (STR)	0.75	1						
Detached Leaf (DLA)	0.75	0.60	1					
Detached stem (DSA)	0.68	0.67	0.80	1				
Mycelium spray (MSI)	0.75	0.71	0.75	0.73	1			
Petiole inoculation (PIT)	0.81	0.60	0.87	0.82	0.81	1		
Petal inoculation (PET)	0.98	0.74	0.76	0.67	0.73	0.79	1	
Oxalic acid (OAA)	0.74	0.75	0.71	0.68	0.78	0.74	0.77	1

[1]All correlation coefficients are significant at P <0.0001

matter which inoculation techniques or isolate was used in the evaluation. Independent analysis of variance of each study (greenhouse and laboratory) showed significant differences between the lines, two isolates, and the inoculation techniques (Table 3.2 and 3.3). Based on the reaction of isolates on lines in individual inoculation methods, PIT and STR didn't have any interaction between resistance level of lines and virulence level of isolates.

Table 3.2. Analysis of variance of three inoculation techniques i.e. detached leaf assay, detached stem assay, oxalic acid assay to screen for sclerotinia stem rot resistance in canola in laboratory conditions.

Source	**Degree of Freedom**	**Mean Square**	**Probability>F**
Exp	1	58.97	<0.0001
Rep (exp)	6	2.77	0.5481
Method	2	451.06	<0.0001
Line	1	13954.39	<0.0001
Isolate	1	180.18	<0.0001
Exp*method	2	39.51	<0.0001
Exp*line	1	12.17	0.0600
Exp*Isolate	1	22.78	0.0110
Line*Isolate	1	2.63	0.3766
Method*Isolate	2	71.49	<0.0001
Method*line	2	1148.76	<0.0001
Method*line*Isolate	2	51.26	<0.0001
Exp*method*line*Isolate	7	17.31	<0.0001
Error	66	3.32	
Total	95		

Table 3.3. Analysis of variance five inoculation techniques: mycelium spray inoculation, petiole inoculation, petal inoculation, stem-piercing with toothpick, and straw test to screen for sclerotinia stem rot resistance in canola in greenhouse conditions.

Source of variation	**Degree of Freedom**	**Mean Square Error**	**Probability > F**
Exp	1	12.9	0.48
Rep(exp)	6	16.81	0.69
Method	4	92.72	0.01
Line	1	64532.30	<0.0001
Isolate	1	293.28	0.001
exp*method	4	32.35	0.2903
exp*line	1	1.40	0.8157
exp*Isolate	1	6.84	0.6069
line*Isolate	1	18.02	0.404
method*Isolate	4	95.38	0.007
method*line	4	975.25	<0.0001
method*line*Isolate	4	34.89	0.2531
exp*method*line*Isolate	13	7.81	0.9904
Error	114	25.69	
Total	159		

3.5. Discussion

Resistance to sclerotinia stem rot resistance in canola is polygenic and additive (Zhao and Meng, 2003); therefore, it is difficult to discriminate between moderately resistant and susceptible materials. The variability observed in the magnitude of the reaction of plants to disease is associated with differences in genotypes within the same cultivar or breeding line, the environments and their interaction. Two other important sources of variability are biases introduced by personnel measuring and recording the reaction of plants to the pathogen and the inoculation method used to challenge the plants. Reliable inoculation techniques validated by testing them on genetically uniform plant materials in a controlled environment could help discriminate small differences in sclerotinia stem rot resistance. In this study, we used genetically uniform sclerotinia stem rot moderately resistant and susceptible lines to compare five different whole plant inoculation methods in greenhouse conditions and three different detached assays in laboratory conditions. Previous studies that evaluated inoculation methods used canola cultivars, breeding lines, and/or plant introductions produced from bulk seeds found inconsistent results in PIT (Bradley et al., 2006; Nelson et al., 1991; Zabala, 2008). These homozygous clones used in our study showed consistent results between two independent experiments and among replicates within experiment in greenhouse conditions.

Three different detached plant part assays; DLA, DSA and OAA performed in laboratory discriminated between the susceptible and moderately resistant lines. Detached assays can be completed in 4 to 5 days and large numbers of materials could be screened in small area in a laboratory. Moreover, highly significant rank correlations (rho = 0.6-0.98, $P<0.001$) were observed among detached assays and whole plant inoculation methods. Therefore, detached assays could be useful to screen large numbers of germplasm in initial

stage for biparental population screening for sclerotinia stem rot resistance if verification with suitable whole plant assays could be performed with a subset of randomly selected plants from the main population or in later stages. The ability of laboratory assays to discriminate between the partial resistance and susceptible lines was lower compared to greenhouse assays and also laboratory studies were less consistent compared to greenhouse study. This could be due to asynchronous initiation of lesion development, and the fact that detached plant materials are already dying and therefore some physiological process may no longer be conducted compared to what happened in living plants. The DLA and DSA have been used to screen soybean germplasm for sclerotinia stem rot resistance with some success (Graef et al., 2008). However, in these trials both methods proved to be of marginal utility. The OAA is an indirect evaluation of resistance through measurement of leaf discoloration due to oxalic acid. Oxalic acid is a putative virulence factor associated with pathogenesis by *S. sclerotiorum*. Researchers have used oxalic acid with varying success to challenge plants in efforts to screen accessions of various host species for resistance to *S. sclerotiorum* (Wegulo et al., 1998). The use of oxalic acid rather than the pathogen isolates has been perceived to reduce variability associated with the interaction between plant and pathogen.

Among the five greenhouse assays evaluated, MSI and PIT were most suitable and reliable to discriminate between the moderately resistant and susceptible materials, when either highly or moderately aggressive isolates of *S. sclerotiorum* were used (Figure 3.2). Both methods showed consistent results and clearly discriminated between susceptible and moderately resistant materials compared to other three methods. MSI is easiest and fastest to apply because many plants could be sprayed within short time. However, careful attention would be needed to ensure uniform distribution of the inoculum in each plant in addition to

requiring a misting system. Although PIT is labour intensive and time consuming compared to MSI, the PIT showed larger differences between susceptible and moderately resistant clones than MSI. Further, PIT is done on early stages of plant development compared to PET, STR and SPT methods, and the PIT method killed the plants whereas the latter three did not. As a result, plants that tolerate the infection originated using the PIT can still be taken to seed production to further advance the populations. In addition, PIT allows breeders to screen both spring and winter type canola without vernalization, since other tested methods usually require stem/stalk for inoculation including MSI. Furthermore, PET, STR and SPT methods did not show high difference between susceptible and moderately resistant clones as shown by PIT. This could be due to inoculation of fungus at different plant stages between the PIT and other three methods. In PIT, the fungus is inoculated at 4-5 leaf stage, while the fungus is inoculated close to flowering stage in these three other methods. During flowering stage, hard/tough nodes are developed on stems, which could act as physical/structural barriers and reduce the lesion size.

Among the eight inoculation methods, PIT and STR didn't show any interaction between isolates and lines. While MSI and DLA showed little deviation i.e. likely to have interaction if more numbers of lines and isolates were used. In DSA, SPT and PET, there was a clear interaction between isolates and lines which could be due to the influence of resistance level of lines depends on the virulence level of isolates or vice versa.

Of the many methods used to screen canola materials, the PIT has been considered one of the best. Bradley et al. (2006) compared PIT, OAA and DLA by using 19 *B. napus* cultivars in greenhouse and lab conditions. Significant differences among cultivars were detected using PIT and OAA but not DLA. Zhao et al. (2004) evaluated 47 *B. napus*

germplasm using PIT. The responses were consistent among experiments and among evaluations criteria; days to wilt and lesion phenotype index. Chen and Wang (2005) evaluated PIT, spray mycelium and drop mycelium inoculation techniques using 18 soybean cultivars. This study reveals that PIT and other two methods are significantly correlated to each other. In our study, Spearman's ranks correlation also showed that the tested techniques were significantly correlated to each other. We also kept plants inoculated using PIT with other methods in the mist chamber with high humidity and similar light condition in this study. The uniform environmental conditions used during incubation and the identical genotypes produced using cloning techniques might have helped us obtain the consistent results observed. We used only two isolates and two *B. napus* lines, which might be the reason for highly significant correlation coefficient. Based on time and expenses calculation, Chen and Wang (2005) also recommended MSI for large scale screening of soybean germplasm since it is cheap, quick and significantly correlated to PIT.

Detached assays allow breeders to maintain the germplasm even in the early stage of breeding program; i.e. if F_2 plants need to be screened, detached methods would allow the breeder to maintain the seed without risking killing the plants. For further verification, whole plant assays like PIT or MSI could be used in successive generations. The PIT allows breeders for early stage screening of *B. napus* germplasm. The advantage of early stage screening through PIT, allowed intensive care to resistance plants for further study. PIT is expensive, laborious and somewhat technical compared to MSI. Whereas, MSI allows breeder to screen large population with low cost and less laborious, but could be problematic for winter type canola. MSI and PIT are highly correlated to each other so in case of large germplasm screening for breeders, MSI could be of similar importance.

3.6. References Cited

Boland G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 16:93-108.

Bradley, C. A., Henson, R. A., LeGare, D. G., del Rio, L. E., and Khot, S. D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Dis. 90:215-219.

Chen, Y., and Wang, D. 2005. Two convenient methods to evaluate soybean for resistance to *Sclerotinia sclerotiorum.* Plant Dis. 89:1268-1272.

Chun, D., Kao, L. B., and Lockwood, J. L. 1987. Laboratory and field assessment of resistance in soybean to stem rot caused by *Sclerotinia sclerotiorum*. Plant Dis. 71:811–815.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., and Pang, E. C. K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142:169-196.

del Rio, L. E., Kurtzweil, N. C., and Grau, C. R. 2001. Petiole inoculation as a tool to screen soybean germplasm for resistance to *Sclerotinia sclerotiorum.* Phytopathology 91:S176.

Ender, M., Terpstra, K., and Kelly, J. D. 2008. Marker-assisted selection for white mold resistance in common bean. Mol. Breed. 21:149-157

Graef, G. L., Clemente, T. E., Steadman, J. R., and Jackson, T. 2008. Sclerotinia resistance enhanced by accumulation of QTL and transgenic approaches. Jan 23- 25, 2008 National sclerotinia initiative, Annual Meeting, MN, USA.

Kelly, J. D., Gepts, P., Miklas, P. N., and Coyne, D. P. 2003. Tagging and mapping of genes and QTL and molecular mapping of economic importance in bean and cowpea. Field Crop Res. 82:135-154

Kharbanda, P. D., and Tewari, J. P. 1996. Integrated management of canola diseases using cultural methods. Can. J. Plant Pathol. 18:168-175.

Khot, S. 2006. Evaluation of *Brassica napus* accessions for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis, NDSU, USA.

Kull, L. S., Vuong, T. D., Powers, K. S., Eskridge, K. M., Steadman, J. R., and Hartman G. L. 2003. Evaluation of resistance screening methods for Sclerotinia stem rot of soybean and dry bean. Plant Dis. 87:1471-1476.

Lamari, L. 2002. ASSESS: Image analysis software for plant disease quantification. The American Phytopathological Society, MN, USA.

Lamey, A., and Bradley, C. A. 2003. Sclerotinia stem rot of canola: Biology and management. N. D. State Univ. Ext. Rep. No. Pp-1201.

Lamey, A., Knodel, J. E. G., Andol, K., Ashley, R., Barondeau, D., Craig, B., Crary, V., Fore, Z., Johnson, N., Knoke, S., Liane, M., Lykken, T., Melaas, R., Miller, M., Nyegaard, C., Person, H., Peterson, N., and Severson, R. 2001. Canola disease survey in Minnesota & North Dakota. available at http://www.ext.nodak.edu/extpubs/plantsci/crops/er71w.htm: accessed on July 17, 2009.

Miklas, P. N. 2007. Marker-assisted backcrossing QTL for partial resistance to sclerotinia white mold in dry bean. Crop Sci. 47:935-942.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Nelson, B. D., Helms, T. C., and Olson, M. A. 1991. Comparison of laboratory and field evaluations of resistance in soybean to *Sclerotinia sclerotiorum*. Plant Dis. 75:662-665.

Northern Canola Growers Association. 2009. *Sclerotinia* risk forecast program for North Dakota. Available from http://www.northerncanola.com/maps/index.asp: Accessed July 17, 2009.

Peltier, A. J., and Grau, T. 2006. Use of oxalic acid to characterize soybean accessions for partial resistance to *Sclerotinia sclerotiorum.* Phytopathology 97:S163.

Petzoldt, R., and M. H. Dickson. 1996. Straw test for resistance to white mold in beans. Annu. Rep. Bean Improv. Coop. 39:142-143.

Purdy, L. H. 1979. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 69:875-880.

Saharan, G. S. and Mehta, Y. N. 2008. Sclerotinia diseases of crop plants: biology, ecology and disease management. Springer, Dordrecht, UK.

SAS Institute. 2010. SAS user's guide: Statistics. 9th ed. SAS Inst., Cary, NC.

Su, Y. 2009. Double haploid production of canola (*Brassica napus* L.) with improved resistance to *Sclerotinia sclerotiorum* via microspore culture. M.S. Thesis, NDSU, USA.

Vuong, T. D., Hoffman, D. D., Diers, B. W., Miller, J. F., Steadman, J. R., and Hartman, G. L. 2004. Evaluation of soybean, dry bean, and sunflower for resistance to *Sclerotinia sclerotiorum*. Crop Sci. 44:777-783.

Wegulo, S. N., Yang, X. B., and Martinson, C. A. 1998. Soybean cultivar responses to *Sclerotinia sclerotiorum* in field and controlled environment studies. Plant Dis. 82:1264-1270.

Zabala, F. 2008. Screening the USDA-NGRP *Brassica rapa* Collection for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis, NDSU, USA.

Zhao, J., and Meng, J. 2003. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 106:759-764.

Zhao, J., Peltier, A. J., Meng, J., Osborn, T. C., and Grau, C. R. 2004. Evaluation of Sclerotinia stem rot resistance in oilseed *Brassica napus* using a petiole inoculation technique under greenhouse conditions. Plant Dis. 88:1033-1039.

Zhao, J., Udall, J. A., Quijada, P. A., Grau, C. R., Meng, J., and Osborn, T. C. 2006. Quantitative trait loci for resistance to *Sclerotinia sclerotiorum* and its association with a homologues non-reciprocal transposition in *Brassica napus* L. Theor. Appl. Genet. 112:509-516.

CHAPTER 4. DEVELOPMENT OF ADVANCE BREEDING LINES WITH RESISTANCE TO MULTIPLE CANOLA DISEASES

4.1. Abstract

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum*, blackleg (BL) caused by *Leptosphaeria maculans*, and brown girdling root rot (BGRR) caused by *Rhizoctonia solani* are three major fungal diseases affecting canola production worldwide. Genetic resistance to sclerotinia stem rot and BGRR is polygenic. Resistance to BL is monogenic. Use of genetic resistance against sclerotinia stem rot and BGRR has not seen as successful component of disease management programs in canola but it has been against BL. However, resistance against BL has lately been faltering due to development of new *L. maculans* strains. The objective of this study was to develop *B. napus* breeding lines with acceptable levels of resistance against these three diseases. A total of 230 F_2 plants were developed by crossing *B. napus* plant introductions 458939 and 649136 from Germany and Japan, respectively. These plants were evaluated for their reaction to *S. sclerotiorum* using the petiole inoculation technique in greenhouse. Surviving plants were allowed to self-pollinate and their progenies screened again until F_6 generations. This cycle was repeated until the F_6 generation was produced. Thirty three F_5 seedlings were evaluated for their reaction to pathogenicity groups (PG) 3 and 4 of *L. maculans*, as well as to AG2-2 isolates of *R. solani* in replicated experiments. Petiole inoculation technique and a slightly modified version of the sand corn-meal inoculum layer method were used to inoculate seedlings for blackleg and BGRR screening, respectively. Plants were scored in 0-9 scale for PG 3 and PG 4, and 0-7 scale for

BGRR. Plants rating 6.0 to 9.0 were categorized as susceptible to BL and root ratings 4.0 to 7.0 were categorized as susceptible to BGRR. Eight lines (16.1, 20, 28, 72, 140, 160, 178 and 186) were resistant to sclerotinia stem rot (slow molding) and survived successive cycles of screening from F_2 to F_6. lines 30, 53, 71,153 and 160.3 were more resistant than a parental line PI 458939; and 16.1, 28, 140, 160, 178 and 186 were resistant to BGRR. Sixty five percent and 63% of F_5 lines were moderately resistant to PG-3 and PG-4 strains of *L. maculans*, respectively, at the seedling stage, but only 27% of all the moderately resistant plants survived to produce seed. These nine survived lines were again screened for BL in F_6 generations, four lines survived from the adult stage infection also showed higher resistance at seedling stage than both parents. The average BGRR scoring was ranged from 0 to 7.0 and plant weight was ranged from 0.64 to 4.2. Approximately, 45, 28 and 27 percent of 33 F_5 progenies were found resistance, intermediate resistance and susceptible to brown girdling root rot, respectively. These results suggest that the resistant genotypes identified in this study possess high levels of resistance to multiple diseases and could be valuable sources for canola improvement programs. The resistant RILs identified in this study should be useful for the future improvement of canola resistance in spring type.

4.2. Introduction

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, and blackleg (BL), caused by *Leptosphaeria maculans* (Desm.) Ces. Et. De Not., are two economically important diseases affecting canola (*Brassica napus* L.) production in North America and other areas of the world (Fitt et al., 2006; Salisbury et al., 1995). While yield loss reports due to the combined effect of these diseases are not available, each of them is capable of reducing

yields significantly. Sclerotinia stem rot is endemic in canola growing areas of North Dakota with annual incidences ranging from 1 to 59% (del Rio et al., 2007). Markell et al. (2008) reported that BL symptomatic plants can be found in all canola growing fields in North Dakota and in favorable environments growers could experience serious yield losses. Besides sclerotinia stem rot and BL, brown girdling root rot (BGRR), which is caused by *Rhizoctonia solani* AG2-2, is also capable of causing significant yield loss in Brassicas (Klein-Gebbinck and Woods, 2002; Thomas, 2003). In Alberta, where the disease has been well established, BGRR causes up to 30% yield losses (Sippell et al., 1985). BGRR has been detected in North Dakota, although its distribution in the state is not known. Preliminary research conducted to assess the effect of *R. solani* AG2-2 on canola germination and plant stand revealed 20% reduction in seedling germination and 30% reduction in plant stands in infested soil compared to sterile soil (Nepal and del Rio, 2010). Each percentage unit of sclerotinia stem rot incidence has been associated with a 0.5% reduction in potential yield of canola in North Dakota (del Rio et al., 2007). In Canada, more than 50% yield reduction in canola has been reported in fields severely affected by blackleg (Gugel and Petrie 1992). Surveys conducted in North Dakota between 1991 and 2007 revealed the mean incidences of blackleg infected plants were increasing every year (Markell et al., 2008).

The most effective way to manage canola diseases is through the use of genetically resistant cultivars. Unfortunately, canola cultivars with resistance to multiple diseases are not available. While most canola cultivars were resistant or moderately resistant to BL strains prevalent in early 2000, the discovery of new strains with the ability to infect these cultivars (Bradley et al., 2005) has increased the risk of BL outbreaks and make more urgent the use of fungicide treatments, planting disease-free seeds and using four year crop rotation with non-

hosts (Markell et al., 2008). Sclerotinia stem rot management in canola is difficult and relies on fungicides because canola cultivars with acceptable levels of genetic resistance are not available for growers (Bradley et al., 2006; Zhao et al., 2004). Short crop rotations have reduced effect on the pathogen because sclerotia can survive in the soil 7- 8 years (Ben-Yephet et al., 1993). Moderately resistant reaction to *S. sclerotiorum* has been observed in some *B. napus* accessions collected from Germany and Japan (Khot, 2006). Traditionally, breeding programs have crossed a source of resistance with a susceptible parent and made selections from the segregating populations; however, crossing two resistant parents also could lead to the development of segregating populations. Use of appropriate selection criteria (inoculation method) could help identify segregating individuals. The incorporation of resistance from two different sources could widen the genetic basis of the resistance and improve the levels of control. Gugel et al. (1987) found BGRR disease is less severe on mature plants than seedlings. Data revealed an inverse relationship between the percentage disease rating and the age of Westar plants at the time of inoculation. A disease rating of 70% was recorded for one week-old seedlings inoculated with *R. solani* isolate compared with 12% for mature plants inoculated at 10 weeks of age. In addition, some resistance has been identified in progenies from *B. napus* cv. *DSN11* and cv. *Midas* (Kataria and Verma, 1992; Yang and Verma, 1992). To our knowledge, there are no cultivars with comprehensive package for resistance to sclerotinia stem rot, BL and BGRR.

