ANALYSIS AND IDENTIFICATION OF QTL FOR RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* IN PEA (*PISUM SATIVUM* L.)

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

White mold caused by *Sclerotinia sclerotiorum* is one of the most devastating diseases infecting field pea (*Pisum sativum* L.) which causes severe yield loss worldwide. Population 17 (Lifter/ PI240515), and Population 19 (PI169603/ Medora) were developed by single seed descent and screened by greenhouse evaluation and detached stem assay to identify potential sources of white mold resistance. Twenty-two partial resistant inbred lines were identified with short internode which met at least two resistance criteria based on lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI). To find SNPs (single nucleotide polymorphism) responsible for white mold resistance, Populations 17 and 19 were genotyped using GBS (genotyping by sequencing) methodology and analyzed with the GBS-SNP-CROP pipeline. Linkage maps were constructed for each population and a composite map based on shared SNPs between the two populations was also generated. Nineteen QTL were identified as contributing to resistance to white mold. Seventeen were associated with LEI and two were associated with NTI. The QTL responsible for lesion expansion on LG VII were duplicated in the short internode subset of both populations. Partially resistant inbred lines and QTL responsible for white mold resistance identified in this study can be useful as resources for resistance to *S. sclerotiorum* in further experiments aimed at developing resistant cultivars.

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LIST OF ABBREVIATIONS

Add .....Additive effect

AFLP.....Amplified fragment length polymorphism

ANOVA .....Analysis of variance

*Ape*KI .....*Aeropyrum pernix* K1

AUDPC.....Area under disease progress curve

BAM .....Binary version of a SAM file

BLASTN .....Basic local alignment search tool nucleotide

Bp.....Base pair

BWA .....Burrows-Wheeler aligner

CA .....Composite agar

CIM .....Composite interval mapping

cM .....Centimorgan

CSFL .....Cool season food legume

CTAB.....cetyl trimethylammonium bromide

CV .....Coefficient of variation

DArT .....Diversity array technology

DNA.....Deoxyribonucleic acid

dpi .....Days post inoculation

DSA.....Detached stem assay

EST .....Expressed sequence tag

GBS.....Genotyping by sequencing

GBS-SNP-CROP .....GBS SNP-calling reference optional pipeline

GWAS.....Genome wide association mapping

LEI .....Lesion expansion inhibition

LG .....Linkage group

LOD .....Logarithm of the odds

LS .....Least square

MAF .....Minor allele frequency

MAS .....Marker assisted selection

MLM .....Mixed linear model

NTI .....Nodal transmission inhibition

OA .....Oxalate acid

PCR .....Polymerase chain reaction

PDA .....Potato dextrose agar

PEAR .....Paired-end read merger

PI .....Plant introduction

QTL .....Quantitative trait loci

R^2 .....Phenotypic variation explained

RAPD .....Random amplification of polymorphic DNA

RCBD .....Randomized complete block

RE .....Restriction enzyme

RFLP .....Restriction fragment length polymorphism

RIL .....Recombinant inbred lines

RNA .....Ribonucleic acid

SAM .....Sequence alignment map

SAS .....Statistical analysis system

SNP .....Single nucleotide polymorphism

SSR .....Single sequence repeats

Std.Dev.....Standard deviation

Std.Error.....Standard error

STM .....Sequenced tagged microsatellite site

TASSEL.....Trait analysis by association, evolution and linkage

TM.....Transcriptome-based mapping

UNEAK.....Universal network enabled analysis kit

USDA-ARS.....United States Department of Agriculture-Agricultural Research Service

vcftools.....Variant call format tools

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CHAPTER 1: LITERATURE REVIEW

Pea and its origin

Pea is a member of the Leguminosae family, subfamily Faboideae tribe: Fabeae, and the Pisum genus. Taxonomy of *Pisum sativum* L. is divided into five subspecies and five varieties (Table 1-1) (Zong et al. 2009). Morphology of field pea: is typically normal leaf and long vine (0.9-1.8 meter) types, or semi-leafless or afila with short, vine types (0.6-1.2 meter); however, all combinations of leaf type and vine length are present in the germplasm. Primary gene pool consists of *P. sativum* including wild *P. sativum* ssp. *Elatius,* secondary gene pool includes *P. fulvum* and tertiary gene pool is comprised of *Vavilovia Formosa* (Smykal et al. 2015).

Table 1-1: Taxonomy of the Pisum genus (Zong et al. 2009)

Species	Subspecies	Variety
Pisum sativum L.	*ssp. Sativum*	*var. sativum* *var. arvense (L.)* Poir.
	ssp. Elatius	*var. elatius (M. Bieb.)* Alef. *var. pumilio* Mzikle *var. humile Boiss* and Noe
	ssp. Abyssinicum *ssp. Asiaticum* *ssp. transcaucasicum*	
Pisum fulvum Sibth and Sm.		

The center of origin for pea encompasses Turkmenistan, Iran, southern Europe and northern Africa (Maxted and Ambrose 2001). Peas were one of the oldest domesticated crops in the Fertile Crescent about 10,000 years ago with barley (*Hordeum vulgare*), wheat (Triticum) and lentil (*Lens culinaris*) (Jing et al. 2010). Two types of wild Pisum (*P. elatius* Beib, and *P. humile* Boiss. and Noe) are genetically related to domesticated pea, and *P. humile* has the same number of chromosomes and standard karyotype of domesticated pea (Zohary et al. 2012).

According to Weeden (1987) *Pisum sativum* ssp. *elatius*, is thought to be the wild ancestor of the cultivated pea and the starting point for the domestication process.

Genetic diversity was reduced during domestication and seed size and seed weight in domesticated peas have increased (Zohary et al. 2012). Other traits selected during domestication include pods that do not dehisce during maturity (wild pea pods shatter and scatter their seeds out), absence of seed dormancy, larger seed and reduced seed pigmentation. Further selection and breeding resulted in reducing vine length in modern pea cultivars for ease of mechanical harvest. Wild type had small and poor seed quality, long- day photoperiod flowering and many basal branches compared with domesticated pea cultivars (Weeden 2007).

Pea, along with lentil and bean, was a significant food for most people in the Middle East, North Africa and Europe during the Medieval Period (5th to the 15th century). Eating fresh green peas, became a luxury food of early modern Europe in the 17th and 18th centuries. The popularity of green peas spread to North America and other regions of the world by European colonization. Thomas Jefferson was known for his love of pea and grew more than 30 cultivars of peas on his land (Falaschi 2013).

Pea market classes

Peas are grown for their edible seed or pods either in the mature or immature state. There are several different market classes of peas. They are broadly divided into field dry pea and garden or green peas. Garden or green peas are harvested when seeds are immature for the fresh market or for processing into frozen or canned peas a few hours after harvest (Biddle and Cattlin 2007). Development of the canning and frozen food market caused peas to become an important vegetable for consumption on their own or in mixed vegetable or in ready-meal packages. The fresh pea market classes are the garden or English pea, the oriental or snow pea and the sugar

snap pea. Edible pod peas (sugar snap peas and snow peas) lack “parchment”, a fibrous layer including lignified sclerenchyma cells in the inner pod wall (McGee and Baggett 1992).

The dry pea market classes are Dunn, marrowfat, maple, yellow pea, green pea, Austrian winter peas, and wrinkled seed peas (Janzen et al. 2006). Field peas are harvested when seeds are mature and dry, and they are used for human consumption as whole or split peas (variety that halves are held loosely together). For example, green marrowfat varieties are used for mushy peas and the wasabi snack peas which are mostly in Asian market. Field dry peas can be used as livestock consumption like Maple peas are largely used as bird feed. Increasing demand for vegetable protein in the food industry has resulted in field peas receiving greater research focus throughout the world (Boye et al. 2010).

Nutritional composition

Pea seeds contains 23-31% protein (legumin, vicilin, and lectins) with high concentrations of the essential amino acids lysine and tryptophan, starch, sugars, fiber, minerals and vitamins (riboflavin, folate). Field pea has low fat and is cholesterol-free making it a heart healthy food. Legume proteins compliment cereal grain protein based on amino acid composition. Legumes also contain secondary metabolites such as isoflavonoids which have antioxidant properties valuable for human health (Boye et al. 2010).

Pea morphology and agronomy

The pea plant has a vine length ranging from 0.6-2.7 meter (Tilton 2009). Leaves are alternate and pinnately compound with the rachis ending in a branched tendril. The dicotyledonous field pea has hypogeal emergence (Hanley et al. 2004). Pea flowers have five green attached sepals and five white, purple or pink petals with different sizes. In each flower, there are multiple stamens (anther and filament) and one carpel (ovary, style and stigma), which

develops into a pod (fruit) with several peas. Flowers are borne in the leaf axils and are cleistogamous favoring self-pollination. Pea seeds are round, smooth or wrinkled, with different colors (green, yellow, beige, brown, red-orange, blue-red, dark violet to almost black, or mottled) (Oelke et al. 1991).

Peas grow well on fertile, light-textured, well-drained soils and are vulnerable to soil salinity and acidity. Optimum soil pH for pea cultivation ranges from 5.5 to 7.0. Annual precipitation of 40.6 to 99.1 cm is the optimum range for pea production (Pavek 2012). Pea seed is planted at a depth of 3.8 to 7.6 cm in rows ranging from 15.2 to 30.4 cm apart (Pavek 2012). Spring-planted peas bloom 30 to 50 days after planting and fall-planted peas flower around 250 days after planting. The growing season for spring-planted peas ranges from 60 to 150 days and for fall-planted peas 300 to 320 days (Pavek 2012).

If green-cotyledon pea harvest is belated, bleaching happens mostly due to the precipitation at maturity, high moisture, direct sunshine and high temperatures (Schatz and Endres 2009). Bleaching, admixture and earth tag (soil adhered to the seed) during harvest downgrade seed for human consumption market. Many factors are required for market acceptance and must be considered in breeding and pea production. For example, seed color, seed size, seed shape, surface dimpling, splitting efficiency, damaged seed criteria like (bleaching, cracked seed, immature seed), nutritional value (protein, carbohydrate, lipid, and mineral nutrient concentration), and storage condition for cookability.

The pea crop grows fast and provides a nitrogen-fixation benefit to growers, so they can use it as green manure and cover crop. Peas are often grown alone or with cereals for silage and green fodder or be grazed while they are in the field like Austrian winter pea. Austrian winter

peas are the best pea type suited for green manure or cover crop as they are adapted to cold temperatures, they avoid nitrogen losses during the winter period (Chen et al. 2004).

Pea production

Based on FAO data world field pea harvested area was 8,459,444 ha and dry pea production was 15,557,228 tons across 98 countries in the world in 2016 (http://www.fao.org). Pea production worldwide increased in 2016 compare to 2015 which was 13,310,280 tons. Currently, Canada is the main producer and exporter of dry peas. Russia, China, India, United states, Ukraine, and France also had significant dry pea production in 2017. India consumes the most and is the world's major importer of pea.

In the United States, Montana, North Dakota, and Washington lead the nation in dry pea production. Planted area of dry edible pea in the US was 456,485.41 ha, harvested area was 425,122.27 ha in 2017. The average yield was 1512 kg ha^{-1} and total production of 0.8 MT in 2017, which was decreased compared to 2016. North Dakota ranks second after Montana with 171,991 ha planted and 165,921 hectares harvested versus 212,459 ha planted and 190,202 ha harvested in Montana in 2017. Average yield per hectare in North Dakota was higher than Montana in 2017, with 2016 kg ha^{-1} versus 918.4 kg ha^{-1} and total production in ND was 413,280 versus 215,824 tons in Montana (USDA-NASS 2017). The USDA data excluded wrinkled seed and Austrian winter pea from these data.

Wrinkled seed production is limited to Idaho, and Washington state with 19,992 tons in 2017. Austrian winter pea is planted in Idaho, Montana and Oregon on 11,736 ha with harvested area of 6475 ha in 2017. The average winter pea yield per hectare was 1490 kg ha^{-1} with total winter pea production of 7,000 tons in 2017 in United states. Austrian winter pea production in 2017 was decreased about three times compared to 2016 production. Green pea had a harvested

area of 4,112,297 ha in 86 countries worldwide in 2016 with overall production of 32.1 MT, these data were increased slightly comparing to 2015 green pea production around the world. (http://www.fao.org). China, India, United States, France and Egypt had significant green pea production among other countries in 2017.

Nitrogen fixation and crop rotation

Pisum sativum is a cool season legume crop grown in many parts of the world for dry seed production, green manure and cover crops because they grow quickly and contribute nitrogen to the soil. High temperatures and dry soils between late vegetative and early reproductive periods has a negative effect on nitrogen fixation (Schatz and Endres 2009).

The bacteria *Rhizobium leguminosarum* infects root hairs on the pea root system and forms nodules where they convert atmospheric nitrogen (N_2) to ammonia (NH_3) (Smykal et al. 2012). Young nodules are white and grey and do not fix nitrogen at this point. When nodules mature and become active, they turn pink or reddish due to the presence of leghemoglobin (a hemoprotein that carries oxygen to the bacteria in root nodules of legume plants). The Rhizobium bacteria contains the nitrogenase enzyme which is responsible for fixation of atmospheric nitrogen. Nitrogenase is very sensitive to oxygen and leghemoglobin reduces the oxygen concentration to activate nitrogenase (Downie 2005). The ability to fix atmospheric nitrogen is a primary benefit of legumes, which allows them to contribute to a sustainable production system and minimize the use of inorganic nitrogen fertilizer. Grain legumes, through deposition of residual nitrogen can increase production of subsequent crops such as cereals in low-N environments. Legumes add nitrogen to soil and is considered a N credit and can be measured by fertilizer replacement value (Bundy et al. 1993). Field pea has highest nitrogen credit to the soil in crop rotation with wheat in comparison with other legume (chickpea, soybean

and bean) (Przednowek et al. 2004). Nitrogen credit of field pea to the subsequent wheat crop in southern Manitoba was 16 kg N ha^{-1} per 1000 kg (Przednowek et al. 2004). Peas in rotation with cereals can improve soil nitrogen level, recover soil microbe variety, and improve soil aggregation, as well as preserve water in the soil (Chen et al. 2006). In general, energy cost of N_2 fixation with legume-based crop rotation is much cheaper than the Haber–Bosch process.

Distribution and adaptation

Pisum sativum is grown in temperate regions or during cool seasons in warm regions of the world and can be grown at high elevations. Optimum soil temperatures for pea production is 24°C but they tolerate temperatures as low as 5°C (Schatz and Endres 2009). Temperatures during flowering greater than 30°C may decrease seed set or stop flower and pod production in dry pea (Pavek 2012). High temperatures five days before and 15 days after full bloom decrease fresh weight, the number of seeds and yield in garden pea (Alaska pea) (Lambert and Linck 1958).

Winter hardiness in peas is associated with prostrate growth, branching and reduced height. Winter hardy types of field peas, particularly Austrian winter peas, can tolerate temperatures as low as -12.2°C and with snow adequate cover, they may tolerate -30°C (Fiebelkorn 2013). Winter hardy cultivars should be planted early enough in the fall to have sufficient growth before the soil freezes. In Northern climates the best time for sowing Austrian winter hardy is late-August. Seeds should be sown at a depth of 1.9 to 3.2 cm (Fiebelkorn 2013). In North Dakota pea cultivation is restricted to spring planted types due to harsh winter conditions. However, potential benefit for growers due to advantages of N-credit in rotation as a fall-sown cover crop and green manure, encourage breeders to try to adapt winter pea to more harsh climates (Holdt 2017; Fiebelkorn 2013).

According to (Fiebelkorn 2013) Melrose is one of the most winter hardy pea genotypes and can survive at -8°C. Cold acclimation is related to many physiological and metabolic changes such as concentration of soluble sugars in the leaves (Bourion et al. 2003). Increasing ABA level is related to increased winter hardiness in pea and may play a role in the induction of cold-regulated (COR) proteins (Welbaum et al. 1997). *Pisum fulvum* accessions in the secondary Pisum genepool were superior to most *P. sativum* accessions and can completely survive in -6°C with no damage to its leaves (Ceyhan 2006).

Genetics

Pisum sativum L. is an annual, diploid, self-pollinated crop and its karyotype consists of seven chromosomes (2n = 2x = 14), and nuclear genome size is 4.45×10^9 bp (4450 Mb) (Dolezel and Greilhuber. 2010). The pea genome contains many different transposable elements groups that have been used in various genomic studies (Vershinin et al. 2003). The GC content is 37.4% with a high rate of repetitive sequences (Ellis and Poyser 2002). Despite the size and repetitive nature, the pea genome is being sequenced through an international consortium for pea genome sequencing (PGS) led by France Génomique (https://www.france-genomique.org). Also, transcriptome, proteome and metabolome resources for *Pisum sativum* are being developed for biochemical, physiological, and cell biological experiments.

The first linkage map of pea was developed by Lamprecht in 1948 and included 37 genes disseminated across seven chromosomes (Folkeson 1984). Second genetic map created by Blixt in 1972 had seven linkage groups containing morphological traits across 160 mutant individuals (Weeden et al. 1998). Weeden in 1987 used isozyme markers to establish the genetic map and align them to the previous map. In 1992, Ellis et al. constructed a genetic map from a population

from the cross JI 281× JI 399 comprising 151 morphological characters, or RFLP markers, covering 1700 cM in nine linkage groups (Ellis et al. 1992).

With the advent of PCR-based marker systems, coverage of the genetic map improved in pea. The first PCR- based and hybridized-based marker (AFLP, RAPD and RFLP) linkage map of pea was created on F_2 plants of a cross between Primo× OSU442-15 (Gilpin et al. 1997). This linkage map contains 209 markers and covers 1330 cM in nine linkage groups (Gilpin et al. 1997). In 1998, Laucou et al. scored RAPD markers on a population of 139 recombinant inbred lines (RIL) from the cross of Terese× K586. This genetic map comprising 240 RAPD markers spanned 1139 cM over nine linkage groups. This map aligned to the Ellis et al. (1992) map using RAPD markers (Laucou et al. 1998).

In 1998, the first consensus map was created by combination of two maps. The first map was created by 51 F_{10}-derived recombinant inbred lines from the cross JI1794×Slow and had 465 different molecular and morphological markers (RFLP, RAPD, SSR, and isozyme and morphological markers) across seven linkage groups and covered 800 cM with a saturation of one marker per 2 cM. The second map consisted of a known marker which although did not segregate in JI1794×Slow population, their positions relative to segregated markers in first map are well known to estimate their location on the primary map (Weeden et al. 1998).

In 2001, Irzykowska et al. generated a genetic linkage map comprised of 204 molecular and physiological markers across 2416 cM, with an average distance of 12 cM. Nine linkage groups were created which eight of them have been associated to Weeden consensus map (Irzykowska and Wolko 2004). They used this data for interval mapping of QTL responsible for seed number, pod number, 1000-seed weight, 1000-yield, and seed protein content.

The first composite genetic map was developed based on three different populations covering 1439.2 cM and containing 581 markers including 243 STMS markers (Loridon et al. 2005). Three segregating populations consist of 139 RILs resulting by single seed descent from the crosses Terese× K586, a 164 F_8 RILs from Champagne× Terese, and 187 F_7 RILs from Shawnee× Bohatyr were used to build a composite map. Seven linkage groups were generated with a mean distance between contiguous SSR markers of 5.9 cM (Loridon et al. 2005).

In 2006, Aubert et al. constructed another composite genetic map based on the two pea RIL populations (population 1: Terese× K586, population 2: Terese× Champagne) using mapping knowledge from the previous composite map of Loridon et al. (2005). It shares several RAPD markers with the Laucou et al. (1998) map. This new composite genetic map includes 363 gene-anchored markers, SSR and morphological markers and covers 1458 cM, with 4 cM between adjacent markers (Aubert et al. 2006).

In 2010, Deulvot et al. developed the first genetic map based on SNP markers from RIL population derived from Cameor× China. The 91 SNPs covered over 680 cM on 8 linkage groups (Deulvot et al. 2010). The second pea consensus map developed by (Bordat et al. 2011) from six different RIL populations and 536 markers including morphological, SSR, RAPD, RFLP, and SNP markers. This consensus map spanned 1389 cM over 7 linkage groups with marker intervals under 10 cM and LOD of 30. Markers from previous study by (Aubert et al. 2006) were used as anchors. The third pea consensus map was made of 586 RILs from five mapping populations using 1536 polymorphic SNP loci. SNPs were derived from transcriptome sequencing of 8 diverse Pisum accessions, six *Pisum sativum* cultivars and two wild accessions. The 939 SNPs mapped across seven LGs with a total coverage of 771.6 cM. Ninety-four SSR

markers from (Bordat et al. 2011) were used to anchor this map with the previous consensus map to enable comparative analysis (Sindhu et al. 2014).

The latest pea consensus map was developed by (Sudheesh et al. 2015) included maps based on single nucleotide polymorphisms (SNP) and simple sequence repeat (SSR) markers from two RIL populations generated by crossing phenotypically divergent parental genotypes Kaspa× Yarrum and Kaspa×ps1771. Data from each map were combined with bridging markers previous published studies to generate a consensus map including 2028 loci disseminated across seven linkage groups covering 2387 cM and average density of one marker per 1.2 cM (Sudheesh et al. 2015). A major QTL correspond to *er1* gene was identified on LG VI of these two populations. They also found another QTL associated with boron tolerance on Kaspa × ps1771 population that overlapped with the powdery mildew resistance locus, which permit to use co-select strategy for these desirable traits.

Application of next generation sequencing and *de novo* assembly contributed to assembly of reads to produce a transcriptome database for non-reference genomes (Dassanayake et al. 2009). Transcriptome analysis aids in the characterization of genes that are differentially expressed in different cells, time and condition. Transcriptome sequencing of *Pisum sativum*, the garden pea, resulted in total of 2,209,735 EST reads for assembly of 42,000 contigs derived from above-ground organs of pea. Pea transcriptome data is available through next generation sequencing, Roche/454 platform, and *de novo* assembly (Franssen et al. 2011). Alves et al. (2015) produced 46,099 contigs from more than a billion short reads corresponding to almost 100 Gb sequence using Illumina sequencing from 20 pea cDNA libraries (Alves-Carvalho et al. 2015). Transcriptome analysing on two genotype of pea (Kaspa and Parafield) in 2015 from 23 cDNA libraries were performed using Illumina sequencing. A total of 407 and 352 million

paired-end reads were assembled into 129,282 and 149,272 contigs, respectively, from Kaspa and Parafield (Sudheesh et al. 2015). Kerr et al. (2017) assembled a pea axillary bud transcriptome into 81,774 transcripts containing 194,067 isoforms using Illumina MiSeq technology (Kerr et al. 2017).

Proteome analysis identifies differentiation of large protein-abundance and post-translational modifications using the Mass spectrometry (MS) (Aebersold and Mann 2003). Proteomics analysis of *Pisum sativum* gives biological information about different physiological processes, disease and stress tolerance. Proteomics was used to study powdery mildew (*Erysiphe pisi*) (Curto et al. 2006), *Didymella pinodes* (Desalegn et al. 2016), *Mycosphaerella pinodes* in pea (Castillejo et al. 2010). Proteome analysis was also used to interpret osmotic stress in seed germination (Brosowska-Arendt et al. 2014), loss of desiccation tolerance in the embryonic axis of pea seed during germination (Wang et al. 2012), chilling response from cold acclimation (Dumont et al. 2011), salinity response in the roots of pea (Kav et al. 2004), and root growth indicators in different pea cultivars (Meisrimler et al. 2017).

Pea breeding

Grain yield improvement is essential for pea to be a good option in crop rotations for cereals compared to other legume crops. Yield improvement requires attention to many biotic (fungal diseases, various insects, and viruses) and abiotic stresses (drought, heat stress, frost and salinity) using different approaches such as selecting parents from diverse germplasm, selection under stress conditions, and yield testing in different environments. Presence of vast diversity in cultivated and wild peas provides the necessary variation for improvement of the crop.

Traditional breeding has improved grain yield over time. Some pea quality traits should be considered and selected visually. Improving qualitative traits like some of the disease

resistance is possible by traditional breeding (Lejeune-Hénaut et al. 2008). Introgression of favorable genes using traditional breeding practices is an approved method to improve many species. Selecting parents from non-native plant introduction (PI) can introgress genetic diversity into the cultivated germplasm. One example is the use of PI269818 from the United Kingdom as a source of resistance to pea seed borne mosaic virus Pathotype-1 (*sbm-1*gene) and Pathotype -2 (*sbm-2* gene) isolates, and PI347492 accession of *Pisum sativum* from India has been used to confer PSbMV-P4 to susceptible pea cultivars (Johansen et al. 2001). The secondary gene pool (*P. fulvum*) has been used to improve resistance to pea weevil (*Bruchus pisorum* L.) (Aryamanesh et al. 2012) and powdery mildew by introgression of the dominant *Er-3* allele (Fondevilla et al. 2007). Also, improving lodging resistance through introduction of the *afila* trait for upright growth (Pesic and Djordjevic 2013), and introgression of the *Hr* allele from a forage line which delays flowering until after freezing periods (Lejeune-Hénaut et al. 2008).

By developing genomic tools and our knowledge about the pea genome and molecular markers associated with quantitative trait loci (QTL), marker assisted selection (MAS) could help breeders accelerate crop improvement (Tayeh et al. 2015). Using MAS can help select target traits more efficiently in less time and expense (Collard and Mackill 2008). For example, using different markers associated with physiological traits such as, frost tolerance (Liu et al. 2017) flowering time (*HR*, *SN*, *LF*, and *E* loci) (Weller and Ortega 2015) lodging resistance (Zhang et al. 2006) can accelerate progress in breeding programs. Another important MAS aid in speeding up the breeding process is using markers associated with resistance to diseases such as powdery mildew (Cardoso et al. 2017), pea enation mosaic virus (PEMV) (Jain et al. 2014), pea seed-borne mosaic virus (PSbMV) (Scegura 2017) fusarium wilt (Shalu Jain et al. 2015) rust (Singh et al. 2015), and Ascochyta blight (*Mycosphaerella pinodes*) (Prioul-Gervais et al. 2007).

Pests, disease and potential problems

Pea is challenged with different harmful living organisms or biotic stress (pests, pathogens and weeds) and non-living factors or abiotic stress in various conditions. Different pathogens infect the pea plant and cause disease. Specific examples of disease caused by pathogens in pea include fungi such as *Sclerotinia sclerotiorum* (white mold), *Fusarium oxysporum* f. sp. *pisi* (wilt), *Peronospora viciae* (downy mildew), complex of *Mycosphaerella pinodes*, *Phoma medicaginis*, *Ascochyta pisi*, and/or *Phoma koolunga* (Ascochyta blight), bacteria like *Pseudomonas syringae* pv. *pisi* (bacterial blight). Viral pathogens include pea enation mosaic virus (PEMV), pea seed-borne mosaic virus (PSbMV) and pea streak virus (PeSV) (Johansen et al. 1994). Several insects cause damage to pea plants and the most important is the pea aphid (*Acyrthosiphon pisum*) due to the viruses (PSbMV, PEMV, and PeSV) they transmit. Others include the pea leaf weevil (*Sitona lineatus*), lygus bug (*Lygus lineolaris*), and grasshoppers (Caelifera). Perennial and annual weeds that emerge in the field compete with pea for resources or cause difficulty with harvest. Examples include common lambsquarters (*Chenopodium album*), kochia (*Bassia scoparia*), volunteer grain, wild mustard (*Sinapis arvensis*), wild oat (*Avena fatua*), Russian thistle (*Kali tragus*), nightshade (Solanaceae) and wild buckwheat (*Fagopyrum esculentum*).

On the other hand, abiotic stresses also, constraining pea production and need to be considered. Specific examples of abiotic stress include extremes of soil moisture (drought or saturation logging), temperature (heat stress or cold temperature), and imbalances in soil fertility (nutrient deficiencies or toxicity including salinity) (Wang et al. 2003)

Pathogen biology and disease cycle

Sclerotinia sclerotiorum (Lib.) de Bary is a member of kingdom Fungi, phylum Ascomycota, class Discomycetes, order Helotiales, family Sclerotiniaceae, and genus Sclerotinia (Bolton et al. 2006). *Sclerotinia sclerotiorum* is a soil-borne, hemi-biotrophic fungus and has both biotrophic and necrotrophic characteristics, initially attacking living cells before switching to a necrotrophic lifestyle in which nutrients are attained from dead host cells. *S. sclerotiorum* virulence correlates with mycelial age through decreasing oxalate biosynthesis (Wang et al. 2016). *S. sclerotiorum* causes disease in over 400 plant species including most of the dicotyledonous and some monocotyledonous plants including onion and tulip. Disease caused by this pathogen is often referred to as cottony rot, watery soft rot, stem rot, and, perhaps most common, white mold. Annual losses from *S. sclerotiorum* in the United States in pulse crop exceeded $12 million in 2016 (USDA-ARS 2016).

Sclerotinia species are homothallic (self-fertile), and sclerotia of *S. sclerotiorum* germinate by carpogenic (apothecial) germination or myceliogenic (hyphal) germination methods. Following the period of conditioning in a near-saturated, moderately cool environment around 10 to 20°C, sclerotia in soil depths of up to 2 cm produce a mushroom-like fruiting body called an apothecium to reach the soil surface. Each apothecia produces around 10 million ascospores over a period of several days, as a result of a sexual process and are blown by wind to the aerial portions of plants. Most often, this happens after a significant rain or irrigation, and is supported by a shaded, slow-drying soil surface. Long humidity periods about 16 to 48 hours with low temperatures (12° to 24°C) provides optimum conditions for ascospore germination and infection initiation. Ascospores fall on the leaves or the stem of a susceptible plant and germinate to hypha, and then produce a special flattened, hyphal pressing organ called an appressorium to

punch the host tissue cuticle and infect the healthy plant. The penetration process of appressoria (referred as infection cushions) is through an enzymatic digestion process of the cuticle. A large vesicle formed at the appressorium tip before penetration, seems to be discharged into the host cuticle during infiltration (Tariq and Jeffries 1984).

Since ascospores lack the energy needed to infect healthy host tissues directly, ascospores first attack weak host tissue such as flower petals or senescing leaves. Infected weak tissues then aid as a food base to grow mycelium with adequate energy to penetrate healthy plant parts. Since senescing petals frequently drop, the main infection often coincides with bloom and post-bloom periods. Fluffy white mycelium is produced by *S. sclerotiorum* on and in infected plant parts and aggregates into 2 to 5 mm in diameter and up to 25 mm in length sclerotium. Sclerotia consist of a black melanin rind surrounding a compact mass of white-colored hyphae. The sclerotia form on the plant tissue and when plant tissues fall to the soil, the sclerotia drop to the soil as well where they can survive in the absence of a host and remain viable for up to 5 years.

S. sclerotiorum rarely germinates myceliogenically, unlike *S. minor* which usually infects through hyphal germination (Heffer and Johnson 2007). The *S. sclerotiorum* life cycle mostly occurs in the soil as sclerotia, which can directly produce hyphae and can directly attack any plant tissue (Bolton et al. 2006). Effective pathogenesis of *S. sclerotiorum* requires mycelium to secrete pathogenicity factors such as oxalic acid and extracellular lytic enzymes (Fernando et al. 2004). Understanding pathogenicity factors can provide a better perception about disease and resistance mechanism.