While *S. sclerotiorum* isolates can vary in aggressiveness, a more structured partition of the pathogen population, e.g. pathogenicity groups, biotypes, or races, have not been reported. In contrast to this, pathogenicity groups (PG) and races have been reported in *L. maculans* populations. PG classification of *L. maculans* is based on their ability to cause

disease on three differential cultivars, Westar, Glacier, and Quinta (Mengistu, et al., 1991). PG-1 and PG-2 were most prevalent in ND in the late 1990s. Recently, PG3 and PG-4 strains of *L. maculans* strains were reported in some fields of North Dakota (Bradley et al., 2005). These new strains pose a serious threat to the canola industry due to the increased prevalence of isolates with virulence profiles that render the varieties currently grown with resistance to PG-1 and PG-2 (Bradley et al., 2005; Chen and Fernando, 2006). Loci controlling resistance to PG-1 and PG-2 have been mapped to the N7 linkage group of the A genome in *B. napus* genotypes (Ferreira et al., 1995; Mayerhofer et al., 1997; Rimmer, 2006; Zhu and Rimmer, 2003). To combat the challenges from these new pathogenic groups of the pathogen, new sources of resistance are needed. The goal of this study was to develop *B. napus* germplasms with durable resistance against sclerotinia stem rot, BL, and BGRR

4.3. Materials and Methods

4.3.1.Population development

Two *B. napus* plant introductions, 458939 and 649136 (i.e Ames 26628), from the USDA-GRIN collection considered to be moderately resistant to sclerotinia stem rot (Khot, 2006) were selected as parental lines. Two seeds from each were planted in 20 cm diameter plastic containers with pasteurized greenhouse soil-less Sunshine Mix #1 (Fison Horticulture, Vancouver, B. C.). Plants were grown in greenhouse rooms maintained at 21 ± 2°C with supplemental light (400 W Lucalox LU400 sodium, General Electric Co., Cleveland, OH) to obtain 16 h day lengths throughout the experiment. Plants were watered daily and fertilized with 3 g/liter of 15-30-15 Miracle Gro (Scotts, Pot Washington, NY). Crosses were made just

before opening of buds. One F_1 seed from the cross was planted to produce F_2 seeds to further the study.

4.3.1.1. F_2 generation

Two hundred and thirty F_2 seedlings and twenty four seedlings from their parental lines were grown in individual 10 cm X 10 cm X 8.9 cm. plastic containers and watered daily. This population and its parental lines were screened for resistance to sclerotinia stem rot using the petiole inoculation technique (PIT) when seedlings were at the 4-5 leaf growth stage. Plants that were still alive two weeks after inoculation were transferred to 20 cm diameter plastic containers without disturbing the root system. These plants were not protected using fungicides and at flowering they were covered with individual pollinating bags for seed production. The F_3 seeds produced by these plants were harvested and kept separately. Each surviving plant and their seeds were considered a line.

4.3.1.2. F_3 and F_4 generation

Six F_3 seeds from each line and the original parental lines were planted individually in pots filled with Sunshine Mix #1 soil-less mix as described before. Seedlings were screened for sclerotinia stem rot resistance using the same inoculation methodology described for the F_2 generation. Plants that survived this inoculation were transplanted and allowed to produce seeds as described. This cycle was repeated with F_4 seedlings.

4.3.1.3. F_5 generation

Seeds from 33 F_5 lines and the original parental lines were screened for BL and BGRR resistance in addition to being screened for resistance to sclerotinia stem rot. For sclerotinia stem rot screening seeds of each line were planted individually as described. For BL and BGRR seeds were planted in small plastic inserts (each cell; 5.7 cm X 5.1 cm X 8.3 cm) using

the same soil-less mix. Six seedlings from each line were screened for resistance to sclerotinia stem rot. Resistance to BL and BGRR was tested on 24 seedlings per line. Surviving plants from each disease were transplanted as described and allowed to produce seed by self-pollination.

4.3.1.4. F_6 generation

Seeds produced by 22 and nine F_5 lines, that survived from sclerotinia stem rot and BL screenings, respectively, were planted as described and the resulting seedlings were screened again. Twenty-four seedlings from each line and the parental lines were screened at this stage for sclerotinia stem rot resistance and blackleg resistance, respectively.

4.3.2. Inoculum preparation

S. sclerotiorum isolate WM031, collected in North Dakota was used for inoculation. This isolate is well characterized and had been used by other researchers in previous studies (Khot, 2006; Zabala, 2008). Sclerotia that develop from a single ascospore culture were used to produce the hyphal tips used for the inoculation of F_2 seedlings. Inoculation of F_3 to F_6 seedlings were conducted using hyphal tips produced by sclerotia retrieved from inoculated plants from the previous generations. After collection from plant tissues, sclerotia were air dried and stored at room temperature (21 ± 2^oC) in dark. When mycelium was needed, a single sclerotium was surface disinfested by submerging it in a 0.5% (v/v) NaOCl aqueous solution for 10 seconds and rinsed with sterilized distilled water twice. Then, small pieces of the sclerotium were partially submerged on half-strength potato dextrose agar (HSPDA; Becton Dickinson and Company, Sparks, MD) in 10 cm diameter plastic petri dishes and

incubated for four days at 21 ± 2°C with daily 10 and 14 h light and dark, respectively. Hyphal tips of 4-5 days-old colonies were used to inoculate the canola plants.

L. maculans isolates BL729, BL730, BL731 and BL732 of PG3 and BL736 of PG4 were used for all inoculations. These isolates were retrieved from infected canola grown in North Dakota. Single spore cultures were grown on V8 agar medium (200 ml of Campbell V8 juice (Camden, NJ), 0.75 g of $CaCO_3$, and 15 g of agar in 800 ml of distilled water). The isolates were incubated for 14 days under continuous cool-white fluorescent light at 20ºC. Sporulating cultures were flooded with 10 to 15 ml of sterile distilled water and gently scraped with a flamed glass rod to release spores from pycnidia. Pycnidiospore suspensions were filtered through sterilized cheesecloth and centrifuged at 9,000 rpm for 20 min. The supernatant was decanted and the spore pellet was re-suspended in approximately 1 ml of sterile distilled water and stored as a concentrate at –20ºC. Each pycnidiospore suspension was adjusted to 1×10^7 spores/ml and inoculated once on seedlings of cv. Westar which is susceptible to BL to maintain their virulence. Once symptoms were expressed, isolates were retrieved from plant tissues and cultured as described to produce the inoculum to be used in the screening trials.

An AG2-2 isolate of *R. solani* originally obtained from a North Dakota field was grown on HPDA for 6 days in 12 h light and dark cycles at 21 ± 2°C. This isolate was chosen to be used in the trials due to its high level of aggressiveness on canola as observed in a preliminary trial with other *R. solani* AG 2-2 isolates (del Rio, *personal communication*). Inoculum was produced as described by Nepal and del Rio (2010). Briefly, eight 5-mm plugs of the culture were placed in 1000-ml conical flasks containing 250 g of previously sterilized canola seeds. Seeds had been mixed with 10 ml of distilled water and autoclaved at 121°C

with 103 kPa for 20 minutes; 24 h after they were autoclaved again as described. Flasks were kept at room temperature for seven days and were shaken daily by hand to allow the fungus to grow throughout the contents of the flask. The flasks were stored in the dark at 4°C until use.

4.3.3. Inoculation and incubation procedures

Sclerotinia sclerotiorum. Seedlings were inoculated following the protocol for the PIT described by del Rio et al. (2001). Briefly, hyphal tips of 3 to 5 day-old culture of *S. sclerotiorum* growing on HPDA were loaded on 200 µL sterile pipette tips (VWR Scientific Co., San Diego, CA) by cutting the mycelium with the broad base of the tips. The petiole of the fourth fully-expanded canola leaf on each plant was cut off with a razor blade approximately 2 cm away from the main stem. Then the petiole tip was capped with an inoculum-loaded pipette. Care was taken to ensure the petiole tip was flush with the side of the agar that contained fungal growth. After inoculation, plants were moved to mist chambers for 48 hr to maintain consistent incubation conditions and then returned to a greenhouse room where temperature was kept at 25 $\pm$ 2°C. A total of 230 F_2 plants were initially screened. This inoculation procedure was repeated on seedlings of surviving plants until the F_5 generation without replications due to the limited number of seeds. A total of 24 seedlings from each F_6 line were inoculated. Seedlings were arranged in groups of six plants per replication with four replications following the same protocol aforementioned.

Leptosphaeria maculans. Blackleg inoculations were conducted only on seedlings of the F_5 and F_6 generations. These seedlings were produced from seeds derived from plants that survived inoculations of *S. sclerotiorum* in the F_2, F_3, and F_4 generations. Similarly, F_5 plants that survived blackleg screening were allowed to produce F_6 seeds that were used for an

additional round of screenings. We followed the procedure described by Marino (2011) to inoculate the seedlings. Briefly, a tiny puncture wound was made with sterile needles on the cotyledons of 10-day-old plants. Seedlings were evaluated in four replicated trials in greenhouse conditions. A 10 µl aliquot of a blend of 3- 4 isolates of each PG (pycnidiospore suspension: 1 x 10^7spores ml^{-1}) was deposited on the wounds. Also, a set of Westar seedlings of similar age were inoculated using sterile water (mock inoculation). Inoculated plants were incubated for 24 h in a misting chamber at 21 ± 2^oC in the dark and returned the next day to the greenhouse where they were incubated for another 10 days prior to disease recording.

Rhizoctonia solani. A slightly modified version of the sand corn-meal inoculum layer method used by Strausbaugh et al. (2004) to evaluate pathogenicity of *Fusarium* isolates on roots of wheat (*Triticum aestivum* L.) seedlings was used to evaluate the effect of *R. solani* on the F_5 *B. napus* lines. In this study, inoculum was grown on canola seed (cv. Westar) following the same protocol. Seeds from each line were planted in replicated trials in greenhouse soil mix infested or not with canola seeds colonized by an *R. solani* AG 2-2 isolate. In plastic inserts with small holes at the base for water drainage, 25 g of Sunshine Mix were placed and compressed lightly; then one teaspoon of inoculum was deposited forming a layer on top of the soil-less mix. The layer of inoculum was covered with 8 g of Sunshine Mix. Two seeds of each line were placed on the top Sunshine Mix layer and another 2-g of Sunshine Mix was added to cover the seeds. Plastic inserts with seed were watered daily. A daily cycle of 14 h light and 10 h dark, with temperatures of 21 ± 2°C was maintained in the greenhouse room. Germination and plant standing were quantified 14 and 20 days after planting, respectively. Root rot severity was evaluated 20 days after planting.

4.3.4. Data collection

Sclerotinia sclerotiorum inoculated plants were examined for wilting symptoms daily starting eight days after inoculation and for the next 28 days. A plant was considered dead when the plant was irreversibly wilted.

Blackleg severity was recorded ten days after inoculation using the 0 to 9 scale developed by Delwiche (1980). The disease ratings were: 0= no darkening around the wound; 1= limited darkening around the wound, lesion diameter 0.5-1.5mm; 3= dark necrotic lesion 1.5 – 3.0 mm diameter; 5 = non-sporulating lesion 3.0-5.0 mm diameter; 7 = grey-green tissue collapse lesion 3.0-5.0 mm diameter; and 9= tissue collapse at about 10 days, profuse sporulation in large (>5.0 mm) lesion with a diffuse margin. Once the readings were taken, the median for each line was calculated for further analysis and median ratings for each line was calculate to categorize the resistance level of each line. Disease average ratings 0 to 2, 3 to 6, and 7 to 9 were categorized as resistant, intermediate and susceptible to blackleg, respectively. Some plants that showed a resistant reaction as seedlings showed susceptible reaction once they reached the bolting stage. These plants started wilting and had cankers at the collar region with some visible pycnidia. Disease severities in adult plants were rated as resistant (R), moderately resistant (MR) or susceptible (S) based on visual inspection of symptoms. MR plants had stem cankers and some degree of wilting but produced a few seeds; resistant plants produced good number of healthy seeds without showing wilting symptoms or have small stem cankers; whereas susceptible plants died before producing seeds.

BGRR was rated using the 1-7 scale described by Schneider and Kelly (2000), where 1 = healthy roots with no discoloration of root and no reduction in root mass; 2 = 0.1- to 0.2-cm small reddish brown lesions of root, with normal root mass and size; 3 = increase in

intensity and size and coalescing of localized root lesions approximately 180° around the stem, with lesions from 0.5 to 1 cm and 10 to 20% root discoloration but no reduction in root mass size; 4 = increase in intensity of discoloration and size of lesions, with lesions extending and completely encircling the stem, 5 to 10% root mass reduction, and 95% of the roots discolored; 5 = increasingly discolored and extended lesions, with 100% of the roots intensely reddish-brown and 20 to 50% root mass reduction; 6 = lesions encircling the stem extending up to 2 cm, intense root mass discoloration, and 50 to 80% root mass reduction; and 7 = pithy or hollow stem and very extended lesions, 80 to 100% root mass reduction, and dead. Root rating 0 to 2.0, 2.1 to 4.0, and 4.1 to 7.0 were categorized as resistant, intermediate, and susceptible to BGRR, respectively. Fresh weight of whole plants including root was recorded.

4.3.5. Experimental design and statistical analyses

Screenings of F_2 through F_5 seedlings for resistance to Sclerotinia stem rot were conducted using non-replicated trials. Whereas screening of materials from F_6 generations were conducted using a randomized complete block (RCB) design with four replications and six plants per replication. Cultivar Westar was screened in each generation as a susceptible check. Screening of F_5 and F_6 materials for resistance to blackleg and BGRR were conducted using an RCB design with four replications and six plants per replication. Six plants per experimental unit were inoculated for each generation when screening for sclerotinia stem rot.

Percentages of plant mortality were recorded when plants were screened for resistance to sclerotinia and the data was used to calculate the area under the mortality curve (AUMC). The AUMC was calculated adapting the equation of the area under the disease progress curve (Shaner and Finney, 1977). The equation used was:

$$\text{AUMC} = \sum_{i=1}^{n-1} \frac{Y_i + Y_{i+1}}{2} (t_{i+1} - t_i)$$

where y_i is the number of dead plants at time t_i and n is the total number of observations. Median disease severities were calculated for blackleg and BGRR and means were calculated for fresh plant weight when screening for resistance against BGRR. Analyses of variance were performed using SAS 9.2 to compare AUMC of different lines in the F_6 generation, and for fresh plant weight of the F_5 generation screened for resistance against BGRR; Fisher's protected least significant difference (LSD) at $P = 0.05$ was calculated to differentiate among lines within each generation. Variance-type statistic was performed using nonparametric analysis (Brunner and Puri, 2001; Shah and Madden, 2004) to compare disease medians and to determine whether F_5 lines differed in resistance to blackleg and/or BGRR. Nonparametric analyses were performed using the PROC MIXED procedure of SAS with LSMEANS option (Version 9.2, SAS Institute Inc., Cary, NC). Fixed effects, mean values, relative treatment effects (pij), variances, and their 95% confidence intervals (CI) were calculated using LD_CI macro (Brunner and Puri, 2001) to determine whether the differences between lines were statistically significant or not.

4.4. Results

4.4.1. Reaction to *Sclerotinia sclerotiorum*

The symptoms observed in susceptible plants in each generation were wilting, water soaked lesions with or without external fluffy mycelium growth, and finally death (Figure 4.1). Plants considered moderately resistant to sclerotinia stem rot had a pinkish pigmentation produced on tissues located in the area where the inoculated petiole meets the stem. Allowing

plants that survived an inoculation and advancing their seeds may have contributed to a delayed wilting symptom expression i.e 60.4% of F_3 seedlings inoculated died within 14 days from inoculation whereas only 27.1% of F_6 seedlings showed wilting symptoms within the same period of time after inoculation (Figure 4.1). F_2 seedlings started to show wilting symptoms after three days of inoculation.

Figure 4.1. Pictorial view of slow molding symptom of sclerotinia stem rot under greenhouse conditions using PIT in F_5 generation after 32 days of inoculation with *Sclerotinia sclerotiorum*.

Dead plants were observed starting five days after inoculation. All susceptible plants died within 23 days after inoculations (Figure 4.2). Of the 230 F_2 plants evaluated, 161 plants died and 69 plants survived. Resistance improved as the AUMC recorded in each successive generation declined. These reductions indicated that ever longer periods of time were required to kill the plants with each succeeding generation (Figure 4.2).

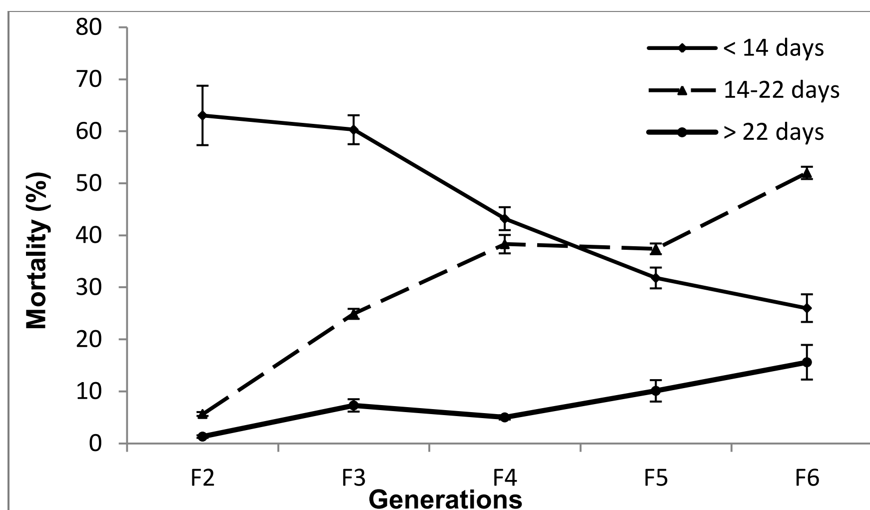

Figure 4.2. Effect of selection for resistance to *Sclerotinia sclerotiorum* on the percentage of plant mortality occurring at <14, 15-22, and >22 days after inoculation using the petiole inoculation technique under greenhouse conditions. The population was produced by the cross of two *Brassica napus* plant introduction materials, PI458939 and PI 649136 (i.e. Ames 26628). Error bars represent standard errors of the means.

The average AUMC at the F_3 generation was 1289 units (Table 4.1). The average AUMC in the F_4 generation was 80.1% that of the F_3 and the average AUMC at the F_5 and F_6 generations were reduced by 23.8% and 26% with respect to that of the F_3 generation. The

Table 4.1. Reaction of different generations of plants from a cross between *B. napus* plant introductions 458939 and 649136 to *Sclerotinia sclerotiorum*[1].

Generation	AUMC range	AUMC Average	CI	Difference with previous generation
F3	550-1983	1289	(1077-1502)	-
F4	650-1583	1032	(956-1108)	257
F5	650-1400	982	(904-1060)	50
F6	417-1367	954	(844-1064)	28

AUMC= Area under mortality curve
CI= 95% confidence interval and the format is (lower-upper) limits.
[1]Inoculations using petiole inoculation technique.

number of survivor lines decreased with each succeeding generations and by the time the F_6 generation was evaluated, only eight lines were left (Figure 4.3). These eight resistant lines (16.1, 20, 28, 72, 140, 160, 178 and 186) had consistently low AUMC values throughout generations (Table 4.2).

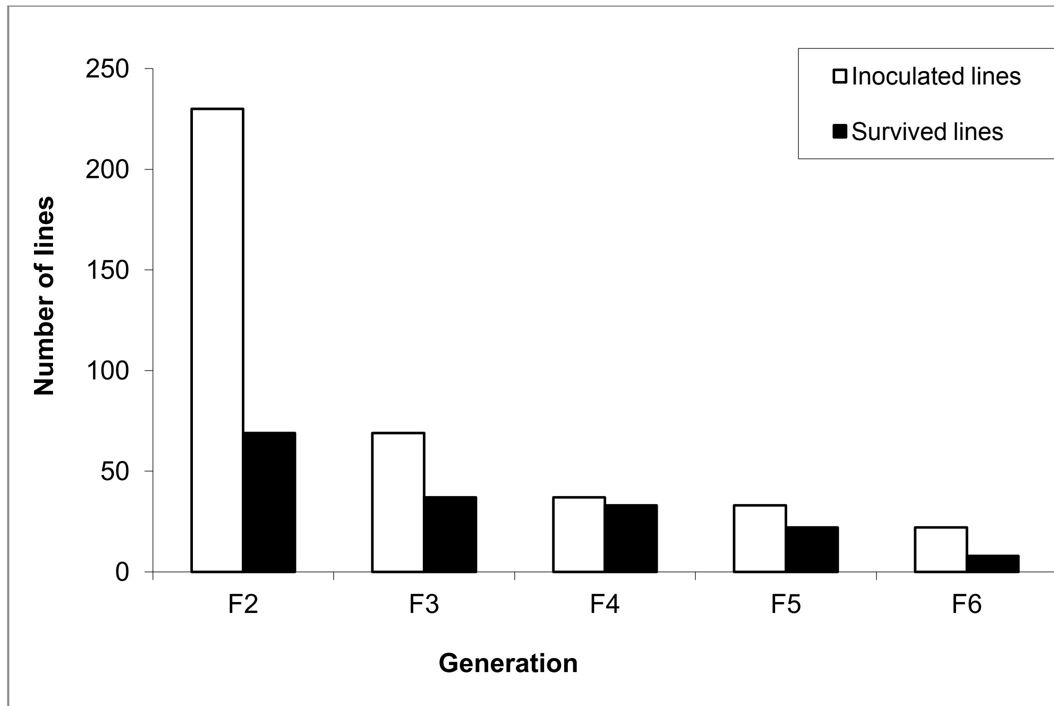

Figure 4.3. Number of breeding lines, produced by the cross of PI458939 and PI 649136, inoculated with *S. sclerotiorum* and lines that survived in different generations under greenhouse conditions using PIT.

In F_6 generation, six lines (16.1, 28, 140, 160, 178 and 186) had lower AUMC than both parents indicating the transgressive segregation (Table 4.2) whereas four lines (16.1, 28, 160 and 186) has even significantly higher level of resistance to sclerotinia stem rot resistance level compared to parental lines (PI458939 and PI 649136) based on AUMC values.

Table 4.2. Reaction of 22 F_5 *B. napus* breeding lines produced by the cross of PI458939 and PI 649136 to inoculation with *Sclerotinia sclerotiorum* using the PIT[a].

Lines	**F_3**	**F_4**	**F_5**	**F_6 [b]**
16.1	1283	983	700	563 l
160.3	900	1250	733	1100 def
20	1117	1033	950	846 hij
28	1100	1050	983	521 l
30	1017	867	800	1058 efg
41	983	867	1000	975 fgh
51	1250	1083	1183	1238 bc
71	683	950	917	1150 bcde
72	1133	800	650	875 hij
77	1150	650	850	1092 def
90	1017	683	717	933 ghi
106	867	650	933	1100 def
111	1000	1117	917	1200 bcd
129	600	1433	767	1033 efg
140	983	933	750	746 jk
153	1117	1117	883	1283 b
160	1050	817	717	621 kl
178	1350	1200	800	783 j
179	1167	1250	717	1142 cde
186	1200	1133	967	496 l
206	1633	900	1033	1100 def
218	1067	1283	883	1125 cde
Westar	2000	2000	2000	2000 a
PI 458939	1033	933	933	833 ij
PI 649136	900	883	833	792 j

a PIT= petiole inoculation technique

[b]same letters within a column indicate values are not statistically different from each other at α=0.05

4.4.2. Reaction to *Leptosphaeria maculans*

L. maculans caused round to irregular-shaped lesions with a tan or buffed color appearance on inoculated cotyledon leaves (Figure 4.4). In very susceptible plants, like cv. Westar, pycnidia were observed in the centre of old lesions approximately after 10 days after inoculations. Lesions were smaller and did not increase as much in moderately resistance seedlings compared to susceptible seedlings. Inoculated seedlings of susceptible lines that

Figure 4.4. Typical blackleg leaf symptom developing on an F_5 plant inoculated with *Leptosphaeria maculans* at cotyledon stage.

reached the adult stage experienced wilting and developed cankers on the base of stem that sometimes completely girdled the stem. Sometimes pycnidia were also observed in leaves and stems of highly susceptible plants (Figure 4.5).

Figure 4.5. Typical stem lesion symptom with pycnidia on an F_5 plant inoculated with *Leptosphaeria maculans* at cotyledon stage.

Most of the sclerotinia stem rot resistance lines were also moderately resistant to blackleg and resistant to brown girdling root rot (Table 4.3, 4.4, and 4.5). Sixty-five and 63

Table 4.3. Reaction of 33 F_5 *B. napus* breeding lines produced by the cross of PI458939 and PI 649136, to inoculation with pathogenicity groups 3 and 4 strains of *Leptosphaeria maculans* at the seedling stage, under greenhouse condition.

	PG3[a]			PG4[a]		
Lines	**Median**	**Treatment relative effect**	**CI[b] (Lower - Upper)**	**Median**	**Treatment relative effect**	**CI (Lower-Upper)**
1	5.5	0.43	(0.30-0.57)	6.0	0.43	(0.30-0.58)
16	5.0	0.28	(0.17-0.44)	5.5	0.28	(0.17-0.44)
16.1	4.0	0.20	(0.07-0.48)	4.5	0.20	(0.07-0.48)
20	6.5	0.59	(0.48-0.69)	6.5	0.59	(0.48-0.69)
28	7.0	0.56	(0.31-0.78)	7.0	0.56	(0.31-0.78)
30	5.0	0.21	(0.18-0.25)	5.5	0.21	(0.18-0.25)
37	6.5	0.61	(0.47-0.73)	5.0	0.61	(0.47-0.73)
41	6.5	0.68	(0.33-0.89)	6.5	0.68	(0.33-0.89)
51	6.0	0.43	(0.30-0.57)	6.0	0.43	(0.30-0.57)
53	5.0	0.21	(0.18-0.26)	5.0	0.21	(0.18-0.25)
71	4.5	0.13	(0.06-0.26)	4.5	0.13	(0.06-0.26)
72	7.0	0.62	(0.33-0.84)	5.0	0.62	(0.33-0.84)
77	5.0	0.33	(0.15-0.58)	5.0	0.33	(0.15-0.58)
85	6.0	0.43	(0.30-0.58)	6.0	0.43	(0.30-0.57)
87	9.0	0.82	(0.69-0.90)	9.0	0.82	(0.69-0.90)
90	8.0	0.81	(0.73-0.87)	8.0	0.81	(0.73-0.87)
106	6.0	0.35	(0.22-0.52)	5.5	0.35	(0.22-0.52)
111	7.0	0.66	(0.54-0.76)	7.0	0.66	(0.54-0.76)
114	5.5	0.35	(0.22-0.52)	5.5	0.35	(0.22-0.52)
123	7.0	0.63	(0.30-0.87)	5.0	0.63	(0.30-0.87)
129	6.0	0.50	(0.45-0.54)	6.0	0.50	(0.45-0.54)
140	5.5	0.45	(0.16-0.78)	5.0	0.45	(0.16-0.78)
153	4.5	0.12	(0.05-0.27)	4.5	0.12	(0.05-0.27)
160	8.5	0.80	(0.64-0.89)	8.0	0.80	(0.64-0.89)
178	6.0	0.50	(0.45-0.54)	6.0	0.50	(0.45-0.54)
179	6.0	0.40	(0.21-0.63)	5.0	0.40	(0.21-0.63)
186	9.0	0.91	(0.88-0.93)	9.0	0.91	(0.88-0.93)
206	8.0	0.70	(0.44-0.87)	8.0	0.70	(0.44-0.87)
209	9.0	0.88	(0.79-0.93)	8.0	0.88	(0.79-0.93)

Table 4.3. Reaction of 33 F_5 *B. napus* breeding lines produced by the cross of PI458939 and PI 649136, to inoculation with pathogenicity groups 3 and 4 strains of *Leptosphaeria maculans* at the seedling stage, under greenhouse condition (Continued)

	PG3[a]			PG4[a]		
Lines	**Median**	**Treatment relative effect**	**CI[b] (Lower - Upper)**	**Median**	**Treatment relative effect**	**CI (Lower-Upper)**
210	5.5	0.49	(0.29-0.70)	5.0	0.49	(0.29-0.70)
215	9.0	0.91	(0.88-0.93)	9.0	0.91	(0.88-0.93)
218	4.5	0.23	(0.06-0.62)	6.0	0.23	(0.06-0.62)
160.3	5.0	0.21	(0.18-0.25)	6.0	0.21	(0.18-0.25)
PI 458939	6.0	0.43	(0.30-0.57)	5.5	0.43	(0.30-0.57)
PI 649136	5.0	0.28	(0.17-0.44)	5.0	0.28	(0.17-0.44)
Westar	9.0	0.88	(0.79-0.93)	9.0	0.88	(0.79-0.93)

[a]Blackleg severity of 33 advance lines, inoculated with Pathogenicity Group (PG) 3 and 4, based on scale of 0-9.
[b]CI= 95% confidence interval (lower-upper) limits for relative treatment effects.

Table 4.4. Reaction of nine F_6 *B. napus* breeding lines produced by the cross of PI458939 and PI 649136, to inoculation with pathogenicity groups (PG) 3 and 4 strains of *Leptosphaeria maculans* at the seedling stage, under greenhouse condition.

	PG3[a]			PG4[a]		
Lines	**Median**	**Treatment relative effect**	**CI[b]**	**Median**	**Treatment relative effect**	**CI**
16.1	5.5	0.50	(0.31-0.69)	5	0.35	(0.15-0.63)
20	5.0	0.40	(0.27-0.54)	6	0.51	(0.32-0.70)
30	5.0	0.33	(0.21-0.49)	6	0.51	(0.37-0.64)
53	5.0	0.21	(0.14-0.30)	5	0.24	(0.16-0.35)
71	4.5	0.34	(0.12-0.71)	5	0.37	(0.13-0.71)
77	5.5	0.46	(0.36-0.56)	6	0.56	(0.41-0.70)
106	6.0	0.58	(0.22-0.86)	8	0.70	(0.39-0.88)
153	5.0	0.25	(0.09-0.59)	5	0.32	(0.11-0.70)
218	6.0	0.56	(0.31-0.77)	6	0.49	(0.20-0.79)
PI 458939	6.0	0.70	(0.51-0.83)	6	0.51	(0.32-0.70)
PI 649136	6.0	0.74	(0.63-0.83)	6	0.52	(0.25-0.77)
Westar	9.0	0.93	(0.87-0.95)	9	0.93	(0.87-0.95)

[a]Blackleg severity of 33 advance lines, inoculated with Pathogen Group (PG) 3 and 4, based on scale of 0-9.
[b]CI= 95% confidence interval (lower-upper) limits for relative treatment effects.

percent of F_5 lines showed intermediate levels of resistance in seedling stage to blackleg strains PG3 and PG4, respectively; however of the 20 moderately resistant entries, only nine (27% of all F_5 lines evaluated) survived to produce seeds. Among those nine lines (16.1, 20, 30, 53, 71, 77, 106, 153, and 218), four lines (i.e. 16.1, 71, 77 and 153) survived from the adult stage infection in F_6 generation. Five lines (30, 53, 71,153, and 160.3) showed resistance at seedling stage than one parents. In seedling stage, narrower range of disease severity was seen in the F_6 generation (4.5 to 6.0 and 4.9 to 6.0 for PG3 and PG4 respectively), compared to F_5 lines (4.0 to 9.0 and 4.5 to 9.0 for PG3 and PG4, respectively (Tables 4.3 and 4.4).

4.4.3. Reaction to *Rhizoctonia solani* AG2-2

Resistance to *R. solani* was evaluated in the F_5 generation. Damping off symptoms were observed 10 days after seed sowing. Light-brown lesions on the taproot or main lateral roots along with the hypocotyl were observed. These lesions enlarged, coalesced, became sunken and girdled the taproot (Figure 4.6). As a result plants wilted and toppled down.

Figure 4.6. Typical brown girdling root rot symptom of *B. napus* developing on an F_5 plant inoculated with *Rhizoctonia solani* on 20 days after inoculation.

The percentage of seedlings that were dead within three weeks after inoculation, plant weight and root scoring (Table 4.5) were used in evaluation. In the F_5 generation, mortality rates ranged from 0% to 67% mortality, where susceptible control Westar had 100% mortality. The average root scoring ranged from 0 to 7.0 and plant weight from 0.64 to 4.2 (Table 4.5). Approximately, 45, 28 and 27 percent of the 33 F_5 lines evaluated were considered to be resistant, moderately resistant and susceptible to BGRR, respectively. Eight lines (16.1, 28, 51, 123, 160, 160.3, 209, and 218) showed higher resistance than one parent. Also, most of the sclerotinia stem rot resistance lines were moderately resistant to brown girdling root rot (Table 4.2 and 4.5).

Table 4.5. Reaction of 33 F_5 *B. napus* breeding lines, produced by the cross of PI 458939 and PI 649136, to inoculation with *R. solani,* based on scale of 0-7 and fresh plant weight of inoculated plant at seedling stage (20 days after planting) in greenhouse.

Lines	Disease severity	Treatment relative effect	Confidence Interval at 95% (Lower Limit-Upper Limit)	Fresh plant weight (gm)[c]
1	6.5	0.73	(0.63-0.81)	0.9 k-n
16	3.5	0.57	(0.40-0.72)	1.9 fgh
16.1	0.0	0.17	(0.15-0.19)	1.9 efg
20	2.5	0.51	(0.34-0.68)	1.3 h-m
28	0.0	0.36	(0.22-0.53)	1.2 i-n
30	1.0	0.45	(0.31-0.59)	2.9 bc
37	7.0	0.86	(0.84-0.87)	0.9 lmn
51	0.0	0.29	(0.19-0.41)	3.1 b
53	1.0	0.37	(0.26-0.50)	1.3 i-m
71	7.0	0.72	(0.59-0.83)	0.7 nm
72	2.5	0.56	(0.42-0.70)	2.2 def
77	0.0	0.34	(0.22-0.48)	1.2 i-n
85	1.0	0.44	(0.35-0.53)	1.5 g-k
87	7.0	0.67	(0.505-0.8)	1.0 j-n
90	1.0	0.37	(0.26-0.50)	1.3 i-m
106	2.5	0.57	(0.42-0.71)	2.2 def
111	7.0	0.79	(0.69-0.85)	0.9 lmn
114	6.0	0.65	(0.49-0.78)	0.7 n
123	0.5	0.33	(0.24-0.44)	1.6 g-j
129	7.0	0.76	(0.68-0.83)	1 k-n
140	1.0	0.41	(0.30-0.52)	1.7 f-i
153	4.0	0.60	(0.47-0.71)	0.9 k-n
160	0.0	0.22	(0.16-0.30)	1.6 g-j
178	1.0	0.39	(0.28-0.52)	1.2 i-n
179	6.0	0.60	(0.46-0.73)	1.2 i-n
186	0.5	0.42	(0.27-0.58)	2.5 cde
206	1.0	0.48	(0.32-0.64)	1.3 h-l
209	0.0	0.29	(0.21-0.39)	1.4 g-l
210	1.5	0.50	(0.34-0.66)	0.9 k-n
215	7.0	0.86	(0.84-0.87)	0.6 n
218	1.0	0.42	(0.34-0.49)	1.6 g-j
160.3	0.0	0.29	(0.18-0.42)	2.6 b-d
PI 458939	0.0	0.22	(0.16-0.30)	1.6 g-j
PI 649136	3.0	0.61	(0.49-0.71)	4.2 a
Westar	7.0	0.86	(0.84-0.87)	0.7 n

[c]same letters within a column indicate values are not statistically different from each other at α=0.05

4.5. Discussion

This study reports pre-breeding activities conducted to develop germplasm with enhanced resistance to two important diseases affecting production of canola in North Dakota, sclerotinia stem rot and blackleg, and to an emerging disease, brown girdling root rot. The parental lines used, had been previously identified as being resistant to sclerotinia stem rot (Khot et al., 2006), although no genetic studies have been performed on them to characterize the nature of their resistance in identified new sources of resistance to sclerotinia stem rot, blackleg and brown girdling root rot in *B. napus*. Two diverse plant introduction materials from Japan and Germany were crossed and their progenies were screened up to the F_6 generations for sclerotinia stem rot resistance. In addition, F_5 and F_6 progenies were evaluated for blackleg and F_5 progenies were screened for brown girdling root rot. The population was advanced towards homozygosity by self-pollinating plants that survived a round of inoculations in each generation.

None of the materials evaluated proved to be immune to *S. sclerotiorum* or to have major genes for resistance. While the nature of the resistance detected in some of the lines evaluated has not been characterized yet, it could come from genes not previously identified since the plant introduction materials in which their activity was observed have not been used before as sources of resistance. Identification and characterization of these genes should be the next logical step in this research. Evaluation of canola germplasms for resistance to sclerotinia stem rot and blackleg (Chen and Fernando, 2006) in the field could be complicated due to variability of pathogen inoculum, variability in environmental and microclimatic conditions, and competition between pathogens present in field conditions. In addition, mixed infection of pathogens that produce similar wilting symptoms (disease syndrome) in field

condition makes difficult to evaluate canola germplasm or cultivars. Therefore, screening of canola germplasms in the greenhouse has been found an effective strategy for identifying sources of resistance. In separate study (different inoculation study, Chapter 3), PIT was found efficient method for screening canola germplasms for sclerotinia stem rot. Therefore, PIT was used to screen the progenies for sclerotinia stem rot resistance in this study.

The number of progenies showing resistance to sclerotinia stem rot and surviving inoculation decreased in succeeding generations; however, the ratio or percentage of resistance in each subsequent generation was not consistent. For example progenies from F_3 to F_4 and F_4 to F_5 showed 89% and 67% resistant, respectively, whereas 49% and 36% resistant plants were observed in progenies from F_2 to F_3 and F_5 to F_6, respectively. These differences in reaction could be due to the polygenic nature of resistance or due to partial nature of resistance. Zhao et al. (2003a; 2003b; 2006) has reported several QTLs present in *B. napus* genome associated with sclerotinia stem rot resistance moreover Yin et al. (2010) also reported different QTLs in both AA and CC genomes of *B. napus* associated with sclerotinia stem rot resistance. However, the lines in subsequent generation gained in homozygosity and there are eight individuals with higher levels of resistance within each generation.

Our goal was to develop breeding lines or germplasm with enhanced resistance to for sclerotinia stem rot. Of the 230 progenies developed by crossing two moderately resistant plant accessions from Japan and Germany, eight lines showed to be moderately resistant to sclerotinia stem rot and were able to survive and four lines showed higher levels of resistance than both parents indicating the transgressive segregation. Yin et al. (2010) also reported transgressive segregation in doubled haploid progenies obtained from a cross between two doubled haploid lines (line DH821, derived from Zhongyou 821, as partially resistant to *S.*

sclerotiorum, and DHBao604 as a susceptible parent). Most of these lines (eight lines) showed resistance to *Rhizoctonia* and few of them also showed resistance to blackleg (both seedling and adult stage). These lines could be utilized in canola breeding programs where sclerotinia stem rot; blackleg and brown girdling root rot are severe problems. Previous studies also reported improvement in disease resistance when combined diverse source of resistance. Burlakoti et al. (2010) reported superior FHB resistance in the recombinant inbred line developed from Chinese and Brazilian cultivars. Zhao et al. (2003a; 2003b; 2006) reported that few progenies developed for quantitative trait loci mapping showed the higher level of disease resistance compared to parental lines. Yin et al. (2010) also reported transgressive segregation in doubled haploid progenies obtained from a cross between two doubled haploid lines (line DH821, derived from Zhongyou 821, as partially resistant to *S. sclerotiorum*, and DHBao604 as a susceptible parent).