White mold develops from sclerotia in field soil and ascospores are discharged at ground level under closed canopies. Wind-disseminated ascospores and contaminated machinery and water aid in the spread of disease. Presence of broadleaf weeds in the field which are hosts of the

pathogen can also increase sclerotia in the field. Initial symptoms of *S. sclerotiorum* appear as soft, water-soaked or white cottony to grey lesions on leaves, stems and pods. Mature lesions resemble a "bull's eye" and are easily shredded and sclerotia can be seen inside the infected stems (Bolton et al. 2006).

Annual rise in yield loss due to *S. sclerotiorum*, has increased demands for developing broad management strategies to combat *S. sclerotiorum*. Crop rotations will not entirely eliminate the pathogen since *Sclerotinia* has such a wide host range and the pathogen survives in soil as sclerotia for at least 5 years (Peltier et al. 2012; Jain et al. 2012). Treatment with fungicides is not appropriate and is deleterious for human health by effect on edible parts. Biological controls do not completely remove all sclerotia (Fernando et al. 2004).

Disease and resistance mechanism

Sclerotinia sclerotiorum is a hemi-biotrophic fungus that transits from biotrophy to necrotrophy. Oxalic acid (OA) acidifies host tissues and sequesters calcium from host cell walls to help cell wall degrading enzymes that contribute to pathogenesis (Kabbage et al. 2015). Furthermore, oxalic acid induces reactive oxygen species (ROS) levels in plants that trigger programmed cell death (PCD). OA as an elicitor of PCD in plants induces apoptotic-like reactions which are crucial for fungal pathogenicity (Kim et al. 2008).

Transcriptome analysis of pea-*S. sclerotiorum* interaction showed up-regulation of 95 annotated *S. sclerotiorum* contigs in active pathogen infected tissues compared with inactive pathogen which may involve in pathogenicity (Zhuang et al. 2012). Annotation of these contigs showed that degrading plant cell wall enzymes, such as, exoglucanase 2 precursor, carbohydrate esterase, and enzymes that synthesize the fungal cell wall like chitin, glucan and mannan, increase during the infection and shows that the pathogen is ready to attack. Expression of genes

involved in differentiation of appressoria into infection cushions, and contigs encoding transporters of secondary metabolites in *S. sclerotiorum* rise during invasion of the host plant. Guanine nucleotide-binding protein (G protein) and an importin beta-2 nuclear transporter may also have a role in pathogenicity of *S. sclerotiorum* as well (Zhuang et al. 2012).

On the other hand, 451 unique annotated pea ESTs were found that may have a role in defense mechanism to stress, as they were expressed significantly more during the infection compared with non-infected tissue. Higher expression of genes involved in biosynthesis of plant cell wall and sequestration of fungal cell walls such as germin-like proteins, chitinase, beta-1,3-glucanase and other glycoside hydrolases that degrade fungal cell walls can improve fungal resistance mechanisms. Expression of some transcription factors including the MYB family, the Apetala2/Ethylene responsive element binding protein family and the WRKY family increases during the reaction between the pathogen and host and shows their possible role in the defense mechanism. Also, increasing expression of contigs encoding pathogenesis-related (PR) proteins and signaling molecules such as abscisic acid, auxin, brassinosteroid, calcium ion, ethylene, gibberellic acid, jasmonic acid, salicylic acid and small GTPase can be the sign that the plant is preparing itself to confront an invading pathogen (Zhuang et al. 2012).

As mentioned above germins and germin-like proteins (GLPs) which are cell wall glycoproteins may have a key role in resistance mechanisms. GLPs are resistant to degradation with detergent, heat, and proteases and have N-terminal secretory signals, which may confirm their role in defense mechanism against pathogens or cell wall function (Zimmermann et al. 2006). Three cDNAs encoding germin-like proteins (PsGER1, PsGER2a, and PsGER2b) were separated from *Pisum sativum*. Cereals which are resistant to *S. sclerotiorum* have a germin gene encoding oxalate oxidase which oxidizes oxalic acid to CO_2 and hydrogen peroxide (H_2O_2) while

the coding sequence of PsGER1 in pea nodules expresses proteins with superoxide dismutase activity and no oxalate oxidase activity was detected (Gucciardo et al. 2007).

When plants were inoculated with *S. sclerotiorum*, SSITL (*Sclerotinia sclerotiorum integrin-like* gene) was highly expressed during the initial stages of infection. SSITL is likely to be an effector and suppressor of the jasmonic/ethylene (JA/ET) signal pathway and induces systemic acquired resistance at the initial stage of infection. Targeted silencing of SSITL in *S. sclerotiorum* reduced the virulence, hyphal polarity, sclerotia size, sclerotia number and carpogenic germination (Zhu et al. 2013).

Oxidative burst, is the prompt discharge of reactive oxygen species including superoxide radicals ($O^{-2)}$ and hydrogen peroxide production, which is an early event that is correlated to a hypersensitive response (HR) in plant-pathogen interactions. H_2O_2 may reinforce the structure of plant cell walls and activates lipid peroxide and salicylic acid (SA) synthesis. Also, H_2O_2 has roles in the signal transduction pathway that organizes many defense mechanism responses, such as HR and synthesis of pathogenesis-related (PR) proteins and phytoalexin induction (Hu et al. 2003).

Disease control and resistance available in pea

Inheritance of resistance to *S. sclerotiorum* is polygenic with partial resistance to *S. sclerotiorum* involving many genes; each of them making a relatively small contribution to resistance (Davar et al. 2013). Porter et al. (2009) showed that partial resistance to *S. sclerotiorum* is quantitative and is expressed in two forms, lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI). Lesion expansion inhibition (LEI) can be seen as a slowed rate of lesion progression while NTI restricts the path of the pathogen through the node.

Porter et al. (2009) screened 504 genotypes from the *Pisum* core collection for resistance to *S. sclerotiorum* and identified 5 genotypes (Plant Introduction 103709, PI166084, PI169603, PI240515 and PI270536) with highly quantitative partial resistance based on nodal transmission inhibition and five with parti al resistance base on lesion expansion (about 0-1cm) 3 days after inoculation. It was noted that some traits, for instance thick stem diameter, short internode and the afila (semi-leafless) leaf morphology, should also be effective in developing white mold-resistant cultivars.

A previous study by Tashtemirov (2011) screened 848 accessions across the world to find resistant genotypes to white mold which resulted in identifying 13 accessions with both LEI and NTI with partial resistance to *S. sclerotiorum* from which five of them were the same as Porter study (Porter et al. 2009). He based the criteria on lesion expansion of 2 cm or less and NTI less than two nodes from the inoculation point on node four.

PI240515 (seed coat color: green/white, flower color: white, origin: India) and PI169603 (seed coat color: pigmented, flower color: white, origin: Turkey) possess the greatest partial resistance and are suggested to breeders as a good germplasm source in severe infections of *S. sclerotiorum* (Porter 2012a). PI169603 has the best partial resistance to *S. sclerotiorum* across the widest temperature and high relative humidity and also is the best single genotype for plant breeders in order to develop future cultivars with better partial resistance to *S. sclerotiorum* based on stem lesion expansion (Porter 2012b).

Wild pea genotypes and early varieties had tall climbing vines (Smykal et al. 2012) and selection for shorter vines led to reduced internode length which may result in more resistant types through avoidance mechanisms. The data can be biased based on the observed variation in plant height and internode length especially when scoring lesion expansion. While the fungus

will grow rapidly through the internode, disease progress slows down at the node. Thus, plants with long internodes could be rated more susceptible than plants with short internodes if disease evaluation is based merely on lesion expansion. Therefore, it is crucial to develop and optimize a screening process to accurately classify resistant genotypes regardless of vine phenotype.

Molecular analysis of pea collections has shown that, although considerable variation exists within the cultivated gene pool, wild material offers the opportunity to incorporate original traits that may have been unintentionally eliminated. Even thought, backcrossing with wild germplasm may have drawback consequences of dragging linked undesirable genes as well. Association mapping analysis of sixteen major world pea germplasm collections with over 1000 pea germplasm accessions could determine genetic variation related to desirable agronomic traits, which are hard to breed for in a traditional manner (Smykal et al. 2012).

One method to control *S. sclerotiorum* in some crop species has involved the use of transgenes which specifically destroy oxalic acid. The bacterial oxalyl-CoA decarboxylase enzyme will transform oxalate into carbon monoxide (CO) and formic acid (HCOOH) which can be engineered for Sclerotinia control through genetic transformation (Lu. 2003); however, it may have a toxic effect on plant cells. Therefore, the focus is currently on the wheat germin gene encoding oxalate oxidase that results in catalytic oxidation of oxalic acid to CO_2 and hydrogen peroxide (H_2O_2)

$Oxalate + O2 + 2H+ \rightleftharpoons 2CO2 + H2O2$ (Lane et al. 1993).

Only true cereals, are mostly grasses and differ from non-grasses pseudocereals, (such as barley (*Hordeum vulgare*), maize (*Zea mays*), oat (*Avena sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), and wheat) and pine (*Pinus sylvestris*) germins appear to have OXO (Oxalate oxidase) activity (Dunwell et al. 2000). Moreover, the germin gene from barley shows both OXO and

superoxide dismutase activities that result in production of the defense-inducing molecule H_2O_2 ($2H^+ + O^{-2} + O^{-2} \rightarrow H_2O_2 + O_2$) (Hu et al. 2003).

Development of varieties resistant to white mold should include tolerance to oxalic acid, a key component of physiological resistance (Kolkman and Kelly 2003). This is a long-term solution and includes detection of pea genotypes with significant resistance. However, an effective disease management plan incorporating several control methods including cultural practices, varietal resistance, as well as chemical applications, biological control, upright plant growth, wider row spacing and deep ploughing in infested soils should be implemented. At present, there are no pea genotypes with complete resistance to this pathogen.

Molecular markers

Molecular markers are known sequences of DNA that can be associated with a particular sequence of the genome. Development of reliable and cost effective molecular markers has significantly advanced plant breeding by providing many options, such as evaluating genetic variations within germplasm, creating linkage maps, QTL mapping, and association mapping and marker-assisted selection (MAS) (Zargar et al. 2015). Molecular markers are divided into two categories hybridization-based markers like Restriction Fragment Length Polymorphism (RFLP) and PCR (polymerase chain reaction)-based marker. The earliest molecular marker technique was restriction fragment length polymorphism (RFLP) which developed by (Grodzicker et al. 1974; Botstein et al. 1980). (Grodzicker et al. 1974). There are many PCR-based marker techniques which were developed in chronological order as following sequenced tagged microsatellite site (STM) (Beckmann and Soller 1990), random amplified polymorphic DNA (RAPD) (Williams et al. 1990), simple sequence repeat (SSR) or microsatellites (Akkaya et al. 1992), cleaved amplified polymorphic sequence (CAPS) (Akopyanz et al. 1992), amplified

fragment length polymorphism (AFLP) (Vos et al. 1995). And finally, single nucleotide polymorphism (SNP), that were first described by (Jordan and Humphries 1994), have become the marker of choice thanks to massive parallel sequencing techniques that allows thousands of loci to be evaluated within a single experiment (Wani et al. 2013).

Microsatellites are 2–7 bp random sequences that are repeated fewer than 100 times. Repeats at a certain locus may differ resulting in alleles with different lengths among individuals and species. Microsatellites with 3 bp repeats are most abundant and among them, GAA was most abundant within pea genome (Gong et al. 2010). There have been many attempts to develop SSR markers for pea, for example, database-derived SSR markers were developed by searching the pea sequence of Genbank/EMBL databases for 2 or 3 bp repeated motifs using the FINDPATTERN program from the GCC Wisconsin package (Burstin et al. 2001). Burstin et al. (2001) identified one hundred seventy-one SSRs among 663 sequences and 31 of the 43 SSR for which primers were designed showed polymorphism among 12 pea genotypes.

An EST (Expressed sequence tag) database with 18,552 pea ESTs was assembled using DNASTAR software into 10,086 unigenes (Gong et al. 2010). They identified 586 microsatellites among 530 unigenes using the SSRIT (sequence repeat identification tool) software. Primers were designed for forty-nine SSRs and screened on 10 Chinese cultivars yielding nine polymorphic loci. Kaur et al. (2012) sequenced cDNA samples from field pea genotypes and after *de novo* assembly with Next Gene software aligned consensus contigs with the *Medicago truncatula* genome using BLASTN. A total of 2397 SSR were identified among 22,057 unigenes using Batch Primer3 software and SSR primers were developed for field pea. Among 96 EST-SSR markers 86 revealed polymorphism among six genotypes (Kaur et al. 2012).

One thousand eight hundred unigenes were detected among 18,522 pea ESTs and were screened using microsatellite identification software MISA and revealed 2612 microsatellites in 2395 SSR-containing ESTs (Mishra et al. 2012). DNA from 24 pea accessions sequenced with Illumina HiSeq 2500 were scanned for SSRs with MISA software. A total of 8899 SSR sequences were found and from those 1644 SSRs were used to design primers to screen 24 genotypes of *P. sativum* and P. *fulvum* (Yang et al. 2015).

De Caire et al. (2012) used a JAVA-based SSR-finding algorithm program to search 6327 mRNA sequences and identified 96 gene-based SSR markers of which 45 were polymorphic among 13 pea lines. Three thousand nine hundred twenty SSR markers are curated on the CSFL website for *Pisum sativum* and 1187 of these have been mapped in pea (De Caire et al. 2012)

Single nucleotide polymorphism discovery in pea began when Aubert et al. (2006) reported the development of SNPs in three cultivars, Terese, Champagne, and K586. The SNPs were discovered using four approaches. First, primers were designed for 38-49 genes based on EST pea gene sequences. Comparing the amplified sequences revealed SNPs among genotypes. Second, orthologous sequence of the pea genes was identified in *M. truncatula* EST databases and specific primers were designed for *M. truncatula*. The PCR products were sequenced directly and screened for polymorphism (Thoquet et al. 2002). The third approach involved the reverse strategy of searching pea EST database to find homologous sequences of *M. trancatula* gene markers, design primers to amplify homolog sequences and detect polymorphism between the corresponding pea sequences. The fourth approach used capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) to detect sequence variations through electrophoretic mobility differences. Primers were designed from EST sequences and a standard size ladder was used to align data to detect SNPs (Aubert et al. 2006). Aubert et al. (2006)

developed, 63 SNP markers and 15 single-stranded conformation polymorphism (SSCP) markers. Deulvot et al. (2010) used the same SNP development pipeline as described in (Aubert et al. 2006) and designed primers from 334 different genes to amplify, directly sequence, and align sequences using ClustalW. Genomic sequence was obtained from at least two genotypes from Genbank for each gene and by comparing these sequences 2850 SNP in 308 genes were identified (Deulvot et al. 2010).

Sindhu et al. (2014) identified 1,536 SNPs among 8 diverse pea accessions using transcriptome sequencing. High throughput sequencing allowed Boutet et al. (2016) to identify 419,024 SNPs among four pea lines using the discoSnp tool (Boutet et al. 2016). Introduction of genotyping by sequencing (GBS) for SNP discovery has resulted in many reports of SNPs in pea. Annicchiarico et al. (2017) discovered 95,740 SNP markers across three RIL populations using the UNEAK pipeline. There are 2,797 SNPs housed on the CSFL website and among them 1,355 SNPs have been mapped to different linkage group maps (https://www.coolseasonfoodlegume.org).

Genotype by sequencing

Genotype by sequencing (GBS) is a sequencing method to discover single nucleotide polymorphisms (SNP) across a given species genome based on decreasing genome complexity with restriction enzymes (REs) (Elshire et al. 2011). This method is cost-effective, simple, rapid, very precise, highly repeatable, and may sequence significant genome regions. Using restriction enzymes which are sensitive to methylation, lower copy regions would be more targeted than repetitive regions with higher efficiency. Using insensitive methylation restriction enzymes can help access non-coding DNA, transposable elements and repeat regions where genes for quantitative and economically important traits are frequently positioned.

Genotyping by sequencing simplifies computational analysis of species with large genomes and high repetitive sequences and species without a reference genome. Genotyping by sequencing analysis involves digesting the genome, sequencing individual reads and assembling them into consensus contigs. The contigs are ideally aligned with a reference genome to identify SNPs. In the absence of a reference genome, sequenced reads are assembled with different *de novo* assembly methods and SNPs are discovered by SNP calling algorithms.

Torkamaneh et al (2016) compared seven GBS bioinformatics pipelines that process raw GBS sequence data into SNP genotypes on re-sequenced soybean lines. TASSEL-GBS, Stacks, IGST, and Fast-GBS require a reference genome, while UNEAK and Stacks do not require a reference genome. They compared the results with re-sequencing data to assess their accuracy in calling SNPs with these pipelines (Torkamaneh et al. 2016).

Development of the UNEAK and Stacks pipeline helped develop GBS for non-model genome crops. Berthouly-Salazar et al. (2016) directly mapped GBS reads on transcriptomes in non-model pearl millet populations and compared their results with the UNEAK pipeline. Both methods produced several tens of thousands of SNPs. A transcriptome-based mapping (TM) pipeline uses transcriptome data as a reference and is a less biased way since the UNEAK pipeline searches coding and non-coding regions while the TM pipeline only explores coding regions. Using *Ape*KI resulted in greater SNP discovery in coding regions. In TM, reads are mapped to the assembly with BWA version 0.7.5 for a maximum number of 3 mismatches. SNPs are called using UnifiedGenotyper GATK version 2.4.7.

Melo et al. (2016) used the GBS-SNP-CROP pipeline (GBS SNP-Calling Reference Optional Pipeline) in tetraploid kiwiberry (*Actinidia argute*) which lacks a reference genome. GBS-SNP-CROP applies a clustering strategy to form a “Mock Reference” for SNP calling and

genotyping. GBS-SNP-CROP eliminates unnecessary data culling due to imposed read-length uniformity and maximizes data usage. GBS-SNP-CROP extracts large numbers of additional high-quality SNPs and higher average read depth with lower genotyping error rate. It may give more information per sequencing dollar spent.

Genotyping by sequencing has been applied in pea and used to conduct GWAS analyses for seed yield (Annicchiarico et al. 2017), QTL mapping of mineral concentration (Ma et al. 2017) and GWAS for *S. sclerotiorum* resistance (Chang et al. 2017). Annicchiarico et al. (2017) used GBS for genomic selection and GWAS studies on 315 pea genotypes from 3 connected populations (from crosses between Attika, Isard and Kaspa) for grain yield under severe terminal drought. Sequencing provided an average of 551,210 reads per sample and the UNEAK pipeline produced a total of 95,740 SNP markers. GWAS of the three RIL populations discovered 26 GBS-generated markers associated with the beginning of flowering and grain yield and 21 markers related with the adjusted grain yield. Ma et al. (2017) used GBS method to identify SNPs from 158 recombinant inbred lines from cross between Aragorn and Kiflica. A total of 3095 SNPs were found using the UNEAK pipeline to construct a linkage map and QTL mapping of for seed mineral concentrations (boron, calcium, iron, potassium, magnesium, manganese, molybdenum, phosphorous, sulfur, and zinc) and contents in pea (Ma et al. 2017). Chang et al. (2017) applied GBS to 282 pea plant introduction lines from Porter et al (2009). A total of 35,658 SNPs were identified and used to study genes underlying white mold disease with genome wide association mapping using PLINK (Purcell et al. 2007).

Current status of research

The current aim of research to understand the genetics of disease resistance, molecular nature of defense mechanisms and the pea-*S. sclerotiorum* interaction to establish a method to

control *S. sclerotiorum* in *Pisum sativum*. From the breeding point of view, development of a resistant variety to *S. sclerotiorum* is the goal and application of marker assisted selection (MAS) offers the potential to shorten the breeding cycles required to attain the goal once useful genetic variation can be identified. Although MAS is not widely used in pea due to the lack of a reference genome and a limited number of molecular markers (Jain and McPhee 2013; Smykal et al. 2012), development of new technologies offers more possibilities to generate SNPs and aid QTL mapping.

Zhuang et al. (2012) identified exclusive transcriptome sequence (RNAseq) information from the pea-*S. sclerotiorum* interaction acquired by 454 pyrosequencing and demonstrated a specific EST resource for the pea-*S. sclerotiorum* interaction. This method analyzed host and pathogen ESTs efficiently without the prerequisite for reference genomes using the tBLASTx program against 3 legume genomes (proxy reference) by comparison to non-interaction EST libraries to find candidate resistance and pathogenicity genes. Contigs encoding proteins were found by the SignalP3.0 program and 145 proteins secreted by *S. sclerotiorum* were classified and may play a key role in the expansion of plant disease. Chang et al. (2017) studied genome wide association mapping and RNA sequencing analysis of pea-*S. sclerotiorum* interaction (Chang et al. 2017). The GWAS applied on phenotypic data of 282 lines from (Porter et al. 2009) and genotypic data from GBS method, which showed 206 and 118 significant SNPs were associated with lesion expansion and nodal transmission inhibition, respectively (Chang et al. 2017). RNA sequencing analysis was conducted using Lifter and PI240515 (Population 17 parents) on tissue samples collected 12, 24 and 48 hours post inoculation (hpi) and *de novo* assembly resulted in 60,598 high quality transcripts. GWAS analysis showed only one SNP common in both lesion expansion and nodal transmission inhibition which BLASTN analysis

revealed a putative glutathione S-transferase. GWAS and RNA sequencing revealed that leucine rich-repeat (LRR)-containing transcripts, Armadillo (ARM) repeat superfamily protein, oxidoreductase, UDP arabinopyranose mutase, ATP-binding cassette (ABC) transporter and cytochrome b5 were associated with lesion restriction (Chang et al. 2017). Also, five differentially expressed annotated transcripts were found to be significantly associated with nodal transmission inhibition as ACT domain repeat protein, VQ (Valine-glutamine) motif-containing protein, β-glucosidase, myo-inositol oxygenase, and cytochrome b-561 (Chang et al. 2017). Among these transcripts, the most interesting transcripts were the ones were upregulated in PI240515 and down regulated in Lifter. Only coiled-coil nucleotide-binding site leucine rich repeat (CC-NBS-LRR) protein and cytochrome b-561 seemed to be the most interesting transcripts associated with lesion resistance and nodal transmission inhibition, respectively, the remaining transcripts were upregulated in Lifter and not in PI240515 (Chang et al. 2017).

The only QTL mapping in pea regarding resistance to *S. sclerotiorum* was established based on a population of 189 F_2-derived lines from the cross Lifter/ PI240515. Four hundred nine molecular markers including 239 SSR markers, 146 RAPDs and 24 cleaved amplified polymorphic sequences (CAPS) were tested on the population. Sixty-seven of these markers were assigned to nine linkage group and covered 734 cM with a minimum LOD of 4. The QTL responsible for NTI was placed on linkage group LG II, close to marker PSMPSAA255_230 with explained 34.1% of the phenotypic variation. A QTL for LEI was located on LG III close to marker PSMPSAD73 and explained 2.5% of the phenotypic variation (Tashtemirov 2012).

Molecular breeding has contributed to modern pea breeding programs through genomic knowledge of crop species. There is potential for finding current genetic variation preserved in germplasm, which can be efficiently identified and introduced into current pea cultivars. The

combination of molecular techniques and applied plant breeding with the common goal of increased yield and disease resistance bodes well for the future of pea breeding. In this study the previous work by (Tashtemirov 2012) was expanded with the goal of validating two QTL for *S. sclerotiorum* resistance on pea using RIL populations derived from the same F_2 populations.

CHAPTER 2: GENOTYPING BY SEQUENCING ANALYSIS IN *PISUM SATIVUM* L.

Abstract

Plant breeding research has been improved by next generation sequencing by developing many single nucleotide polymorphisms (SNPs) for marker assisted selection (MAS). Genotyping by sequencing is a cost-effective and simple way to dissect genomes of different species even without reference despite the low coverage and missing data. To analyze field pea (*Pisum sativum*) genome as a non-reference crop, we need to have a suitable analyzing procedure. Sequencing dataset of two RIL population (Population 17 and Population 19) were used to analyze with three different pipelines to call SNPs. We compared two reference free methods (UNEAK and GBS-SNP-CROP), and one reference-based method (transcriptome-based mapping) base on the number of SNPs, accuracy of SNPs and number of missing data in *P. sativum* genome sequencing data. Transcriptome base mapping (TM) had lower number of SNPs compared to non-reference pipelines, which could be due to partiality to coding regions. Although, GBS-SNP-CROP had lower number of SNPs compared to UNEAK and extracts high-quality SNPs base on phred quality score and lower genotyping error rate. Although, each pipeline follows different algorithm, and should produce distinct sets of SNPs, the shared SNPs between GBS-SNP-CROP and TM were considerably high. To combat the low coverage and genotyping error of GBS method, we need to choose appropriate pipeline for further mapping population procedures. To consider that, we choose GBS-SNP-CROP pipeline, which has given high number of SNPs with high phred quality score.

Introduction

Genotype by sequencing (GBS) is a next generation sequencing method to discover single nucleotide polymorphisms (SNPs) across the genomes of different species for genotyping

studies. Genotyping by sequencing was first introduced by Elshire et al. (2011) and is a technique based on preparing libraries to decrease genome complexity using restriction enzymes (REs). This method uses restriction enzymes to prepare a library followed by next generation sequencing. GBS is an excellent technique to simplify analysis of species with large genomes and high repetitive sequence content or species without a complete genome sequence or reference genome (Berthouly-Salazar et al. 2016). This is a cost effective and simple approach for whole genome marker discovery and genotyping which is required for genetic and genomic studies. Pea (*Pisum sativum*) has a large genome with a high amount of repetitive sequences (Macas et al. 2007) and a complete genome sequence is not yet available for pea. GBS using the methylation sensitive enzyme, *Ape*KI (*Aeropyrum pernix* K1), provides access to low copy genic regions.

The most challenging issue with GBS is large amount of missing data which can be due to many reasons. First, the restriction site may not be in one of the samples due to the biological nature of the sequence (insertion and deletion) (Swarts et al. 2014). Second, genotype by sequencing provides a random sample of all loci next to restriction enzyme digestion sites and many loci might be missed in any individual due to under-sampling. Third, it is possible that the DNA was not uniformly digested during the digestion or due to DNA quality or other technical problems. Fourth, low coverage at particular positions, which could be due to different factors such as read length, genome size, repetitive sequences, and error rate in sequencing method or the assembly algorithm. The amount of missing data could be reduced by first increasing sequence coverage which will allow additional sequences to be represented, but this will result in increased cost and presents a computational challenge. Secondly, improving the GBS library preparation protocol using selective primers to focus on sequencing a smaller set of fragments

obtained from digestion will increase depth of coverage (Sonah et al. 2013). Third, using new combinations of restriction enzymes may increase the depth of coverage (Fu et al. 2016).

GBS data analysis can be a difficult task due to low and variable depth of sequence coverage, different read lengths and sample demultiplexing issues. It is essential for any GBS pipeline to filter out low quality reads, break down multiplex data based on individual barcodes and call variants based on *de novo* analysis or align reads on a reference genome to detect SNPs. There are two types of pipelines to work with GBS data, *de novo*-based and reference-based (Torkamaneh et al. 2016). The most direct method is to align individual reads directly on the reference genome. In the absence of a reference genome, using a clustering of sequence can help find minor alleles (Melo et al. 2016). There are many reference-based and *de novo*-based pipelines designed to handle GBS data which sacrifices depth of coverage to increase the number of SNPs with different approaches (Glaubitz et al. 2014). In this study we compared three GBS methods to filter, sort and align sequences of two RIL populations from *Pisum sativum*. The first pipeline was the reference free GBS SNP calling pipeline, UNEAK, which is an extension of the Java program, TASSEL3.0. In this pipeline, alignment of tags to a reference genome is substituted by formation of a pair of tags and network filtering to allow for SNP discovery (Lu et al. 2013). This pipeline removes the barcode and trims reads to 64 bp sequences (including cut site residue) to minimize the consequences of sequencing error and to reduce database size and memory usage. The UNEAK SNP calling is based on a ML genotyping algorithm, (Hohenlohe et al. 2010). The UNEAK estimates the likelihood for each probable genotype at a given locus and chooses the one with the major likelihood. The total number of each four-possible nucleotide at that site(n), likelihoods of a homozygote genotype (1/1), heterozygote one (1/2) and sequencing error rate (ε) are shown as follow:

$$L(1/1) = P(n_1, n_2, n_3, n_4|1/1) = \frac{n_1}{n_1! n_2! n_3! n_4!} \left(1 - 3\varepsilon/4\right)^{n_1} \left(\varepsilon/4\right)^{n_1+n_3+n_4}$$

$$L(1/2) = P(n_1, n_2, n_3, n_4|1/2) = \frac{n_1}{n_1! n_2! n_3! n_4!} \left(0.5 - \varepsilon/4\right)^{n_1+n_2} \left(\varepsilon/4\right)^{n_3+n_4}$$

The second pipeline, GBS-SNP-CROP (GBS SNP-Calling Reference Optional Pipeline) was generated by Melo et al. (2016) (https://github.com/halelab/GBS-SNP-CROP) and used in tetraploid kiwiberry (*Actinidia argute*) which lacks a reference genome. The GBS-SNP-CROP pipeline is seven Perl scripts that execute parsing, filtering and SNP calling with practical bioinformatic tools such as Trimmomatic, PEAR, USEARCH, and SAMtools. The GBS-SNP-CROP eliminates unnecessary data culling due to imposed read-length uniformity and maximizes data usage. The GBS-SNP-CROP extracts large numbers of additional high-quality SNPs base on phred quality score and higher average read depth with lower genotyping error rate. A phred score (or Q score) indicates the estimated probability of an incorrect loci while P is the error probability, $Q = -10\ log_{10}(P)$, for example quality score of 30 means 1 in 1000 probability of error with base call accuracy of 99.9% (Illumina 2011). It may give more information per sequencing dollar spent.

The third approach was transcriptome-based mapping (TM) in which raw GBS reads are trimmed with Trimmomatic and aligned to a transcriptome reference using TopHat (http://ccb.jhu.edu/software/tophat/downloads/) and SNPs were called using SAMtools (DePristo et al. 2011). TopHat is a software package designed to align reads of RNA-Seq to a reference genome and uses unmapped reads of Bowtie to step and look for reads that span junctions with a seed-and-extend strategy. Seeds are reads, or fragments generated from reads, that are extended to align the reference genome without gaps. Long seeds speed up the process while shorter seeds

have more accuracy. Aligning these seeds to a reference can reveal the junctions or introns in the reference genome.

In this study we compared three different GBS methods to filter, sort and align sequences of two RIL populations from *Pisum sativum*. We compared two *de novo*-based pipelines (UNEAK and GBS-SNP-CROP), and one reference-based method (transcriptome-based mapping), based on number of SNPs, missing data, precision, speed and ease. Finally, we measured the number of shared SNPs and overlap reads between pipelines.

Materials and methods

Plant material

Population 17 consists of 192 recombinant inbred lines (RIL) developed from the cross Lifter/ PI240515 and advanced to the F_7 by single seed descent. Lifter is a short internode variety with normal leaf morphology, white flowers and green seed, developed by the USDA-ARS in cooperation with Washington Agricultural Research Center in Pullman, WA, and the Idaho Agricultural Experiment Station, Moscow, ID (McPhee and Muehlbauer 2002). PI240515 is a plant introduction with long internode vines, normal leaf types, white flower and yellowish green seed color from India (https://npgsweb.arsgrin.gov/gringlobal/accessiondetail.aspx?id=1187486). PI240515 has partial resistance to white mold according to (Porter et al. 2009) and Lifter was susceptible.