Some lines showed sclerotinia stem rot resistance in earlier generations but could not survive in later generations; however, these lines showed ability to delay disease development. The number of days required to kill the plants increased in each generation. Therefore, the lines which delays the disease development in F_4 or F_5 generation can be crossed with other resistant germplasm and their progenies could give durable resistance for sclerotinia stem rot. Slow rusting wheat cultivars developed from parents having slow rusting genes gave near-immunity to leaf and stripe rusts in wheat and used for several years (Singh et al., 2000). Previous studies reported that same resistant canola lines have the ability to delay sclerotinia stem rot development (Wang and Fristensky, 2001).

Sixty-five percent and 63 percent of F_5 progenies showed intermediate levels of resistance to blackleg at the seedling stage. While only 27 percent expressed adult stage

resistance and remained canker-free. Similarly, 45, 28 and 27 percent of 33 F_5 progenies were resistance, intermediate resistance and susceptible to brown girdling root rot, respectively.

Large proportion of F_5 lines showed high levels of resistance to brown girdling root rot (45% of lines) and intermediate seedling resistance (>60% progenies) and adult plant resistance (27% progenies) to blackleg. In F_6 generation, 6 lines showed high levels of seedling resistance to blackleg and four of them survived to produce seeds. Few lines also showed higher resistance to both blackleg (four lines) and *Rhizoctonia* root rot (eight lines) than both parents showing the evidence of transgressive segregation. The result suggests that these lines will be very effective and can be utilized in blackleg and *Rhizoctonia* root rot resistance breeding programs. Furthermore, the progenies showing consistent resistance to sclerotinia stem rot from (F_2 to F_6) and also showing resistance to blackleg and/or BGRR could be very useful sources of durable resistance to these diseases.

Resistance to multiple diseases is not very common, although there are few examples in *B. napus*. Transgenic lines with DRR206 from spring *B. napus* cultivar Westar, has previously been reported to be resistant to *L. maculans* PG3 and PG4 and BGRR with slow lesion development that delays sclerotinia stem rot development (Wang and Fristensky, 2001). Mitchell-Olds et al. (1995) screened genetically polymorphic populations of *B. rapa* for white rust (*Albugo candida*), downy mildew (*Peronospora parasitica*) and blackleg (*L. maculans*) resistance. The data from 9518 plants showed the positive genetic correlation between resistance to *P. parasitica* and *L. maculans*.

Although resistance to blackleg has been reported to be controlled by multiple genes for adult stage (Delourme et al., 2006; Pilet et al., 1998) and by single genes for seedling stage (Delourme et al., 2006; Ferreira et al., 1995), resistance breeding that combines seedling stage

resistance and adult plant resistance could be more durable to blackleg resistance in field conditions (Chevre et al., 2008). In our study, plants were inoculated at the seedling stage, but evaluations were conducted both at seedling and adult plant stages. This approach allowed us to identify resistant and susceptible germplasm (Mengistu et al., 1991). Since all plants were kept in the greenhouse conditions, reaction of genotypes in field condition is still unknown.

The expression of blackleg symptoms depends on the interaction between isolate and canola cultivar (Mengistu et al., 1991). Most evaluations of *B. napus* germplasm conducted by other researchers have used only one pathogen at a time (Yin et al., 2010; Zhao and Meng 2003a; Zhao and Meng, 2003b; Zhao et al., 2006) or multiple strains of one (Marino, 2011). Polygenic resistance to blackleg has been identified in *B. napus* cv. Dunkeld (Ananga et al., 2006; Gororo et al., 2004). On the other hand, the resistance available in cultivars planted in this state has been defeated by *L. maculans* strains of PG3 and PG4 (Bradley et al., 2005; Marino, 2011). In this study *B. napus* lines were evaluated using mixture of four different isolates of PG3 and a highly virulent isolate of PG4. Whether these blackleg-resistant genotypes identified in the present study are resistant or susceptible to several other PG isolates prevalent in other canola growing areas deserves further studies.

Similarly, our study also revealed that the progenies which had good levels of adult plant resistance to blackleg also showed good levels of resistance to sclerotinia stem rot and brown girdling root rot. These lines could be the effective sources of resistance to breeding programs of the region.

4.6. References Cited

Ananga, A. O., Cebert, E., Soliman, K., Kantety, R., , Konan, K., and , Pacumbaba, R. 2006. Association mapping of resistance to blackleg disease (*Leptosphaeria maculans*) in canola. PAG:XIV conference, 14th-18th Jan. 2006, CA, USA.

Ben-Yephet, Y., Genizi, A., and Siti, E. 1993. Sclerotial survival and apothecial production by *Sclerotinia sclerotiorum* following outbreaks of lettuce drop. Phytopathology 83:509-513.

Bradley, C. A., Henson, R. A., LeGare, D. G., del Rio, L. E., and Khot, S. D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Dis. 90:215-219.

Bradley, C. A., Parks, P. S., Chen, Y., and Fernando, W. G. D. 2005. First report of pathogenicity groups 3 and 4 of *Leptosphaeria maculans* on Canola in North Dakota. Plant Dis. 89:776.

Brunner, E., and Puri, M. L. 2001. Nonparametric methods in factorial designs. Stat. Pap. 42:1-52.

Burlakoti, R. R., Mergoum, M., Kianian, S. F., and Adhikari, T. B. 2010. Combining different resistance components enhances resistance to *Fusarium* head blight in spring wheat. Euphytica 172:197-205.

Chen, Y., and Fernando, W. G. D. 2006. Induced resistance to blackleg (*Leptosphaeria maculans*) disease of canola (*Brassica napus*) caused by a weakly virulent isolate of L*eptosphaeria biglobosa*. Plant Dis. 90:1059-1064.

Chèvre, A.-M., Brun, H., Eber, F., Letanneur, J.-C., Vallee, P., Ermel, M., Glais, I., Hua Li, Sivasithamparam, K., and Barbetti, M. J. 2008. Stabilization of resistance to *Leptosphaeria maculans* in *Brassica napus–B. juncea* recombinant lines and its introgression into spring-type *Brassica napus*. Plant Dis. 92:1208-1214.

del Rio, L. E., Bradley, C. A., Henson, R. A., Endres, G. J., Hanson, B. K., McKay, K., Halvorson, M., Porter, P. M., LeGare, D. G., and Lamey, H. A. 2007. Impact of sclerotinia stem rot on yield of canola. Plant Dis. 91:191-194.

del Rio, L. E., Kurtzweil, N. C., and Grau, C. R. 2001. Petiole inoculation as a tool to screen soybean germplasm for resistance to *Sclerotinia sclerotiorum.* Phytopathology 91:S176.

Delourme, R., Chevre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., and Dias, J. S. 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 114:41–52.

Delwiche, P. A. 1980. Genetic aspects of blackleg (*Leptosphaeria maculans*) resistance in rapeseed (*Brassica napus*). Ph.D. Thesis, Univ. of Wisconsin, Madison, USA.

Ferreira, M. E., Rommer, S. R., Williams, P. H., and Osborn, T. C. 1995. Mapping loci controlling brassica resistance to *L. maculans* under different screening conditions. Phytopathology 85:213–217.

Fitt, B. D. L., Brun, H., Barbetti, M. J., and Rimmer, S. R. 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 114:3–15.

Gororo, N., Salisbury, P., and Marcroft, S. 2004. Dunkeld and Rainbow: A tale of two victorian canola cultivars. In: Proc. 4th Int. Crop Sci. Cong. Brisbane, Queensland, Australia.

Gugel, R. K., Petrie, G. A., 1992. History, occurrence, impact and control of blackleg of rapeseed. Canadian J. Plant Pathol. 14:36-45.

Gugel, R. K., Yitbarek, S. M., Verma, P. R., Morall, R. A. A., and Sadasivaiah, R. S. 1987. Etiology of the rhizoctonia root rot complex of canola in the Peace River region of Alberta. Can. J. Plant Pathol. 9:119-128.

Kataria, H. R., and Verma, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. Crop Prot. 11:8-13.

Khot, S. D. 2006. Evaluation of *Brassica napus* accessions for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis. NDSU, USA.

Klein-Gebbinck, H. W., and Woods, D. L. 2002. Yield loss assessment in canola: Effects of brown girdling root rot and maggot damage on single plant yield. Plant Dis. 86:1005-1010.

Lamey, H. A., and Hershman, D. E. 1993. Canola (*Brassica napus* L.) blackleg caused by *Leptosphaeria maculans* in North Dakota. Plant Dis. 77:1263.

Marino, D. 2011. Screening of plant introduction materials from the *Brassica species* for resistance against PG3 and PG4 isolates of Blackleg. M.S. Thesis, NDSU, USA.

Markell, S., del Rio, L., Halley, S., Mazurek, S., Mathew, F., and Lamey, A. 2008. Blackleg of Canola. NDSU Extension Service, NDSU. PP-1367.

Mayerhofer, R., Bansal, V. K., Thiagarajah, G. R., Stringam, G. R., and Good, A. G. 1997. Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. Genome 40:294–301.

Mengistu, A., Rimmer, S. R., Koch, E., and Williams, P. H. 1991. Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling Brassicas. Plant Dis. 75:129-1282.

Mitchell-Olds, T., Vaughn James, A., Palmer, M. J., Williams, P. H. 1995. Genetics of *Brassica rapa* (syn. campestris).2. Multiple disease resistance to three fungal pathogens: *Peronospora parasitica*, *Albugo candida* and *Leptosphaeria maculans.* Heredity 75:362-369.

Nepal, A., and del Rio-Mendoza, L. E. 2010. Brown girdling root rot as a potential threat to canola production in North Dakota. Phytopathology100:S89.

Pilet, M. L., Delourme, R., Foisset, N., and Renard, M. 1998. Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in winter rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 96:23–30.

Rimmer, S. R. 2006. Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Can. J. Plant Pathol. 28:s288–s297.

Salisbury, P. A., Ballinger, D. J., Wratten, N., Plummer, K. M., and Howlett, B. J. 1995. Blackleg disease on oilseed brassica in Australia: a review. Aust. J. Exp. Agric. 35:665–672.

Schneider, K. A., and Kelly, J. D. 2000. A greenhouse screening protocol for fusarium root rot in bean. HortScience 35:1095-1098.

Shah, D. A., and Madden, L. V. 2004. Nonparametric analysis of ordinal data in designed factorial experiments. Phytopathology 94:33-43.

Shaner E., and Finney, R. E. 1977. The effect of nitrogen fertilization on the expression of slow mildewing in Knox wheat. Phytopathology 67:1051-1056.

Singh, R. P., Huerta-Espino, J., Rajaram, S. 2000. Achieving near-immunity to leaf and stripe rusts in wheat by combining slow rusting resistance genes. Acta Phytopathol. Hungarica 35:133-139.

Sippell, D. W., Davidson, J. G. N., and Sadasivaiah, R. S. 1985. *Rhizoctonia* root rot of rapeseed in the Peace River region of Alberta. Can. J. Plant Pathol. 7:184-186.

Strausbaugh, C. A., Bradley, C. A., Koehn, A.C., and Forster, R. L. 2004. Survey of root diseases of wheat and barley in southeastern Idaho. Can. J. Plant Pathol. 26:167-176.

Thomas, P. 2003. Canola growers manual. The Canola Council of Canada, Canada Available from http://www.canolacouncil.org/contents2.aspx: Accessed March 17, 2011.

Wang, Y., and Fristensky, B. 2001. Transgenic canola lines expressing pea defense gene DRR206 have resistance to aggressive blackleg isolates and to *Rhizoctonia solani.* Mol. Breed. 8:263-271.

Yang, J., and Verma, P. R. 1992. Screening genotypes for resistance to preemergence damping-off and post-emergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG2-1. Crop Prot. 11:443-448.

Yin, X., Yi, B., Chen, W., Weijie, Z., Tu, J., Dilantha Fernando, W. G., Fu, T. 2010. Mapping of QTLs detected in a *Brassica napus* DH population for resistance to *Sclerotinia sclerotiorum* in multiple environments. Euphytica 173:25-35.

Zabala, F. 2008. Screening the USDA-NGRP *Brassica rapa* Collection for resistance to *Sclerotinia sclerotiorum*. M.S. Thesis, NDSU, USA.

Zhao J., and Meng, J. 2003a. Detection of loci controlling seed glucosinolate content and their association with Sclerotinia resistance in *Brassica napus*. Plant Breed. 122:19–23.

Zhao, J., and Meng, J. 2003b. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 106:759–764.

Zhao, J., Peltier, A. J., Meng, J., Osborn, T. C., and Grau, C. R. 2004. Evaluation of Sclerotinia stem rot resistance in oilseed *Brassica napus* using a petiole inoculation technique under greenhouse conditions. Plant Dis. 88:1033-1039.

Zhao, J., Udall, J. A., Quijada, P. A., Grau, C. R., Meng, J., and Osborn, T. C. 2006. Quantitative trait loci for resistance to *Sclerotinia sclerotiorum* and its association with a homologues non-reciprocal transposition in *Brassica napus* L. Theor. Appl. Genet. 112:509-516.

Zhu, B., and Rimmer, S. R. 2003. Inheritance of resistance to *L. maculans* in two accessions of *B. napus*. Can. J. Plant Pathol. 3:98–103.

CHAPTER 5. IDENTIFICATION OF DART MARKERS LINKED TO SCLEROTINIA STEM ROT RESISTANCE IN *BRASSICA NAPUS* USING GENOME-WIDE ASSOCIATION MAPPING APPROACH

5.1. Abstract

Association mapping approach is a powerful tool to identify genes/loci that contributes to phenotypic variation. This method is important because it provides the means to identify and map multiple loci contributing to trait variation (i.e. disease resistance) in natural populations. Diversity Array Technology (DArT) is used to construct genetic maps allowing determining genetic diversity and performing association mapping in diverse crops where biparental populations are difficult or expensive to develop because they are easy to use and the markers are present in the coding region of gene itself. The objectives of this study were to assess genetic diversity, and identify QTLs associated with sclerotinia stem rot resistance in a diverse group of *B. napus* accessions. Accessions from twenty-one countries from around the world, classified into nine sub-populations, were analyzed. The accessions collected from South Korea, Japan, China, France, Pakistan, Sweden, Poland, and Germany have higher percentage of sclerotinia stem rot resistance. The accessions were screened for sclerotinia stem rot resistance in the greenhouse using petiole inoculation technique (PIT). The area under mortality curve (AUMC) value ranged from 0 to 1675 with an average of 604. About 41.4 % of the DArT markers were polymorphic in the population. Associations between phenotypes and genotypes were assessed through a set of mixed linear models accounting for

population structure and kinship relatedness, leading to the identification of DArT markers significantly associated with resistance. We identified a total of 32 significant markers for sclerotinia stem rot resistance that explain about 1.5 to 4.6% of the variation. Three markers had high association ($P<0.001$) and eight markers had an association ($P<0.01$) with sclerotinia stem rot resistance. The significant markers are distributed among the eight chromosomes of *B. napus* but chr A5 had the highest number of loci associated with sclerotinia stem rot resistance. Homologous sequences of significant markers were observed on all chromosomes of *A. thaliana* and on eight (A1, A2, A3, A4, A5, A6, A9, and A10) chromosomes of *B. rapa.* Blastn search indicates that similar nucleotide sequences are distributed throughout the genomes of *B. napus, B. oleracea, B. rapa*, and *A. thaliana.* The current findings indicate that association mapping could be an excellent tool to exploit the diversity present in accessions and to identify of QTLs.

5.2. Introduction

Brassica napus, *B. rapa*, *B. oleracea* and *Arabidopsis thaliana* belong to the same family, Cruciferae. These two genera have a common ancestor but differentiated about 14.5 to 20.4 million years ago (Yang et al., 1999). U (1935) described the cytogenetic relationships of major *Brassica* species that is currently accepted. Each diploid species contain its own specific genome (e.g. AA for *B. rapa*, BB for *B. nigra,* and CC for *B. oleracea*), and chromosome number (i.e., n =10 for AA, n= 8 for BB, and n=9 for CC). *Brassica napus* (AACC, n=19), is an amphidiploid thought to have originated from a cross where the maternal donor was closely related to two diploid species, *B. rapa* (A genome donor) and *B. oleracea* (C genome donor). Several genetic and morphological variations have occurred in

amphidiploid crops due to the natural hybridization of *Brassica* species i.e. polyploidy. Extensive mixtures of single, duplicated, and triplicated genomes have been found in comparative studies among *Brassica* species and *A. thaliana* conducted at genome level (Babula et al., 2003; Kowalski et al., 1994; Lagercrantz and Lydiate, 1996; Lan et al., 2000; Lukens et al., 2003; Parkin et al., 2003; Schmidt et al., 2001). Genomes of naturally occurring *B. napus* are similar to those of modern-day *B. rapa* and *B. oleracea* (Parkin and Lydiate, 1997; Udall et al., 2005); further, Parkin et al. (2005) speculated that *B. napus* could be the result of replication and rearrangement of the genome of *A. thaliana*.

Sclerotinia stem rot of canola is one of the two most important diseases affecting canola production in North Dakota. *Sclerotinia sclerotiorum* (Lib.) de Bary, an ascomycetous fungus, is causal agent of sclerotinia stem rot (Lamey and Bradley, 2003). Sclerotinia stem rot is endemic in canola growing areas of North Dakota with annual incidences ranging from 1 to 59%. Yield reductions attributed to this disease average approximately 12.75 kg/ha for each incidence unit (del Rio et al., 2007). None of the commercial canola varieties currently in use in the region has acceptable levels of resistance to sclerotinia stem rot (Bradley, 2006).

Resistance to *S. sclerotiorum* is polygenic in nature and reported to be partial or incomplete (Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2006). Quantitative trait loci (QTL) associated with resistance to sclerotinia stem rot have been identified in *B. napus* and in related species (Mei et al., 2011; Yin et al., 2010; Zhao et al., 2003a; Zhao and Meng, 2003b; Zhao et al., 2006). Most of them were identified using biparental populations. These findings strengthen the possibilities of using marker assisted selection to incorporate these genes into commercial cultivars.

The use of biparental populations to detect QTL has some limitations. Maps produced using this approach usually have low resolution due to the relatively low numbers of recombination events occurring in biparental crosses, and usually provide the opportunity to study only two alleles at any given locus at once (Flint-Garcia et al., 2003). Further, the lines selected from these populations might not be 100% compatible with each other since markers linked to QTL are not always transferable to other backgrounds (Snowdon and Friedt, 2004).

Association mapping (AM) can overcome some of the limitations of biparental populations to map QTL. AM uses the concept of linkage disequilibrium (LD) and population structure of the germplasm used to identify significant associations between a trait and molecular markers (Ersoz et al., 2008). The pattern of LD dictate the appropriateness for candidate gene or whole genome mapping while LD basically depends on the population used in the study. Because AM uses historical and evolutionary recombinant populations, many alleles can be evaluated simultaneously. AM has been used in several crops to identify markers associated with diversified phenotypic traits such as flowering time of *A. thaliana* (Aranzana et al., 2005), genome organization of maize (Yu et al., 2006), yield and its component in rice (Agrama et al., 2007), and kernel size in wheat (Breseghello et al., 2006). In *Brassica* spp., AM has been used to identify phenological, morphological and quality traits in winter type *B. napus* (Honsdorf et al., 2010), glucosinolate content and seed oil content in *B. napus* (Hasan et al., 2008; Zou et al., 2010); seed coat color in *B. juncea* (Sabharwal et al., 2004); and leaf traits, flowering time and phytate content in *B. rapa* (Zhao et al., 2007); QTL linked to blackleg resistance QTL (Ananga et al., 2006; Jestin et al., 2010). AM has been also used to identify QTL linked to resistance to Fusarium head blight (Massman et al., 2010) and

Stagonospora nodorum blotch of wheat (Tomassini et al., 2007), and leaf rust of barley (Steffenson et al., 2007).

The development of techniques like AM and more recently, diversity array technology (DArT) for *Brassica* species offer an opportunity to significantly increase the number of primers used to screen genomic DNA for markers associated with any trait including diseases resistance (Jestin et al., 2010; Steffenson et al., 2007). DArT can detect and type DNA variations at several hundred genomic loci in parallel without relying on sequence information. DArT markers in *B. napus* have been recently developed (Raman et al., 2011) but the position of all DArT markers in the species is yet to be determined. This temporary limitation can be overcome, however, because genomic sequences for *Arabidopsis thaliana*, *B. rapa*, and *B. oleracea,* which are all closely related to *B. napus*, are publicly available; thus, comparative mapping among these four species could be used to determine the genetic position of DArT markers linked to sclerotinia stem rot resistance in the latter.

To our knowledge, the DArT has not been used to identify markers linked to sclerotinia stem rot resistance in *Brassica spp.* In this study, we intend to use DArT to identify markers linked to sclerotinia stem rot resistance in a worldwide collection of *B. napus* accessions. The specific objectives of this study were to: (a) analyze the population structure of *B. napus* plant introductions from worldwide collections, (ii) evaluate these plant materials for their reaction to sclerotinia stem rot, (iii) identify DArT markers linked to resistance to *S. sclerotiorum*, and (iv) compare the genome of *A. thaliana, B. oleracea* and *B. rapa* with DArT markers significantly linked to resistance to *S. sclerotiorum* in *B. napus*.

5.3. Materials and Methods

5.3.1. Plant materials

Two hundred and seventy eight *B. napus* plant introductions were used to study the marker-trait association. These materials were collected from at least 21 countries from four continents (Table 5.1). These materials were chosen because they could represent a large spectrum of responses to *S. sclerotiorum* infection, ranging from susceptibility to high levels of resistance (Khot, 2006). This collection also represents a wide range of winter and spring type canola. Two to three seeds of each accession and Westar (susceptible check) were planted in six cone plastic inserts (each cell; 5.7 cm X 5.1 cm X 8.3 cm) with pasteurized Sunshine Mix #1 (Fison Horticulture, Vancouver, B. C.) in greenhouse. Plants were thinned down to a single plant in each insert 10 days after planting. Greenhouse temperatures were maintained at 21 ± 2°C and supplemental light (400 W Lucalox LU400 sodium, General Electric Co., Cleveland, OH) was provided to obtain 16 h day lengths. Plants were fertilized with 3 g/plant of 15-30-15 (Miracle Gro, Scotts, Pot Washington, NY) once in a month.

Table 5.1. Origin of *Brassica napus* plant introductions materials used for the association mapping study.

Country	Plant introductions	Country	Plant introductions
India	1	Nepal	1
Mongolia	1	New Zealand	7
Bangladesh	4	Norway	1
Canada	10	Pakistan	3
China	3	Poland	12
Czech Republic	1	Sweden	9
France	24	Turkey	1
Germany	20	United Kingdom	1
Italy	1	United States	6
Japan	7	Serbia	1
South Korea	164	Total	278

5.3.2. Isolate and inoculum preparation

Well characterized *S. sclerotiorum* isolate WM031, from North Dakota, was used for all inoculations. The isolate WM031 has been widely used in other studies as well. Mycelia of this isolate were produced from a single ascospore culture in half-strength potato dextrose agar (HPDA) medium. At maturity, sclerotia were stored in a refrigerator at 4°C until use. When needed, sclerotia were seeded in Petri dishes containing a 7 mm thick layer of HPDA and incubated for four days at room temperature (21 ± 2°C) with 10 h of light daily. Hyphal tips of these cultures were loaded on 200 µL sterile pipette tips (VWR Scientific Co., San Diego, CA) by cutting the mycelium with the broad base of the tips. Loaded pipettes were taken immediately to the greenhouse to inoculate plants.

5.3.3. Phenotypic evaluation of reaction to *S. sclerotiorum* and data analyses

Seedlings at the 3-4 leaf stage were inoculated using the PIT (del Rio et al., 2001). Briefly, the petiole of the fourth fully-expanded canola leaf on each plant was cut off with a razor blade approximately 2 cm away from the main stem. Then the petiole tip was capped with an inoculum-loaded pipette. Care was taken to ensure the petiole tip was flush with the side of the agar that contained fungal growth. After inoculation, plants were moved to mist chambers for 48 hr to maintain consistent incubation conditions and then returned to a greenhouse room. The experiment was conducted using a randomized complete block design with four replications and three samples per replication. Inoculated plants were incubated in greenhouse rooms at 16 hour light and 22°C daily, and rated for wilting every two days for 21 days after inoculation. The experiment was repeated once. The principle of the area under the disease progress curve (AUDPC) (Shaner and Finney, 1977) was used to calculate an area

under mortality curve (AUMC) for each plant introduction. Analysis of variance was performed using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC) for AUMC after verifying assumptions for normality of data and homogeneity of variances. Two independent experiments were included as a fixed effect in our model for further analysis. Additionally, susceptible check served as a reciprocal confirmation population for identification of associated markers.

5.3.4. DNA extraction and marker analysis

DNA samples were extracted from one day old fully expanded top leaves of 2-4 pooled 15 days old plants per genotype, using a modified sodium dodecyl sulfate (SDS) protocol (Sambrook and Russell, 2001). Leaves were collected from the plants inoculated in the first trial. In brief; samples were collected in 2 ml micro-centrifuge tubes, lyophilized at -80 °C for 48 h and grounded to a fine powder using a high-speed mixer (Model MM301, Retsch Inc., Newtown, PA). DNA extraction buffer (200 mM Tris–HCl, pH 8.0; 1.25 mM NaCl and 0.25 mM EDTA; 0.5% sds 50 µl of b-mercaptoethanol) was prepared. In contrast to phenol-chloroform method, twenty to twenty-five milligram of polyvinyl pyrrolidone (PVP-40) (Sigma-Aldrich, St. Louis, MO), was added to 500 to 600 mg of ground leaf tissues in addition to extraction buffer. Nine hundred and fifty µl of warm (65°C) DNA extraction buffer was added to that mixture in a two ml vial. The contents were mixed well by inversion and gentle vortexing. The mixture were incubated at 65°C for 20 min. Organic compounds from each sample was separated by adding 800 µl of a phenol: chloroform:isoamyl alcohol (25:24:1) solution. The mixture was centrifuged at 13,200 rpm for 15 min and the resulting supernatant transferred to a clean 2 ml vial. This step was repeated. The resulting supernatant

from repeated step was transferred to sterile vial. The DNA was precipitated by adding 3 M sodium acetate (pH 5.2) to the supernatant in the vial at a 1:10 (vol/vol) ratio of supernatant and 2X volume of icy cold absolute ethanol. The mixture was incubated for 5 min at room temperature (21 ± 2°C) and centrifuged for 10 min at 8,000 rpm. The DNA pellet was washed with icecold 75% ethanol, dried and re-suspended in 80 µL of 1 X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA in each sample was quantified using a NanoDrop spectrophotometer (Model ND-1000, Thermo Scientific Inc., Waltham, MA), and the concentration was adjusted to 80 ng/ µL by adding molecular grade water. One hundred µl of DNA from each sample was sent to DArT Pty. Ltd., Australia for genotyping. Genotyping procedure was conducted according to Raman et al. (2011).

5.3.5. DNA marker data analyses

FastPHASE 1.3 (Scheet and Stephens, 2006) was used to impute data for missing genotype values. We have 3.17% missing data for 1272 DNA-based markers. Minor allelic frequency (MAF) was calculated and values < 10% were removed (328 markers has less than 10% MAF). LD was calculated and markers with $r^2 > 0.5$ (632 markers) were discarded from further analysis used to control for population structure (Structure, PCA, Kinship models). The remaining markers (312 markers) which had LD < 0.5 with every other marker, were used to find the number of subpopulations within the genotypes of Plant Introduction (PI) lines by using Structure 2.3.3 software (Pritchard et al., 2000). We estimated the number of subpopulations using two methods: Structure and NPAR1WAY. We tested for 1 to 15 numbers of sub-populations with 5 replicated for each using admixture model. Each run used 100,000 iterations as burn in followed with 500,000 iterations in Monte Carlo Markov chain

replications. Delta k approach by Evanno et al. (2005) was used to determine the optimum number of sub-populations using Structure Harvester (http://taylor0. biology. ucla.edu/struct_harvest). In addition, PROC NPAR1WAY in SAS 9.2 was used to run Wilcoxon two sample tests as described by Rosenberg et al. (2001). For this, we compared the posterior probabilities from all runs of *K* sequentially with the probabilities values of *K*+1. i.e. K=1 vs. K=2; K=2 vs. K=3, and so on. The smallest *K* value in the first non-significant Wilcoxon test was considered as the number of sub-populations (Mamidi et al., 2011). The columns are equal to the number of subpopulations and also give the probability of membership of each individual in a sub-population.

Structure (Q) matrix with one column less than the number of sub populations was used to achieve linear independence in the mixed model. Principal component analysis was conducted to determine the genetic variability among the populations. PROC PRINCOMP was used for PCA analysis and principal components that explain about 25% variability were used as P matrix in our mixed model. Kinship matrix with Loiselle coefficients (Loiselle et al., 1995) was calculated using the SPAGeDi (Spatial Pattern Analysis of Genetic Diversity) software (Hardy and Vakemans, 2002). All negative Kinship values were set to zero as suggested by Yu et al. (2006). A similarity matrix (K_2) was calculated using PROC DISTANCE to estimate the shared percentage of alleles between the two individuals. Nine different mixed models (Table 5.2) were tested to determine the associations between genotypes and phenotypes. These models account for population structure (Q matrix) and PCA matrix and are used as fixed effects and genetic relatedness matrices (kinship or K matrix and similarity matrix K_2) as a random effect as described by Yu et al. (2006). The inferred ancestry output from STRUCTURE that accounts for coarse population structure is Q

Table 5.2. Summary of the statistical models used to test for marker-trait associations.

Model	Statistical model	Information captured
Naive	$y = X\alpha + \varepsilon$	y is related to X, without correction for structure (Q or PCA) or relatedness (K or K*)
K	$y = X\alpha + Kv + \varepsilon$	y is related to X, with correction for K
K*	$y = X\alpha + K^{*}v + \varepsilon$	y is related to X, with correction K*
Q	$y = X\alpha + Q\beta + \varepsilon$	y is related to X, with correction for Q
PCA	$y = X\alpha + P\beta + \varepsilon$	y is related to X , with correction for PCA
Q+K	$y = X\alpha + Q\beta + Kv + \varepsilon$	y is related to X, with correction for Q and K
Q+K*	$y = X\alpha + Q\beta + K^{*}v + \varepsilon$	y is related to X, with correction for Q and K*
PCA+K	$y = X\alpha + P\beta + Kv + \varepsilon$	y is related to X, along with correction for PCA and K
PCA+K*	$y = X\alpha + P\beta + K^{*}v + \varepsilon$	y is related to X, along with correction for PCA and K*

y = Vector of phenotypic values,
β = Vector of fixed effects regarding population structure
α = Fixed effect for the candidate marker
v = A vector of the random effects pertaining to co ancestry and
ε = A vector of residuals. P is a matrix of the significant PCA that explain 25% variability.
Q = A structure matrix with one column less than the number of subpopulations to achieve linear independence.

matrix. X is the vector of genotypes at the candidate marker, and I is an identity matrix. The variances of the random effects are assumed to be : Var(v) = $2KV_g$ and Var(e) = IV_R where K represents the kinship matrix , V_g represents the genetic variance, and V_R the residual variance. The K matrix is a measure of relative kinship and quantifies the probability that two homologous genes are identical by descent. For each model, all marker p-values were ranked from smallest to largest, and the mean square difference (*MSD*) was calculated as:

$$MSD = \frac{\sum_{i=1}^{n}(p_i - \frac{i}{n})^2}{n}$$

where i is the rank number, p_i is the probability of the ith ranked p-value, and n is the number of markers (Stich et al., 2008). The model with the lowest MSD was used to identify the markers associated with sclerotinia stem rot resistance. For the best model p-value for multiple comparisons was corrected using 10,000 permutation test. This will provide a test of significance corresponding to our experiment-wise error. This P-value was used to define the significance level of marker effects on phenotypic traits. The R^2_{LR} values for the significant loci were calculated using the PROC MIXED procedure in SAS 9.2 (Sun et al., 2010).

5.3.6. Blastn analysis

The nucleotide sequences of significant DArT markers (Courtesy: DArT Inc.) associated with *S. sclerotiorum* resistance in *B. napus*, were aligned with Arabidopsis genome sequences using the blastn program in TAIR (WWW.arabidopsis.org, data accessed on November, 2011). The 10 chromosomes from version 1.1 of the *B. rapa* and EST, GSS sequences of *B. oleracea* were accessed through http://brassicadb.org on November, 2011. This was used for homolog identification and finding position on chromosomes using a standalone blast. Functional characterization of genes was also performed in TAIR.

5.4. Results

5.4.1. Phenotypic evaluation of *B. napus* plant introductions

The plant introductions evaluated for Sclerotinia stem rot resistance using the PIT exhibit wide variability in disease ratings. The more susceptible plant introductions and the susceptible check cv. Westar, died within 13 DAI whereas resistant introductions did not. Analysis of variance showed significant effects of genotypes and experiments as well as genotypes by experiment interaction effect ($P < 0.001$). The Plant introductions collected from South Korea, Japan, China, France, Pakistan, Sweden, Poland, Germany showed higher levels of resistance to sclerotinia stem rot than accessions from other countries. AUMC values ranged from 0 to 1675 with an average of 604. The frequency distribution of resistance to sclerotinia stem rot was skewed slightly towards susceptibility (Figure 5.1). Among the 278 accessions evaluated, 7, 7, 29, and 12 plant introductions showed 0-10, 11-20, 21-30 and 31-40 percent mortality, respectively (Figure 5.2). PIs 436556, 458922, 469812, 469873 and 469920 showed consistent resistant reaction to *S. sclerotiorum* in both trials.

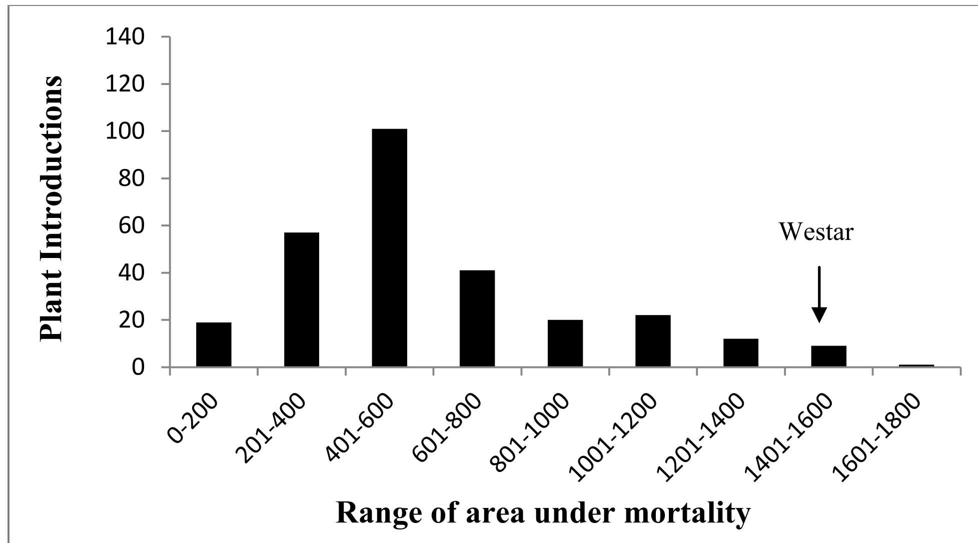

Figure 5.1. Frequency distribution of AUMC of 278 Plant introductions of *B. napus.* AUMC was calculated from two independent experiments.

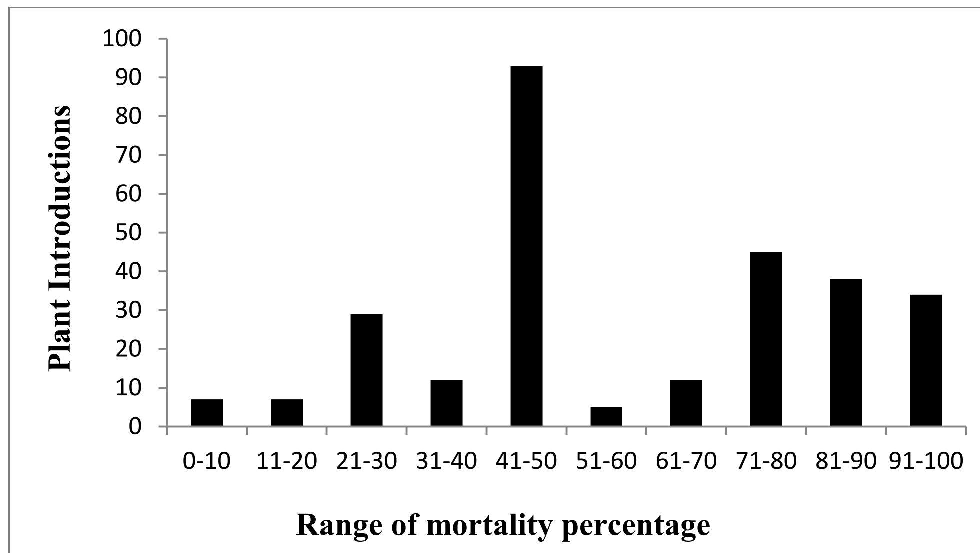

Figure 5.2. Frequency distribution of mortality percentages of 278 Plant introductions of *B. napus.* Mortality percentages was calculated from two independent experiments.

5.4.2. DArT marker analysis

DArT marker information was collected in each population at 3072 informative loci using Diversity Array Technology. Out of the 3072 markers tested 1272 were found polymorphic among the population. Of the 1272 DArT polymorphic marker loci, 944 markers

had a MAF > 10%, and therefore were used for AM analysis. The expected heterozygosity is generally low for DArT markers because of their bi-allelic nature and the self pollinating nature of *B. napus*.

5.4.3. Population structure and genetic diversity

Population structure was estimated with the software program, STRUCTURE, using the linkage based model for the multilocus genotype data (Pritchard et al., 2000) with the subpopulation number selection criteria of Rosenberg et al. (2001), and Δk approach (Evanno et al., 2005). Nine sub populations, based on standard deviations of the logarithm of the probability of data for different *k* tested were detected (Table 5.3 and Figure 5.3). Principal component analysis was implemented to evaluate population structure. Twenty-five percent of the variation was explained by four principal components.

Table 5.3. Averages and standard deviations of the logarithm of the probability of data {LnP(D)} for different *k* tested.

K	Average LnP(D)	Standard deviation
1	-96620.36	0.416
2	-85038.56	1.647
3	-79921.08	1703.169
4	-77094.52	23.479
5	-75332.58	265.970
6	-74001.10	30.265
7	-72556.94	190.330
8	-71413.10	446.997
9	-70004.14	9.151
10	-69437.98	558.950
11	-68610.98	523.100
12	-69085.58	2302.089
13	-66713.14	184.079
14	-66863.30	822.258
15	-65751.58	652.400

5.4.4. Marker-trait associations

Independent marker-trait associations were conducted for 944 markers. Approximately 74% of the 1272 markers evaluated had a MAF > 10% in the populations. The association between genotypic and AUMC data was evaluated using nine different models as described by Zhao et al. (2007); models are listed in Table 5.2. These models are often used in plant association mapping. The ideal model exhibits a uniform distribution when cumulative *p* values are regressed on observed *p*-values. Mean square deviations (MSD) were calculated to observe the degree to which the statistical results for each model deviated from the expected distribution. Models that contained a structure component and shared allele kinship component had the lowest MSD values. These models were chosen to select those significant DArT marker/trait associations.