Population 19 consists of 324 RILs developed from the cross of PI169603/Medora. Medora has short internodes with afila leaf morphology, white flowers and smooth green seed color. It was developed for Midwest production at Spillman Research Farm and released in 2007 (GLGP 2007). PI169603 is an exotic accession, originating from Turkey with long internode vines, normal leaf types, white flower and yellow seed color (https://npgsweb.ars-

grin.gov/gringlobal/accessiondetail.aspx?id=1145629). PI169603 was recognized as having partial resistance to white mold Porter et al. (Porter et al. 2009) and Medora was susceptible. Population 19 was advanced by single seed descent to the F_7 generation.

DNA extraction and GBS library construction

Young leaves from individual F_7-derived plants of each RIL for Population 17 were collected for DNA extraction. DNA was extracted using a standard CTAB protocol (Doyle et al. 1987). Two 96-plex samples were prepared for library preparation based on the GBS protocol of Elshire et al. (2011). Type II restriction endonuclease enzyme, *Ape*KI which has partial methylation sensitivity, was used to construct the library. Considering ligating with sticky ends and the middle wobble base of the *Ape*KI cut site, 5' GCWGC, adapters were designed based on the paired end sequences (http://seqanswers.com/forums/showpost.php?p=1576&postcount=7) which can work with single read or paired end reads. The 7-8 bp barcodes were ligated to one end of the DNA fragment and a common adapter to the other end. Barcodes were provided by Dr. Buckler's lab (http://www.maizegenetics.net/ genotyping-by-sequencing-gbs/384-barcodes-apeki.xls) and made by Deena's Bioinformatics (http://www.deenabio.com/services/gbs-ad). Tagged DNA fragments were sent to the McDermott Center (http://www.utsouthwestern.edu/labs/dna-genotyping-core/) and sequenced on an Illumina HiSeq 2000. The quality of raw single-end reads from Illumina sequencing was checked by fastqc_v0.11.5 (Andrews 2013).

Young leaves from each RIL of Population 19 were collected for DNA extraction from field-grown plants at the Prosper (47°0 ′ N, 97°3′ W, elevation 280 m) associated with the North Dakota Agricultural Experimental Station at Fargo in 2014. DNA was extracted using the DArt protocol (https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf). Two 96-plex DNA

samples were sent to Genomic Facility of Cornell University Biotechnology Resource Center (BRC) for library preparation and sequencing.

GBS analysis and SNP calling

Two *de novo* (UNEAK, and GBS-SNP-CROP) and one reference-based methods (Transcriptome base mapping) were used to call variants (Lu et al. 2013; Melo et al. 2016; DePristo et al. 2011). All methods were run using the same parameters of minimum depth of coverage (minDP 3), maximum number of mismatch in alignment (n= 1), maximum missing data (max-missing 0.5), and minor allele frequency (maf 0.05).

UNEAK (Universal Network Enabled Analysis Kit) pipeline

Raw reads were analyzed with the UNEAK data pipeline in TASSEL 3.0, a reference free GBS SNP calling pipeline. In this pipeline, individual tags (identical 64 bp reads) generate a pair of tags and network filtering used to identify tags with only single base pair mismatches (Lu et al. 2013). The network filter with an error tolerance rate of 0.03 were used to remove repeats, paralogs and sequencing errors, to recognize reciprocal tag pairs. We set the minimum and maximum call rate at a lowest value (mnC 0, mxC 1) as the cutoff in the HapMap file which shows how many individuals have been covered by at least one tag (Huang et al. 2014).

GBS-SNP-CROP pipeline

The GBS-SNP-CROP pipeline (https://github.com/halelab/GBS-SNP-CROP) applies a clustering strategy to form a "Mock Reference" for SNP calling and genotyping. The GBS-SNP-CROP eliminates unnecessary data culling due to imposed read-length uniformity and maximizes data usage. VSEARCH has been substituted in this study as an alternative to USEARCH. VSEARCH handles large databases greater than 4 GB of memory. Our single-end fastq file was directly fed into VSEARCH version 2.4.4 (https://github.com/torognes/vsearch) to

cluster reads based on nucleotide identity values of 0.93 and a read length of 150 bp. VSEARCH uses all fastq reads from all genotypes to produce a list of non-redundant consensus sequences or centroids which attached together by poly-A borders to create a Mock Reference. Reads of each genotype align to a Mock Reference using BWA mem algorithm, (Li and Durbin 2009). Finally, SAMtools version 1.2 (http://samtools.sourceforge.net/) was used to filter mapped reads with minimum mapping quality of 30 to decrease false SNPs due to misalignment. SAMtools discarded reads with more than one mismatch and indels from the data. At the end, the pipeline filters out false SNPs and PCR error by setting some criteria which depend on ploidy level of species and confidence level (mnHoDepth0 3 -mnHoDepth1 5 -mnHetDepth 2 -altStrength 0.96 -mnAlleleRatio 0.25 -mnCall 0.5 -mnAvgDepth 3 -mxAvgDepth 200) and calls SNPs and genotypes. (Melo et al. 2016).

Transcriptome-based mapping (TM)

The GBS raw reads were trimmed with Trimmomatic and aligned to the *Pisum sativum* reference transcriptome (*P. sativum*_CSFL_RefTrans V1) by TopHat version 2.0.14 (Trapnell et al. 2009) (http://ccb.jhu.edu/software/tophat) which uses Bowtie version 1.1.2.0 and SNPs were called using SAMtools version 1.3 (DePristo et al. 2011). The *P. sativum*_CSFL_RefTrans V1, was created using the RefTrans (1.0) method and the *de novo* RNA assembly program, Trinity. It contains 45,727 contigs with a total of 157 million RNA- Seq reads in fasta format and was const ructed on 04/13/2016 (https://www.coolseasonfoodlegume.org/analysis/143). After calling variants, indels were removed from the dataset using vcftools, also minor allele counts were (mac 3) filtered out, which is relative to genotypes and number of times SNPs were called over individuals.

Overlap between the datasets

Shared reads or SNPs between transcriptome-based mapping and UNEAK were analyzed with TASSEL v 3.0 (TagCountToFastqPlugin) (Glaubitz et al. 2014) to convert tag count files from the UNEAK pipeline to fastq files and then aligned them to the transcriptome reference sequences with BWA (Burrows-Wheeler Aligner) software (Li and Durbin 2009). The UNEAK tags were aligned with Mock Reference to find out shared SNPs between UNEAK and GBS-SNP-CROP pipeline. Fastq files of tag counts from the UNEAK pipeline aligned with BWA on Mock Reference. To find shared reads or SNPs between the GBS-SNP-CROP pipeline and transcriptome-based mapping (TM), we aligned bam files derived from GBS-SNP-CROP pipeline and converted to fastq file format with SAMtools-1.2 (bam2fq option) and then aligned them with BWA on the transcriptome reference.

GBS-SNP-CROP pipeline

From a total of 179,944,894 raw reads for Population 17, 92,192,041 of the reads had no identifiable restriction site and 5,339,934 reads had no identifiable barcode and were discarded. After removing barcodes 165,961,867 reads remained. The total number of 3,110,407 centroids were attached together with poly-A to create a mock reference. Running all scripts of the GBS-SNP-CROP pipeline resulted in 16,383 raw SNPs. After filtering the SNPs to remove the heterozygotes, monomorphic markers and those SNPs distorted from the expected 1:1 ratio, 1440 polymorphic markers were identified. SNPs missing greater than 50% data were removed leaving 1397 SNPs from 192 genotypes.

Population 19 had 363,977,809 raw reads and 146,893,952 reads were usable since 210,080,602 of the reads had no identifiable restriction site and 7,003,255 reads had no identifiable barcode and were discarded. A total of 3,292,860 centroids attached were together

with poly-A to make a mock reference. 18,713 raw SNPs were identified, and 1499 SNPs remained after being filtered for missing data, heterozygotes, monomorphic, and distorted markers. Finally, we exclude markers with > 50% missing data leaving 1482 polymorphic SNPs for 186 individuals from Population 19 (Figure 2-3).

Results

After reads were sequenced, we checked raw fastq file quality of Population 17 and Population 19 using FastQC software (Andrews 2013). A total of 179,944,894 raw sequences were generated for Population 17 with average sequence lengths of 35-100 bp and 45% GC content. The sequence quality had a phred score ranging from 29-38 (Figure 2-1). Missing content across all bases was low and percent of duplicated sequences was 24.7%. There were no overrepresented sequences in the raw data. The adapter and k-mer content were high and should be trimmed.

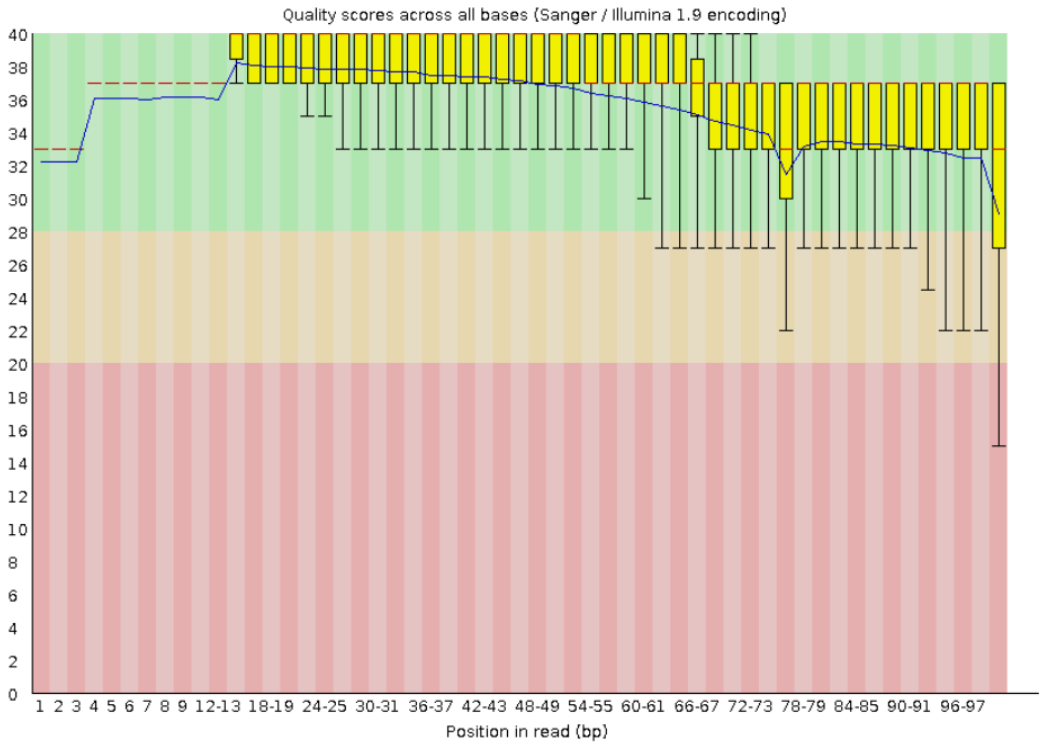


Figure 2-1: Box-and-Whisker plots for per base sequencing quality scores from FastQC for Population 17. The y-axis on the graph shows the quality scores. The blue line denotes the mean quality score for each base and red lines show medians. Yellow boxes show the range of difference between third and first quartiles (25-75%) and shows the middle 50% values. The upper and lower whiskers show 10% and 90% scores, respectively.

The total raw sequences for Population 19 was 363,977,809 with average sequence lengths of 101 bp and 48% GC content. The sequence quality has a range of 24-38 phred score (Figure 2-2). Missing data content across all bases were low and duplicated sequence percentage was 23.1%. There were no overrepresented sequences in the raw data. The adapter and k-mer content were high and should be clipped (Andrews 2013).

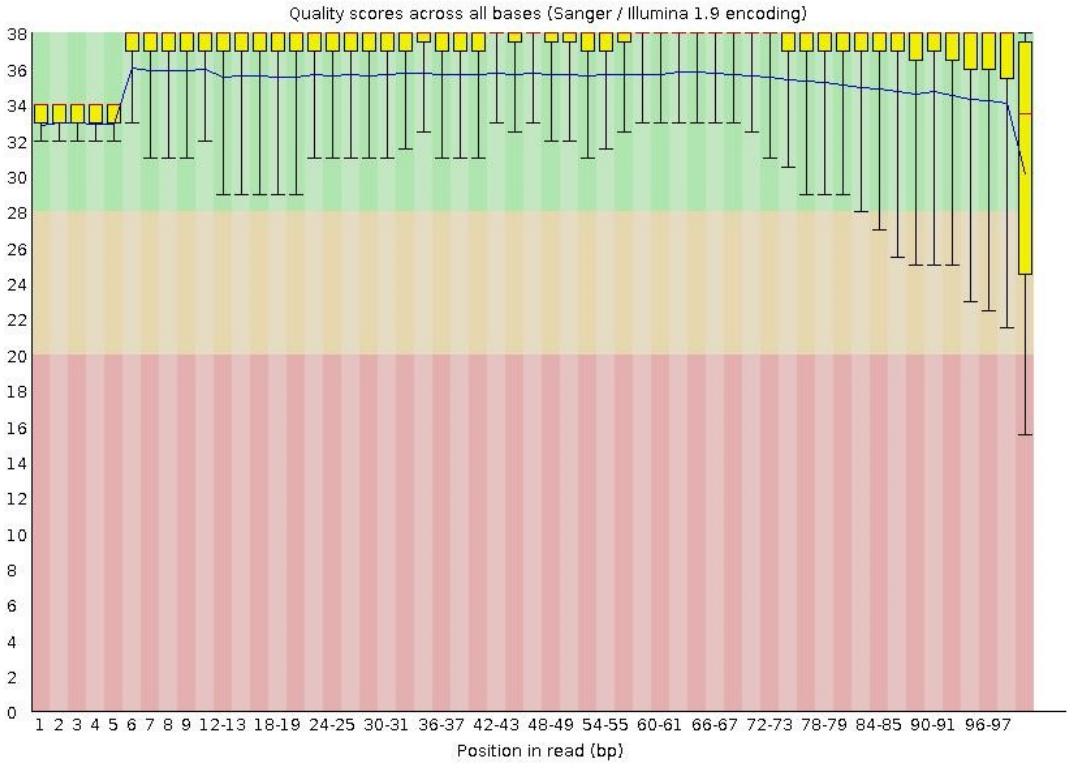


Figure 2-2: Box-and-Whisker plots for per base sequencing quality scores made by FastQC for Population-19. The y-axis on the graph shows the quality scores. The blue line denotes the mean quality score for each base and the red lines show medians. Yellow boxes show the range of difference between the third and first quartiles (25-75%) and shows the middle 50% values. The upper and lower whiskers show 10% and 90% scores, respectively.

UNEAK pipeline

Population 17 had 3,159,951 tags after merging multiple tag count files from the same individuals. After implementing pairwise alignment of tags the total number of reciprocal tag pairs was 371,502 and the number of raw SNPs called by UNEAK was 123,656. Inspection of the raw data showed that average missing data per site and average frequency of missing data on a per individual basis was 0.85 and 0.84, respectively. One sample from Population 17 was removed because of a high proportion of missing data. We filtered genotypes that called less than 50% across genotypes, and SNPs that have a minor allele count (number of times that allele appears across all genotypes at loci) below 3. After filtering, the total number of remaining SNPs was 11,945. Eighty-three SNPs below minimum depth of 3 and minor allele frequency of 0.05 or

more were discarded, and 11,862 SNPs remained for further analysis. After excluding heterozygotes, missing sites, and monomorphic SNPs, the remaining markers were filtered for distortion from the expected 1:1 ratio. Finally, 3430 markers were obtained for the 192 individuals of Population 17.

Population 19 had 1,629,805 tags after merging all duplicate individuals. A total of 144,444 tag pairs were identified across individuals. The total number of raw SNP markers was 40,932. SNPs with missing data per site of less than 50% and minimum minor allele count of 3 were excluded resulting in 8653 SNPs. SNPs were filtered with minor allele frequency and minimum depth criteria resulting in 8619 SNPs. Finally, data from 187 genotypes filtered out for missing, heterozygote and monomorphic parents, and distortion from the expected 1:1 ratio which led to 2131 markers for 187 genotypes (Figure 2-3).

Transcriptome-based mapping

Raw Population 17 sequences were trimmed using Trimmomatic. These sequences were aligned to the transcriptome using TopHat version 2.0.14 and used to call variants via SAMtools. A total of 50.25% of Population 17 reads mapped to the transcriptome. A total of 32,506 raw variants were called from 179 individuals. From those raw SNPs, 189 SNPs were multiallelic (specific locus in a genome that contains three or more observed variant alleles), 22,318 were from transitions (substitution of a purine with another purine or a pyrimidine with another pyrimidine), 10,188 were due to transversions (substitution of a purine for a pyrimidine or vice versa), and 130 were indels. Indels are not applicable and were removed from the dataset. There were no consistent or inconsistent repeats and no missing data was observed in this reference-based method. We cleaned our dataset for minimum locus quality score and depth of coverage and 25,044 SNPs remained. The data were filtered for minor allele count leaving 15,605 SNPs.

Also, 9927 SNPs remained when SNPs were cleaned for minor allele frequency. After filtering for heterozygotes and monomorphic loci and SNPs distorted from the expected 1:1 ratio, a total of 932 markers were selected for 179 individuals.

A total of 37.0% of Population 19 reads aligned to the transcriptome. 48,321 raw SNPs were called from 186 individuals. Among these 380 were multiallelic SNPs, 28,087 were due to transitions, 20,234 from transversions, and 118 were indels (Indels removed from the dataset). The dataset was filtered for minQ and minDP leaving 31,755 SNPs. The SNPs were cleaned for minor allele count leaving 16,223 SNPs that passed the criteria. SNPs with minor allele frequency of 0.05 (maf 0.05) left 9655 SNPs. After filtering out missing data, heterozygotes and monomorphic markers based on parent genotype and SNPs distorted from the expected 1:1 ratio across the population, 896 SNPs remained (Table 2-1, Figure 2-3).

Table 2-1: Number of raw and good SNPs in addition to number of SNPs removed in each applied filter from three datasets of GBS-SNP-CROP, UNEAK and TM in both population.

	GBS-SNP-CROP		UNEAK		TM	
	Population 17	Population 19	Population 17	Population 19	Population 17	Population 19
Raw SNPs	16,383	18,713	123,656.00	40,932	32,506	48,321
Distorted Ratio 1:1	420	30	10	20	0	0
non-Polymorphic	6236	4695	5962	2378	1530	2095
Missing & heterozygote parents	8286	12490	2460	4090	7426	6518
maf0.05	0	0	83	34	5678	6568
mac3	0	0	0	1	9439	15532
max-missing %50	0	0	111711	32278	0	0
minDP3	0	0	0	0	0	0
minQ30	0	0	.	.	7269	16173
Indel	0	0	0	0	193	393
Good SNPs	1441	1498	3430	2131	971	1042

***** minQ did not apply for UNEAK dataset, none of SNPs from UNEAK dataset will remain if minQ30 applies.

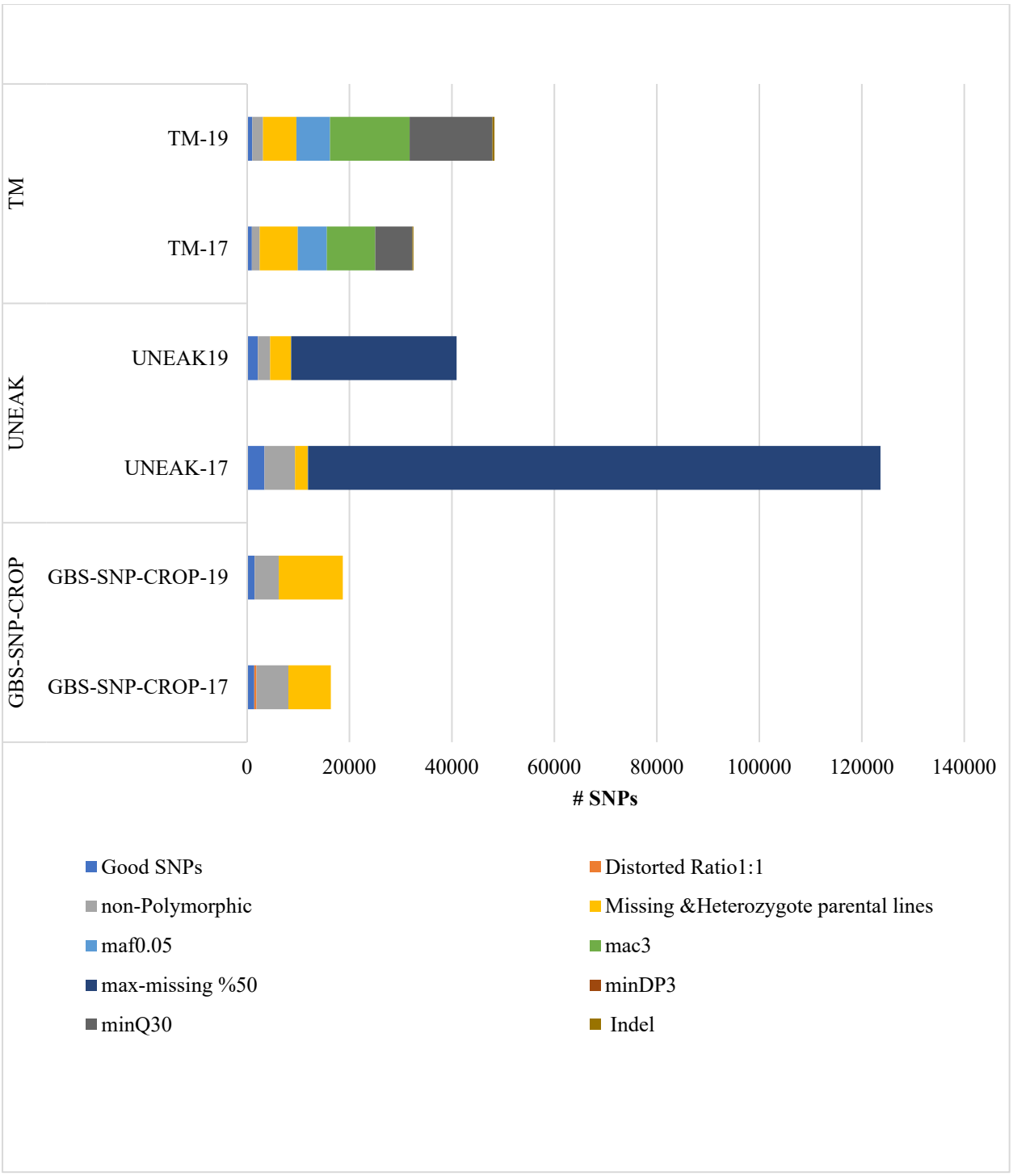


Figure 2-3: Number of SNPs removed for each applied filter in 3 datasets of GBS-SNP-CROP, UNEAK and TM in both population.

Shared tags and SNPs between datasets

Total number of mapped UNEAK tags on the transcriptome in Population 17 were 911,540 while a total of 2,255,550 tags could not be mapped on the transcriptome. In other words, 28.7% of tags from the UNEAK pipeline aligned to transcriptome sequences and 226 shared SNPs were called by SAMtools on 192 individuals (Table 2-2). In Population 19 total number of 1,865,605 UNEAK tags were mapped on transcriptome reads and 4,675,971 UNEAK tags were not mapped resulting in 28.5% of UNEAK tags being mapped. One hundred forty-six SNP markers were shared between these two pipelines across 186 individuals from Population 19.

Eleven percent of the UNEAK tags from Population 17 were mapped on the Mock Reference from GBS-SNP-CROP and 14 shared SNPs were detected between the two pipelines (UNEAK and GBS-SNP-CROP). In Population 19, 1,215,816 UNEAK tags were mapped on the mock reference and 5,325,760 tags remained unmapped. Nineteen percent of the UNEAK tags were mapped on the mock reference and 137 shared SNPs between UNEAK and GBS-SNP-CROP were identified on 186 individuals (Table 2-2).

The total number of reads from GBS-SNP-CROP that mapped to the transcriptome in Population 17 were 107,171,933, while a total of 46,735,845 reads could not be mapped. Sixty-nine percent (69%) percent of the total reads that mapped on the mock reference had overlap with transcriptome references. Three thousand seventy-three shared SNPs were found between these two methods in 192 individuals of Population 17. In Population 19, total number of 104,166,262 reads (which come from bam files and already aligned to mock reference) mapped to the transcriptome, and 153,316,343 of them were unmapped. Forty-one percent of the total

GBS-SNP-CROP reads were mapped on the transcriptome and 4,843 shared SNPs were detected in 186 individuals in Population 19 (Table 2-2).

Table 2-2: Total shared SNPs between three different pipelines

	UNEAK&TM		UNEAK&GBS-SNP-CROP		GBS-SNP-CROP&TM	
Population	#SNPs	Mapped%	#SNPs	Mapped%	#SNPs	Mapped%
Population 17	226	28.7%	14	11.3%	3,073	69%
Population 19	146	28.5%	137	18.5%	4,843	40.5%

Discussion

Thanks to significant advances in next-generation sequencing and high-throughput genotyping technologies, *Pisum sativum* genetics progressed more rapidly to discover molecular bases of agronomical traits and improve breeding. Sequencing large genome of pea with reparative nature would be challenging since there is no reference genome. Genotyping by sequencing is a rapid and simple constructing library approach for next-generation sequencing with low coverage which keeps costs low to achieve a large number of markers. GBS may be considered a good method to dissect pea genome and detect novel and known variants within a given population (Ibeagha-Awemu et al. 2016). However, there are certain drawbacks associated with low coverage including large amount of missing data (Lu et al. 2013). We used methylation sensitive restriction enzyme, *Ape*KI, in library preparation of high GC abundant pea genome (Ellis et al. 2002). The *Ape*KI will not cut if the 3' base of the recognition site on the forward and reverse strands is 5-methylcytosine and avoid repetitive sequence and reduce complexity. Elshire et al. (2011) used e methylation-sensitive enzyme (*Ape*KI) for genotyping large and full of transposable elements maize genome to minimize the repetitive sequences. Knowing the genome

content helps us to determine the most suitable enzyme. For example, in common bean with a small genome, 26% of the genes are located close to the centromere of chromosomes. Ariani et al. (2016) used the methylation insensitive *Cvi*AII enzyme which has more restriction sites present.

To dissect pea genome with no reference available to discover high reliable SNPs, it needs to have a suitable analyzing procedure. To find out which analyzing algorithm to use in pea genome SNP discovery for downstream analyses, we compared two *de novo*- base (UNEAK and GBS-SNP-CROP) and one reference-based (Transcriptome-based mapping) GBS pipelines and also studied their impact on the number of SNPs, accuracy of SNPs and number of missing data in two RIL populations of *P. sativum*. Different GBS analyzing algorithms have different approaches to compensate for low coverage and improve the statistical analysis of read loss by filtering out sequencing errors more effectively. Pipelines with different methods for SNP calling can produce distinct groups of SNPs, to the degree that in all cases, less number of SNPs were shared. Although, there was high number of SNPs in all three pipelines, UNEAK showed a higher number of SNPs and higher missing SNP percentage across populations. It might be due to less quality control in UNEAK in selecting contigs which lead many inferior quality contigs contribute to tag alignments. UNEAK methodology to control false SNPs instead of quality check is based on how many times tags are observed in the dataset (Lu et al. 2013). UNEAK uses population genetics parameters of minor allele frequency to filter out false SNPs due to sequencing error (Glaubitz et al. 2014). UNEAK is most commonly used pipeline in non-reference GBS studies which mostly applied in pea genome GBS sequencing data as well (Jha et al. 2017; Ma et al. 2017; Annicchiarico et al. 2017). Holdsworth et al. 2017 used union of two none-reference UNEAK and Stacks pipelines SNPs to assess genetic diversity and GWAS of pea

single plant collection (Holdsworth et al. 2017). *De novo* assemblies are not biased toward a reference genome and uses all the available sequences to creates larger number of SNPs with novel contigs from no prior knowledge.

The GBS-SNP-CROP pipeline has a high-quality trimming control and reads are trimmed based on phred quality score and read length to increase the accuracy of analysis. Although, quality control is not able to reduce PCR errors, it still reduces random sequencing errors and improves the accuracy of the analysis (Eren et al. 2013). Transcriptome-based mapping used the same quality trimming criteria and had a benefit of reference-based pipelines as well. Reference-based pipelines have more access to a larger sequence around SNPs for validation and annotation of SNPs and more possibility of finding high quality variants (Berthouly-Salazar et al. 2016). On the other hand, transcriptome-based mapping is biased toward transcriptome and only browses coding regions of DNA, while the other two pipelines scan both coding and non-coding DNA (Torkamaneh et al. 2016). Almost half of the reads in both populations were mapped to the transcriptome (*P. sativum*_CSFL_RefTrans V1) and showed despite using methylation sensitive enzyme some of the repeated sequences and non-coding areas were still in our dataset. Transcriptome-based SNP discovery has been already used in GBS data sequencing analyzing of alfalfa (*Medicago sativa*) (Hawkins and Yu 2018), and olive (*Olea europaea*) (Ipek et al. 2017). GBS-SNP-CROP took a fortnight to create a mock reference once using 100% of read samples, while each UNEAK plugin takes couple of hours.

To find overlapping markers between different methods or different populations, there should be a universal marker name. Reference-based pipelines name SNPs based on alignment position which simplify the comparisons process. But in *de novo*-based pipelines naming is based on arbitrary numbers to markers as there is no other information for naming loci. SNPs

comparison between the UNEAK pipeline and two others requires tags or reads comparisons. Low percentage of UNEAK tags were aligned to the mock reference than transcriptome reference and number of shared SNPs were lower when UNEAK tags aligned to the mock reference than transcriptome as well. We expected the number of overlap reads and SNPs to be low when it comes to comparison between pipelines since they follow different algorithms to call SNPs. But surprisingly, the total number of overlapped reads and shared SNPs of GBS-SNP-CROP and the transcriptome-base method were significantly high. SNPs which are called by more than a single pipeline are mostly more precise than SNPs called by only one pipeline (Torkamaneh et al. 2016). Berthouly-Salazar et al. (2016) compared transcriptome-based mapping with UNEAK in GBS sequencing data to analyze population structure and diversity of pear millet (Berthouly-Salazar et al. 2016). They found similarly high number of SNPs in both pipelines and 8% of UNEAK tags mapped to transcriptome contigs which confirms our data (Berthouly-Salazar et al. 2016).

Although, GBS can be a cost-effective method for calling and mapping of large numbers of SNPs for pea genome, the quality of the relative linkage maps is slightly restricted by genotyping errors and missing data (due to low coverage). To control this limitation to obtain more accurate and complete data for mapping population, we need to choose a good analyzing approach for SNPs calling. Pipelines with differing algorithm for SNP calling can yield different sets of SNPs (Holdsworth et al. 2017). In this study, we tried to show the effect of three different pipelines on pea GBS data for SNPs discovery. Although, UNEAK can produce more SNPs, their quality is under question. Percentage of missing data in the UNEAK pipeline is more compared to GBS-SNP-CROP or TM. Also, transcriptome-based SNP calling was biased toward coding regain and had slightly smaller number of SNPs compared to other two pipelines

(Berthouly-Salazar et al. 2016). In consideration of the foregoing, we chose GBS-SNP-CROP pipeline that produced large number of high-quality SNPs for future pea populations mapping.