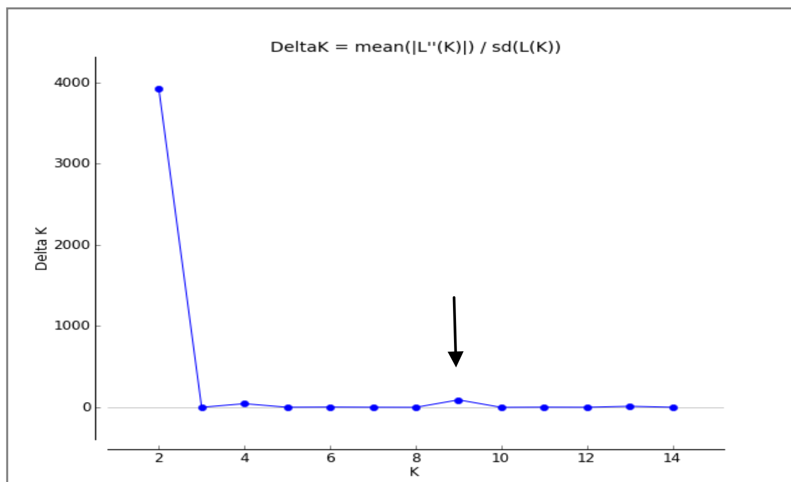

Figure 5.3. A graph to determine the number of subpopulations (Evanno et al., 2005) with DeltaK and number of subpopulations. The peak (indicated by the arrow) represents the appropriate number of subpopulations.

Two hundred and seventy eight independent *B. napus* populations were used to represent different genetic backgrounds from worldwide. These populations included breeding lines of unknown pedigree as well as local varieties and wild accessions collected

from 21 countries. None of the lines contained the same DArT marker haplotypes, thus they were considered to be an independent reciprocal confirmation populations for any significant markers discovered in the other population. Significant markers (*P<0.05*) explained about 1.5 to 4.22% of the phenotypic variation. Whole genome scanning of *B. napus* using 3072 DArT markers in a set of 278 *B. napus* lines identified a total of 32 significant (*P <0.05)* markers for Sclerotinia stem rot resistance (Table 5.4). Among them, three markers showed very high association (*P*<0.001) and eight markers showed high association (*P* < 0.01) with sclerotinia stem rot resistance.

Table 5.4. List of significant DArT markers associated with Sclerotinia stem rot resistance in *B. napus*.

Marker	Chromosome[a]	Genome[b]	raw_*P*	Significance[c]	MAF	R^2_{LR}
brPb-838446		A	0.0005	***	29.9	4.16
brPb-660286		A	0.0005	***	30.2	4.22
brPb-658100	A03		0.0007	***	31.6	4.16
brPb-662889		C	0.0014	**	49.6	3.60
brPb-838519	A02	A, C	0.0014	**	49.6	3.60
brPb-840085	A06	A, C	0.0022	**	13.7	3.22
brPb-663029	A06	A	0.0032	**	18.3	3.005
brPb-670835	A06	A, C	0.0045	**	28.8	3.01
brPb-659694		A, C	0.0052	**	24.5	2.84
brPb-661063	A05	A	0.0058	**	39.2	2.77
brPb-839883		C	0.0087	**	17.0	2.28
brPb-663526			0.0106	*	26.6	2.17
brPb-839131			0.0147	*	23.7	2.31
brPb-661510			0.0147	*	23.7	2.31
brPb-840115		A	0.0147	*	23.7	2.31
brPb-662267			0.0147	*	23.7	2.31
brPb-658137	A06	C	0.0151	*	18.3	2.21
brPb-809259	A06	C	0.0151	*	18.3	2.21

Table 5.4. List of significant DArT markers associated with Sclerotinia stem rot resistance in *B. napus* (Continued)

Marker	Chromosome[a]	Genome[b]	raw_*P*	Significance[c]	MAF	R^2_{LR}
brPb-663449		C	0.0151	*	18.3	2.21
brPb-671217		C	0.0162	*	18.7	2.24
brPb-663342	A09		0.0171	*	38.8	2.28
brPb-661991	A09		0.0171	*	38.8	2.28
brPb-808829			0.0183	*	42.4	2.06
brPb-659904	C08	A, C	0.0205	*	13.7	2.17
brPb-658598		A	0.0211	*	15.5	1.85
brPb-657650		A, C	0.0211	*	15.5	1.85
brPb-838947			0.0219	*	23.4	2.06
brPb-659743	A10	A, C	0.0221	*	35.3	1.82
brPb-840715		A, C	0.0227	*	10.4	1.92
brPb-658734	A09		0.0228	*	27.7	2.06
brPb-663260		C	0.0256	*	10.1	1.82
brPb-841326	A07		0.0264	*	48.9	1.57

[a] Chromosome numbers based on Raman et al. (2011).
[b] Genome determined by blasting marker sequences with a combined dataset of *B. rapa* (A-genome) gene models and *B. oleracea* ESTs and GSS (C-genome). The cutoff criteria used was a lower e-value (E-10) with at least 50% query match to the hit sequence.
[c] Markers are statistically significant at $P < 0.0006$ (99.9% CI, ***); $P < 0.0025$ (99% CI, **); or $P < 0.0267$ (95% CI, *)

5.4.5. Comparative genomics

Based on the sequence query of significant markers from *B. napus* with *A. thaliana, B. rapa*, and *B. oleracea* it was determined that similar regions exist on all four species. Among the 32 queries; 20, 21 and 19 nucleotide sequences were found having corresponding loci distributed on multiple chromosomes in *A. thaliana* (Table 5.5)*, B. rapa* (Table 5.6), and *B. oleracea* (Table 5.6 and Table 5.7), respectively. Some chromosomes, i.e. chromosome 9, contained up to six copies of nucleotide clusters (brPb-658598 in chr 9) (Table 5.5). In total,

Table 5.5. Gene models, genomic locations and estimated copy number of loci detected in *B. rapa* based on sequences of Sclerotinia stem rot resistance DArT markers of *B. napus*.

B. napus	***Brassica rapa***			
DArT Loci[a]	**Gene models[b]**	**Number of copies [c]**	**Chromosome[b]**	**Position (mbp)[b]**
brPb-657650	Bra027547, Bra027549, Bra027552	3	9	13433575, 13412126, 13402332
	Bra033743	1	6	25306542
brPb-658100	Bra029179	1	3	6720925
brPb-658137	Bra020005	1	6	3309173
	Bra031676	1	9	35288654
brPb-658598	Bra006964, Bra027547, Bra027549, Bra027552, Bra027553, Bra027554	6	9	26558382, 13433575, 13412126, 13402332, 13366579, 13381952
brPb-659694	Bra004959	1	5	2659421
	Bra039292	1	4	18731137
brPb-659743	Bra015200	1	10	3603713
brPb-659904	Bra025859, Bra026184	2	6	8506231, 5695778
	Bra026800	1	9	33766174
brPb-660286	Bra029593	1	5	23098128
brPb-661063	Bra005190	1	5	3905536,
	Bra017182	1	4	16167473
brPb-662889	Bra000802	1	3	13230286,
	Bra018503	1	5	8728827
	Bra029511	1	9	17455019
brPb-663029	Bra018935	1	6	1158755
brPb-663449	Bra020005	1	6	3309173
	Bra031676	1	9	35288654
brPb-671217	Bra002677	1	10	8289067
	Bra006786	1	3	5045709
brPb-809259	Bra020005	1	6	3309173
	Bra031676	1	9	35288654
brPb-838446	Bra029593	1	5	23098128
brPb-838519	Bra000802,	1	3	13230286
	Bra018503	1	5	8728827
	Bra029511	1	9	17455019
brPb-840085	Bra024342	1	6	15075864
	Bra028333	1	1	18442389
	Bra031891, Bra031892	2	2	27249648, 27246329
brPb-840115	Bra009639	1	10	17549157
	Bra028804	1	2	1967721
brPb-662889	Bra018503	1	5	8728827
	Bra000802	1	3	13230286

Table 5.5. Gene models, genomic locations and estimated copy number of loci detected in *B. rapa* based on sequences of Sclerotinia stem rot resistance DArT markers of *B. napus* (Continued)

B. napus	***Brassica rapa***			
DArT Loci[a]	**Gene models[b]**	**Number of copies [c]**	**Chromosome[b]**	**Position (mbp)[b]**
	Bra029511	1	9	17455019
brPb-840715	Bra028804	1	2	1967721

[a] Sclerotinia stem rot resistance associated significant DArT Markers of *Brassica napus*.
[b] Gene model, chromosome number and position in *Brassica rapa* as determined in www.brassicadb.org.
[c] Estimated from the number of repetition of homology sequences of corresponding DArT marker of *B. napus* in *B. rapa*.

Table 5.6. Gene symbols, genomic locations and homologous DArT loci detected in *A. thaliana* based on sequences of Sclerotinia stem rot resistance DArT markers of *B. napus.*

DArT Loci[a]	**Gene symbol[b]**	**Arabidopsis hit[b]**	**Chromosome[b]**	**Position[b]**	**E-value[b]**
brPb-657650	K9L2.19, K9L2_19	AT5G44390.1	5	17883431	9.00E-51
		AT5G44390.1	5	17883431	2.00E-10
brPb-658100	AT5G51240, EMF2, ATEMF2, CYR.1 MWD22.18, VEF2	AT5G51230.3	5		2.00E-06
brPb-658137	F21M12.3, F21M12_3	AT1G09640.1	1	3121506	5.00E-101
		AT1G09640.1	1	21379275	1.00E-57
		AT1G57720.2	1	3121506	3.00E-19
brPb-658598	K9L2.19, K9L2_19	AT5G44390.1	5	17882732	0
		AT5G44390.1	5	17879280	1.00E-104
		AT5G44390.1	5	17886765	1.00E-103
		AT5G44390.1	5	17882732	3.00E-79
brPb-659694	ATMPK12, MAPK12, MPK12, T3F17.28	AT2G46070.1	2	18946581	2.00E-62
		AT2G46070.2	2	18946581	1.00E-24
brPb-659904	ATMAP70-4, F10B6.24, F10B6_24, MAP70-4	AT1G14840.1	1	5113619	2.00E-63
		AT1G14840.1	1	5113619	4.00E-13

Table 5.6. Table 5.6. Gene symbols, genomic locations and homologous DArT loci detected in *A. thaliana* based on sequences of Sclerotinia stem rot resistance DArT markers of *B. napus* (Continued)

DArT Loci[a]	**Gene symbol**[b]	**Arabidopsis hit**[b]	**Chromosome**[b]	**Position**[b]	**E-value**[b]
brPb-660286	F5E6.5, F5E6_5	AT3G06620.1	3		1.00E-12
brPb-661063	F3G5.29, F3G5_29	AT2G37500.1	2	15742707	4.00E-83
		AT2G37500.1	2	15742707	4.00E-34
brPb-662889	ATPC1, T19J18.4, T19J18_4	AT4G04640.1	4	2351649	5.00E-53
brPb-663029		AT1G51860.1	1		1.00E-15
brPb-663342		AT3G58930.1	3		7.00E-48
brPb-663449	F21M12.3, F21M12_3	AT1G09640.1	1	3121506	1.00E-96
		AT1G09640.1	1	21379275	1.00E-57
		AT1G57720.2	1	3121506	1.00E-17
brPb-671217	ATFIP1[V], ATFIPS5, FIP1[V], FIPS5, K21L19.3	AT5G58040.1	5	23489602	1.00E-27
brPb-809259	F21M12.3, F21M12_3	AT1G09640.1	1	3121888	3.00E-99
		AT1G09640.1	1	21379431	2.00E-55
		AT1G57720.2	1	3121888	1.00E-19
brPb-838446	F5E6.5, F5E6_5	AT3G06620.1	3		1.00E-12
brPb-838519	ATPC1, T19J18.4, T19J18_4	AT4G04640.1	4	2351649	4.00E-48
		AT4G04640.1	4	2351649	6.00E-08
brPb-839883		AT1G20960.2	1		1.00E-14
brPb-840085	CDKC2, MXK3.19 MXK3_19	AT5G64960.2	5		3.00E-14
brPb-840115	APRATAXIN-LIKE, APTX, T10O8.20	AT5G01310.1	5		6.00E-10
brPb-840715			5	1104525	7.00E-25
brPb-841326			1	8336641	8.00E-29

[a] Sclerotinia stem rot resistance associated significant DArT Markers of *Brassica napus*.
[b] Gene model, chromosome number and position in *Arabidopsis thaliana* as determined in www.arabidopsis.org.

Table 5.7. Homologous loci in *B. oleracea* and their E-value based of DArT loci of *B. napus.*

DArT Loci	Homologous Loci[b]	E-Value[b]
brPb-657650	gi|224904748|gb|FI746087.1|FI746087	1.00E-63
brPb-657650	gi|224913609|gb|FI753933.1|FI753933	9.00E-31
brPb-658100	gi|23417201|gb|BH937135.1|BH937135	2.00E-72
brPb-658137	gi|224868367|gb|FI709797.1|FI709797	3.00E-113
brPb-658137	gi|18829241|gb|BH724082.1|BH724082	3.00E-113
brPb-658137	gi|224882715|gb|FI723819.1|FI723819	2.00E-36
brPb-658137	gi|17802709|gb|BH550929.1|BH550929	2.00E-30
brPb-658598	gi|224859904|gb|FI698713.1|FI698713	4.00E-114
brPb-658598	gi|224904748|gb|FI746087.1|FI746087	1.00E-44
brPb-659694	gi|23397928|gb|BH919860.1|BH919860	1.00E-64
brPb-659743	gi|224900744|gb|FI741809.1|FI741809	8.00E-60
brPb-659743	gi|224866761|gb|FI708228.1|FI708228	5.00E-37
brPb-659904	gi|17616272|gb|BH430551.1|BH430551	2.00E-79
brPb-659904	gi|26785816|gb|BZ483418.1|BZ483418	5.00E-60
brPb-659904	gi|18737836|gb|BH672167.1|BH672167	3.00E-32
brPb-662889	gi|224852399|gb|FI692730.1|FI692730	5.00E-98
brPb-662889	gi|224854131|gb|FI697838.1|FI697838	2.00E-96
brPb-662889	gi|224905262|gb|FI747077.1|FI747077	9.00E-06
brPb-662889	gi|224840506|gb|FI681176.1|FI681176	9.00E-06
brPb-663260	gi|17616419|gb|BH430698.1|BH430698	1.00E-75
brPb-663260	gi|17606070|gb|BH420342.1|BH420342	2.00E-52
brPb-663449	gi|224868367|gb|FI709797.1|FI709797	8.00E-109
brPb-663449	gi|18829241|gb|BH724082.1|BH724082	8.00E-109
brPb-663449	gi|224882715|gb|FI723819.1|FI723819	2.00E-36
brPb-663449	gi|17802709|gb|BH550929.1|BH550929	2.00E-30
brPb-670835	gi|33815029|gb|CC964570.1|CC964570	5.00E-58
brPb-671217	gi|17716125|gb|BH508028.1|BH508028	3.00E-113
brPb-671217	gi|18721532|gb|BH662660.1|BH662660	3.00E-49
brPb-671217	gi|27028084|gb|BZ506746.1|BZ506746	3.00E-24
brPb-809259	gi|18829241|gb|BH724082.1|BH724082	2.00E-111
brPb-809259	gi|224868367|gb|FI709797.1|FI709797	6.00E-111
brPb-809259	gi|224882715|gb|FI723819.1|FI723819	3.00E-35
brPb-809259	gi|17802709|gb|BH550929.1|BH550929	4.00E-28
brPb-838519	gi|224852399|gb|FI692730.1|FI692730	2.00E-96
brPb-838519	gi|224854131|gb|FI697838.1|FI697838	9.00E-95
brPb-839883	gi|224905645|gb|FI747495.1|FI747495	5.00E-120
brPb-839883	gi|224853323|gb|FI694881.1|FI694881	5.00E-120
brPb-839883	gi|27045734|gb|BZ516324.1|BZ516324	1.00E-12
brPb-839883	gi|26697168|gb|BZ440461.1|BZ440461	1.00E-12
brPb-840085	gi|17073827|gb|BH250251.1|BH250251	3.00E-114
brPb-840085	gi|224845737|gb|FI688548.1|FI688548	7.00E-56
brPb-840085	gi|17746724|gb|BH530323.1|BH530323	2.00E-32
brPb-840085	gi|18829378|gb|BH724144.1|BH724144	6.00E-32
brPb-840085	gi|23443177|gb|BH961951.1|BH961951	2.00E-16
brPb-840085	gi|224853316|gb|FI694506.1|FI694506	5.00E-08
brPb-840715	gi|17620207|gb|BH434486.1|BH434486	2.00E-69

Table 5.7. Homologous loci in *B. oleracea* and their E-value based of DArT loci of *B. napus* (Continued)

DArT Loci	Homologous Loci[b]	E-Value[b]
brPb-840715	gi|224887575|gb|FI727842.1|FI727842	9.00E-24
brPb-840715	gi|224874402|gb|FI716972.1|FI716972	9.00E-24
brPb-841326	gi|23631247|gb|BZ041300.1|BZ041300	3.00E-08
brPb-658137	gi|95860552|gb|DY029014.1|DY029014	4.00E-78
brPb-658137	gi|150919404|gb|ES949867.1|ES949867	8.00E-51
brPb-658137	gi|150916939|gb|ES947400.1|ES947400	2.00E-42
brPb-659904	gi|150911305|gb|ES941763.1|ES941763	1.00E-27
brPb-661063	gi|95856354|gb|DY026903.1|DY026903	8.00E-32
brPb-661063	gi|150130640|gb|EE531612.1|EE531612	5.00E-29
brPb-663449	gi|95860552|gb|DY029014.1|DY029014	4.00E-78
brPb-841326	gi|150917251|gb|ES947712.1|ES947712	5.00E-27
brPb-841326	gi|150912065|gb|ES942523.1|ES942523	5.00E-27

[b] Homologous loci and their E-values in *B. oleracea* as determined in www.arabidopsis.org.

resistance nucleotide clusters are distributed and repeated on 38 location of all chromosomes of *A. thaliana*, and 37 locations of eight chromosomes of *B. rapa* (Table 5.5). These results support the relationship and evolution concepts of species of cruciferae family i.e. same ancestors and genome from *A. thaliana* are distributed over the different chromosomes of *B. napus, B. rapa,* and *B. oleracea* by either replication duplication or so on. *A. thaliana* has the highest and lowest resistance nucleotide clusters on chromosomes 1 (14 loci) and 3 (3 loci), respectively, while *B. rapa* genome has them in chromosomes 9 (9 loci) and 1 (1 locus), respectively. Using blast search of *B. napus* DArT loci, 47.6 % (10/21) composed a single homologous region ($E > 10^{-5}$) in the *A. thaliana*, while the other loci represented >2 loci i.e. 28.5% composed double, 9.5% triple and 14.4% quadruple (Table 5.6). Similarly, 30% (6/20), 35% (7/20), 20% (4/20), 10% (2/20) and 5% (1/20) composed a single, double, triple, quadruple and sextuple homologous region in *B. rapa* (Table 5.5). Based on this result, it seems like five chromosomes of *A. thaliana* were dispersed in various regions in *B. rapa* and *B. napus* genome. This results supports the relationship and evolution of species of cruciferae

family are from same ancestors and genome from *A. thaliana* are distributed over the different chromosomes of *B. napus, B. rapa* and *B. oleracea* by either replication duplication or so on. Three highly significant markers (Table 5.4) i.e. brPb-838446, brPb-660286, and brPb-658100 were observed on chromosomes 5, 5 and 3, respectively on *B. rapa* (Table 5.5) and on chromosomes 3, 3 and 5 respectively on *A. thaliana* (Table 5.6).

5.5. Discussion

A total of 278 plant introductions of *B. napus* collected from 21 different countries were evaluated for sclerotinia stem rot resistance and DArT markers were used for whole genome scanning of this population to identify markers linked to the disease resistance with association mapping approach. To our knowledge, this is the first whole genome association analysis in *B. napus* to identify the DArT markers linked to sclerotinia stem rot resistance. The plant introductions showed wide range of Sclerotinia stem rot resistance and genotypes collected from South Korea, Japan, China, France, Pakistan, Sweden, Poland, Germany showed higher percentage of sclerotinia stem rot resistance than the PI from other countries. As expected, variability in disease rating was observed between two independent experiments. The frequency curve of AUMC and percentage mortality curve of PI for first experiment was skewed towards resistance while second experiment showed relatively normal distribution. The significant interaction between PI and experiments suggesting that disease resistance is influenced by environments. This further substantiates that both genetic and environmental factors influence the sclerotinia stem rot response in *B. napus*. The PI with high percentage of dead plant had high AUMC values indicating that both percent mortality and area under

mortality curve can be used to quantify the disease severity in phenotypic evaluation. Some of the susceptible PI, which has high mortality percentage, had relatively low AUMC value because of delay disease progress i.e. slow molding. In wheat, slow rusting cultivars were used for durable resistance (Skovmand et al., 1978). The resistance factors associated with slow molding in *B. napus* could be also useful in canola breeding program. The highest AUMC value was higher than the susceptible check and relatively lower AUMC value was seen on some PIs.

Several previous studies reported that sclerotinia stem rot resistance in *B. napus* is polygenic or quantitative in nature and strongly influenced by environment. Quantitative trait loci for sclerotinia stem rot resistance have been identified in Chinese source of resistance (Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2006). QTL linked to sclerotinia stem rot resistance has been also reported to link with other agronomic traits such as plant growth stage or stem morphology, and these traits are correlated with sclerotinia stem rot severity (Yin et al., 2010; *del Rio personal communication*). Although complete durability may not be possible in most of plant diseases, identification of QTL can be used in resistant breeding program to reduce the sclerotinia stem rot pressure in field. Polygenic or quantitative resistance is more durable than qualitative resistance because the pathogen overcomes it more slowly than a single major resistance gene (Boyd, 2006; Lindhout, 2002).

Use of association mapping or analysis has been increasing in crop science and could be very effective tool in disease resistance breeding program in addition with biparental mapping approach. Association mapping could be useful to identify multiple QTLs for

diverse trait because of diversified plant materials are used whereas in biparental population is normally developed targeting for few specific traits. Moreover, association mapping provide higher resolution map than biparental mapping. Tomassini et al. (2007) reported that 390 fold higher marker resolutions can be achieved by AM of the *Stagonospora nodorum* blotch (SNB) resistance gene (*QSng.sfr-3BS*) in winter wheat using 44 varieties compared to QTL mapping using 240 RILs. AM uses the linkage disequilibrium (LD) pattern in a large population of unrelated individuals (Risch, 2000) and it can identify common genetic variants that control a common phenotype. Population structure can result in spurious correlation, leading to an elevated false-positive rate for marker-trait association (Marchini et al., 2004 and Zhao et al., 2007). Different statistical methods (Devlin and Roeder, 1999; Price et al., 2006; Pritchard et al., 2000; Yu et al., 2006) that account for population structure and/or family relatedness can be used to reduce the false positives and thus increase the power of AM (Zhao et al., 2007). In this study, we used different AM models to account population structure and family relatedness (Q+K) using both STRUCTURE (Pritchard et al., 2000) and principal component analysis (Price et al., 2006). The diversified *B. napus* population used in this study showed nine sub-populations by STRUCTURE suggesting that the population might have diversified sources of resistance to sclerotinia stem rot. High numbers of subpopulations were identified in our study because the population consists of plant introductions from 21 different countries with diverse genetic backgrounds and admixed ancestry or pedigree. The PIs in this study are predominantly spring type of *B. napus* including varieties and breeding lines from worldwide collection. Admixture observed in this study might have impacted on overestimation of number of *k* groups in our study. Therefore,

the knowledge of PIs pedigrees could help to refine and/or to validate the population structure. Jestin et al. (2010) also identified nine sub-populations in *B. napus* population, which has predominantly winter oilseed rape varieties/line. The models used in identification of DArT marker and sclerotinia stem rot resistance association uses either sub-population from structure, or result from PCA with kinship matrixes. The use of sub-population information in model also helped to decrease the number of false positives.

Since Sclerotinia stem rot disease of *B. napus* is complex in nature and resistance is governed by multiple factors, AM seems to be an appropriate tool to identify molecular markers linked to them. Indeed, previous mapping study in *B. napus* using a limited number of SSR or AFLP markers identified few markers linked to sclerotinia stem rot resistance (Yin et al., 2010; Zhao et al., 2003a; Zhao et al., 2003b; Zhao et al., 2006). The sequences for those markers are not publicly available and, therefore difficult to validate these markers in new mapping population. DArT has developed markers for many crops (Jaccoud et al., 2001; Wenzl et al., 2004) and has been widely used to map QTLs in several crops. Recently, DArT has developed markers for *B. napus* (Raman et al., 2011). This technology does not require sequence information and can be deployed on a microarray platform. DArT uses an array of individualized clones from a genomic representation prepared from amplified restriction fragments. Labeled genomic representations of individuals to be genotyped, such as the progeny of a segregating population, are then hybridized to the arrays. The polymorphisms scored are the presence versus absence of hybridization to individual array elements. The platform allows high-throughput screening of hundreds of molecular markers in parallel, and is especially suited for the generation of genome-wide markers for e.g. genetic linkage

mapping. The initial proof-of-concept was provided for a species with a relatively simple genome i.e. rice (Jaccoud et al., 2001), and later for a species with a more complex genome, i.e. barley (Wenzl et al., 2004).

In this study, a total of 32 significant DArT markers linked to Sclerotinia stem rot resistance were identified from a genome-wide association analysis from using the 3072 DArT markers set (www.diversityarrays.com). Among them, three DArT markers were significant at $P<0.001$ and eight markers were significant at P value <0.01. DArT markers have been used to identify diverse traits including disease resistance in many crops including model plant such as barley (Steffenson et al., 2007), wheat (Tomassini et al., 2007), *A. thaliana* (Atwell et al., 2010). DArT markers have been recently developed for *B. napus* and construction of consensus map is available to public. The DArT markers were developed using several different populations and found very informative and polymorphic (Raman et al., 2011). In our population, 41.4 % of total DArT markers were polymorphic. The percentage of polymorphic markers revealed that our collection was diverse in genotype. Furthermore, 2.5 % of polymorphic markers were linked to sclerotinia stem rot resistance in *B. napus*.

The r^2 value of the individual markers (phenotypic variability explained by each marker) identified in this study were relatively low compared to that obtained when using biparental populations (Table 5.4). The low r^2 values of these markers may be an indication that the contribution of the genes to which they are associated may be small, however, if one considers that resistance to *S. sclerotiorum* is epistatic Zhao and Meng, 2003b), the large number of markers identified in this study may provide an important contribution. Besides

this, however, there may be several other reasons for this. One of them is the diversified mix of genotypes used in this study. These accessions constitute a relatively large and diverse population compared to what has been normally used in conventional studies in which QTL in *B. napus* were associated with sclerotinia stem rot resistance (Yin et al., 2010; Zhao and Meng, 2003a; Zhao and Meng, 2003b; Zhao et al., 2006). Lower marker densities in any chromosomal region could also contribute to production of low r^2 values. Moreover, the resistance allele present at more than one locus in any one PI could affect the other loci. Nevertheless, further studies are needed to identify the chromosome localization and verify the true contribution of the markers identified in this study.

Multiple genetic factors are involved in *S. sclerotiorum*- *B. napus* interaction; for example, the expression of cell wall degrading enzymes from *B. napus* is necessary for the competition and restricts the distribution of fungus through the plant (Sharan and Mehta, 2008). Several studies have been conducted to understand the genetic nature of *S. sclerotiorum*- *B. napus* interaction whereas several QTL has been identified on multiple *B. napus* chromosomes (Yin et al., 2010; Zhao et al., 2003a; Zhao et al., 2003b; Zhao et al., 2006). Glucosinolate content at the seedling stage has been associated with *B. napus* resistance to sclerotinia stem rot (Zhao and Meng, 2003a). Of the nine QTL identified for total glucosinolate content in *B. napus*, one was found to be associated with Sclerotinia resistance on the leaf at the seedling stage, and one locus, responsible for 3-indolyl-methyl glucosinolate content, was thought to be linked to Sclerotinia resistance on the stem of the maturing plant. QTL linked to sclerotinia stem rot resistance have been identified using biparental mapping

approach. Yin et al., (2010) identified few (1-10) QTLs associated with Sclerotinia stem rot resistance in double haploid *B. napus* in seedling stage.

Zhao and Meng (2003b) reported three QTL for resistance at the seedling stage and three different QTL for stem resistance at the adult plant stage in an $F_{2:3}$ populations. Zhao et al. (2006) detected additional QTL linked to sclerotinia stem rot in two segregating populations of doubled haploid (DH) lines, the line RV289 from HUA population and the Major x Stellar (MS) population. A total of eight genomic regions affecting resistance were detected in the HUA population, with four of these regions affecting both measures of resistance. Only one region affecting both measurements was detected in the MS population. Individual QTL explained 6–22% of the variance. The number of markers linked to Sclerotinia stem rot resistance is comparatively lower in biparental studies compared to our association study. Thus, AM in world-wide collection of plant introduction could be capable of identifying more (both new and previously described) regions associated with Sclerotinia stem rot resistance. According to linkage map of *B. napus* (Raman et al., 2011) significant markers from this study were found on eight (A02, A03, A05, A06, A07, A09, A19 and C08) chromosomes although most of them were detected in A 05 and A 09 chromosomes of *B. napus*. The comparative analysis between significant sclerotinia stem rot resistance markers detected in this study and genome of *B. rapa* suggested that these markers were also present on eight chromosomes of *B. rapa* (Table 5.5). Previous studies have reported the presence of markers associated with sclerotinia stem rot resistance in chromosomes A1, A2, A3, A4, A5, A6, A7, and A10 (Yin et al., 2010; Zhao et al., 2003a; Zhao et al., 2003b; Zhao et al., 2006), our study detected markers in chromosome A9 as well. Similarly, homologous sequences of

significant markers were also observed on *B. oleracea* genome (Table 5.8). This result suggests that C genomes might have loci that are associated with *S. sclerotiorum* resistance in *B. napus*.

Our results showed that sequences of significant DArT markers are scattered throughout the five chromosome of *A. thaliana* (38 locations) or 10 chromosomes of *B. rapa* (37 locations). Sillito et al., (2000) also reported that R-ESTs are mapped on all 5 chromosomes of *A. thaliana* ranging from 5 in Chr2 to 17 in Chr1. Our results also suggest that Chr1 has the highest (14 loci) and chr3 has the lowest (3 loci) loci associated with sclerotinia stem rot resistance homology sequences. Results from Suwabe et al. (2006) showed that few genomic segments of *A. thaliana* are scattered throughout the genome of *B. rapa* and two of the three identified QTLs for clubroot resistance are mapped in Chr5 which belongs to disease-resistance clusters of genome of *A. thaliana.* Similarly, among the three most significant DArT markers, we observed one marker in Chr5 and two markers in Chr3 of *A. thaliana*.

Results from the present study and from literature review (Yin et al., 2010; Zhao and Meng 2003a; Zhao and Meng 2003b; Zhao et al., 2006) indicated both biparental and association approach are suitable tools for mapping of QTL with strong effects on sclerotinia stem rot resistance in *B. napus*. The conventional QTL study had reported QTL associated with sclerotinia stem rot are widely present in both A and C genome (i.e. in A1, A2, A3, A4, A5, A6, A7, A10, C1, C2, C4, C5, C6, C7 and C9 chromosomes). Each biparental approach study discovered some different QTLs i.e. different resistance germplasm might contain different QTL for sclerotinia stem rot resistance. Therefore, it is wise to study all resistance

sources at the same time, a feat that is possible only through association mapping approach. For future research related to QTL identification and mapping might greatly facilitate by the recent study. With this findings, the use of elite PIs in AM offers the advantage that results can be easily used in future breeding programs and marker assisted selection.

5.6. References Cited

Agrama, H. A., Eizenga G. C., and Yan. W. 2007. Association mapping of yield and its components in rice cultivars. Mol. Breed. 19:341–356.

Ananga, A. O., Cebert, E., Soliman, K., Kantety, R., Pacumbaba, R. P., and Konan, K. 2006. RAPD markers associated with resistance to blackleg disease in *Brassica species*. Afr. J. Biotechnol. 5:2041-2048.

Aranzana, M. J., Kim, S., Zhao, K. Y., Bakker, E., Horton, M., Jakob, K., Lister, C., Molitor, J., Shindo, C., Tang, C. L., Toomajian, C., Traw, B., Zheng, H. G., Bergelson, J., Dean, C., Marjoram, P., and Nordborg, M. 2005. Genome-wide association mapping in Arabidopsis identifies previously known flowering time and pathogen resistance genes. PLOS Genet. 1:531–539.

Atwell, S., Huang, Y. S., Vilhjalmsson, B. J., Willems, G., Horton, M., Li, Y., Meng, D., Platt, A. A. M., Tarone, A., Hu, T. T., Jiang, R., Muliyati, N. W., Zhang, X., Amer, M. A., Baxter, I., Brachi, B., Chory, J., Dean, C., Debieu, M., de Meaux, J., Ecker, J. R., Faure, N., Kniskern, J. M., Jones, J. D., Michael, T., Nemri, A., Roux, F., Salt, D. E., Tang, C., Todesco, M., Traw, M. B., Weigel, D., Marjoram, P., Borevitz, J. O., Bergelson, J., Nordborg, M. 2010. Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. Nature 465:627-631.

Babula, D., Kaczmarek, M., Barakat, A., Delseny, M., Quiros, C. F., and Sadowski, J. 2003. Chromosomal mapping of *Brassica oleracea* based on ESTs from *Arabidopsis thaliana*: complexity of the comparative map. Mol. Genet. Genomics 268:656–665.

Boyd, L. A. 2006. Can the durability of resistance be predicted? J. Sci. Food Agric. 86:2523–2526.

Bradley, C. A., Henson, R. A., LeGare, D. G., del Rio, L. E., and Khot, S. D. 2006. Response of canola cultivars to *Sclerotinia sclerotiorum* in controlled and field environments. Plant Dis. 90:215-219.

Breseghello, F., and Sorrells, M. E. 2006. Association analysis as a strategy for improvement of quantitative traits in plants. Crop Sci. 46:1323–1330.

del Rio, L. E., Bradley, C. A., Henson, R. A., Endres, G. J., Hanson, B. K., McKay, K., Halvoson, M., Porter, P. M., LeGare, D. G., and Lamey, H. A. 2007. Impact of Sclerotinia stem rot on yield of canola. Plant Dis. 91:191-194.

del Rio, L. E., Kurtzweil, N. C., and Grau, C. R. 2001. Petiole inoculation as a tool to screen soybean germplasm for resistance to *Sclerotinia sclerotiorum.* Phytopathology 91:S176.

Devlin, B., and Roeder, K. 1999. Genomic control for association studies. Biometrics 55:997–1004.

Ersoz, E., S., Yu, J., and Buckler, E. S. 2008. Application of Linkage disequilibrium and association mapping in crop plants. p 97-120. *In* R. Varshney and R. Tuberosa (ed.) Genomic assisted crop improvement: Vol. I: Genomics approaches and platforms. Springer Verlag, Germany.

Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecology 14:2611-2620.

Flint-Garcia, S. A, Thornsberry, J. M., and Buckler, E. S. 2003. Structure of linkage disequilibrium in plants. Annu. Rev. Plant Biol. 54:357–374

Hardy, Q. J., and Vekemans, X. 2002. SPAGeDi: a versatile computer program to analyze spatial genetic structure at the individual or population levels. Mol. Eco. Res. 2:618-620.

Hasan, M., Friedt, W., Pons-Kuehnemann, J., Freitag, N. M., Link, K., and Snowdon, R. J. 2008. Association of gene-linked SSR markers to seed glucosinolate content in oilseed rape (*Brassica napus* ssp. *napus*). Theor. Appl. Genet. 116:1035–1049.

Honsdorf N., Becker, H. C, and Ecke, W. 2010. Association mapping for phenological, morphological, and quality traits in canola quality winter rapeseed (*Brassica napus* L.). Genome 53:899-907.

Jaccoud, D., Peng, K., Feinstein, D., and Kilian, A. 2001. Diversity Arrays: a solid state technology for sequence information independent genotyping. Nucleic Acids Res. 29:e25.

Jestin, C., Lode, M., Vallee, P., Domin, C., Falentin, C., Horvais, R., Coedel, S., Manzanares-Daulex, M. J., and Delourme, R. 2010. Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Mol. Breed. 27:271–287

Khot, S. 2006. Evaluation of *Brassica napus* accessions for resistance to *Sclerotinia sclerotiorum*. MS Thesis, NDSU, USA.

Kowalski, S. D., Lan, T. H., Feldmann, K. A., and Paterson, A. H. 1994. Comparative mapping of *Arabidopsis thaliana* and *Brassica oleracea* chromosome reveals islands of conserved gene order. Genetics 138:499-510.

Lagercrantz, U., and Lydiate, D. 1996. Comparative genome mapping in Brassica. Genetics 144:1903-1910.

Lamey, A. and Bradley, C.A. 2003. *Sclerotinia* stem rot of canola biology and management. N. D. State Univ. Ext. Rep. No. PP-1201.

Lan, T. H., Delmonte, T. A., Reischmann, K. P., Hyman, J., Kowalski, S. P., McFerson, J., Kresovich, S., and Paterson, A. H. 2000. An EST-enriched comparative map of *Brassica oleracea* and *Arabidopsis thaliana.* Genome Res. 10**:**776–788.

Lindhout, P. 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. Euphytica 124:217–226.

Loiselle, B. A., Sork, V. L., Nason, J., and Graham, C. 1995. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). Am. J. Bot. 82:1420-1425.

Lukens, L., Zou, F., Parkin, I., Lydiate, D., and Osborn, T. 2003. Comparison of the *Brassica oleracea* genetic map with the *Arabidopsis thaliana* physical map. Genetics 164**:**359–372.

Mamidi, S., Chikara, S., Goos, R. J., Hyten, D. L ., Annam, D., Moghaddam, S. M., Lee, R. K., Cregan, P. B., and McClean, P. E. 2011. Genome-wide association analysis identifies candidate genes associated with iron deficiency chlorosis in soybean. The Plant Genome 4:1-11.

Marchini, J., Cardon, L. R., Phillips, M. S., and Donnelly, P. 2004. The effects of human population structure on large genetic association studies. Nat. Genet. 36:512–517.

Massman, J., Cooper, B., Horsley, R., Schwarz, P., Dong, Y., Neate, S., Dill-Macky, R., Franckowiak, J., Muehlbauer, G., Chao, S., and Smith, K. 2010. Association mapping of Fusarium head blight resistance QTL in contemporary barley breeding germplasm. Mol. Breed. 27:439-454.

Mei J., Qian, L., Disi, J. O., Yang, X., Li, Q., Li, J., Frauen, M., Cai, D., and Qian, W. 2011. Identification of resistant sources against *Sclerotinia sclerotiorum* in *Brassica* species with emphasis on *B. oleracea*. Euphytica 177:393-399.

Parkin, I. A. P., and Lydiate, D. J. 1997. Conserved patterns of chromosome pairing and recombination in *Brassica napus* crosses. Genome 40:496-504.

Parkin, I. A. P., Gulden, S. M., Sharpe, A. G., Lukens, L., Trick, M., Osborn, T. C., and Lydiate, D. J. 2005. Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana.* Genetics 171:765-781.

Parkin, I. A., Sharpe, A. G., and Lydiate, D. J. 2003. Patterns of genome duplication within the *Brassica napus* genome. Genome 46**:**291–303.

Price, A. L., Patterson, N. J., Plenge, R. M., Weinblatt, M. E., and Shadick, N. A. 2006. Principal components analysis corrects for stratification in genome-wide association studies. Nat. Genet. 38:904–909.

Pritchard J. K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Raman, H., Raman, R., Nelson, M. N., Aslam, M. N., Rajasekaran, R., Wratten, N., Cowling, W. A., Kilian, A., Sharpe, A. G., and Schondelmaier, J. 2011. Diversity array technology markers: genetic diversity analyses and linkage map construction in rapeseed (*Brassica napus* L.). DNA research pp:1-15. doi:10.1093/dnares/dsr041

Risch, N. J. 2000. Searching for genetic determination in the new millennium. Nature 405:847-855.

Rosenberg, N. A., Burke, T., Elo, K., Feldman, M. W., Freidlin, P. J., Groenen, M. A. M., Hillel, J., Maki-Tanila, A., Tixer-Boichard, M., Vignal, L., Wimmers, K., and Weigend, S. 2001. Empirical evaluations of genetic clustering methods using multilocus genotypes from 20 chicken breeds. Genetics 159:699-713.