CHAPTER 3: PHENOTYPING OF TWO DIFFERENT POPULATIONS OF *PISUM SATIVUM* FOR RESISTANCE TO WHITE MOLD

Abstract

White mold caused by *Sclerotinia sclerotiorum* is an important constraint to field pea (*Pisum sativum* L.) production worldwide. To breed resistant pea cultivars to *Sclerotinia*, development of a reliable phenotyping method is required to identify resistant pea germplasm. Two partially resistant plant introductions were crossed with susceptible pea cultivars to create two recombinant inbred line populations. Population 17 was developed by crossing PI240515 with Lifter followed by single seed decent to the F_7. Population 19 was created by crossing PI169603 with Medora followed by single seed decent to the F_7. Both populations were screened in the greenhouse as 11 day old plants and inoculated with mycelial agar plugs using a dental amalgam carrier. To avoid height variation as a confounding factor, genotypes were separated into short and tall categories for analysis. None of the individuals showed complete resistant, however, 22 short genotypes demonstrated partial resistance based on having at least two resistance criteria in the greenhouse evaluation and detached stem assay. Only two pea genotypes with quantitative partial resistance to *S. sclerotiorum* (PRIL19-18 and PRIL19-124) had both afila and short internode. There was no significant correlation between pea stem strength and lesion expansion measured in the greenhouse evaluation.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a hemi-biotrophic fungus that attacks more than 400 plant species, including pulse crops like field pea (*Pisum sativum* L.). White mold, caused by *S. sclerotiorum*, is one of the most damaging diseases for most dicotyledonous crops. White mold preferably develops in cool, cloudy, wet, and humid weather during flowering

(Mueller 2014). Narrow row spacing, and an early canopy closure typically by long-vine plants also make the condition ideal for mycelium germination and development of disease (Kraft and Pfleger 2001). The pathogen infects the stem, leaf or pod tissue of plants and lesions are water-soaked and produces a white cottony mass of mycelium on the surface (Mueller 2014).

Annual losses from *S. sclerotiorum* in six crops participating in the ARS National Sclerotinia Research Initiative (sunflower, soybean, dry bean, canola, lentil and pea crops) is high as $482 million in the United States (USDA-ARS 2016). White mold, is a major barrier to field pea production in the Northwest and Midwest areas of the USA and worldwide (Porter et al. 2009). Limited options for agronomic management of white mold requires development of resistant varieties of field pea to *S. sclerotiorum* as an efficient, economic and environment-friendly means of control (Peltier et al. 2012; Jain et al. 2012, Fernando et al. 2004).

Identifying sources of resistance and characterizing the mode of inheritance is crucial to the success of resistance breeding. Studies on sunflower show that resistance to *S. sclerotiorum* is quantitatively inherited, including many genes with small contributions to partial resistance (Gentzbittel et al. 1999; Davar et al. 2013). Similarly, the polygenic nature of the trait in field pea showed that resistance to *S. sclerotiorum* is expressed in two forms, lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI) and we call them lesion expansion and nodal resistance respectively from now on. LEI can slow the rate of lesion progression while NTI blocks the path of the pathogen through the node (Porter et al. 2009). Traits such as thick stem diameter, short internode and leaf morphology (semi-leafless), could be important in white mold-resistant cultivars (Porter et al. 2009). In this case, identifying novel sources of quantitative partial resistance to *S. sclerotiorum* in Pisum germplasm collections would be valuable to pea breeders. To develop resistant cultivars, germplasm should be screened to find individuals with

partial resistance to *S. sclerotiorum.* Research conducted on eleven-day-old plants using the colonized oat kernel inoculation method in greenhouse identified 39 plant identification accessions from the Pisum collection with quantitative partial resistant (Blanchette and Auld 1978). Porter et al. (2009) screened 504 pea accessions from the Pisum core collection in the greenhouse and laboratory by mycelial agar plugs technique. Five accessions (Plant Introduction 103709, PI166084, PI169603, PI240515 and PI270536) showed highly quantitative partial resistance based on nodal resistance of 1 or greater, and survival of 50% or greater. In another study 848 accessions across the world were screened and identified 13 accessions with both LEI and NTI with partial resistance to *S. sclerotiorum*. Eight of these genotypes were not reported by Porter et al. (2009) (Tashtemirov 2012).

To finding new sources of resistance breeders and to develop cultivars with resistance to *S. sclerotiorum* in field pea, PI169603 and PI240515 (with quantitative partial resistance to LEI and NTI from Porter study) were crossed with two susceptible pea cultivars, Medora and Lifter, respectively. Screening methodology is important since the environment has a profound impact on the expression of the trait (Ender and Kelly 2005). The objective of this research is to develop a reliable screening method and find novel sources with substantial level of partial resistance to white mold within two populations that could be used in pea breeding program and future QTL mapping.

Material and methods

Plant materials

Two different recombinant inbred lines (RIL) populations of field pea were used in this study. Population 17 had 192 F_7 individuals derived from the cross Lifter/PI240515 and developed by single seed decent. Population 19 was developed by single seed decent and

comprised a set of 324 F_7 genotypes derived from the cross PI169603/Medora. Lifter and Medora are susceptible to *S. sclerotiorum* while PI240515 and PI169603 have partial resistance reactions (Porter et al. 2009). Lifter was developed by the USDA-ARS in cooperation with Washington Agricultural Research Centre Pullman, WA, and the Idaho Agricultural Experiment Station, Moscow, ID (McPhee and Muehlbauer 2002). Lifter has short internodes with normal leaf morphology, white flowers and green seed. Medora was developed at the Spillman Research Farm near Pullman, WA, and released in 2007 for production in the Midwest region (GLGP 2007). Medora has short internode with afila leaf morphology, white flowers and smooth green seed color. PI240515 is an exotic accession from India with long internodes, normal leaf types, white flowers and yellowish green seed color (https://npgsweb.ars- grin.gov/gringlobal/accession detail.aspx? id=1187486). The PI169603 originated in Turkey and has long internodes, normal leaf type, white flowers and yellow seed color (https://npgsweb.ars- grin.gov/gringlobal/accessio ndetail.aspx?id=1145629). PI240515, and PI169603 were identified in 2009 as partial resistance genotypes (Porter et al. 2009).

Plant preparation

A single pea seed of each genotype was planted at a depth of 1 cm in PRO-MIX® LP 15 potting media (Premier Tech Horticulture, Quakertown, PA) contained in an 11.4 cm square plastic pot. Plants were grown under greenhouse conditions for 14 days with natural sunlight supplemented, 600-Watt Pressure Sodium Lamps (P. L Light Systems, Inc., Beamsville, Ontario, Canada) to maintain a 16:8 hour photoperiod and temperatures from 20-25°C in day time and 20°C in nighttime.

Inoculum preparation

S. sclerotiorum sclerotia of isolate Sc102 were obtained from pea cultivar named 'Snake' in 2003 by Dr. Lyndon Porter in Quincy, WA, and used to screen the genotypes. Sclerotia were kept at 4ºC until used. To break sclerotia dormancy, we placed them in a 10% bleach solution for 20 minutes followed by 3 rinses with sterile distilled water. The rinsed sclerotia were put in 95% ethanol for one minute, removed and briefly flamed. Surface sterilized sclerotia were cultured on sterile composite agar (CA) media containing Difco™ PDA and Difco™ oatmeal agar for three days prior to inoculation. Mycelium from the leading edge of the colony was used to inoculate pea genotypes. CA media was prepared by mixing 18.5 g Difco™ PDA and 8.75 Difco™ oatmeal in 0.5 L distilled water and autoclaved at 121ºC for 20 min (Tashtemirov 2012). Pasteurized media was poured into 100 × 15 mm petri dishes in a laminar flow hood and allowed to cool. Each petri dish contained 20 ml of media and was sealed with parafilm and placed in complete darkness with 21-23ºC until the fungus colonized half the surface of the agar (Figure 3-1b).

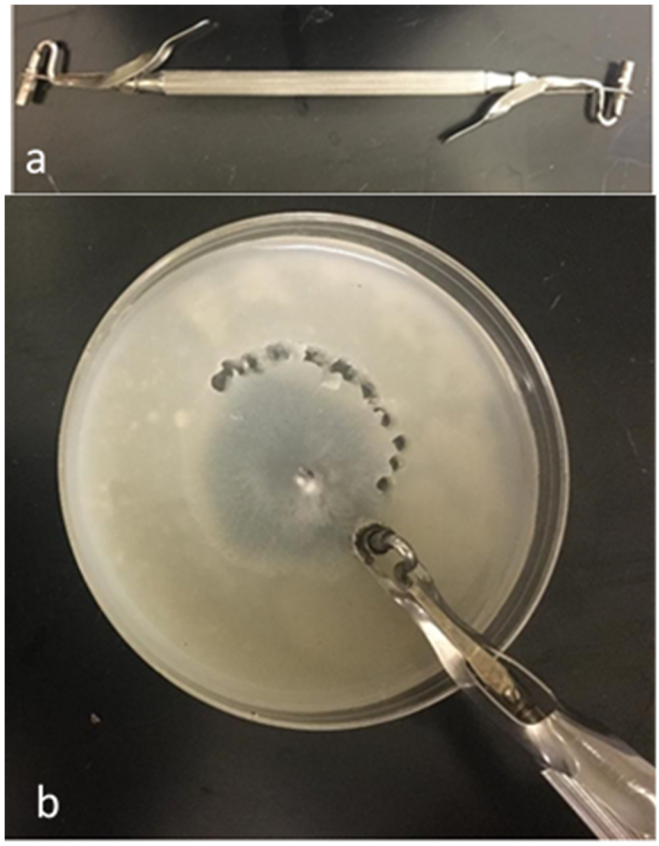


Figure 3-1: A pulpdent amalgam carrier (a) and an image showing collection of mycelium from the leading edge of the fungal mat (b).

Greenhouse evaluation

Fourteen-day old plants were inoculated by placing the colonized media plug in the leaf axis at the 4th node using a jumbo-sized pulpdent amalgam carrier (Figure 3-1a) (Safco Dental Supply Co., USA). The 4th node was defined as the fourth node above the soil surface (Figure 3-2c). After inoculation, plants were transferred to a mist chamber with 100% humidity and temperature ranging from 19 to 21°C for 3 days in the dark (Figure 3-2a). White mold lesions were measured 3 days after inoculation with a digital caliper (Figure 3-3b).

Plants were removed from the mist chamber after 3 days and placed in a mist room with 80% humidity and 14:10 day: night photoperiod with supplemental lighting from 400-Watt High Pressure Sodium Lamps (P. L Light Systems, Inc., Beamsville, Ontario, Canada) for another 11

days (Figure 3-4a). Lesion expansion and nodal resistance were measured at seven and 14 days post inoculation. NTI was scored on a scale of 0-4 (Table 3-1).

Table 3-1: Nodal transmission inhibition scale for *S. sclerotiorum* progress in *P. sativum* (Porter et al. 2009)

Scale	NTI development of white mold
0	Plant did not survive
0.5	Lesion expanded down the stem from the 4th inoculated node to the internode between root and 1st node
1	Lesion expanded down the stem from the 4th inoculated node to the 1st node
1.5	Lesion expanded down the stem from the 4th inoculated node to the internode between 1st and 2nd node
2	Lesion grew from the 4th to the 2nd node
2.5	Lesion expanded down the stem from the 4th inoculated node to the internode between 2nd and 3rd node
3	Lesion extended from the 4th to the 3rd node
3.5	Lesion expanded down the stem from the 4th inoculated node to the internode between 3rd and 4th node
4	Lesions did not develop from the initial inoculation spot at the 4th node

The experiment was arranged in a randomized complete block (RCBD) with 4 replicates in each run and the whole experiment was repeated 3 times. 184 genotypes from Population 17 and 123 individuals from Population 19 were used in this experiment with 5 repeated checks comprising both parents, Stirling (susceptible), Bohatyr and Shawnee in each set. We analyzed 62 genotypes plus 5 checks in each set, overall took 15 sets to screen 184 individuals of Population 17 and 123 individuals of Population 19. Mean lesion expansion and nodal resistance score were calculated using ANOVA and PROC MIXED procedures in SAS Enterprise Guide 7.1 (SAS Institute Inc. USA). In the statistical analysis, genotypes were assumed as fixed effects, while runs, replication within runs, and run per genotype were treated as random effects. Multiple observations of 3, 7 and 14 day post inoculation lesion progress were used to calculate

area under disease progress curves (AUDPC) in Excel for each genotype with following formula while y is disease level at different time (t) (Simko and Piepho 2012).

$$AUDPC = \sum_{i=1}^{N_i-1} \frac{(y_i+y_{i+1})}{2}(t_{i+1} - t_i)$$

Figure 3-2: Whole plant screening of pea populations for reaction to *S. sclerotiorum*, a) 14-day old inoculated plants were maintained in a mist chamber for 3 days in darkness, b) Infected pea plants with white fluffy mycelial growth 3 days post inoculation, c) Lesion of an inoculated plant expanded from 4th node down to 3rd node 3 days post inoculation.

Resistant genotypes to white mold were identified based on having one or more of the three criteria. Those criteria include mean lesion expansion 3 days post inoculation equal or less than 25 mm, mean nodal resistance after 2 weeks equal or greater than 1, and survival rate of 25% or greater.

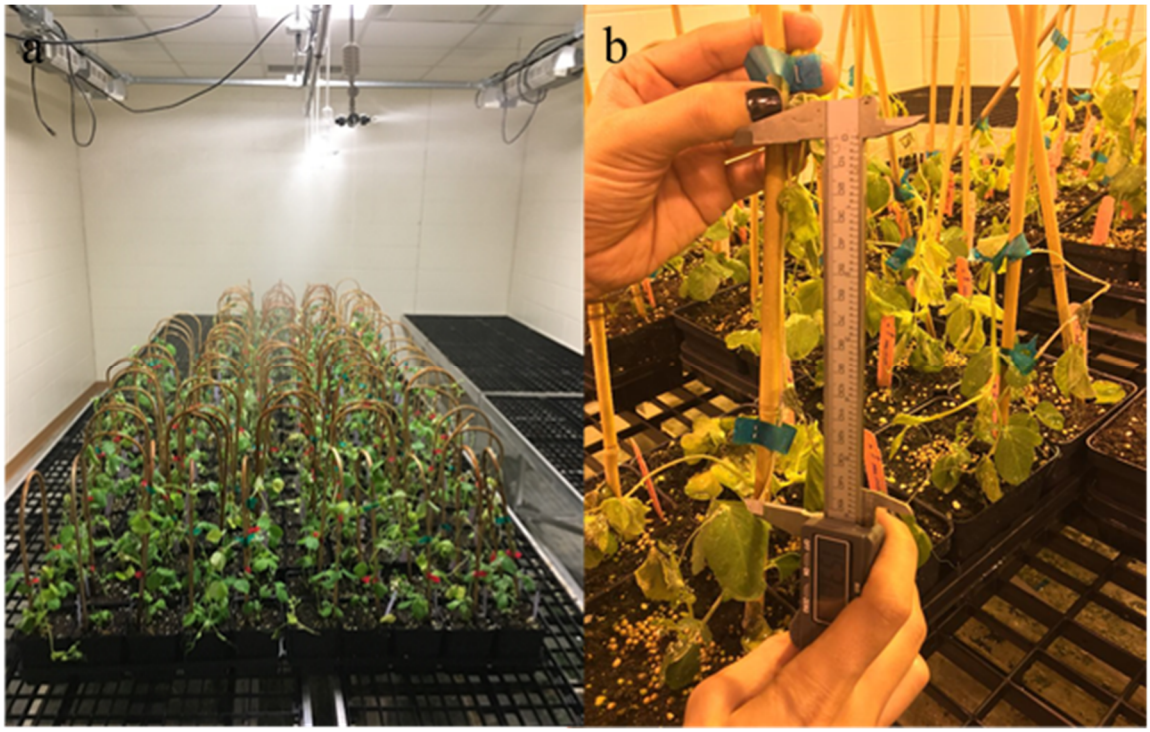


Figure 3-3: Plant maintenance in a mist chamber, a) and measurement of disease progression using a digital caliper.

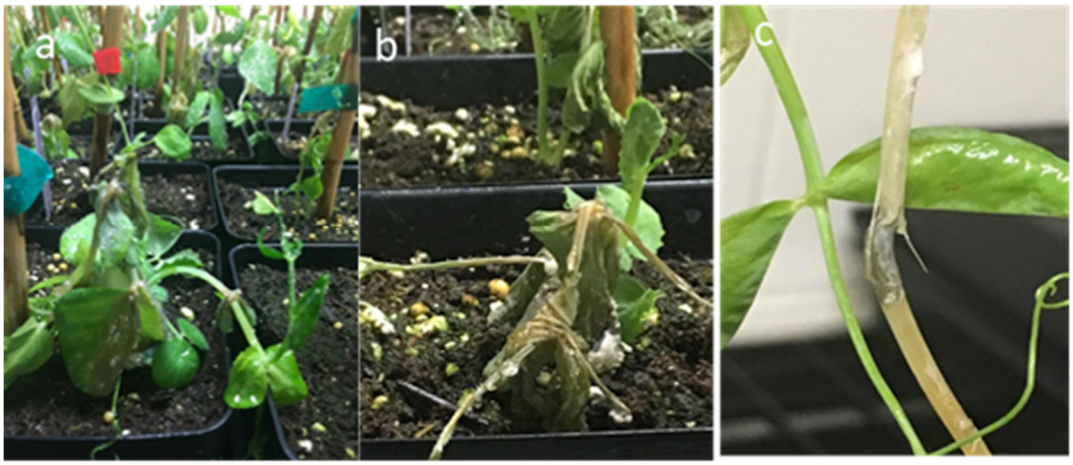


Figure 3-4: Disease progression on pea plants in controlled condition, a) Infected pea plant after 7 days, b) infected pea plant after 14 days, and c) sclerotia seen inside the infected stem.

Detached stem assay

A detached stem screening method was developed to quantify stem resistance of *P. sativum* genotypes to *S. sclerotiorum* under controlled conditions. Approximately 7 cm of stem beginning 1 cm above the 5th node was collected from each plant using a scalpel. The stem at the 5th node was pressed into infected agar plugs. The inoculated stems were placed on a plastic tray (28× 54.3 cm) covered with germination paper of PG1218 30.5×45.7cm (anchorpaper.com). Trays were placed in a mist chamber in the dark for 3 days at 21-25°C. Lesion expansion (mm) was measured 3 days after inoculation (Figure 3-5a). Stems were arranged in a RCBD with 4 replicates in each run and the whole experiment was repeated 2 times. One hundred seventy-nine genotypes from Population 17 and 137 genotypes from Population 19 were used in this experiment. Both parents and Stirling were used as checks. Means for lesion expansion were calculated using ANOVA and PROC Mixed procedures in SAS Enterprise Guide 7.1 (SAS Institute Inc. USA). In the statistical analysis, genotypes were assumed as fixed effects, while runs, replication within runs, and run per genotype were treated as random effects.

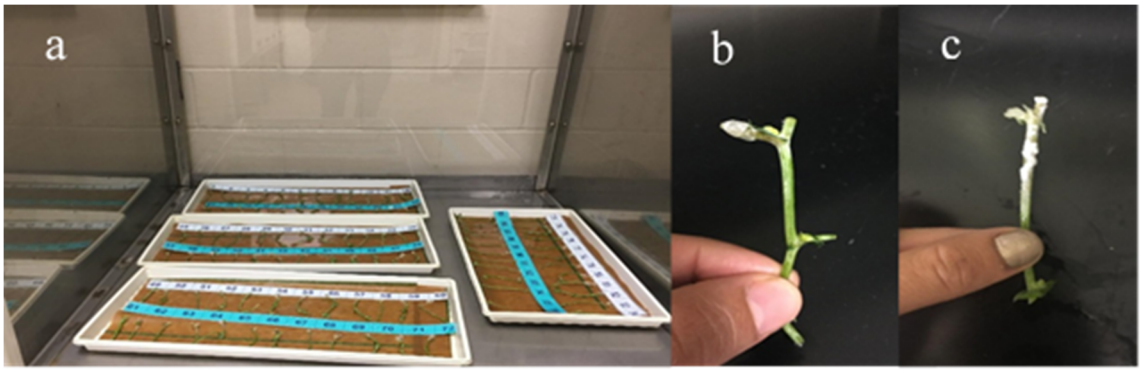


Figure 3-5: Trays containing inoculated stems were transferred to a mist chamber for 3 days in the dark (a), stem inoculated at the axillary branch of the 5th node (b), lesion growth after 3 days (c).

Texture analyzing

Mature stems of 188 individuals from Population 19 were cut from the field at harvest in 2014. Four stems from each genotype were analyzed for hardness using a CT-3 Texture Analyzer (www.brookfieldengineering.com) with a TA53 probe (Figure 3-6) and fixture of TA-BT-KIT. Machine parameters were set at a test speed of 1.00 mm/s and trigger load of 0.10 N with target distance of 10 mm. Stems were cut with the TA53 blade in the center of the 5th internode. Mean hardness recorded in Newtons for each stem was analyzed using PROC GLM SAS Enterprise Guide 7.1 (SAS Institute Inc. USA).

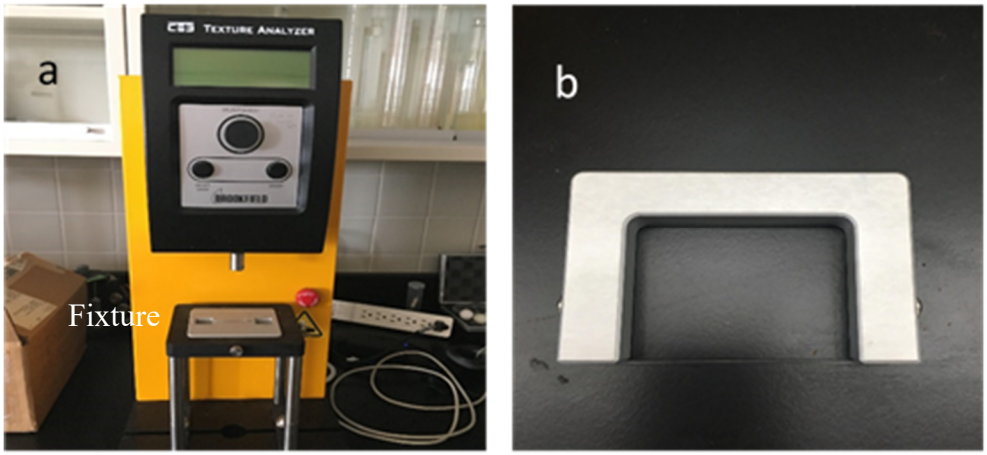


Figure 3-6: Instruments used to evaluate stem strength, a) CT-3 Texture analyzer b) TA53 blade.

Results

Greenhouse evaluation

Population 17 had 81 short internode lines and 104 long internode lines. Also, PRIL17-134 was variable for plant height. Among the 122 genotypes of Population 19 that were analyzed, 54 had short internodes and 68 had long internodes. Population 19 also segregated for leaf type normal leaflets vs. semi-leafless. Seventy-five lines had the semi-leafless character and 46 lines were both short and semi-leafless. Analysis of variance of checks across the runs

showed no significant difference (P>0.05) (Table 3-2, Table 3-3), suggesting the data for different runs could be combined for analysis for each population.

There was positive correlation between lesion expansion and nodal resistance and height (Table 3-4, Table 3-5). This may suggest that height might be a confounding factor and may lead us to an erroneous conclusion, therefore, short and long internode individuals of each population were analyzed separately.

Table 3-2: ANOVA of lesion expansion (mm) at 3 days post inoculation for five check genotypes (Lifter, PI240515, Stirling, Bohatyr and Shawnee) tested with Population 17 across three experimental runs.

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Rep	3	567.4	189.2	1.29	0.3606 ^{ns}
Run	2	204.4	102.2	0.38	0.7012 ^{ns}
Genotype	3	382	127.3	0.52	0.6856 ^{ns}
Rep(Run)	6	881.3	146.9	1.26	0.2979 ^{ns}
Run*Genotype	6	1478.8	246.5	2.12	0.0742 ^{ns}
Error	37	4302.8	116.3		

^{ns} not significant

Table 3-3: ANOVA of lesion expansion (mm) at 3 days post inoculation for five check genotypes (Medora, PI169603 Stirling, Bohatyr and Shawnee) tested with Population 19 across three experimental runs.

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Rep	3	693.5	231.2	1.81	0.2449 ^{ns}
Run	2	41.1	20.6	0.20	0.8306 ^{ns}
Genotype	3	2247.2	749.1	9.05	0.0120**
Rep(Run)	6	765.8	127.6	1.20	0.3264 ^{ns}
Run*Genotype	6	496.6	82.8	0.78	0.590 ^{ns}
Error	37	3924.4	106.1		

^{ns} not significant,
**p<0.01.

Table 3-4: Pearson's correlation coefficient for correlations between lesion expansion, nodal resistance and plant height for Population 17. *dpi: days post inoculation. N=2268

	Lesion expansion (3 dpi*)	Lesion expansion (7 dpi)	Nodal resistance (7 dpi)	Lesion expansion (14 dpi)	Nodal resistance (14 dpi)
Height	0.28**	0.56**	0.48**	0.75**	0.60**

**p<0.01

Table 3-5: Pearson's correlation coefficient for correlations between lesion expansion, nodal resistance and plant height for Population 19. *dpi: days post inoculation. N=1531.

	Lesion expansion (3 dpi*)	Lesion expansion (7 dpi)	Nodal resistance (7 dpi)	Lesion expansion (14 dpi)	Nodal resistance (14 dpi)
Height	0.45**	0.60**	0.55**	0.72**	0.61**

**p<0.01

Table 3-6: ANOVA of lesion expansion (mm) at 3 dpi for 185 individuals of Population 17 across 3 experimental runs.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	3	2031.9	677.3	0.8	0.5278 ns
Rep	2	3776.1	1888.1	2.2	0.1855 ns
Genotype	183	268585	1467.7	11.6	<.0001***
Rep(Run)	6	4946.2	824.4	8.3	<.0001***
Genotype*Run	366	46268	126.1	1.3	0.0011**
Error	1648	167877.7	101.9		

ns not significant,
**p<0.01
***p<0.0001.

Table 3-7: ANOVA for lesion expansion (mm) at 3 dpi for 123 individuals of Population 19 across 3 experimental runs.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Run	2	750.2	375.1	0.3	0.7588 ns
Rep	3	11994	3998.0	3.2	0.1025 ns
Genotype	122	394904	3236.9	13.7	<.0001***
Rep(Run)	6	7397	1232.8	7.4	<.0001***
Genotype*Run	244	57852	237.1	1.4	0.0001***
Error	1101	189195	171.8		

ns not significant,
***p<0.0001.

Table 3-8: Pearson's correlation coefficient and *p*-value for correlations between lesion expansion, nodal resistance data in short subset of Population 17 and Population 19.

	Population 17 N=2256		Population 19 N=1531	
	Lesion expansion (7 dpi[a])	Lesion expansion (14 dpi)	Lesion expansion (7 dpi)	Lesion expansion (14 dpi)
Nodal resistance (7 dpi)	-0.09 **		-0.14 ***	
Nodal resistance (14 dpi)		-0.18 ***		-0.23 ***

[a] day post inoculation,
**p<0.01,
***p<0.0001

The ANOVA results (Table 3-6, Table 3-7) show significance level of differences of lesion expansion (3 dpi) mean in most variables in green house evaluation. Short and long individuals of each population were separated. Genotypes with partial resistance were identified using the criteria of Porter et al. (2009). Analysis of the short internode subset of Population 17 demonstrated that 6 individuals had lesion expansion equal or less than 25 mm. Thirty-three short individuals of Population 17 had survival rate ([height-lesion expansion in 14 dpi/height] ×100) of equal or more than 25% and 47 genotypes showed nodal resistance equal or greater than 1. The AUDPC ranged from 486.9 to 2269.9 in short varieties of Population 17 (Table A-1). The AUDPC of Population 17 parents, Lifter and PI240515, were 776.7 and 1119. Only PRIL17-181 among the short internode subset of Population 17 showed all three of Porter's resistant criteria together and low AUDPC (486.9) and sixteen short individuals of Population 17 showed two of the resistant criteria together. Pearson correlation coefficient between lesion expansion and nodal resistance shows a negative correlation in the short subset of Population 17 and Population 19 (Table 3-8).

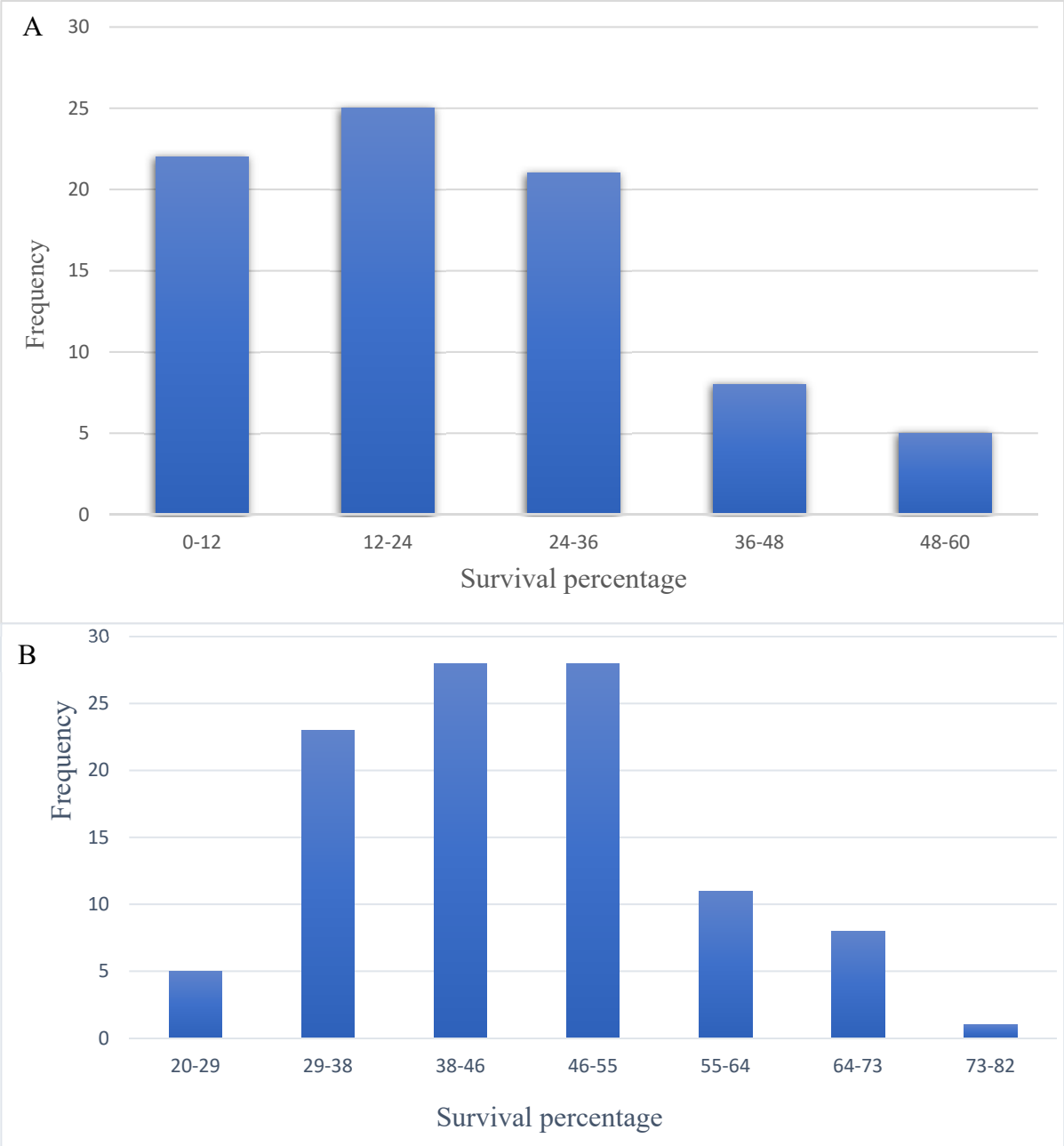
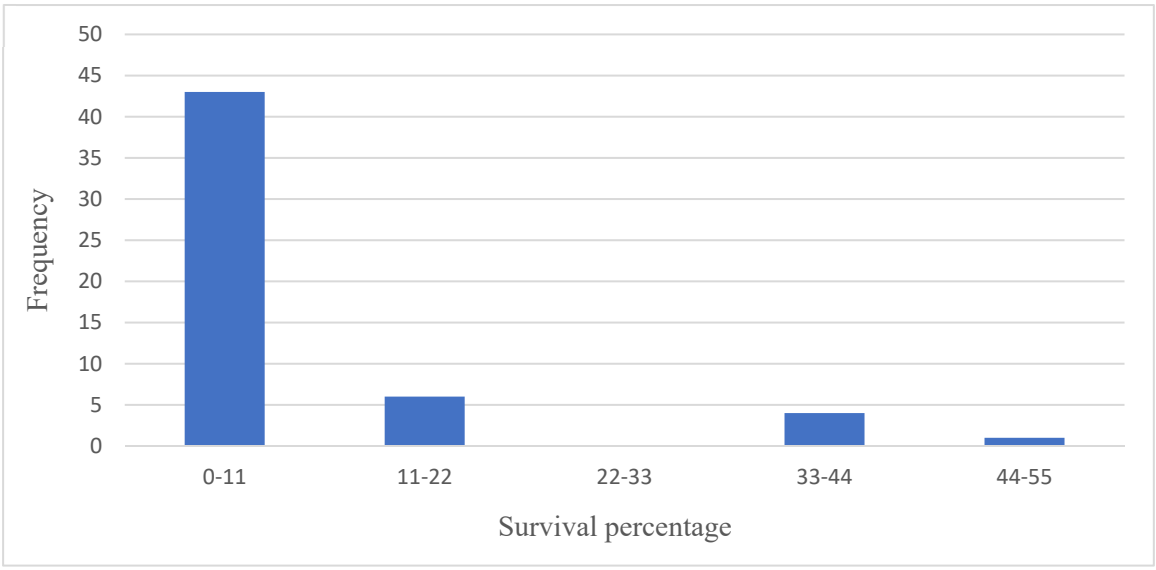


Figure 3-7: Histogram for mean survival percentage recorded 14 dpi for 81 short internode individuals of Population 17 challenged with *S. sclerotiorum* (a) and mean survival percentage recorded 14 dpi for 102 individuals in the tall subset of Population 17 (b).