Sabharwal, V., Negi, M. S., Banga, S. S., and Lakshmikumaran, M. 2004. Mapping of AFLP markers linked to seed coat color loci in *Brassica juncea* (L.) Czern. Theor. Appl. Genet. 109:160-166.

Saharan, G. S. and Mehta, N. 2008. *Sclerotinia* diseases of crop plants: biology, ecology and disease management. Springer, Dordrecht, UK.

SAS Institute. 2002. SAS/STAT user's guide. Version 9.1.SAS Institute Inc. Cary, NC.

Sambrook, J. and Russell, D. 2001. Molecular Cloning: A Laboratory Manual, vol. I. 2ndedition. Cold Spring Harbor Laboratory Press, NY, USA.

Schmidt, R., Acarkan, A., and Boivin, K. 2001. Comparative structural genomics in the Brassicaceae family. Plant Physiol. Bio-chem.39:253-262.

Scheet, P., and Stephens, M. 2006. A fast and flexible statistical model for large-scale Population genotype data: Applications to inferring missing genotypes and haplotypic phase. Am. J. Hum. Genet.78:629–644.

Shaner, E. and Finey, R. E. 1977. The effect of nitrogen fertilization on the expression of slow mildewing in knox wheat. Phytopathology 67:1051-1056.

Sillito, D., Parkin, I. A., Mayerhofer, R., Lydiate, D. J., and Good, A. G. 2000 *Arabidopsis thaliana*: a source of candidate disease resistance genes for *Brassica napus*. Genome 43:452–460.

Skovmand, B., Wilcoxson, R. D., Shearer, B. L., and Strucker, R. E. 1978. Inheritance of slow rusting to stem rust in wheat. Euphytica 27:95-107.

Snowdon, R. J., and Friedt, W. 2004. Molecular markers in Brassica oilseed breeding: current status and future possibilities. Plant Breed. 123:1–8.

Steffenson, B., J., Olivera, P., Roy, J., K., Jin, Y., Smith, K. P., and Muehlbauer, G. J. 2007. A walk on the wild side: mining wild wheat and barley collections for rust resistance genes. Aust. J. Agric. Res. 58:532-544.

Stich, B., Mohring, J., Piepho, H. P., Heckenberger, M., Buckler, E. S., and Melchinger, A. E. 2008. Comparison of mixed-model approaches for association mapping. Genetics 178:1745–1754.

Sun, G., Zhu, C., Yang, S., Song, W., Kramer, M. H, Piepho, H. P., and Yu, J. 2010. Variation explained in mixed model association mapping. Heredity 105:333-340.

Suwabe, K., Tsukazaki, H., Iketani, H., Hatakeyama, K., Kondo, M., Fujimura, M., Nunome, T., Fukuoka, H., Hirai, M., Matsumoto, S. 2006. Simple sequence repeat-based comparative genomics between *Brassica rapa* and *Arabidopsis thaliana*: the genetic origin of clubroot resistance. Genetics 173:309-319.

Tomassini, L., Schnurbusch, T., Fossati, D., Mascher, F., and Keller, B. 2007. Association mapping of *Stagonospora nodorum* blotch resistance in modern European winter wheat varieties. Theor. Appl. Genet. 115:697-708.

U., Nagaharu. 1935. Genomic analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn. J. Bot. 7:389–452.

Udall, J. A., Quijada, P. A., and Osborn, T. C. 2005. Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* L. Genetics 169:967-979.

Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhofs, A., and Kilian, A. 2004. Diversity arrays technology (DArT) for whole-genome profiling of barley. PNAS 101:9915-9920.

Yang, Y. W., Kai, K. N., Tai, P. Y., and Li, W. H. 1999. Rates of nucleotide substitution in angiosperm mitochondrial DNA sequences and dates if divergence between *Brassica* and other angiosperm lineages. J. Mol. Evol. 48:597-604.

Yin, X., Yi, B., Chen, W., Zhang, W., Tu, J., Fernando, D. W. G., and Fu, T. 2010. Mapping of QTLs detected in a *Brassica napus* DH population for resistance to *Sclerotinia sclerotiorum* in multiple environments. Euphytica 173:25–35.

Yu, J., Pressoir, G., Briggs, W. H., Bi, I. V., Yamasaki, M., Doebley, J. F., McMullen, M. D., Gaut, B. G., Nielsen, D. M., Holland, J. B., Kresovich S., and Buckler, E. S.2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat. Genet. 38:203–208.

Zhao J., and Meng, J. 2003a. Detection of loci controlling seed glucosinolate content and their association with Sclerotinia resistance in *Brassica napus*. Plant Breed. 122:19–23.

Zhao, J., and Meng, J. 2003b. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 106:759–764.

Zhao, J., Udall, J. A., Quijada, P. A., Grau, C. R., Meng, J., and Osborn, T. C. 2006. Quantitative trait loci for resistance to *Sclerotinia sclerotiorum* and its association with a homeologous nonreciprocal transposition in *Brassica napus* L. Theor. Appl. Genet. 112:509–516.

Zhao, K., Aranzana, M. J., Kim, S., Lister, C., Shindo, C., Tang, C., Toomajian, C., Zheng, H., Dean, C., Marjoram, P., and Magnus, N. 2007. An Arabidopsis example of association mapping in structured samples. PLoS Genet 3:71–82.

Zou, J. Jiang, C., Cao, Z., Li, R., Long, Y., Chen, S., and Meng, J. 2010. Association mapping of seed oil content in *Brassica napus* and comparison with quantitative trait loci identified from linkage mapping. Genome 53:908-916.

APPENDIX

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach.

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
1	Ames 18935	United Kingdom	52.5	657.5
2	Ames 21490	United States	50.0	500.0
3	Ames 22547	Poland	89.6	1043.8
4	Ames 22548	Poland	50.0	630.0
5	Ames 22549	Poland	89.6	981.3
6	Ames 22550	Poland	87.5	991.7
7	Ames 23093	Italy	100.0	1450.0
8	Ames 23232	Mongolia	29.2	387.5
9	Ames 24226	Sweden	50.0	500.0
10	Ames 26626	United States	50.0	516.7
11	Ames 26627	United States	87.5	1091.7
12	Ames 26635	United States	50.0	525.0
13	Ames 26638	Not available	50.0	516.7
14	Ames 26639	Not available	29.2	295.8
15	PI 169075	Turkey	50.0	666.7
16	PI 169080	Turkey	50.0	508.3
17	PI 221971	Japan	50.0	525.0
18	PI 250135	Pakistan	77.1	664.6
19	PI 251236	Pakistan	91.7	625.0
20	PI 251614	Yugoslavia, Serbia	50.0	433.3
21	PI 269449	Pakistan	12.5	154.2
22	PI 271452	India	62.5	329.2
23	PI 282571	Japan	29.2	187.5
24	PI 284859	Poland	20.8	262.5
25	PI 286418	Nepal	45.8	387.5
26	PI 305278	Sweden	50.0	483.3
27	PI 305279	Sweden	50.0	550.0
28	PI 305281	Sweden	12.5	70.8
29	PI 305282	Sweden	81.3	556.3
30	PI 311727	Poland	29.2	337.5
31	PI 311728	Poland	50.0	391.7
32	PI 311729	Poland	83.3	375.0
33	PI 311730	Poland	50.0	566.7
34	PI 311731	Poland	50.0	616.7

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
35	PI 311732	Poland	100.0	700.0
36	PI 311733	Poland	80.4	496.3
37	PI 365644	Canada	83.3	491.7
38	PI 383422	France	79.2	529.2
39	PI 384536	Sweden	83.3	466.7
40	PI 391553	China	29.2	229.2
41	PI 399418	Czech Republic	29.2	279.2
42	PI 409022	Germany	20.8	262.5
43	PI 409024	Germany	79.2	740.8
44	PI 431571	Canada	50.0	375.0
45	PI 431572	Canada	29.2	287.5
46	PI 431574	Canada	72.9	572.9
47	PI 432391	Bangladesh	100.0	645.8
48	PI 432392	Bangladesh	50.0	625.0
49	PI 432394	Bangladesh	83.3	533.3
50	PI 432395	Bangladesh	50.0	433.3
51	PI 436556	China	0.0	0.0
52	PI 436557	China	12.5	179.2
53	PI 443015	Norway	50.0	391.7
54	PI 458605	New Zealand	37.5	312.5
55	PI 458606	New Zealand	50.0	491.7
56	PI 458607	New Zealand	72.9	306.3
57	PI 458608	New Zealand	50.0	591.7
58	PI 458609	New Zealand	45.8	270.8
59	PI 458918	France	50.0	400.0
60	PI 458919	France	77.1	485.4
61	PI 458920	France	72.9	727.1
62	PI 458922	France	0.0	0.0
63	PI 458923	France	89.6	552.1
64	PI 458924	France	100.0	587.5
65	PI 458925	France	75.0	475.0
66	PI 458930	Canada	50.0	600.0
67	PI 458935	Sweden	72.9	377.1
68	PI 458936	Sweden	89.6	406.3
69	PI 458937	Sweden	100.0	1000.0
70	PI 458939	Japan	29.2	312.5
71	PI 458940	Japan	29.2	237.5

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
72	PI 458941	Japan	29.2	320.8
73	PI 458944	Germany	62.5	470.8
74	PI 458945	Germany	58.3	354.2
75	PI 458946	Germany	87.5	495.8
76	PI 458947	Germany	29.2	295.8
77	PI 458948	Germany	50.0	458.3
78	PI 458949	Germany	45.8	445.8
79	PI 458950	Germany	33.3	241.7
80	PI 458951	Germany	50.0	408.3
81	PI 458952	Germany	79.2	445.8
82	PI 458953	Germany	66.7	366.7
83	PI 458954	Germany	50.0	483.3
84	PI 458955	Germany	50.0	416.7
85	PI 458956	Germany	60.4	331.3
86	PI 458957	Germany	29.2	229.2
87	PI 458958	Germany	29.2	237.5
88	PI 458959	Germany	100.0	1187.5
89	PI 458964	New Zealand	93.8	877.1
90	PI 458965	New Zealand	93.8	1077.1
91	PI 458966	France	77.1	481.3
92	PI 458967	France	45.8	479.2
93	PI 458968	France	70.8	418.8
94	PI 458969	France	29.2	229.2
95	PI 458970	France	81.3	647.9
96	PI 458971	France	50.0	650.0
97	PI 458979	United States	41.7	150.0
98	PI 458980	United States	37.5	458.3
99	PI 469724	South Korea	50.0	575.0
100	PI 469725	South Korea	50.0	675.0
101	PI 469727	South Korea	100.0	1208.3
102	PI 469728	South Korea	79.2	495.8
103	PI 469729	South Korea	29.2	187.5
104	PI 469730	South Korea	50.0	550.0
105	PI 469731	South Korea	58.3	566.7
106	PI 469732	South Korea	50.0	508.3
107	PI 469733	South Korea	50.0	483.3
108	PI 469734	South Korea	83.3	600.0

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
109	PI 469735	South Korea	79.2	595.8
110	PI 469736	South Korea	33.3	275.0
111	PI 469738	South Korea	83.3	550.0
112	PI 469740	South Korea	89.6	618.8
113	PI 469741	South Korea	77.1	339.6
114	PI 469742	South Korea	72.9	302.1
115	PI 469743	South Korea	89.6	377.1
116	PI 469744	South Korea	83.3	783.3
117	PI 469745	South Korea	83.3	283.3
118	PI 469746	South Korea	20.8	229.2
119	PI 469747	South Korea	50.0	508.3
120	PI 469748	South Korea	79.2	662.5
121	PI 469749	South Korea	20.8	212.5
122	PI 469750	South Korea	50.0	566.7
123	PI 469751	South Korea	37.5	362.5
124	PI 469752	South Korea	37.5	370.8
125	PI 469753	South Korea	79.2	670.8
126	PI 469754	South Korea	50.0	533.3
127	PI 469755	South Korea	89.6	868.8
128	PI 469756	South Korea	50.0	533.3
129	PI 469757	South Korea	45.8	454.2
130	PI 469758	South Korea	100.0	500.0
131	PI 469759	South Korea	89.6	710.4
132	PI 469760	South Korea	91.7	1208.3
133	PI 469761	South Korea	45.8	570.8
134	PI 469762	South Korea	87.5	1241.7
135	PI 469763	South Korea	45.8	412.5
136	PI 469764	South Korea	95.8	1262.5
137	PI 469765	South Korea	100.0	1516.7
138	PI 469766	South Korea	100.0	1537.5
139	PI 469767	South Korea	91.7	1370.8
140	PI 469768	South Korea	50.0	575.0
141	PI 469769	South Korea	100.0	1000.0
142	PI 469771	South Korea	50.0	550.0
143	PI 469772	South Korea	89.6	1410.4
144	PI 469773	South Korea	75.0	1220.8
145	PI 469774	South Korea	12.5	162.5

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
146	PI 469775	South Korea	100.0	1579.2
147	PI 469776	South Korea	64.6	660.4
148	PI 469777	South Korea	45.8	595.8
149	PI 469778	South Korea	45.8	520.8
150	PI 469779	South Korea	100.0	1543.8
151	PI 469780	South Korea	83.3	1170.8
152	PI 469781	South Korea	50.0	550.0
153	PI 469782	South Korea	79.2	1150.0
154	PI 469783	South Korea	100.0	1502.1
155	PI 469784	South Korea	50.0	641.7
156	PI 469785	South Korea	45.8	420.8
157	PI 469786	South Korea	79.2	1170.8
158	PI 469787	South Korea	68.8	1027.1
159	PI 469788	South Korea	100.0	1216.7
160	PI 469789	South Korea	89.6	1235.4
161	PI 469790	South Korea	52.1	577.1
162	PI 469791	France	45.8	420.8
163	PI 469792	France	89.6	756.3
164	PI 469793	France	72.9	952.1
165	PI 469794	France	45.8	495.8
166	PI 469795	France	41.7	500.0
167	PI 469796	France	81.3	1239.6
168	PI 469797	France	72.9	897.9
169	PI 469798	France	45.8	520.8
170	PI 469799	France	100.0	1508.3
171	PI 469800	France	89.6	1114.6
172	PI 469801	South Korea	75.0	1129.2
173	PI 469803	Germany	45.8	479.2
174	PI 469805	South Korea	50.0	433.3
175	PI 469806	South Korea	41.7	375.0
176	PI 469807	South Korea	75.0	825.0
177	PI 469808	South Korea	89.6	856.3
178	PI 469809	South Korea	83.3	700.0
179	PI 469810	South Korea	100.0	1633.3
180	PI 469811	South Korea	89.6	1072.9
181	PI 469812	South Korea	0.0	0.0
182	PI 469813	South Korea	50.0	625.0

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
183	PI 469815	South Korea	50.0	466.7
184	PI 469816	South Korea	41.7	366.7
185	PI 469818	South Korea	70.8	754.2
186	PI 469819	South Korea	85.4	747.9
187	PI 469821	South Korea	78.3	1056.7
188	PI 469822	South Korea	87.5	1025.0
189	PI 469823	South Korea	75.0	1022.9
190	PI 469825	South Korea	93.8	1285.4
191	PI 469826	South Korea	58.3	825.0
192	PI 469827	South Korea	50.0	416.7
193	PI 469828	South Korea	100.0	1308.3
194	PI 469829	South Korea	50.0	566.7
195	PI 469830	South Korea	100.0	1133.3
196	PI 469831	South Korea	72.9	860.4
197	PI 469832	South Korea	29.2	379.2
198	PI 469833	South Korea	100.0	875.0
199	PI 469834	South Korea	50.0	525.0
200	PI 469835	South Korea	77.1	706.3
201	PI 469836	South Korea	16.7	183.3
202	PI 469837	South Korea	41.7	625.0
203	PI 469838	South Korea	50.0	491.7
204	PI 469839	South Korea	33.3	191.7
205	PI 469840	South Korea	45.8	512.5
206	PI 469841	South Korea	66.7	877.1
207	PI 469842	South Korea	50.0	550.0
208	PI 469843	South Korea	8.3	75.0
209	PI 469844	South Korea	16.7	233.3
210	PI 469845	South Korea	50.0	625.0
211	PI 469846	South Korea	12.5	170.8
212	PI 469847	South Korea	100.0	1075.0
213	PI 469848	South Korea	72.9	822.9
214	PI 469850	South Korea	89.6	777.1
215	PI 469851	South Korea	89.6	931.3
216	PI 469852	South Korea	64.6	556.3
217	PI 469853	South Korea	66.7	558.3
218	PI 469854	South Korea	8.3	41.7
219	PI 469855	South Korea	45.8	462.5

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
220	PI 469856	South Korea	50.0	500.0
221	PI 469857	South Korea	50.0	541.7
222	PI 469858	South Korea	100.0	1016.7
223	PI 469859	South Korea	79.2	1002.5
224	PI 469860	South Korea	66.7	558.3
225	PI 469861	South Korea	45.8	445.8
226	PI 469862	South Korea	45.8	379.2
227	PI 469863	South Korea	37.5	379.2
228	PI 469864	South Korea	50.0	425.0
229	PI 469865	South Korea	79.2	843.3
230	PI 469866	South Korea	33.3	375.0
231	PI 469867	South Korea	50.0	516.7
232	PI 469868	South Korea	70.8	730.8
233	PI 469869	South Korea	50.0	391.7
234	PI 469870	South Korea	33.3	466.7
235	PI 469871	South Korea	29.2	395.8
236	PI 469872	South Korea	56.3	531.3
237	PI 469873	South Korea	0.0	0.0
238	PI 469874	South Korea	50.0	475.0
239	PI 469875	South Korea	89.6	747.9
240	PI 469876	South Korea	64.6	547.9
241	PI 469877	South Korea	75.0	683.3
242	PI 469878	South Korea	50.0	583.3
243	PI 469879	South Korea	37.5	329.2
244	PI 469880	South Korea	50.0	491.7
245	PI 469881	South Korea	33.3	241.7
246	PI 469884	South Korea	50.0	591.7
247	PI 469885	South Korea	100.0	1358.3
248	PI 469886	South Korea	41.7	518.1
249	PI 469887	South Korea	50.0	600.0
250	PI 469888	South Korea	75.0	1029.2
251	PI 469889	South Korea	25.0	258.3
252	PI 469890	South Korea	20.8	237.5
253	PI 469891	South Korea	87.5	845.8
254	PI 469896	South Korea	79.2	466.7
255	PI 469897	South Korea	100.0	1066.7
256	PI 469898	South Korea	20.8	254.2

Table A1. List of Plant Introductions used in identification of DArT markers linked to sclerotinia stem rot resistance in *Brassica napus* using genome-wide association mapping approach (Continued)

S.No.	PI Number	Country of origin	Mortality percentage	AUMC
257	PI 469899	South Korea	75.0	833.3
258	PI 469900	South Korea	70.8	762.5
259	PI 469901	South Korea	75.0	1025.0
260	PI 469903	South Korea	77.1	718.8
261	PI 469904	South Korea	100.0	1025.0
262	PI 469905	South Korea	50.0	500.0
263	PI 469906	South Korea	72.9	714.6
264	PI 469907	South Korea	50.0	516.7
265	PI 469908	South Korea	20.8	237.5
266	PI 469909	South Korea	20.8	229.2
267	PI 469910	South Korea	41.7	425.0
268	PI 469911	South Korea	50.0	441.7
269	PI 469913	South Korea	45.8	604.2
270	PI 469914	South Korea	41.7	408.3
271	PI 469915	Not available	72.9	810.4
272	PI 469920	South Korea	0.0	0.0
273	PI 469928	South Korea	45.8	345.8
274	PI 469930	South Korea	20.8	229.2
275	PI 469960	Japan	25.0	175.0
276	PI 469963	Japan	50.0	700.0
277	PI 535865	Germany	20.8	187.5
278	Glacier	Canada	91.7	650.0
279	Quinta	Canada	75.0	708.3
280	Regent	Canada	75.0	625.0
281	Topaz	Canada	50.0	500.0
282	Westar	Canada	100.0	1441.7