A



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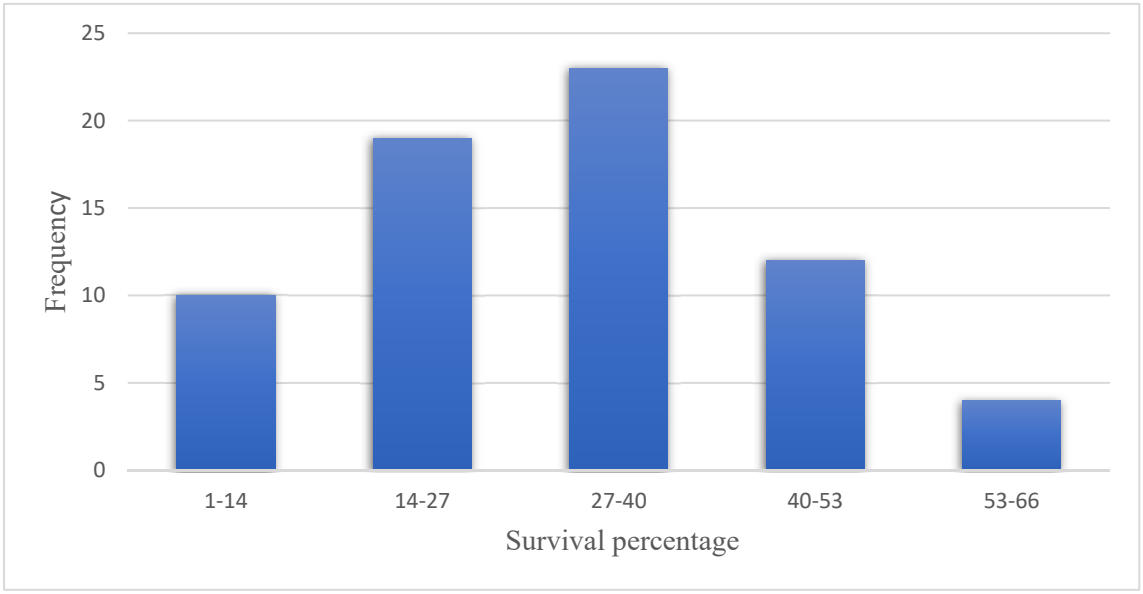

Figure 3-8: Histogram of mean for survival percentage recorded 14 dpi for 54 short individuals of Population 19 with *S. sclerotiorum* (a) Histogram of mean of survival percentage recorded 14 dpi for 68 individuals in tall subset of Population (b)

Table 3-9: Descriptive statistics of lesion expansion 3 day post inoculation of short and long internode subsets of Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	34.0	44.7	42.8	61.2
Std. Dev.	10.6	16.1	15.4	20.9
Std. Error	0.3	0.5	0.6	0.7
Variance	113.1	258.5	236.0	435.3
Minimum	9.7	9.6	14.5	0.8
Maximum	80.1	105.5	91.0	121.0

Table 3-10: Descriptive statistics of lesion expansion 7 day post inoculation of short and long internode subsets of Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	60.0	116.6	85.0	132.7
Std. Dev.	28.5	51.7	36.2	43.8
Std. Error	0.9	1.5	1.5	1.5
Variance	813.3	2668.5	1311.0	1916.3
Minimum	9.7	13.5	14.7	9.6
Maximum	247.0	282.0	290.0	297.0

Table 3-11*:* Descriptive statistics of lesion expansion 14 day post inoculation of short and long internode subsets of Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	82.8	192.3	119.1	200.1
Std. Dev.	39.5	76.9	60.6	66.9
Std. Error	1.3	2.2	2.4	2.4
Variance	1557.0	5917.8	3669.8	4476.2
Minimum	9.7	13.5	30.0	21.5
Maximum	315.0	420.0	531.0	490.0

Table 3-12: Descriptive statistics of nodal resistance 7 day post inoculation of short and long internode subsets of Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	2.2	3.3	1.6	2.7
Std. Dev.	1.1	0.4	0.9	0.6
Std. Error	0.0	0.0	0.0	0.0
Variance	1.2	0.2	0.9	0.3
Minimum	0.0	1.0	0.0	0.0
Maximum	4.0	4.0	3.5	4.0

Table 3-13: Descriptive statistics of nodal resistance 14 day post inoculation of short and long internode subsets of Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	1.3	2.8	0.3	1.8
Std. Dev.	1.4	0.7	0.7	1.0
Std. Error	0.0	0.0	0.0	0.0
Variance	1.8	0.6	0.5	1.1
Minimum	0.0	0.0	0.0	0.0
Maximum	4.0	4.0	3.5	3.5

Table 3-14: Descriptive statistics of survival rate of short and long internode subsets Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	21.7	45.6	6.17	29.2
Std. Dev.	24.9	19.8	14.7	21.7
Std. Error	0.8	0.6	0.6	0.8
Variance	620.8	390.9	216.5	472.5
Minimum	0	0	0	0
Maximum	96	96.9	88.1	95.7

Table 3-15*:* Descriptive statistics of AUDPC of short and long internode subsets Population 17 and Population 19.

	Population 17		Population 19	
Test statistic	Short	Tall	Short	Tall
Mean	1105.4	1918.9	1327.5	2239.3
Std. Dev.	436.9	523.1	259.3	462.8
Std. Error	47.7	52.3	37.0	54.5
Variance	190899.0	273610.8	67236.0	214147.6
Minimum	486.9	412.2	823.7	1106.8
Maximum	2269.9	3594.1	3047.5	3306.3

Among long internode genotypes of Population 17 only 4 genotypes restricted lesion expansion after 3 days post inoculation (≤ 25 mm). However, all long internode genotypes of population 17 showed nodal resistance (≥ 1) and 101 genotypes met the survival rate criteria (≥25%). The AUDPC for tall individuals of Population 17 ranged from 412.2 to 3594.1 (Table 3-15, Table A-2). Only four tall genotypes from Population 17 showed all three criteria together for partial resistance to *S. sclerotiorum* and from those PRIL17-28 also showed low AUDPC of 412.2 (Table A-2).

Analysis of the short internode subset of Population 19 displayed that one individuals had lesion expansion equal or less than 25 mm, five short individuals had survival rate of equal or more than 25% and five genotypes showed nodal resistance equal or greater than 1. The AUDPC range was from 823.7 to 3047.5 in short varieties of Population 19 (Table 3-15,Table A-3). None of the short individuals of Population 19 showed all three criteria and five of them showed two Porter criteria for *S. sclerotiorum* partial resistance.

Twenty-two individuals from short genotypes of Population 17 and 19 showed partial resistance and at least two of the Porter criteria in the greenhouse evaluation and two of them (PRIL19-18 and PRIL19-124) had afila leaf type feature as well (Table 3-17).

Analysis of long internode subset of Population 19 revealed that none of the individuals had lesion expansion equal or less than 25 mm. Fifty-six of the tall subset of Population 19 demonstrated nodal resistance equal or greater than 1 and Forty-nine tall individuals showed survival percentage of equal or more than 25%. The AUDPC for tall individuals of Population 17 ranged from 1106.8 to. 3306.3 (Table A-4).

Detached stem assay

Population 17 and Population 19 genotypes were rescreened using a detached stem assay. Stirling was included as a repeated check in each run. Levene's homogeneity test for lesion expansion (3 dpi) variance of checks plants for each population among experimental runs were calculated to justify combining all experimental and showed no significant difference among runs (Population 17 p= 0.1, Population 19 p= 0.06). Lesion expansion after 3 day post inoculation averaged 32.1 mm and ranged from 13.1 mm to 57.8 mm in Population 17, while lifter and PI240515 lesion expansion were 35.2 and 41 mm respectively (Figure 3-9). The lesion expansion of Population 19 averaged 24.3 mm and ranged from 5.0 mm to 61.6 mm while Medora and PI169603 lesion expansion were 51.7 and 41.6 mm respectively (Figure 3-9). The average of lesion expansion of Stirling as a susceptible check was 33.3 mm. Thirty-two genotypes from Population 17 and eighty-six genotypes from Population 19 demonstrated lesion expansion of 25 mm or less. From those six genotypes had short internode and demonstrated at least two of the partial resistance criteria on greenhouse evaluation (RIL17-129, PRIL19-18, PRIL19-74, PRIL19-86, PRIL19-124, PRIL19-127) (Table 3-17).

Rescreening the genotypes by stem assay showed that responses to *S. sclerotiorum* among Population 17 had no significant correlation with the greenhouse experiment (Pearson's

r= 0.13, p-value=0.09). There was no significant correlation between detached stem assay and greenhouse experiment of Population 19 as well (Pearson's r= 0.08, p-value=0.41).

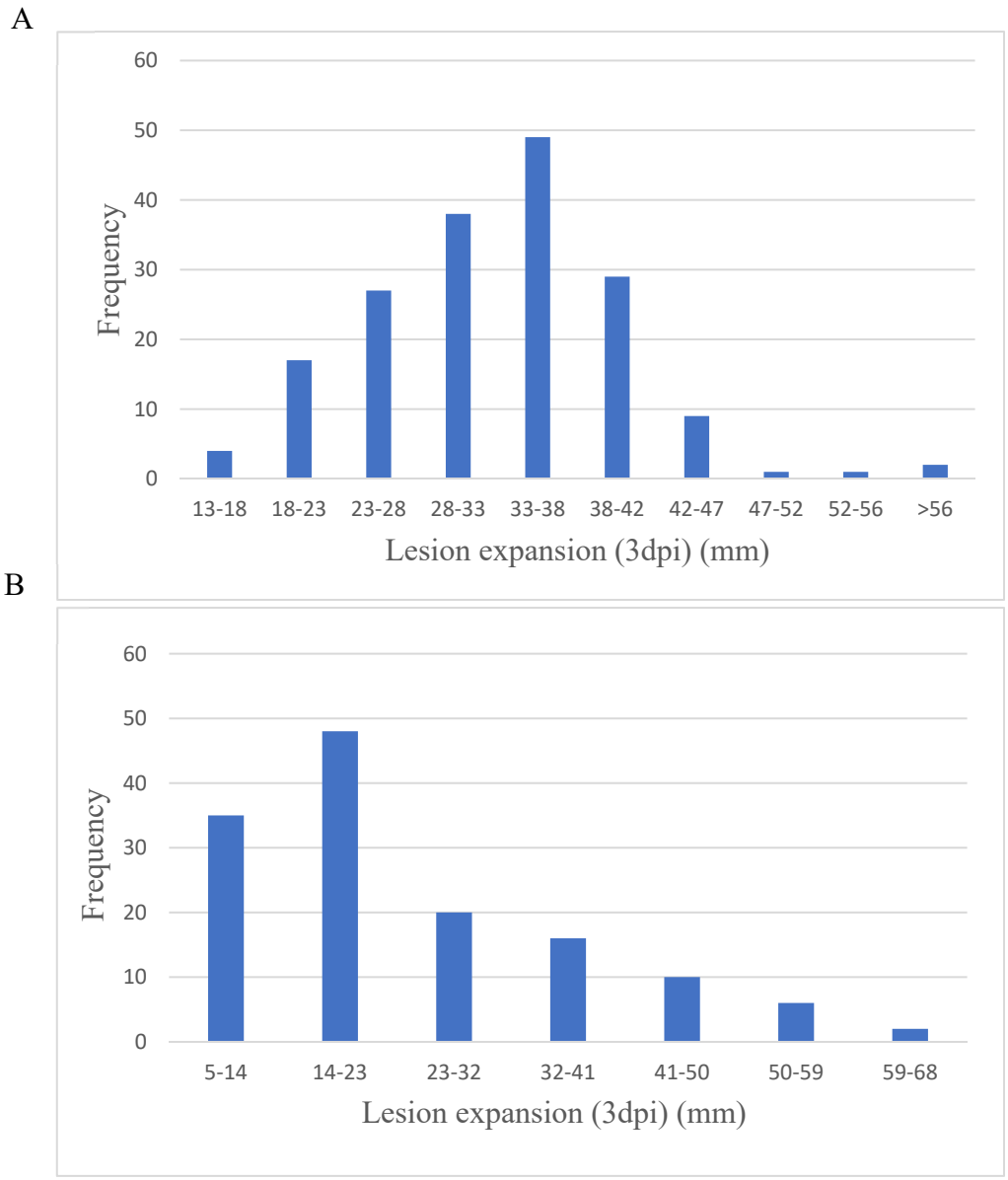

Figure 3-9: Histogram for lesion expansion recorded (3 dpi) (mm) with *S. sclerotiorum* in detached stem assay for (a) Population 17 (b) Population 19.

Stem strength

Stem hardness of the Population 19 stems ranged from 5.7 to 31.4 newtons (Figure 3-10). Analyzing hardness of the stems of Population 19 demonstrated that there is a weak correlation between lesion expansion in detached stem assay and hardness of the stems (Pearson's r= 0.16, p-value=0.05). Also, there was no correlation between stem strength and lesion expansion, nodal resistance, survival rate and AUDPC in the greenhouse evaluation (Table 3-16). Sixteen genotypes of Population 19 showed hardness equal or greater than 20 Newton, and of those six genotypes showed nodal resistance and survival rate of 25% or more in the greenhouse evaluation (PRIL19-98, PRIL19-105, PRIL19-108, PRIL19-110, PRIL19-112, PRIL19-141).

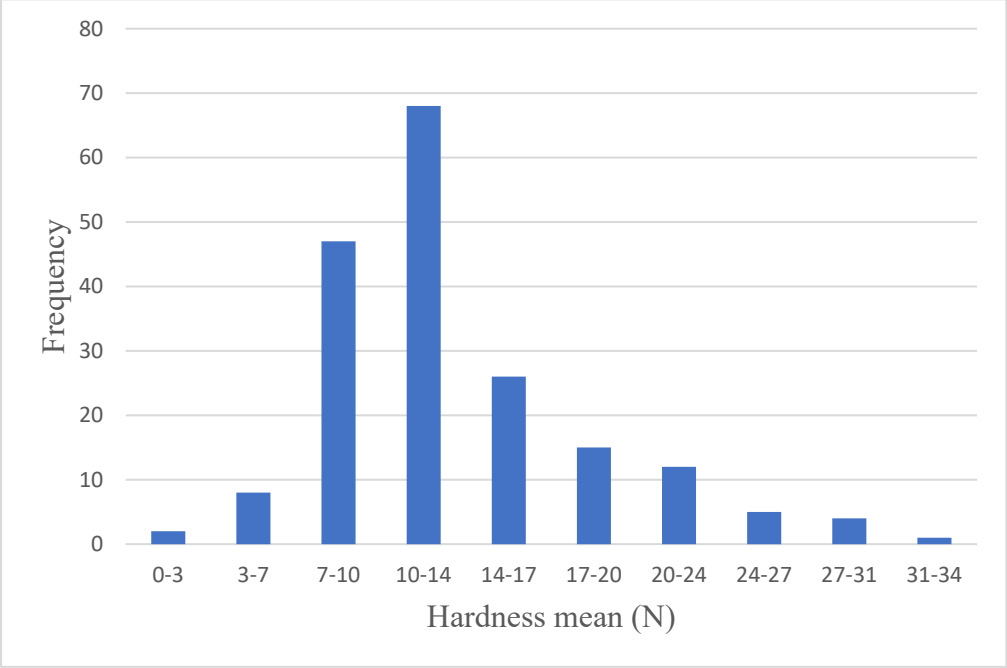

Figure 3-10: Distribution of mean stem hardness (Newton) for 190 individuals of Population 19.

Table 3-16: Pearson correlation between stem hardness and lesion expansion (3, 7, and 14 dpi), nodal resistance (7 and 14 dpi), survival rate and area under disease progress curve.

	Lesion expansion (3 dpi)	Lesion expansion (7 dpi)	Nodal resistance (7 dpi)	Lesion expansion (14 dpi)	Nodal resistance (14 dpi)	Survival rate	AUDPC
Stem hardness	-0.15 ^{ns}	0.07 ^{ns}	0.10 ^{ns}	0.02 ^{ns}	-0.03 ^{ns}	-0.05 ^{ns}	-0.05 ^{ns}

^{ns} not significant.

Table 3-17*:* Lesion expansion 3 dpi, nodal resistance and survival rate after 14 dpi and area under disease progress curve (AUDPC) from greenhouse evaluation and lesion expansion 3 dpi from detached stem assay (DSA) and leaf type of the 22 of the most partial resistant short pea genotypes of Population 17 and 19 to *S. sclerotiorum* which at least have two resistance criteria.

Short internode genotypes	Lesion expansion (3 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC	DSA-lesion expansion (3 dpi) mm	Leaf type
Population 17						
PRIL17-11	23.8	0.2	27.1	651	31.8	Normal
PRIL17-54	50.4	1.4	44.2	2269.9	37.7	Normal
PRIL17-56	24.3	1.5	15.6	654.3	34.1	Normal
PRIL17-58	40.7	1.5	33.2	1231.7	29.7	Normal
PRIL17-71	43.1	1.2	34.4	1293.8	24	Normal
PRIL17-97	40.2	1.4	30.4	1038.2	43.3	Normal
PRIL17-127	40.7	1.4	52.1	1302	36.3	Normal
PRIL17-128	30.4	1	25.2	764	33.9	Normal
PRIL17-129	27.2	2.3	31.7	833.8	28.8	Normal
PRIL17-139	33.8	1.2	31.4	882.7	39	Normal
PRIL17-141	36.1	2.9	29.8	991	30.7	Normal
PRIL17-145	26.7	2.5	53.5	580.7	32.4	Normal
PRIL17-149	45.8	1.3	44.5	1665.8	35.5	Normal
PRIL17-158	26.7	1.8	25.3	727.9	36.9	Normal
PRIL17-166	24.7	0.3	34	654.7	43.9	Normal
PRIL17-180	31	1.6	34.6	837.2	33.9	Normal
PRIL17-181	23.1	1.5	58.2	486.9	31.1	Normal
Population 19						
PRIL19-18	63.1	1.4	33.8	2336.4	16.1	Afila
PRIL19-74	74.1	2.3	52.8	2400.7	14.2	Normal
PRIL19-86	37.7	2.8	35.7	1822.2	17.3	Normal
PRIL19-124	37.3	1.2	34.8	1026.7	16.9	Afila
PRIL19-127	29	1.4	35.3	930.2	14.9	Normal

Discussion

The objective of this experiment was to develop appropriate techniques to evaluate disease resistance as a crucial step to have insight about genetic control of host resistance to pathogen to develop resistant varieties *S. sclerotiorum*. Two partial resistance PI parents in this study were screened in greenhouse study and identified as partial resistance to *S. sclerotiorum* (Porter et al. 2009). Population 17 and Population 19 were screened by two different methods, agar plug technique as described and used by (Porter et al. 2009) and detached stem assay. Detached stem assay technique already used in other crops like rapeseed (*Brassica napus*) and peanut (*Arachis hypogaea*) and is suitable for large-scale evaluation of screening genotypes for resistance against *S. sclerotiorum* (Mei et al. 2012; Melouk et al. 1992). In 1987, oat (*Avena sativa*) kernels infested with *S. sclerotiorum* were used to inoculate eleven-day old pea genotypes in the greenhouse (Blanchette and Auld 1978). The coefficient of variation for lesion expansion (3 dpi) of stem in the detached stem assay experiment in Population 17 and Population 19 were 16.6 % and 21.5 respectively. Coefficient of variation for lesion expansion (3 dpi) in the greenhouse evaluation with agar plug technique in Population 17 and 19, was higher than stem assay technique, 26.0 and 30.7, respectively. Although, this might support the reliability of detached stem assay, we should consider the fact that there was no significant correlation between the two techniques. This might bring up this hypothesis that the pathogen behaves differently in attached stem versus detached stem. Use of detached stem assays is unlikely to reflect the host-pathogen interaction that happens between *S. sclerotiorum* and intact pea plants. Detached stem assays may not precisely mirror what is taking place in whole plant. Gene expression study between attached and detached stems needs to prove this theory. This behavior in detached leaves and intact plants interaction with hemi-biotrophic pathogen has been observed

in Arabidopsis and cereals against *Colletotrichum* spp. (Beirn et al. 2015; Liu et al. 2007). Gene expression analysis revealed that disease symptom developed in detached leaves in Arabidopsis appeared to be strictly associated with senescence other than with defense pathways (Liu et al. 2007).

There was an obvious positive and significant correlation between plant height and lesion expansion and nodal resistance of pea individuals (Table 3-4, Table 3-5). Previous studies on pea accession lines indicated that internode length and lesion expansion were significantly positively correlated and decrease in internode length result in reduction of lesion expansion (Porter et al. 2009). Bazzalo et al. (1991) showed that lesion expansion of sunflower (*Heliantheae annuus*) in *S. sclerotiorum* infection also positively associated with plant height. In soybean (*Glycine max*), two out of three detected QTL of partial resistance to *S. sclerotiorum* were associated to physiological defense such as plant height, lodging, and date of flowering (Bazzalo et al. 1991). We assumed height in this pea- *S. sclerotiorum* study as a confounding factor that might obscure the result of *S. sclerotiorum* infection. To control this confounding factor, individuals of each population divided by plant heights to short and long internode and then analyzed with PROC MIXED model by SAS. The twenty-two of short individuals demonstrated greatest partial resistance to *S. sclerotiorum* based on lesion expansion, nodal resistance and survival rate in greenhouse evaluation and lesion expansion in detached stem assay (Table 3-17). Survival percentage of long internode individual's subsets in Population 17 and 19 were higher than the short internode subsets (Table 3-14) which also can confirm the height confounding assumption. Quantitative partial resistance nature of the trait has been reported by other studies (Gentzbittel et al. 1999; Davar et al. 2013). Partial resistance to *S. sclerotiorum* in field pea is expressed in two forms, lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI) (Porter 2012a).

LEI restricts lesion development while NTI blocks the path of the pathogen through the node. Lesion expansion is an important epidemic component and starts with small lesion size and constricted in size or slowed rate of expansion over the life of the host tissue as a result of high lignin concentrations (Berger et al. 1997). Our results supported Porter et al. (2009) of the negative phenotypic correlation between lesion restriction and nodal resistance which shows different genetic backgrounds between them as reported in GWAS analysis and RNA sequencing of pea-*S. sclerotiorum* interaction study (Chang et al. 2017). Our study noted that small number of pea genotypes restricted lesion advancement and partial resistance was mostly rely on nodal resistance to inhibit lesion development through the stem. Stems showed restricted lesion expansion in detached stem assay, were totally gone after one-week post inoculation. Previous study on pea accessions showed that pea genotypes with restricted lesion development, their lesions continued to enlarge, until the plant was killed (Porter et al. 2009).

Despite a hypothesized direct relationship between stem strength and disease resistance, our finding showed no observed correlation between stem strength and lesion expansion, nodal resistance and survival percentage in greenhouse experiment. In previous study, correlation coefficients for stem crushing and shearing strength was negatively correlated with lesion expansion and there were no significant correlations between nodal resistance and survival, with stem crushing and shearing strength (Porter et al. 2009). Mechanical strength and lodging could associate with stem lignin content and other hardening plant cell compounds (Wu et al. 2017). Soybeans lines, with high stem lignin content were more susceptible to *S. sclerotiorum* (Peltier et al. 2009). There was positive correlation between lignin concentration and nutritional value for pathogen and severity of disease in soybean and could be used as a biological marker to select

for resistance to *S. sclerotiorum* (Peltier et al. 2009). Porter et al. (2009) also reported negative correlation between stem diameter and lesion expansion in a greenhouse trial.

Incorporating physical character such as stem thickness, short internode and the afila leaf type can help to develop a genetically resistant genotype with architectural resistance to *S. sclerotiorum* (Porter 2012a). Only two pea genotypes with quantitative partial resistance to *S. sclerotiorum* (PRIL19-18 and PRIL19-124) had both afila and short internode. Based on the present study, our recommendation to develop resistant pea cultivar to white mold would be to make crosses between best partial resistant pea genotypes of each population together or with other partial resistant PI from previous study that had both lesion expansion and nodal resistance in addition of architectural resistance, to overcome disease development in pea. We need further field testing in multi environments for validating of these genotypes, although small number of partial resistance inbred lines makes field validation less expensive. Also, further research is necessary in this regard to screen pea gene pool to find novel resource for resistance to *S. sclerotiorum.*

CHAPTER 4: IDENTIFICATION OF QTL FOR RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* IN PEA

Abstract

Crop improvement and conventional breeding has been facilitated by development of next-generation sequencing technology following marker-assisted selection to identify loci and markers linked with favorable traits. Resistance to white mold is a crucial trait for many important dicotyledonous crops including *Pisum sativum*. Population 17 was developed by crossing PI240515 with Lifter followed by single seed decent. Population 19 was created by crossing PI169603 with Medora and advancing the F_2 through the F_7 by single seed decent. Both populations were screened in the greenhouse as whole plants and using a detached stem assay. White mold caused by *Sclerotinia sclerotiorum* is one of the most destructive fungal diseases and causes massive yield damage. Two plant identification accessions were crossed to agronomically adapted pea cultivars to transfer and study resistance to *S. sclerotiorum* in field pea. One hundred ninety-two individuals from each population were genotyped by genotyping by sequencing. Twenty-seven QTL were identified from both populations. Eight QTL were associated with plant height (disease avoidance), and 19 QTL were associated with physiological resistance to white mold. From physiological resistance QTL, 17 QTL of *S. sclerotiorum* resistance were associated with lesion expansion inhibition and two QTL were associated with nodal resistance might regulates defense mechanism pathway genes. Using white mold resistance markers in marker assisted breeding may speed up breeding program.

Introduction

Sclerotinia sclerotiorum is a ubiquitous fungus that infects a wide variety of plant species (Zhang 2005; Bolton et al. 2006). Outbreaks of *S. sclerotiorum* in the United States costs over

$200 million per year (Garg et al. 2013). White mold caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is a serious disease of pea (*Pisum sativum*) worldwide (Fernando et al. 2004; Zhang 2005). It is one of the main disease problems on field peas for growers in the Northwest and Midwest areas of the USA (Porter et al. 2009). The ability of melanized sclerotia to survive for a long time in the soil along with a wide host range make it difficult to eradicate *S. sclerotiorum* using cultural practices (Peltier et al. 2012; Jain et al. 2012, Fernando et al. 2004).

White mold growth is favored by cool, cloudy, wet, and humid weather during flowering (Mueller 2014). Wide row spacing, and an open canopy helps reduce damage from several foliar fungal diseases including *Sclerotinia sclerotiorum* (Kraft and Pfleger 2001). The pathogen affects vegetative tissues and forms water-soaked lesions and white cottony mycelium on the surface of green parts of the plants (Mueller 2014).

Development of *Sclerotinia* resistant varieties is the most desirable approach to control the pathogen. Breeding resistant varieties is challenged by a lack of highly resistant germplasm in *P. sativum*. Although, there are many studies about molecular mechanisms of the host-pathogen interaction (Wei 2017; Seifbarghi et al. 2017; Zhuang et al. 2012), interaction of pea-*S. sclerotiorum* has not been completely elucidated due to the lack of *P. sativum* reference genome. There have been a few attempts to screen pea germplasm and attain partially resistant varieties (Blanchette and Auld 1978; Porter et al. 2009; Tashtemirov 2012)

Breeding for *Sclerotinia* resistant varieties is difficult in many crops due to the control by multiple genes with small impact with partial resistance (Porter et al. 2009; Davar et al. 2013). Porter et al. (2009) stated that expression of the partial quantitative white mold resistance on pea might be in two forms of lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI) and we call them lesion expansion and nodal resistance respectively from now on. The

LEI slows the rate of lesion development while NTI blocks passage of the pathogen through the node. Other traits such as stem thickness, short internodes and leaf morphology (semi-leafless), can influence white mold resistance (Porter et al. 2009). In this case, molecular breeding can help the conventional breeder develop resistant varieties more quickly. The emergence of next-generation sequencing technology and identification of quantitative trait loci (QTL) for *S. sclerotiorum* resistance in *P. sativum* brings hope for the genetic improvement of disease resistance. The objective of this study was to detect quantitative trait loci (QTL) associated with *S. sclerotiorum* resistance. Specifically, we aim to identify QTL associated with lesion expansion and nodal resistance factors in *P. sativum* and validate previous research (Tashtemirov 2012).

Materials and methods

Plant materials

Two mapping populations of *Pisum sativum* were used: (i) Population 17 (PRIL-17) comprised a set of 192 recombinant inbred lines (RILs) derived from the cross of Lifter/ PI240515 developed by single seed decent and (ii) Population 19 (PRIL-19) comprised a set of 324 F_7 genotypes derived from cross PI169603/Medora also developed by single seed decent. Lifter and Medora were susceptible cultivars while PI240515 and PI169603 expressed partial resistance. Lifter and Medora used as susceptible parents crossed with two exotic pea accession PI240515 and PI169603 as partial resistance parents. Lifter, developed by the USDA-ARS, has short internodes with normal leaf type, white flower and green seed (McPhee and Muehlbauer 2002). Medora which has developed for Midwest production region, has a short internode with afila leaf type, white flowers and smooth green seed color (GLGP 2007). Two partial resistant plant introductions were selected from previous study (Porter et al. 2009). PI240515 (originated from India) has long internodes, normal leaf morphology, white flowers and yellowish green

seed color. PI169603 originated from Turkey and has long internodes, normal leaf types, white flowers and yellow seed color (https://www.ars-grin.gov/).

Phenotyping

Greenhouse evaluation

Seeds of individual RILs were planted in the greenhouse 14 days prior to inoculation using the jumbo agar plug technique described by (Porter et al. 2009). Plants were grown under natural sunlight supplemented with, 600-Watt Pressure Sodium Lamps (P. L Light Systems, Inc., Beamsville, Ontario, Canada) in greenhouse to maintain a 16:8 hour photoperiod and temperatures from 20-25°C in day time and 20°C in nighttime. The experiment was arranged in a randomized complete block (RCBD) with 4 replicates and the experiment was repeated 3 times. 186 genotypes from Population 17 and 140 individuals from Population 19 in addition to 5 checks were used in this experiment. The agar plug was placed at the 4^{th} node and media pressed into the leaf axis. After inoculation, plants were transferred to a mist chamber with 100% humidity and temperature ranging from 19 to 21°C for 3 days in the dark. Plants were then transferred to a mist room for another 11 days with 80% humidity with a 14:10 day: night photoperiod with supplemental lighting of 400-Watt high pressure sodium lamps (P. L. Light Systems, Inc., Beamsville, Ontario, Canada)

White mold lesions were measured 3 days post inoculation with a digital caliper (mm). Lesion expansion and nodal resistance were measured seven and 14 days post inoculation. Lesion expansion percentage and survival percentage of each genotype were recorded after 14 days. Lesion expansion and survival percentage were calculated 14 dpi by measuring lesion expansion and uninfected part of the main stems over plant height. Nodal resistance was scored

on a scale of 0-4 based on lesion movement from the 4th node down as described in Porter et al. (2009).

Means for lesion expansion, nodal resistance score, lesion expansion and survival percentage were calculated using ANOVA and PROC MIXED procedures in SAS Enterprise Guide 7.1 (SAS Institute Inc. USA). In the statistical analysis, genotypes were assumed as fixed effects, while runs, replication within runs, run per replication per genotype, and run per genotype were treated as random effects.

Detached stem assay

Seeds of individual RILs were planted in the greenhouse under natural sunlight supplemented with, 600-Watt high pressure sodium lamps (P. L Light Systems, Inc., Beamsville, Ontario, Canada) to maintain a 16:8 hour photoperiod and temperatures from 20-25°C in the day and 20°C at night. Plants were grown in the greenhouse for 14 days prior to inoculation using the jumbo agar plug technique described by (Porter et al. 2009). Seven cm stem segments were cut from fourteen-day old plants beginning 1 cm above 5th node using a scalpel. The 5th node was pushed into inoculated agar plugs. The inoculated stems were placed on a plastic tray (28× 54 cm) covered with PG1218, 30 × 46 cm germination paper (anchorpaper.com). Trays were placed in a mist chamber in the dark for 3 days at temperatures of 21-25 °C. Lesion expansion (mm) was measured 3 days after inoculation. Stems were arranged in a RCBD with 4 replicates in each run and the whole experiment was repeated 2 times. The 179 genotypes from Population 17 and 137 genotypes from Population 19 were used in this experiment. Both parents and Stirling were used as checks. Means for lesion expansion were calculated using ANOVA and PROC Mixed procedures in SAS Enterprise Guide 7.1 (SAS Institute Inc. USA). In the statistical analysis,

genotypes were assumed as fixed effects, while runs, replication within runs, run per replication per genotype, and run per genotype were treated as random effects.

Genotyping by sequencing

Two 96-plex samples of Population 17 were prepared for library preparation based on the published GBS protocol of (Elshire et al. 2011) and sent to the McDermott Center (http://www.utsouthwestern.edu/labs/dna-genotyping-core/) and sequenced by Illumina HiSeq 2000.

In Population 19, DNA was extracted using the DArt protocol (https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf) and two 96-plex DNA samples were sent to the Genomic Facility of Cornell University Biotechnology Resource Center (BRC) for library preparation and sequencing with Illumina HiSeq 2000.

GBS analysis and SNP calling

The GBS data for Populations 17 and 19 were analyzed using the GBS-SNP-CROP pipeline (Melo et al. 2016). GBS-SNP-CROP pipeline is seven Perl scripts that implement parsing, filtering and SNP calling with bioinformatic tools such as Trimmomatic, USEARCH, and SAMtools. The GBS data filtered with minimum depth of coverage (minDP 3), maximum number of mismatch in alignment (n = 1), maximum missing data (max-missing 0.5), and minor allele frequency (maf 0.05). Total number of 186 individuals from each population and their parents genotyped by GBS and analyzed using the GBS-SNP-CROP pipeline. SNPs filtered out for heterozygous and monomorphic parents and missing data greater than 50% was excluded.

Shared SNPs between two populations

For creating composite map, we need to find shared SNPs between two populations. For doing that, fastq files of individuals from Population 17 (created in step 3 of GBS-SNP-CROP pipeline) aligned to Mock Reference created for Population 19 (created in step 4 of GBS-SNP-

CROP pipeline) by BWA and followed by calling shared SNPs and genotypes by SAMtools. Also, fastq files of individuals from Population 19 aligned to Mock Reference created for Population 17 to extract shared SNPs. After extracting SNPs in each step, markers filtered out for heterozygous and monomorphic parents and the missing data > 50% were excluded.

Linkage group mapping

Linkage groups were established using JoinMap® 4 (Plant Research International B.V and Kyazma B.V) (Ooijen 2006) for each population. Linkage group formation was based on independence LOD with a start value of 0.5 and end value of 20.0 with steps of 0.5 LOD. The independence LOD score calculated by JoinMap for recombination frequency based on G-tests for independence (maximum likelihood statistical significance $G^2 = 2\sum O \log\frac{O}{E}$). This feature is used to calculate the number of recombinant and non-recombinant gametes from recombination frequency and its LOD score (Ooijen 2006). After linkage groups were determined the linkage map was constructed using the *Monte Carlo maximum likelihood* (ML) mapping algorithm for each group.

Composite map construction

A composite map was created based on 221 shared SNPs between two populations (from aligning Population 17 sequences to Mock Reference of Population19) by JoinMap® 4 (Plant Research International B.V and Kyazma B.V). Regression mapping algorithm using Kosambi mapping function was used to create the composite map.

Anchoring linkage groups with physiologic markers and synteny with *Medicago trancatula*

Physiological markers such as height (*Le*), powdery mildew (*er-1*), *Pl,* green cotyledon (*I*)*,* and afila leaf type (*af)* were used for anchoring linkage groups of I, III and VI. Also, using genome comparative analysis of ten legume species map (Lee et al. 2017) and orthology of

shared genes between pea and *Medicago trancatula*, other pea linkage groups in Population 17 and 19 were anchored. The sequence of 30-60 SNPs from each linkage group of Population 17 and 19 map were used to conduct a BLASTN search against *Medicago truncatula* MedtrA17_4.0 (http://plants.ensembl.org/Medicago_truncatula/Tools/Blast, https://www.ebi.ac.uk/ena/data/view/GCA_000219495.2). The E-value of less than 0.05 used to select the overlapping genes. The information of each SNP was found from "SNP_genotyping.desc.txt" file and then sequences of SNP markers were extracted from the "MockRefName.MockRef_Clusters.fa" file with "xargs samtools faidx" command. (http://samtools.sourceforge.net/). Both files were produced using the GBS-SNP-CROP pipeline.

QTL mapping

QTL analysis was performed using least square means across runs for each population. Each population was separated into tall and short internode individuals and QTL analysis was conducted on each subset. QTL analysis was completed using the Inclusive Composite Interval Mapping (ICIM) method accessible in the software QTL IciMapping V 4.1 (Meng et al. 2015). Only unique loci were included in each linkage group. Permutation tests based on 1000 repeats were performed to determine significant LOD threshold values (α= 0.05 Type I error).

Identification of candidate gene for resistance to *S. sclerotiorum*

The nucleotide sequence of the markers associated with resistance to white mold were used to run a BLASTN search against *Medicago truncatula* MedtrA17_4.0 (http://plants.ensembl.org/Medicago_truncatula/Tools/Blast, https://www.ebi.ac.uk/ena/data/view/GCA_000219495.2). The E-value of less than 0.05 used to select the overlapping genes. The information of each SNP was found from "SNP_genotyping.desc.txt" file and then sequences of SNP markers were extracted from the "MockRefName.MockRef_Clusters.fa" file using the faidx command from SAMtools (http://samtools.sourceforge.net/).

Results

Genetic map construction of two pea populations

Population 17: After filtering the genotypic data, total of 1397 polymorphic SNP markers were selected to construct the linkage map. Out of those markers, a total of 1359 SNPs were mapped into 7 linkage groups. The total map length was 1846.7 cM with a minimum LOD of 8. The 1359 markers (1359 markers from 1397 markers mapped in 7 linkage groups by JoinMap) represented 1120 unique loci with an average distance of 1.7 cM between two loci (Table 4-1). Four anchoring physiological markers (*Le* for height, *er-1* for powdery mildew, *Pl* for hilum color, and *I* for cotyledon color) were used to anchor linkage groups to published consensus maps (Bordat et al. 2011) (Figure 4-1).

Population 19: After filtering the genotypic data, total of 1482 polymorphic SNP markers were selected and assembled into 8 linkage groups. The total map length was 2078 cM with a minimum LOD of 10. The 1473 markers (1473 markers from 1482 markers mapped to 8 linkage groups by JoinMap) represented 1102 unique loci with an average distance of 1.9 cM between

loci. (Table 4-1). Five anchor markers (*Le, er-1*, *Pl, I,* and *af* or afila) were used to anchor our linkage groups to published consensus maps (Bordat et al. 2011).

Table 4-1: Summaries of linkage groups of genetic maps for Population 17 and 19.

Linkage group	Population 17			Linkage group	Population 19		
	#Loci	# Unique loci	Map length cM		#Loci	# Unique loci	Map length cM
LG I	50	44	55.9	LG I (a)	63	41	54.2
LG II	203	176	354.2	LG I (b)	102	78	152.4
LG III	215	168	228.2	LG II	190	128	278.7
LG IV	217	177	268.0	LG III (a)	283	224	242.5
LG V	197	156	166.7	LG III (b)	72	50	58.4
LG VI	142	121	141.8	LG V	204	163	308.6
LG VII	335	278	631.9	LG VI	369	271	663.2
				LG VII	190	147	320.0
Total	1359	1120	1846.7	Total	1473	1102	2078

Composite map construction

From total of 221 shared SNPs between the two populations 198 markers were assembled into 9 linkage groups by JoinMap. The 192 unique loci spanned over 658.3 cM with an average distance of 3.4 cM between loci (Table 4-2).

Table 4-2: Summaries of linkage groups of composite map.

Linkage group	Composite map		
	#Loci	#Unique loci	Map length cM
LG II (a)	18	16	61.3
LG II (b)	16	16	58.3
LG III (a)	35	34	62.0
LG III (b)	13	13	29.9
LG III (c)	16	15	20.9
LG V	33	33	114.4
LG VI (a)	38	36	179.7
LG VI (b)	17	17	64.0
LG VII	12	12	67.9
Total	198	192	658.3

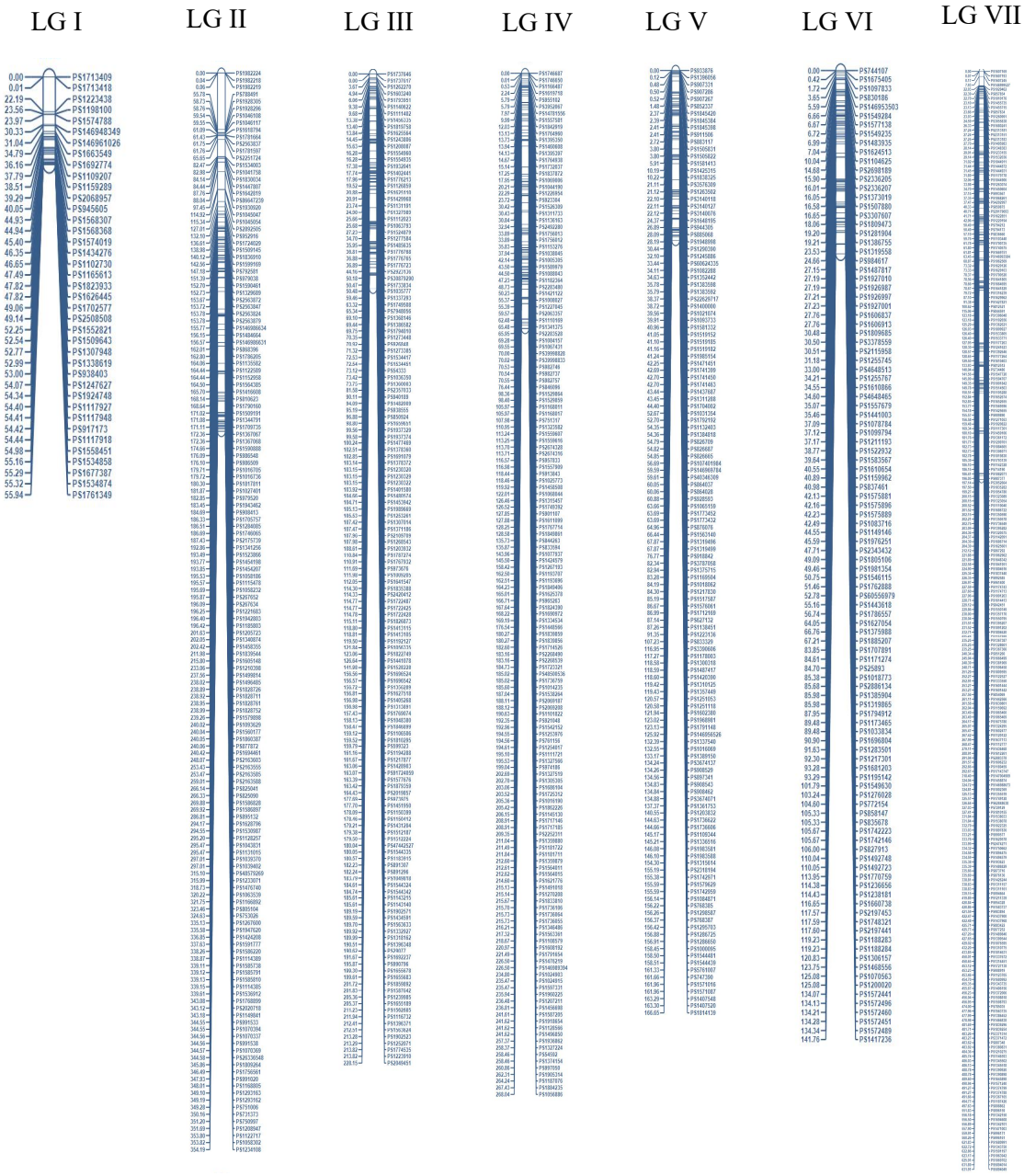

Figure 4-1: Linkage map for Population 17 based on SNPs derived from genotype by sequencing.

Anchoring Pisum linkage groups

Green cotyledon (*I*), internode length (*Le*), powdery mildew (er-*1*), and hilum color (*Pl*), were used to anchor LG I, LG III, and LG VI in Population 17. The *I* locus is located on LG I, *Le* is located on LG III, and er-*1* and *Pl* are located on LG VI (Weeden et al. 1998; Bordat et al.

2011). The remaining linkage groups were anchored based on synteny with *Medicago trancatula* (Lee et al. 2017) (Figure 4-2). *Le* is located on distal end of LG III (265.1 cM) on Weeden's map map (Weeden et al. 1998; Lee et al. 2017) and since *Le* is at position 8 cM of LG III in the Population 17 map, this linkage group shows inverted gene order.

Afila (*af*), internode length (*Le*), powdery mildew (er-*1*), and hilum color (*Pl*) were used to anchor LG I, LG III, and LG VI in the Population 19 map. The *af* locus is located on LG I, *Le* is located on LG III, and er-*1* and *Pl* are located on LG VI (Weeden et al. 1998; Bordat et al. 2011). Based on orthologous gene loci between *Medicago trancatula* and *Pisum sativum*, other linkage groups were identified. Linkage group I might show the inversion in SNP order, since the *af* gene is located on 131.4 cM of LG I on Weeden's consensus map (Weeden et al. 1998; Lee et al. 2017), in Population 19 *af* is at position 10 cM of the LG I (b) between PS126916996 and PS126418717 (Figure 4-3).

Also, composite map created from shared markers between two populations (aligning Population 17 individual's sequences on Mock Reference of Population 19) anchored based on comparison with Population 19 linkage groups.

Lee et al (2011) found total of 110 genomic synteny blocks with different sizes (*L*: large, *M*: moderate and *S*: small) across ten legume genomes including *Pisum sativum and Medicago trancatul*a. The comparative composite map of ten legume constructed to simplify genomic comparison, and identification of conserved genome regions between evolutionary related legume species due to diverging from a common ancestor.

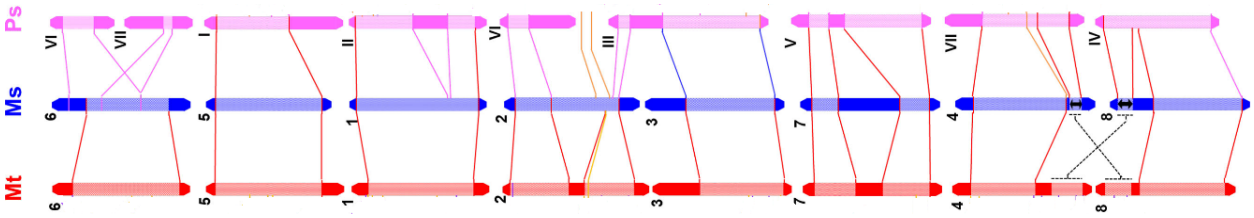

Figure 4-2: Conserved chromosome/LG information of syntenic regions of *Pisum sativum*, *Medicago trancatula*, *Medicago sativa* (Lee et al. 2017).

Table 4-3: Conserved chromosome and linkage group information of syntenic regions (Lee et al. 2017).

	Conserved chromosome and linkage blocks[a]							
Medicago trancatula	1	2	3	4	5	6	7	8
Medicago sativa	1^{L}	2^{L}	3^{L}	4^{L},8^{S}	5^{L}	6^{L}	7^{L}	4^{S}, 8^{L}
Pisum sativum	II^{L}	III^{S}, VI^{M}	III^{L}	IV^{M}, VII^{L}	I^{L}	VI^{M}, VII^{S}	V^{L}	IV^{L}, VII^{S}

[a] Conserved block sizes: *L* large; *M* moderate; *S* small

Quantitative Trait Loci analysis

Ten QTLs for lesion expansion and one for nodal resistance to *S. sclerotiorum* were identified in Population 17 based on the greenhouse evaluation and one QTL for lesion expansion was identified based on data from the detached stem assay (Table 4-4). The QTL based on the detached stem assay was located at position 69 on LG VII with LOD=2.2. This QTL explained 5.58% of the phenotypic variance. One major QTL associated with lesion expansion in greenhouse evaluation (LOD=24.1) was located between SNP markers PS1793951 and PS1140622 on LG III near *Le* and explained 41.6% of the phenotypic variation (Figure 4-5). A QTL explaining 26.2% of the phenotypic variation for nodal resistance (LOD=16.4) was also located near *Le* on LG III between markers PS1262270 and PS1456335 (Figure 4-5).

Other QTLs for lesion expansion were located on LG II, LG III, LG IV, and LG VII with minor effect (LOD = 4-5) and explaining 4-8% of the phenotypic variation (Figure 4-5, Figure 4-6a, Figure 4-8ab). Analysis of the long internode individuals of Population 17 revealed two

QTL on LG II, and LG VII for lesion expansion (LOD= 3.5- 2.5, and phenotypic variation explained (R^2)=13.1- 9.6%) (Table 4-5, Figure 4-6b, Figure 4-7b). In summary, six QTL were detected on LG III and LG VII for lesion expansion and one QTL for nodal resistance on LG II (Table 4-6). In addition, two lesion expansion QTL on LG III based on the short internode subset were the same as the QTL at position 3-4 cM identified with the combined data. Four QTL for lesion expansion were on LG VII with LOD range from 3.1 to 4.7 and R^2 from 13.7 to 17.6% (Figure 4-7a). The nodal resistance QTL in the short subset was placed on LG II at position 318 cM (LOD=2.8, R^2=15.1%).

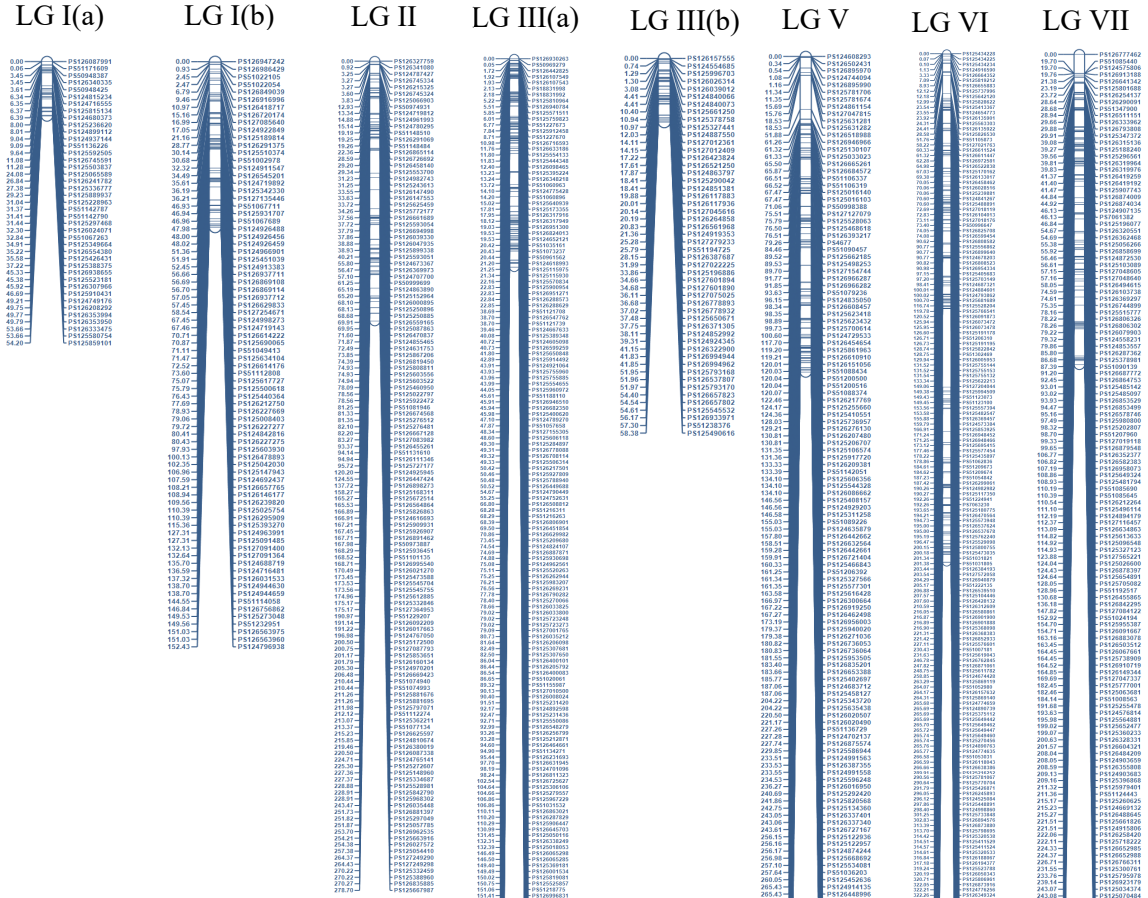

Figure 4-3: Linkage map for Population 19 based on SNPs derived from genotype by sequencing.

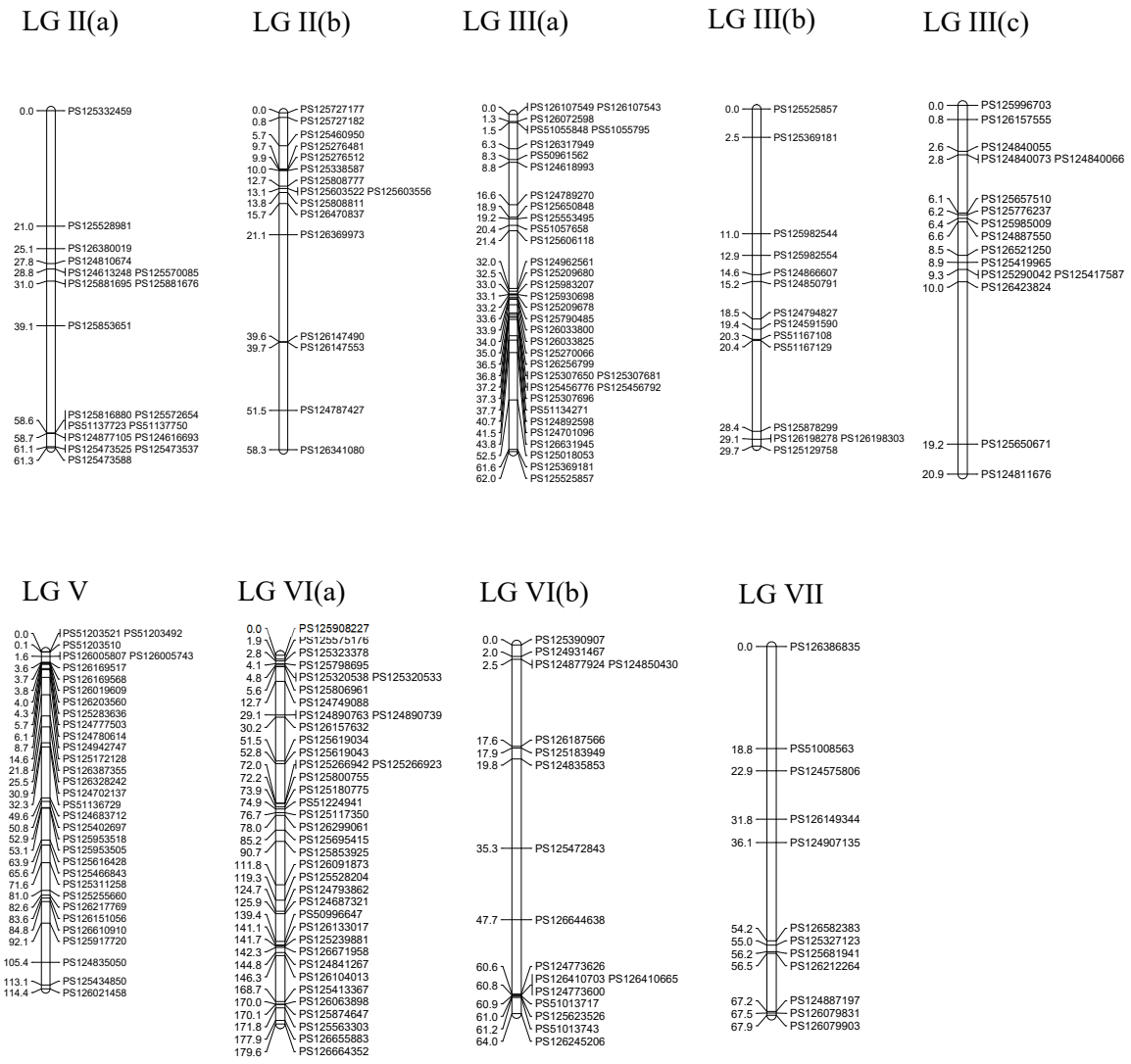

Figure 4-4: Composite map generated based on 221 common SNP markers from Population 17 and Population 19.

Table 4-4: QTLs for reaction to *S. sclerotiorum* infection identified in Population 17 based on all RILs.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2a}	Add[b]
Height (mm)	III	8	PS1793951	PS1140622	25.5	48.2	-99.9
Lesion expansion (14 dpi)	III	8	PS1793951	PS1140622	24.1	41.6	-44.7
Lesion expansion (7 dpi)	III	8	PS1793951	PS1140622	15.2	32.6	-23.6
Lesion expansion (14 dpi) %[1]	III	8	PS1793951	PS1140622	11.9	26.5	9.1
Lesion expansion (3 dpi)	III	8	PS1793951	PS1140622	9.9	18.4	-4.8
AUDPC	III	8	PS1793951	PS1140622	23.3	40	-401.9
Nodal resistance (14 dpi)	III	10	PS1111402	PS1456335	16.4	34.6	-0.6
Nodal resistance (7 dpi)	III	10	PS1111402	PS1456335	11.3	25.1	-0.4
Survival rate[2]	III	4	PS1262270	PS1603240	12.1	26.2	-9.1
Lesion expansion (3 dpi)	III	88	PS2357033	PS940189	3.9	7.5	3
Lesion expansion (3 dpi)	III	91	PS940189	PS1482009	3.5	6.3	2.8
Lesion expansion (14 dpi)	II	0	PS1982224	PS1982218	5.2	6.9	18
Lesion expansion (14 dpi)	VII	261	PS854069	PS1442566	3.7	4.8	15.1
Lesion expansion (3 dpi)	VII	181	PS1459106	PS1361172	4.5	8.2	3.2
AUDPC	VII	261	PS854069	PS1442566	3.9	5.1	142.7
Lesion expansion 3 dpi-DSA	VII	69	PS1062509	PS1629126	2.2	5.6	1.8
Lesion expansion (3 dpi)	IV	241	PS1456698	PS1587205	3.9	6.8	-2.9
AUDPC	IV	257	PS1496850	PS1936862	3.4	4.5	-135.2
AUDPC	IV	258	PS1936862	PS1327224	3.3	4.3	-131.2

[a] Phenotypic variation explained by QTL.
[b] Additive effect.

Table 4-5: QTL for reaction to *S. sclerotiorum* infection identified in Population 17 based on the long internode genotype subset.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2a}	Add[b]
Lesion expansion (14 dpi)	II	0	PS1982224	PS1982218	3.5	13.1	19.7
Lesion expansion (14 dpi)	VII	197	PS987377	PS3052664	2.5	9.6	16.6

[a] Phenotypic variation explained by QTL.
[b] Additive effect.

[1] (Lesion expansion (mm) (14 dpi)/Height (mm)) *100

[2] (Height- Lesion expansion (mm) (14 dpi)// Height (mm)) *100

Table 4-6: QTL for reaction to *S. sclerotiorum* infection identified in Population 17 based on the short internode genotype subset.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2a}	Add[b]
Lesion expansion (7 dpi)	III	4	PS1262270	PS1603240	7.1	28.2	-17.1
Lesion expansion (7 dpi)	III	3	PS1737617	PS1262270	6.6	27.6	-17.3
Lesion expansion (14 dpi)	III	4	PS1262270	PS1603240	5.2	23.3	-22.4
Lesion expansion (14 dpi)	III	3	PS1737617	PS1262270	4.8	22.7	-22.4
Lesion expansion (3 dpi)	VII	243	PS951260	PS1666499	3.1	17.6	3
Lesion expansion (7 dpi)	VII	225	PS1804616	PS1837440	4.7	16.7	8.1
Lesion expansion (7 dpi)	VII	224	PS1041951	PS1804616	4.3	16.6	8.0
Lesion expansion (14 dpi)	VII	243	PS951260	PS1666499	3.2	14.6	10.8
Lesion expansion (14 dpi)	VII	238	PS1367366	PS951260	3.1	13.7	10.5
Nodal resistance (7 dpi)	II	318	PS1233071	PS1476740	2.8	15.1	-0.3

[a] Phenotypic variation explained by QTL.
[b] Additive effect.

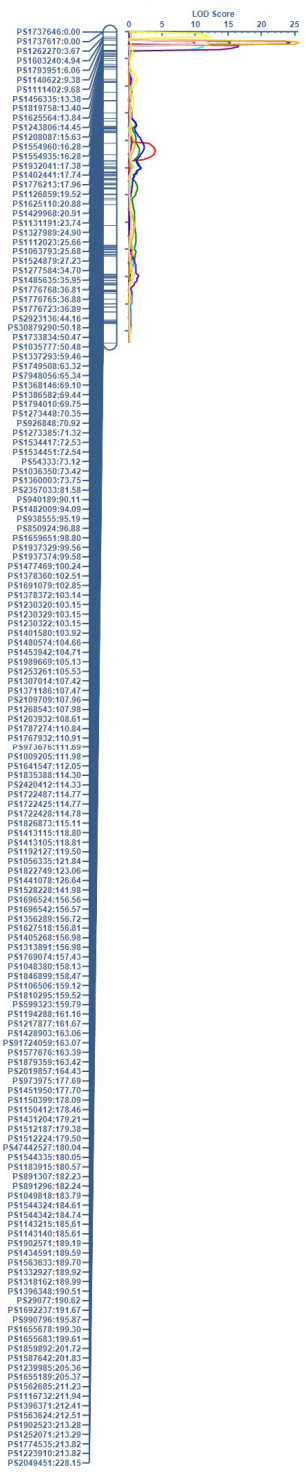

Figure 4-5: QTLs associated with lesion expansion and nodal resistance were detected on linkage group III close to *Le* based on the total data set from Population 17.

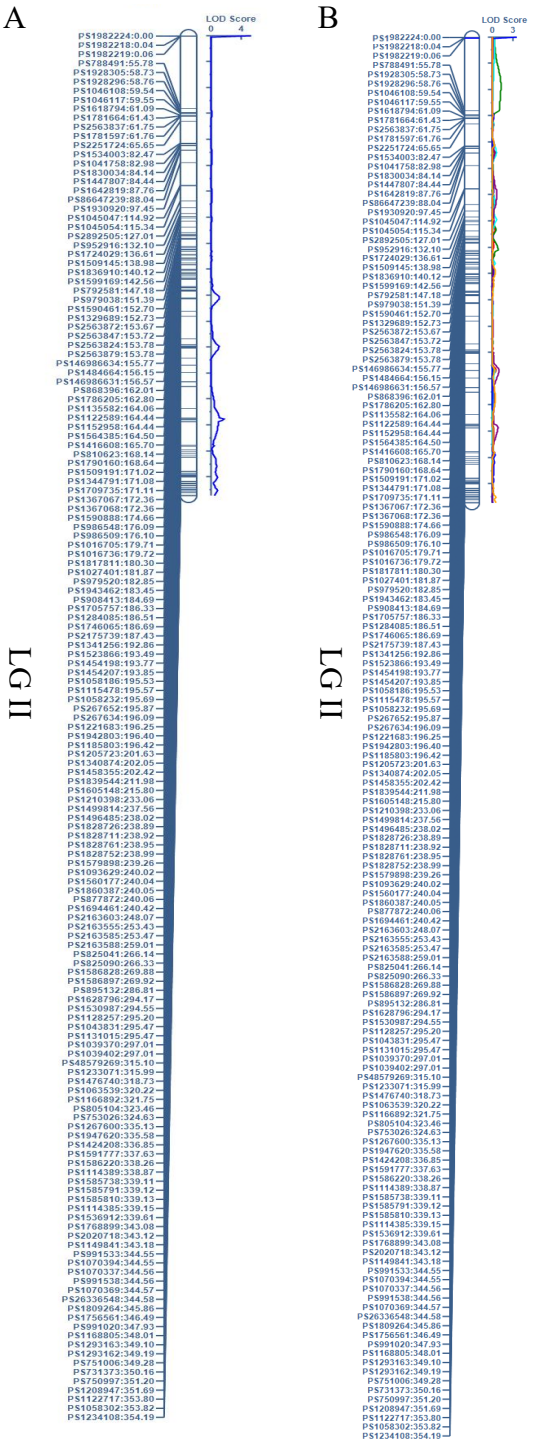

Figure 4-6: QTLs associated with lesion expansion detected on LG II of Population17 based on the total dataset (a) and based on the long internode subset of Population17 (b).

Four QTL for lesion expansion and nodal resistance to *S. sclerotiorum* were identified in Population 19 based on the total dataset (Table 4-7, Figure 4-10). One major QTL associated with lesion expansion (LOD=48.1) was located on LG VI between markers PS51241124 and PS126932560 (Figure 4-9). One major QTL associated with nodal resistance (LOD=19.0) was located between markers PS124554685 and PS125996703 on LG III(b) adjacent to *Le* (Figure 4-10a). The QTL associated with lesion expansion were at position 0-1cM on LG III(b) adjacent to *Le* between markers PS124554685 and PS125996703 with LOD range of 6.8 to 19.0 and explained 20.6 to 51.1% of the phenotypic variation.

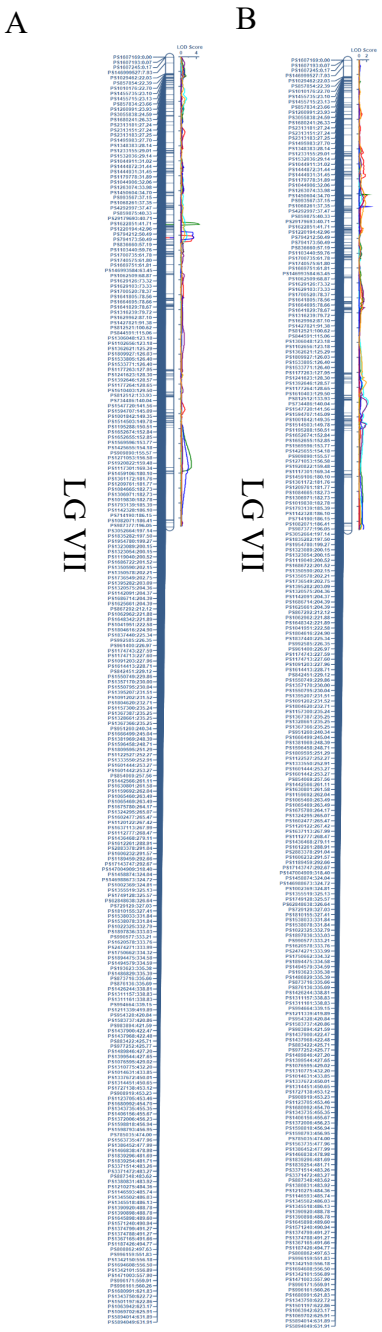

Figure 4-7: QTL associated with lesion expansion detected on LG VII based on the short internode subset of Population 17 (a) and QTL associated with lesion expansion detected on LG VII based on the long internode subset of Population 17 (b).

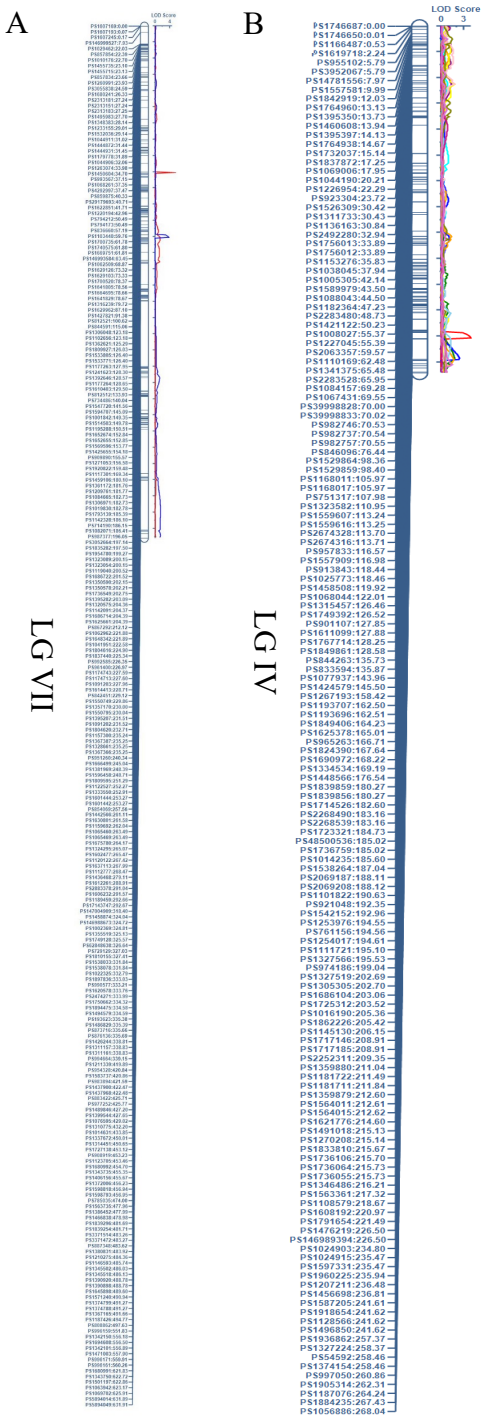

Figure 4-8: QTLs associated with lesion expansion located on LG VII on entire sets of Population 17 (a) and LG IV on entire sets of Population 17 (b).

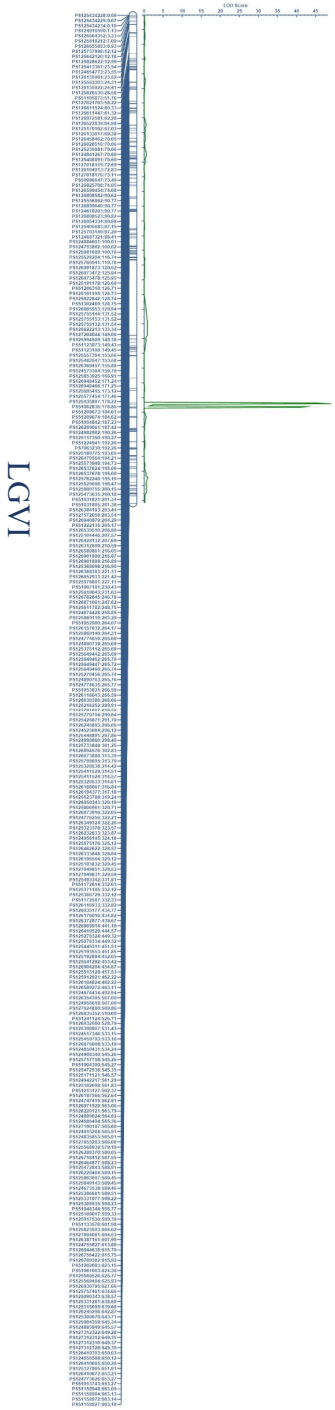

Figure 4-9: QTL associated with lesion expansion on LG VI based on the complete dataset for Population 19.

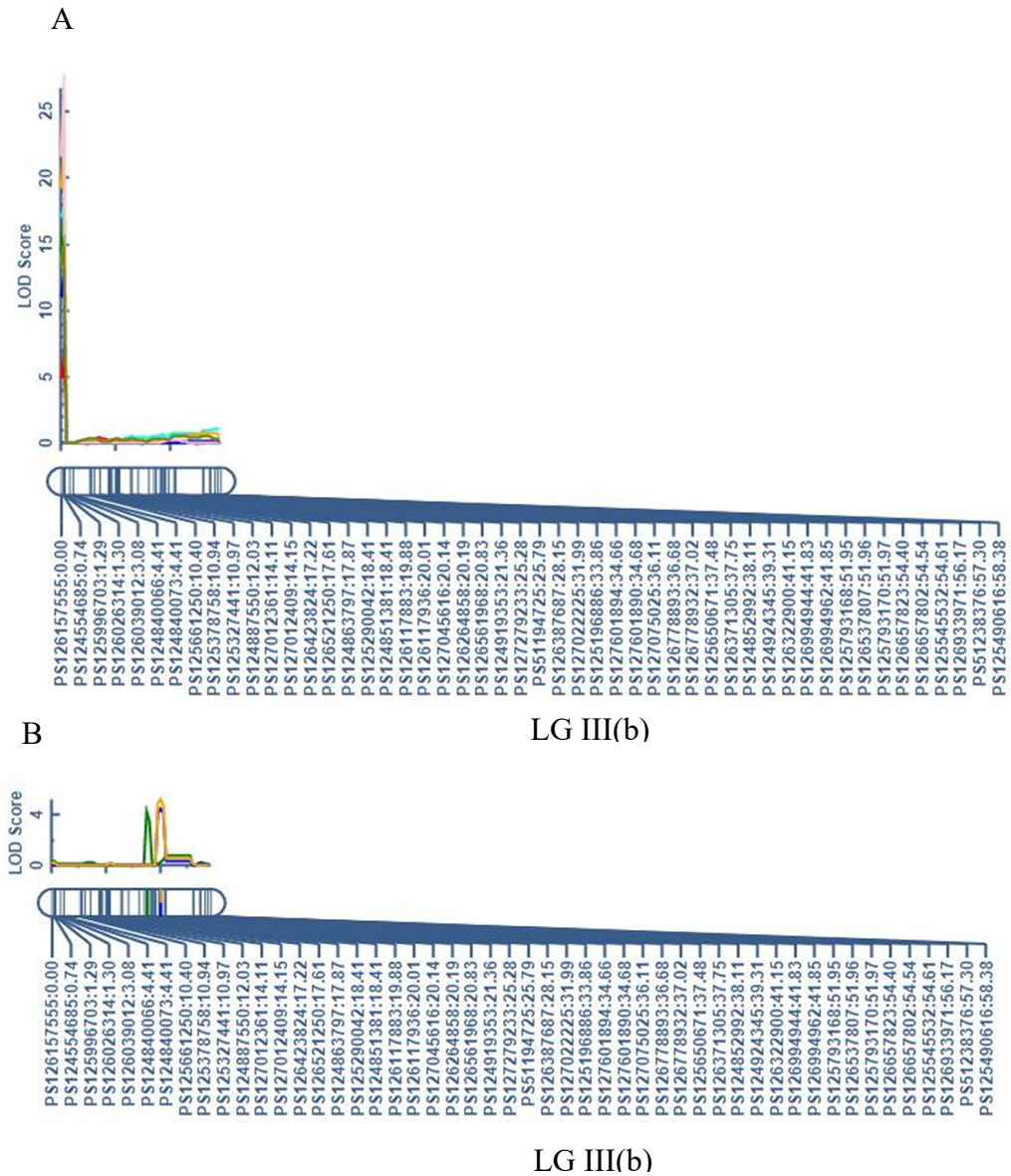

Figure 4-10: QTL associated with lesion expansion at position 0-1 cM on LG III(b) on the same position with *Le*, based on the complete dataset for the Population 19 (pink=height, orange= nodal resistance, green= lesion expansion) (a) and QTL associated with lesion expansion located on LG III(b) neighboring *Le* loci based on the short internode individual's subset of Population 19 (b).

Table 4-7: QTLs identified for reaction to inoculation with *S. sclerotiorum* and four physiological anchor markers based on the complete dataset for Population 19.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2b}	Add[c]
Height (*Le*)	III(b)	1	PS124554685	PS125996703	26.7	63.4	0.4
Height (*Le*)	III(b)	0	PS126157555	PS124554685	21.6	55.5	0.4
Lesion expansion (3 dpi)	III(b)	1	PS124554685	PS125996703	6.8	20.6	7.6
Lesion expansion (3 dpi)	III(b)	0	PS126157555	PS124554685	4.9	15.4	6.6
Lesion expansion (7 dpi)	III(b)	1	PS124554685	PS125996703	17.1	43.2	25.6
Lesion expansion (7 dpi)	III(b)	0	PS126157555	PS124554685	14.3	37.9	24
Nodal resistance (7 dpi)	III(b)	0	PS126157555	PS124554685	17	47.2	0.5
Lesion expansion (14 dpi)	III(b)	1	PS124554685	PS125996703	12.5	37.6	39.2
Lesion expansion (14 dpi)	III(b)	0	PS126157555	PS124554685	11	33.9	37.3
Nodal resistance (14 dpi)	III(b)	1	PS124554685	PS125996703	19	51.1	0.7
Nodal resistance (14 dpi)	III(b)	0	PS126157555	PS124554685	16.6	46.5	0.7
Survival rate	III(b)	1	PS124554685	PS125996703	14.7	42.8	11.4
Survival rate	III(b)	0	PS126157555	PS124554685	13.4	39.8	11
Lesion expansion (14 dpi) %	III(b)	1	PS124554685	PS125996703	14.7	42.8	-11.4
Lesion expansion (14 dpi) %	III(b)	0	PS126157555	PS124554685	13.4	39.8	-11
AUDPC	III(b)	1	PS124554685	PS125996703	14.	41.3	391.3
AUDPC	III(b)	0	PS126157555	PS124554685	11.6	35.5	364.1
Lesion expansion (7 dpi)	VI	528	PS51241124	PS126932560	48.1	254.5	-61.1
Lesion expansion (7 dpi)	VI	532	PS125390907	PS124517346	42.7	194.3	53.4

[a] Phenotypic variation explained by QTL.
[b] Additive effect

Table 4-8: QTLs identified based on the long internode subset of RILs for Population 19.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2a}	Add[b]
Nodal resistance (14 dpi)	III(b)	3	PS126026314	PS126039012	4.4	24.2	0.7
Nodal resistance (14 dpi)	III(b)	4	PS126039012	PS124840066	4.3	23.9	0.7
Nodal resistance (14 dpi)	VI	289	PS126638386	PS125216252	2.9	15.81	-0.3

[a] Phenotypic variation explained by QTL,
[b] Additive effect.

Table 4-9: QTLs identified based on the short internode subset of individuals for Population 19.

Trait name	LG	Position	Left marker	Right marker	LOD	R^{2a}	Add[b]
Lesion expansion (7 dpi)	III(b)	4	PS126039012	PS124840066	5.3	36.3	22.8
Lesion expansion (14 dpi)	III(b)	0	PS126157555	PS124554685	3.4	24.9	0.4
Nodal resistance (14 dpi)	III(b)	1	PS124554685	PS125996703	4.5	31.3	0.5
Nodal resistance (14 dpi)	III(b)	0	PS126157555	PS124554685	3.4	24.9	0.4
Survival percentage	III(b)	1	PS124554685	PS125996703	3.4	24.4	7.9
Lesion expansion (3 dpi)	VII	74	PS126494615	PS126103738	3	22.4	5.6

[a] Phenotypic variation explained by QTL.
[b] Additive effect

Three QTLs associated with nodal resistance were identified on LG III (b) and VI based on the long internode subset of Population 19. Two QTL were located on position 3, and 4 cM on LG III (b) close to *Le* with LOD=4.4, and 4.4, and explained 24.2, and 23.9% of the phenotypic variation in the long internode subset of Population 19 (Table 4-8, Figure 4-10b). The QTL associated with nodal resistance was positioned at 289 cM of LG VI with LOD= 2.9 and R^2=15.8% (Table 4-8).

Four QTL associated with lesion expansion and nodal resistance were found on linkage group III (b) based on the short internode subset of Population 19. The QTL related to lesion expansion located at position 0, 1 and 4 cM on LG III (b) were in the same location as *Le* with LOD of 3.4, 3.4, and 5.3 and explained 24.9, 24.4 and 36.3 of the phenotypic variation (Figure 4-10b). The QTL at position 74 cM of LG VII as associated to lesion expansion with LOD=3 and phenotypic variance explained by that was 22.4%. Two QTL associated with nodal resistance were located in the same place as *Le* gene on position 0 and 1 cM with LOD of 3.4 and 4.5 and explained phenotypic variation of 24.9 and 31.3% (Table 4-9, Figure 4-10b).

Identification of SNP marker sequences associated with partial resistance to *S. sclerotiorum*

A BLAST search of SNP flanking sequences associated with white mold resistance QTL against the *Medicago truncatula* MedtrA17_4.0 database revealed several genes involved in plant defense (Table 1-7).

Table 4-10: Genes found from a BLASTN search against *Medicago truncatula* MedtrA17_4.0 of SNP flanking marker sequences associated with resistance to white mold.

Markers	Overlapping genes	MedtrA17_4.0	E-value
Population 17			
PS1982218	translation elongation factor EF protein	MTR_2g020660	1.9E-24
PS1442566	import component Toc86/159, G and M domain protein	MTR_8g088370	1.9E-18
PS1459106	ubiquitin-conjugating enzyme/RWD-like protein	MTR_4g075250	2.5E-5
PS940189	LRR receptor-like kinase	MTR_2g074980	4.7E-19
PS1456698	group 3 LEA (Late embryogenesis abundant) protein	MTR_4g123950	2.6E-8
PS854069	PPR (pentatricopeptide repeat) containing plant protein	MTR_4g086490	4.7E-19
PS1361172	DHHC-type zinc finger protein	MTR_4g074600	4.9E-22
PS1327224	cyclic nucleotide-gated ion channel-like protein	MTR_4g130820	6.8E-12
PS1936862	transmembrane protein, putative	MTR_4g130250	1.0E-7
PS1629126	nitroreductase family protein	MTR_4g025130	4.9E-22
PS987377	PLC-like phosphodiesterase superfamily protein	MTR_4g085750	8.3E-27
PS1804616	hyccin protein	MTR_4g094262	1.3E-25
PS1837440	copper-transporting ATPase PAA1, putative	MTR_4g094232	9.6E-39
PS1041951	plant/F1M20-13 protein	MTR_4g094375	1.1E-13
PS1233071	type I inositol-1,4,5-trisphosphate 5-phosphatase	MTR_1g071680	1.4E-31
PS1476740	gamete protein	MTR_2g435850	0.0015
Population 19			
PS51241124	RNA polymerase II-binding domain protein	MTR_6g027180	1.7E-9
PS126932560	MATE (Multi-antimicrobial extrusion protein) efflux family	MTR_6g027190	1.2E-16
PS125390907	cationic amino acid transporter 2, vacuolar protein	MTR_6g027630	8.6E-30
PS126638386	lipid transfer protein	MTR_4g029350	0.0061
PS125216252	DUF3527 domain protein	MTR_4g019900	1.7E-12
PS126494615	P-loop nucleoside triphosphate hydrolase superfamily protein	MTR_4g119980	1.2E-19

Discussion

Breeding resistant pea varieties to *S. sclerotiorum* is challenging. There is vague insight into genes related to the pathogen-host interaction (pathogenicity and resistance genes) due to the lack of sequence information of pea genome and more importantly the absence of fully resistant

to *S. sclerotiorum* in pea germplasm. The *S. sclerotiorum* pathogen infects a broad host range including important crops such as soybean, bean, canola, and sunflower with different mechanisms and there is no report of complete resistant in any crop (Bolton et al. 2006). The objective of his study was to map the quantitative trait loci related to partial resistance to *S. sclerotiorum* in pea for future genetic improvement. Our phenotypic data shows the positive correlation between lesion expansion and nodal resistance with plant height (Table 3-2, Table 3-3). Also, survival percentage of long internode individuals' subsets in both Populations noticeably were higher than short internode individuals' subsets (Table 3-7). Porter et al. (2009) indicated that internode length and lesion expansion may be positively correlated in pea accession lines challenged with to *S. sclerotiorum*. Lesion expansion of sunflower challenged with *S. sclerotiorum* infection was also positively associated with plant height (Bazzalo et al. 1991). Two out of three detected QTL contributing to soybean partial resistance to *S. sclerotiorum* were associated with physiological avoidance such as plant height, lodging, and date of flowering (Bazzalo et al. 1991). Height in pea was assumed to be a confounding factor that might obscure the result of real effect of *S. sclerotiorum* infection on pea. To prevent that, QTL analysis was performed on the complete set of data from Population 17 and 19 as well as the tall and short internode subsets of both populations. The inheritance of resistance to *S. sclerotiorum* is partial and quantitative and is obvious from the distribution of phenotypic data for disease resistance and several observed putative QTL from this study. It seems that physiological resistance and morphological avoidance are the components of the partial resistance of pea to white mold. Plant height which was positively correlated with lesion expansion and nodal resistance score may be associated with disease escape as soybean varieties response to *S. sclerotiorum* also depends on disease escape mechanism due to height, maturity

and lodging (Boland and Hall 1986). Although we should consider plant height in the breeding program, it is hard to evaluate the role of these factors in avoidance of infection. Overall, it may be better and more reasonable to select QTL for resistance mechanisms contributing to restrict development of the pathogen in pea.

Although, there have been many reports on QTL identification of *S. sclerotiorum* resistance on sunflower (Micic et al. 2005), soybean (Iquira, Humira, and François 2015; Bastien, Sonah, and Belzile 2014), bean (Miklas 2007; Kolkman and Kelly 2003; Ender and Kelly 2005), rapeseed (*Brassica napus*) (Wu et al. 2013) and *Brassica oleracea* (Mei et al. 2013), there is only one report on *Pisum sativum* (Tashtemirov 2012). Until now, the only QTL mapping in pea concerning resistance to *S. sclerotiorum* was established on F_2 lines from the cross between Lifter and PI240515 (Population 17 parents). Two QTL (SSR markers: AA255 and AD73) were found on LG II and LG III related to NTI and LEI, respectively (Tashtemirov 2012). The RNA sequencing analysis of Lifter and PI240515 on 12, 24 and 48 hours post inoculation (hpi) showed that more leucine rich-repeat containing transcripts and oxidoreductase transcripts were found for lesion resistance, while VQ (Valine-glutamine) motif-containing proteins and a myo-inositol oxygenase were found for nodal resistance (Chang et al. 2017). VQ motif-containing proteins play significant roles in many abiotic and biotic stress responses in plants (Jiang et al. 2018). Chang et al. (2017) also used genotyping by sequencing and phenotypic data of 282 lines from Porter et al. (2009) to apply genome-wide association studies (GWAS). They used GWAS to understand lesion expansion and nodal resistance of *Pisum sativum* to white mold and found 206 and 118 SNPs significantly associated with lesion and nodal resistance, respectively (Chang et al. 2017).

We verified QTL associated with lesion expansion on LG III in both Population 17 (entire set and short subset) and Population 19 (entire set, short and tall subset) and validated a QTL associated with nodal resistance on LG II between PS1233071 and PS1476740 based on the short internode subset of Population 17. Although most of the detected QTL located on LG III, were adjacent to *Le* and associated with physiological avoidance, there were two QTL identified based on the complete dataset of Population 17 at position 88 and 91cM distant to *Le.* Overall, after excluding the eight QTL associated with physiological avoidance, there were 19 QTL associated with *S. sclerotiorum* resistance, on both populations and their short and tall subsets. Seventeen QTL were associated with lesion expansion and two QTL were linked to nodal resistance. There is a noticeable QTL in LG VI of entire set of Population 19 with high LOD (48.1). The QTL associated with lesion expansion on LG VII were duplicated in the short internode subset of both populations. The underlying reason of less number of QTL responsible for NTI should be inefficiency of 0-4 scoring scale of NTI measurement (Porter et al. 2009), that does not give much variation. It has been expected that lesion expansion inhibition data has some measure of nodal transmission inhibition data embedded within it which cannot be separated out. The additive effect of each QTL shows the contribution of each parents on resistance to white mold. The QTL with positive additive effect shows, that allele contributed from first parent and if its additive effect is negative, means loci come from second parent.

BLAST-based sequence analysis of flanking markers of QTL associated with *S. sclerotiorum* resistance against *Medicago truncatula* (MedtrA17_4.0) identified genes that may be involved with resistance. The BLASTN analysis of lesion restriction markers based on the greenhouse evaluation (PS940189 on 88, 89 cM of LG III position), (PS854069 on position 261cM of LG VII) and detached stem assay (PS1629126 on position 69 cM of LG VII) in the

complete dataset of Population 17 revealed LRR receptor-like kinase, pentatricopeptide repeat containing protein and nitroreductase family protein, respectively as described in (Chang et al. 2017). Through BLAST analysis of nodal resistance markers on LG II (PS1233071) from the short internode subset of Population 17, type I inositol-1,4,5-trisphosphate 5-phosphatase was detected and is in accordance with Chang et al. (2017).

Some of the recognized genes from BLASTN analysis have been identified in transcriptome or proteomics analysis of various hosts during *S. sclerotiorum* infection. For example, elongation factors play important roles in providing stress adaptation and down-regulated in the proteomics analysis of pea plants infected with *S. sclerotiorum* (Akansha Jain et al. 2015). Pentatricopeptide repeat proteins which are involved in cell maintenance and development, and LRR containing genes which contribute to R-gene based resistance were differentially expressed during *Sclerotinia sclerotiorum* and *Brassica napus* interaction (Zhao et al. 2007; Wei et al. 2016; Wu et al. 2016). The DHHC-type zinc finger protein (ZFP) identified as candidate genes by GWAS and transcriptome sequencing analysis of resistance to Sclerotinia stem rot in *Brassica napus.* (Wei et al. 2016). MATE transporter (Yang et al. 2007) and amino acid transporter (Yang et al. 2007; Seifbarghi et al. 2017) transcripts were increased in abundance in the *S. sclerotiorum- B.napus* interaction. MATE transporters may be involved in transport of plant-produced metabolites essential for defense such as anthocyanins, nicotine, and salicylic acid (Yang et al. 2007; Rowe et al. 2010). Leucine-rich repeat receptor-like kinase is a pathogen associated molecular pattern recognition receptor and is upregulated during the *S. sclerotiorum-B. napus* interaction (Wu et al. 2016; Zhang et al. 2013). Genetically modified rapeseed (*Brassica napus*) with a lipid transfer protein gene showed better disease resistance to *S. sclerotiorum* and had lower melondialdehyde (MDA) content, higher super oxide dismutase

(SOD), and peroxidase (POD) activity than non-transgenic plants (Fan et al. 2013). The ubiquitin-conjugating enzyme activity induced pattern triggered immunity in tomato and did not affect programmed cell death induced by several effector-triggered immunity elicitors (Zhou et al. 2017).

In this research we developed two genetic maps on Populations 17 and 19 based on SNP markers from GBS data. Linkage groups of each genetic map were assigned base on segregating phenotypic marker loci and synteny with *Medicago truncatula*. Second, we studied the genetics of *S. sclerotiorum* resistance in two pea populations to identify markers for the selection of *S. sclerotiorum* resistance phenotype in breeding programs. Overall nineteen QTL, from Population 17 and 19 and their vine length subsets associated with *S. sclerotiorum* resistance. Seventeen QTL were associated with resistance to white mold with lesion expansion and two QTL were associated with nodal resistance. It is important to relate our genetic maps with consensus Pisum maps to enable comparative analysis of different maps. These QTL should be validated in field experiments and different populations. Using *S. sclerotiorum* resistance markers in marker-assisted selection may facilitate pea breeding programs in developing resistant cultivars.

CHAPTER 5: CONCLUSION

Field pea (*Pisum sativum* L.) is the second most common legume in the world. North Dakota with total production of 413,280 tones which worth $94 million led the nation in pea production in 2017 (USDA-NASS 2017). Among many diseases that infect pea plants white mold caused by *Sclerotinia sclerotiorum,* is one of the most important pathogens of field pea production worldwide. Annual loss in pulse crop was $12 million based on USDA report in 2016 in United States. Management strategies such as crop rotation, biological control and fungicide treatments do not entirely eradicate the pathogen due to the fact that the pathogen can last at least 5 years in the soil and has a wide host range (Peltier et al. 2012; Jain et al. 2012). Development of resistant cultivars to *S. sclerotiorum* is an economical practice to control white mold disease, however, until now there are no pea genotypes with complete resistance to this pathogen (Bolton et al. 2006). Inheritance of resistance to *S. sclerotiorum* is quantitative with partial resistance (Davar et al. 2013) and has been expressed in two forms, lesion expansion inhibition (LEI) and nodal transmission inhibition (NTI) (Porter et al. 2009).

Development of a reliable phenotyping method is required to evaluate pea germplasm to have a better understanding of genetic control of host resistance to *S. sclerotiorum* and breed resistant varieties. Genotyping the field pea genome to develop a linkage map and discover single nucleotide polymorphism associated with *S. sclerotiorum* resistance is the primary objective of this study.

Greenhouse evaluation, detached stem assay and genotyping by sequencing (GBS) of two populations, Population 17 (Lifter/ PI240515), and Population 19 (PI169603/ Medora), were conducted in order to identify genotypes with partial resistance and genomic locations (QTL) responsible for resistance to *S. sclerotiorum.* It is clear that internode length and lesion expansion

and nodal resistance score have been positively correlated. Survival rate was also much higher in long versus short internode plants although short internode plants are favored by growers. To avoid height interfering in our experiment, Population 17 and 19 were divided into short and long internode subsets and then analyzed. Twenty-two inbred lines were identified from short genotypes of Population 17 and 19 which showed partial resistance and met at least two of the resistance criteria in the greenhouse evaluation as proposed by Porter et al. (2009). To decrease the genotyping error of GBS method due to low coverage, three different pipeline methods were used to call SNPs. GBS-SNP-CROP pipeline extracted higher number SNPs with high quality which were used for mapping population. QTL analysis of Population 17 and 19 confirmed our assumption of correlation between height and lesion expansion and nodal resistance score since there were 8 QTL close to *Le* on LG III controlling internode length.

Nineteen QTL were identified as contributing to resistance to *S. sclerotiorum*. Seventeen QTL were associated with lesion expansion inhibition (LEI) and two QTL were associated with nodal transmission inhibition (NTI). BLAST analysis of markers harboring these QTL revealed that genes underlying the QTL in this study have been identified in transcriptome or proteomics analysis of several hosts during *S. sclerotiorum* infection including pea- *S. sclerotiorum* interaction (Akansha Jain et al. 2015). Our results confirmed earlier reports that multiple genes are responsible for white mold resistance (Davar et al. 2013).

This study identified a group of partially resistance inbred lines that can be used in further experiments as resources for resistance to *S. sclerotiorum*. Further field and multiple environment testing is necessary to validate the value of these inbred lines. QTL responsible for resistance to white mold identified in this study should be further validated in field experiments and among different pea populations. The candidate genes associated with significant QTL and

associated SNPs along with future research will lead to a better understanding of white mold resistance mechanisms in pea. Marker-assisted breeding using white mold partial resistance markers may enhance pea breeding programs aiming to develop resistant cultivars.

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APPENDIX

Table A-1: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 short internode subset in green house evaluation.

Short internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-8	31.3	46	1.9	55.6	0.2	9.5	780.9
PRIL17-11	23.8	31.1	3	68.6	0.2	27.1	651
PRIL17-12	31.3	42.9	2	90.7	0.1	6.8	875.6
PRIL17-15	32.7	85.7	2	99.4	0.7	13.9	1298.8
PRIL17-17	29.8	43.2	1.8	55	0	11.3	744.7
PRIL17-19	25.2	78.9	3.5	134.5	0.3	41.8	1319.1
PRIL17-20	36	80.2	2.4	111.8	1.5	18.8	1311.1
PRIL17-22	35.9	62	3.3	84.9	0.5	30.4	1053.3
PRIL17-23	50.9	76.3	2	101.8	1	9.9	1322.6
PRIL17-24	26.6	58.6	3.1	89.2	0.8	14.7	985.7
PRIL17-25	40.9	74.6	2	99.1	0.4	14.4	1242.6
PRIL17-43	46.2	60.5	2.6	67	0.4	32	1032.3
PRIL17-46	25.9	30.5	1.9	54	0.6	15.6	605.9
PRIL17-47	30.7	35	1.9	45.7	0.3	12.9	643.7
PRIL17-49	46.2	77	1.2	104.6	1.7	0	1313
PRIL17-50	46.1	63.5	2.2	97	2.8	17.4	1164.2
PRIL17-54	50.4	133.9	3.2	225	1.4	44.2	2269.9
PRIL17-56	24.3	35.7	2.3	56.9	1.5	15.6	654.3
PRIL17-58	40.7	71	3.2	105.3	1.5	33.2	1231.7
PRIL17-59	46.4	76.6	2.3	94.5	2.8	19.2	1275.5
PRIL17-62	35	76.8	1.8	95.7	3	2.4	1218.5
PRIL17-64	32.6	56.6	1.5	78.3	1.7	14.6	962.9
PRIL17-66	34.5	64.1	1.5	82.2	1.5	13.8	1054.4
PRIL17-71	43.1	79.9	1.8	96.3	1.2	34.4	1293.8
PRIL17-73	40.2	71.6	2.8	95.4	2.4	26.1	1199.3
PRIL17-76	36.1	69.6	2.3	107.5	3	8.4	1200.6
PRIL17-78	41.7	74.2	2.1	111.7	0.8	15.5	1288.4
PRIL17-83	40.6	81.3	1	96.9	3	2.1	1293.7
PRIL17-86	32.5	56.9	1.3	75.3	2.2	11	954.3
PRIL17-87	32.1	76.6	4.1	123	1.6	15.2	1296.4
PRIL17-89	39.4	66	1.8	83.4	1.7	12.6	1102.7

Table A-1: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 short internode subset in green house evaluation (continued).

Short internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-90	49.3	64.5	1.4	77.4	1.5	21	1122.2
PRIL17-92	35.3	49.7	2.5	80.5	1.8	18.7	923.4
PRIL17-93	26	61.3	0.4	68.6	1.2	0	934.9
PRIL17-94	27.4	44	1.6	75	1.3	0.2	809.4
PRIL17-95	34.2	63.5	2.9	83.6	0.8	37	1051.7
PRIL17-97	40.2	60.4	2.2	78.3	1.4	30.4	1038.2
PRIL17-99	38.8	68.4	2.3	108.4	2	6	1208.5
PRIL17-100	32.6	66.1	1.3	82.9	2	4.6	1064.1
PRIL17-101	37	67.2	2	97	0.5	4.7	1148.2
PRIL17-102	37.5	63.9	1.1	78.8	2	1	1057.6
PRIL17-103	28.7	58.9	1.8	79.8	0.9	10.7	967
PRIL17-104	33.8	48.8	0.9	69.4	1.3	0	867.5
PRIL17-105	30.6	51.6	1	63.3	1.1	2.3	853.9
PRIL17-110	26.7	50.9	0.9	63.7	1.8	14.1	827.5
PRIL17-112	42.7	72.4	2.3	98.2	3.3	18.3	1229.9
PRIL17-114	33	56.8	2.4	86.4	2.5	10.6	995
PRIL17-116	29	66.5	1.3	96.8	2.8	7.7	1096.9
PRIL17-119	29.9	59	1.2	77.5	1.3	16.7	965.9
PRIL17-120	29.2	49.3	1.2	74.9	2.4	2.1	866.5
PRIL17-125	27.4	49.5	2.1	57.2	0.8	29.7	796
PRIL17-127	40.7	77.2	3.5	109.5	1.4	52.1	1302
PRIL17-128	30.4	43.3	2.4	59.2	1	25.2	764
PRIL17-129	27.2	51.9	2.3	62.1	2.3	31.7	833.8
PRIL17-131	28.5	42.1	2.1	54.9	0.7	25.5	728.1
PRIL17-132	26.3	34.3	3.2	46.8	0.7	55.6	617.1
PRIL17-136	37.3	143.7	3.5	180.9	0	40	2131.3
PRIL17-139	33.8	49.3	2.6	72.3	1.2	31.4	882.7
PRIL17-141	36.1	55.9	2.5	82.6	2.9	29.8	991
PRIL17-143	28.5	51.7	2	63.5	1.2	23.1	844.7
PRIL17-145	26.7	30.9	2.9	44.4	2.5	53.5	580.7
PRIL17-149	45.8	104.6	3.5	135.1	1.3	44.5	1665.8
PRIL17-150	40.2	63	3.4	79.7	0.3	54.9	1067
PRIL17-151	37.6	56	2.9	78	0.6	31.8	983.1

Table A-1: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 short internode subset in green house evaluation (continued).

Short internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-156	44	64.7	2.8	85.2	0.6	27	1122.5
PRIL17-157	28.1	42.3	2.6	53.3	0.9	38.1	722
PRIL17-158	26.7	42.9	2.3	55.6	1.8	25.3	727.9
PRIL17-161	30.5	50.3	3.1	77	0.5	36.9	889.4
PRIL17-162	28.4	45.9	2.1	62.4	1.1	17.8	787.1
PRIL17-163	28.5	49.3	2	67.7	0.7	30.4	837.1
PRIL17-164	36.5	63.9	2.5	95.2	0.2	16.1	1109
PRIL17-165	22.1	41.8	2.5	57.1	0.5	23.7	697.3
PRIL17-166	24.7	37.8	3	51	0.3	34	654.7
PRIL17-168	34.5	74.3	3	95.9	0.6	25.5	1194.2
PRIL17-173	36.6	61.2	2.8	85	1	29.6	1049.7
PRIL17-174	37.5	83	1.3	88.8	1.1	8.2	1264.3
PRIL17-175	23.5	42.1	2.2	62.9	0	21.4	728.3
PRIL17-176	27.1	53	1.8	65.4	0.1	18.1	855
PRIL17-180	31	45.7	3	73.1	1.6	34.6	837.2
PRIL17-181	23.1	26.9	3.3	33.7	1.5	58.2	486.9
PRIL17-182	34	45.1	3.3	63.8	0.4	44.1	815.9
CV (%)	31.1	47.5	49.1	47.7	107.7	114.7	39.5
LSD	6.7	17.6	0.7	22.9	0.8	16.1	181.3

Table A-2: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 long internode subset in green house evaluation.

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-1	40.3	111.2	3.5	260.3	3.3	37.2	2132.8
PRIL17-2	38.7	88.6	3.5	224	3.3	53.9	1794
PRIL17-3	45.1	82.2	3.5	204.2	3.3	56.4	1702.4
PRIL17-4	37.2	120.7	3.3	311.1	2.9	34.6	2379.1
PRIL17-5	36.7	117.6	3.5	160.9	3.5	68.3	1823.8
PRIL17-6	37.5	92.5	3.5	314.8	3.3	31.4	2140.3
PRIL17-7	27	87.8	3.5	168.5	3	65.7	1528.3
PRIL17-9	46.9	129.6	3.1	209.7	2.5	50.2	2157.6
PRIL17-10	50.1	124.6	3.2	254.7	2.8	30	2288.6
PRIL17-13	31.8	69.9	3.3	169.9	3.2	59.2	1399.3
PRIL17-14	45.1	96.5	3.5	258.8	3.1	47.7	2022.7
PRIL17-16	24	91	3.5	174.3	3.4	65.7	1561.1
PRIL17-18	35.4	98.1	3.5	154	3.4	70.8	1616.9
PRIL17-21	53.2	133.9	3.5	235.1	3.3	53.9	2320.7
PRIL17-26	35.3	80.2	3.4	142.9	2.9	72	1416.3
PRIL17-27	28.1	80.8	3.5	226.4	3	48.1	1673.9
PRIL17-28	24.2	92.5	3.5	222.7	3.4	53.3	1745.2
PRIL17-29	35.4	93.9	3	190.3	2.6	63.6	1705.4
PRIL17-30	70.2	231.3	3.2	322	2.7	29.6	3594.1
PRIL17-31	33.3	224.9	3.5	266.4	2.9	42.3	3139.8
PRIL17-32	33	77	3.5	247.8	3.5	45.3	1741.9
PRIL17-33	29.5	107.8	3.5	145.8	3.1	61.4	1642.6
PRIL17-34	35.8	130.8	3.7	246.6	3	55.5	2237
PRIL17-35	31.6	91.4	3.3	255.4	3	45.8	1890
PRIL17-36	40.5	77.5	3.3	185.8	3	51.7	1570.4
PRIL17-37	45.5	116.7	2.3	209.2	1.7	47.4	2032.7
PRIL17-38	37.9	86	3.3	156.6	3.2	52.6	1530.4
PRIL17-39	34.9	51.7	3.5	157.7	2.7	47.4	1209.3
PRIL17-40	39.9	165.7	3.6	233.4	2.9	49.6	2527.1
PRIL17-41	23.2	67.6	3.6	149.7	3.3	65.2	1259.6
PRIL17-42	45.2	93.3	3.5	190.2	3.2	52.5	1753.9
PRIL17-44	46.3	174.3	3.5	295.6	2.6	36.4	2858.1
PRIL17-45	44.4	165.8	3.5	324.4	3.2	30.1	2871.3

Table A-2: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 long internode subset in green house evaluation (continued).

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-48	30.8	66.5	3.2	151.8	2.7	46.1	1298.7
PRIL17-51	27.6	73	3.5	207.3	3	51	1534.5
PRIL17-52	45.9	78.9	3.1	219.9	2.1	27.2	1732.1
PRIL17-53	32.8	72	3.5	187.7	3.3	43	1485.6
PRIL17-55	37.1	91.3	3.5	203.7	3.5	60.8	1738.7
PRIL17-57	45.7	97.5	3.3	261.1	2.6	38.2	2042.5
PRIL17-60	47.3	157.6	3.5	259.2	3.5	36.4	2585.2
PRIL17-63	49.7	180.4	3.3	247.6	2.3	28.9	2762.9
PRIL17-65	70.8	147.5	2.7	190.2	1.9	36.9	2382.8
PRIL17-67	68.7	148.1	3.4	188.8	2.6	43.4	2371.3
PRIL17-68	66.9	143.6	2.8	249.8	2.5	31.1	2534.6
PRIL17-69	53.8	153.8	2.8	186.4	2.3	36.7	2332.2
PRIL17-70	56.6	176.6	3.3	223.2	2.6	35.2	2682
PRIL17-72	38.1	129.2	3.5	177.1	3.2	53	1992.3
PRIL17-74	51.2	135.9	3.5	175.6	3.1	50.3	2119.5
PRIL17-75	52	115.5	3.3	153.7	3	30.9	1863.9
PRIL17-77	44.5	123.7	3.2	216.3	1.4	19.8	2114.8
PRIL17-79	55.8	158.6	3.5	208.5	2.3	42.6	2464.5
PRIL17-80	64.3	144.6	3.1	188.1	2.3	40.9	2312.7
PRIL17-81	58	153.7	3	256.6	1.2	20.2	2600.5
PRIL17-82	48.4	156.9	3.5	206.6	2.6	42.9	2401.2
PRIL17-84	55.9	154	3	236.6	1.2	22.9	2521.4
PRIL17-85	48	163.5	2.6	237.8	1.7	25.1	2568
PRIL17-88	44.3	121	2.8	160.4	2.2	41.9	1893.8
PRIL17-91	49.8	133.3	3	187	2.8	42.2	2128.2
PRIL17-96	50.6	127.5	3.2	184.5	2.4	36.1	2071.2
PRIL17-98	45.5	102.8	3	126.3	2.3	50	1617.6
PRIL17-106	38.2	161	3.3	204.5	2.7	33.4	2374.7
PRIL17-107	57.2	168.8	3.5	235	2.7	33.2	2656.5
PRIL17-108	49.9	175.1	3.5	214.9	2.7	38.7	2602.1
PRIL17-109	47.6	132.7	3.2	173	3	47.9	2062
PRIL17-111	66.6	118	2.7	152.7	2.5	38.8	1962.6
PRIL17-113	59.2	124.4	2.7	154.2	2.1	33.9	1984.4

Table A-2*:* Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 long internode subset in green house evaluation (continued).

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-115	52.8	130.3	3.2	184.6	3	42.4	2109.4
PRIL17-117	44.8	132.8	3.2	187.3	2.2	42.4	2097.3
PRIL17-118	76.1	156.6	2.3	241.6	2	37.7	2673.3
PRIL17-121	47.5	93.9	3.3	128.4	2.7	45.9	1555.5
PRIL17-122	48.4	160.8	3.5	203.7	3	43	2425.8
PRIL17-123	36.2	91.6	3.5	138.8	3.3	55	1509.8
PRIL17-124	28.3	62.7	3.3	89.5	3.2	67.2	1033.3
PRIL17-125	17.8	23.5	3.6	29.5	3.6	63.9	412.2
PRIL17-126	45.8	129.2	3.3	210.4	3	33.5	2151.4
PRIL17-130	36.4	101.5	3.5	143.9	3.2	48.8	1617.2
PRIL17-133	48.8	87.7	3.5	154.4	3.1	48.1	1598.5
PRIL17-134	43.3	103.9	3.6	134.5	3.6	54.3	1644.1
PRIL17-137	54.3	105.4	3.5	138.8	3.2	58.5	1732.9
PRIL17-138	31.9	69.2	3.5	130.9	3.2	56.5	1256.7
PRIL17-142	58.7	138.5	3.3	183.2	3.2	36.2	2210.5
PRIL17-144	35.5	88.9	3.4	169.8	3.2	48.9	1589.7
PRIL17-146	38.9	105.5	3.5	152.5	3.4	53.7	1696.6
PRIL17-147	38.3	83	3.5	126.2	3.4	53.9	1399.6
PRIL17-152	39.9	98.3	3.3	174.1	2.9	36.7	1713.2
PRIL17-153	38.6	93.7	3.4	132.8	2.9	49.2	1520.1
PRIL17-154	55.6	107.3	3.4	176.8	2.9	37.8	1890.1
PRIL17-155	49.3	107.9	3.5	182.1	3.3	44.8	1879.5
PRIL17-159	41.3	94.9	3.4	170.7	3	43.4	1678.2
PRIL17-160	54.7	113.1	3.3	144.5	2.9	43.5	1824.7
PRIL17-167	33.5	105.9	3.3	142.6	3.1	50.3	1635.9
PRIL17-169	53.5	135.3	3.2	193.2	2.9	44.6	2188.1
PRIL17-170	56.4	111.7	3	182.4	2.7	39.3	1954
PRIL17-171	38.6	84.3	3.1	149.2	3	43.3	1492.9
PRIL17-172	40.3	107.1	3.5	183.7	3.3	45.4	1828.8
PRIL17-177	44.5	73.9	3.5	94	3.4	63.5	1238.6
PRIL17-178	47.6	107.1	3.3	145.2	3.1	48.2	1733.6
PRIL17-179	25.2	31.9	3.5	44.3	3.5	74.4	580.8
PRIL17-183	46.1	107.8	3.3	148.9	3	55.9	1744.8

Table A-2*:* Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 17 long internode subset in green house evaluation (continued).

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL17-184	54.1	184.9	3	216.9	2.6	33.9	2720.7
PRIL17-186	45.6	152.9	3.4	196.1	3	41	2312.6
PRIL17-187	37	77.4	3.5	99.2	3.4	72.2	1247.5
PRIL17-188	41.9	64	2.8	143.4	2.6	52.1	1308.4
PRIL17-189	75.4	158.1	2.6	228.7	2	29.6	2637.7
CV (%)	35.9	44.3	13.1	40	26.2	43.4	27.3
LSD	10.3	32.9	0.3	51.7	0.5	14.2	312.1

Table A-3: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 19 short internode subset in green house evaluation.

Short internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL19-2	28.7	76.5	2	108.2	0	0	1225.1
PRIL19-5	46.7	79.5	2	116.2	0.4	7.5	1378.4
PRIL19-9	56.8	89.5	1.8	138.2	0.5	6.5	1602
PRIL19-11	42.1	98.4	1.7	155.6	0.6	9.1	1662
PRIL19-13	50.5	88.2	1	107.2	0	0	1447
PRIL19-14	39.7	110.3	1.8	137.1	0.6	10.1	1690.4
PRIL19-15	33.9	64.9	1.4	85.5	0	0.1	1070.1
PRIL19-16	31.6	59.8	0.4	69.2	0	0.4	954.7
PRIL19-18	63.1	145	2.3	195.7	1.4	33.8	2336.4
PRIL19-27	35.2	88.1	1.5	113.7	0.2	4.5	1384.6
PRIL19-30	50.3	85.7	1.7	118.9	0.3	3.4	1464.2
PRIL19-32	57.7	80.7	2.2	133.1	0.7	11	1509.7
PRIL19-34	44.1	95.2	2.6	154	0.8	21.6	1638.9
PRIL19-37	56.4	131.6	2	172	0.5	4.1	2096.1
PRIL19-41	57	79.4	1	86.5	0	0	1331.3
PRIL19-43	65.2	184.3	2	294.4	0.4	5.1	3047.5
PRIL19-44	29.7	59.8	1.4	85.7	0	0	1001.4
PRIL19-45	46.4	97.5	1.5	154.7	0	0	1673.8
PRIL19-47	34.8	56.9	2.1	105.7	0.3	7.4	1073.1
PRIL19-49	73.3	173.3	2.5	295	0.4	11.3	2994.8
PRIL19-57	31.9	78.3	0.8	83.6	0	1.3	1172.7
PRIL19-58	59.4	106.9	2	153.1	0.4	6.9	1824.3
PRIL19-59	52.7	72.8	1	117.4	0	0	1356.1
PRIL19-66	42	97.4	1.6	137.9	0.3	7	1590.1
PRIL19-67	38.8	84.9	0.4	89.7	0	0	1291.8
PRIL19-70	59	87.4	1.2	114.9	0.1	3.4	1513
PRIL19-72	55.1	101.5	1.1	125.3	0	0	1655
PRIL19-74	74.1	142.9	2.3	201.9	2.3	52.8	2400.7
PRIL19-75	44.2	95.3	1.6	122.3	0	0.9	1529.1
PRIL19-79	44	92.6	1.7	142.3	0	0	1573.4
PRIL19-81	41.8	71	1.5	111.1	0	0	1257.4
PRIL19-84	43.4	97.6	1.3	143.5	0.1	1.5	1619.3
PRIL19-85	37.4	60.2	2.1	101.7	0	2.4	1103.5

Table A-3*:* Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 19 short internode subset in green house evaluation (continued).

Short internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL19-86	37.7	125	3.3	139.9	2.8	35.7	1822.2
PRIL19-88	38.7	69.5	1.7	94.2	0	0	1167.8
PRIL19-94	33.1	70.3	0.5	76.7	0	0	1083.2
PRIL19-95	32.4	73.7	1	99.2	0	0	1188.4
PRIL19-99	49.8	78.6	2.3	98.7	1	13.1	1326.2
PRIL19-100	40.5	77.5	2.2	116.5	0.8	8.1	1327.6
PRIL19-101	46.6	72.7	1.7	109.2	0.2	2.1	1292.1
PRIL19-102	44.6	82.8	1.2	101.6	0.1	0.4	1346.6
PRIL19-104	30.4	62.7	1.2	74.6	0.2	1.7	992.4
PRIL19-106	32.5	59.6	1	70	0.2	4.9	959.7
PRIL19-109	29.2	67.3	1.5	81.9	0.1	3.3	1053.2
PRIL19-115	29	73.7	1	82.5	0.1	0.8	1111.6
PRIL19-118	44.6	79.4	2.3	121.7	0.8	15	1385.2
PRIL19-124	37.3	59.3	2.3	82.2	1.2	34.8	1026.7
PRIL19-127	29	55.1	2.1	78.5	1.4	35.3	930.2
PRIL19-129	32.6	91.3	1.5	105.2	0.6	11.6	1369
PRIL19-130	46.7	76.7	1	94.4	0.2	2.5	1277.6
PRIL19-132	43.4	72.5	1.8	128.4	0.8	17.9	1340.8
PRIL19-135	24.3	54.2	0.3	57.7	0.1	0.7	823.7
PRIL19-138	38	79	1.5	95.5	0.2	2.5	1253.9
PRIL19-142	26.8	62.2	2.4	144.3	0	0	1211.4
CV (%)	35.9	42.6	59.9	50.9	211.9	238.2	19.5
LSD	10.4	24.7	0.7	39.6	0.5	11.4	223.4

Table A-4: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 19 long internode subset in green house evaluation.

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL19-1	49.1	132	2.8	186.9	1.2	14.9	2112.8
PRIL19-3	75.4	128	2.8	213	1.8	19.2	2311.8
PRIL19-4	47.3	92.4	3	218.1	2.4	27.6	1854.7
PRIL19-6	48.9	127.4	3.4	191.1	2.5	39.6	2084.1
PRIL19-7	54	158.8	2.8	256.7	2.3	35.6	2624.4
PRIL19-8	48.3	142	3.1	190.2	3	43.8	2209.2
PRIL19-10	65.5	150.5	2.8	223.4	2.1	26.9	2496.6
PRIL19-20	61.7	85.1	2.5	130	0.9	9.7	1560.1
PRIL19-21	62.4	155.4	2.6	240.3	0.9	14	2583
PRIL19-22	48.9	99.2	3.2	180.7	2.7	42.3	1793.7
PRIL19-23	59.5	150.6	2.6	249.4	0.9	16.3	2555.3
PRIL19-24	58	166.8	2.7	279.7	0.5	8.3	2798.8
PRIL19-25	69.4	127.1	2.2	154.7	0.5	9.9	2066.5
PRIL19-26	48.8	106.8	2.8	209.4	1.2	18.1	1962.1
PRIL19-28	55.7	92.8	3.2	131.4	2.8	60.8	1601.6
PRIL19-29	61.5	115.8	3	193.3	2.1	46.6	2057.3
PRIL19-31	71.9	167.9	3.5	180.3	3.5	38.7	2537.8
PRIL19-35	77.2	146.3	2.6	236	2.1	20.9	2566.7
PRIL19-36	79.3	162	2.7	236.9	2	37.3	2723.3
PRIL19-38	80.8	149.3	2.8	209.7	2.1	37.2	2521.9
PRIL19-39	63.9	148.7	2.4	215.3	2	39.7	2442.8
PRIL19-40	72.9	162.2	2.6	234.1	0.8	13.5	2680.4
PRIL19-46	80	157.6	2.4	225.3	1.5	30.3	2646.5
PRIL19-51	76.8	175.8	2.6	286.3	2	25.9	3006.7
PRIL19-52	66.9	143.3	2	242.1	1.1	24.1	2504.7
PRIL19-54	60.3	135.4	2.2	178.8	1.8	36.8	2175.7
PRIL19-55	51.6	94.8	2.5	140.4	2.1	54.3	1628.4
PRIL19-60	73.8	194.7	2.2	305.3	0.5	9.3	3226.4
PRIL19-61	60.4	121.3	2.8	203.7	0.3	2.7	2136.9
PRIL19-62	73.8	143.8	2.9	216.4	1.7	30.2	2457.2
PRIL19-63	92.7	183.3	2.7	256.8	1.7	27.4	3058.4
PRIL19-64	61.3	158.4	2.4	250.3	0.4	0.8	2639.4
PRIL19-68	63.5	135.9	2.6	196	1.6	26.2	2258.4

Table A-4: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 19 long internode subset in green house evaluation (continued).

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL19-71	55.4	118	3	197	1.5	22.3	2055.9
PRIL19-74	85	200.5	2.5	295.5	2.1	36.2	3306.3
PRIL19-76	51.1	110.9	3.2	199.2	2	30.7	1976.1
PRIL19-77	94.6	210.3	2.6	242.3	1.5	28.1	3261.4
PRIL19-80	92.5	151.6	2.4	232.5	1.5	22.1	2686.9
PRIL19-82	55.9	109.2	3.2	142.2	2.6	39.1	1787.8
PRIL19-83	32.1	60	3.4	113.4	2.5	35.9	1113.5
PRIL19-86	56.6	130.9	3.3	154.8	3	44.8	2031.5
PRIL19-87	51.1	125.1	2.5	208.6	1.5	19	2136.6
PRIL19-89	43.7	103.3	2.4	139.6	1.8	26.5	1658.5
PRIL19-91	53.5	131.4	2.5	171.4	1.3	15.5	2076.4
PRIL19-92	51.8	123.4	2.9	166.5	1.9	31.3	1978.4
PRIL19-96	47.5	155.2	3	184.6	2.3	40.3	2304.5
PRIL19-97	84.7	119.7	2.7	216.4	2.2	27.3	2300.4
PRIL19-98	66.2	158.9	2.8	206.2	2.7	28.6	2515.6
PRIL19-103	78.3	142.9	2.3	204.8	1.6	21.3	2434.1
PRIL19-105	59.5	117	3	148.7	2.8	46.9	1900.8
PRIL19-108	44.6	88.3	3	113.4	2.5	48.2	1437
PRIL19-110	59.3	109.7	2.8	178.7	1.8	29.9	1938.6
PRIL19-111	77.2	142.8	2.1	247.3	0.1	3.3	2574.9
PRIL19-112	51	124.1	3.3	238.3	1.4	14.8	2231.2
PRIL19-113	51.8	118.4	3	163.3	2.3	51.2	1922.3
PRIL19-116	42	128.6	3.2	241	2.5	43.6	2231.6
PRIL19-117	57.4	186.3	2.5	239.8	1.3	33.1	2832
PRIL19-119	48.9	89.5	3	139.7	2	39.9	1563.1
PRIL19-121	50.1	127.4	2.9	173.8	1.9	30.9	2029.7
PRIL19-122	56.2	128.8	2.4	225.4	1.3	26.5	2257.4
PRIL19-123	29.7	61.1	3.5	112.3	2.6	62.1	1106.8
PRIL19-128	38.1	87.6	3.4	117.5	3.2	63	1408.8
PRIL19-131	52.4	126	2.9	285.6	1.8	23	2421.7
PRIL19-133	71.7	128.2	2	176.3	0.1	1	2165.1
PRIL19-136	71.7	150.7	2.8	222.5	1.6	22.7	2529.6
PRIL19-137	40.3	102.9	2.9	128.1	2.2	47.1	1595.8

Table A-4: Least square means for lesion expansion after 3, 7, and 14 days post inoculation (dpi), nodal resistance after 7 and 14 dpi, percentage of survival after 14 dpi and area under the disease progress curve (AUDPC) in Population 19 long internode subset in green house evaluation (continued).

Long internode genotypes	Lesion expansion (3 dpi) mm	Lesion expansion (7 dpi) mm	Nodal resistance (7 dpi)	Lesion expansion (14 dpi) mm	Nodal resistance (14 dpi)	Survival rate	AUDPC
PRIL19-140	63.3	121.7	2.9	145.6	2.4	43.5	1953.2
PRIL19-141	75.9	129.5	1.7	172.2	0.3	3.1	2185.8
CV (%)	34.1	33	21	33.4	58.9	74.3	20.7
LSD	12.4	26.3	0.4	39.5	0.6	13.1	269.8