GENOME MAPPING AND MOLECULAR MARKERS FOR ASCOCHYTA BLIGHT RESISTANCE IN PEA (*PISUM SATIVUM* L.)

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ABSTRACT

Ascochyta blight is the most common disease of economic importance in peas (*Pisum sativum* L.) in North Dakota. Selection based on molecular markers would greatly facilitate identification of resistant varieties. A mapping population comprised of 394 F_7-derived recombinant inbred line (RILs) and derived from the cross 'Lifter'/'Radley' was developed to study resistance to Ascochyta blight. A genetic map was developed based on 179 loci including SSR, RAPD, and CAPS markers, distributed on seven linkage groups. Phenotyping for reaction to Ascochyta blight was carried out under greenhouse and field conditions. Five replicate plants were scored using a 0 to 5 scale, where 0 = no disease and 5 = plant death. Forty-three lines showed a high level of resistance and QTL analysis identified ten DNA markers associated with Ascochyta blight resistance genes. This genetic map will provide additional insight to localize disease resistance genes/QTLs and aid development of resistant varieties.

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INTRODUCTION

Origin, production and economic importance

Pea (*Pisum sativum L.*) is an important rotational crop in cereal-based cropping systems across the United States northern tier states and production has increased substantially in recent years to over 760,000 Mt on 343,000 hectares (USDA-ERS, 2010). The United States pea production is expanding and is centered in the Midwest region of North Dakota, South Dakota, and eastern Montana; with modest production in the Pacific Northwest region of eastern Washington, northern Idaho, and northeastern Oregon. Continued increase in pea production is expected in the near term. Forty-two percent of pea production is of green cotyledon types, while the remaining 58% is comprised of yellow cotyledon types. North Dakota produces about 57% of the national pea production which is valued at $90 million, followed by Montana with 28% (USDA-ERS, 2010). In addition to its direct economic value, this legume is critical as a rotational crop with cereal grains, because pea fixes nitrogen for subsequent grain crops, breaks disease and insect cycles, enables control of weeds, and improves soil tilth.

Pea is an annual herbaceous crop, adapted to temperate regions. Seeds were found in the Middle East, in archaeological fields and dated back 7000 to 6000 BC (Zohary and Hopf, 1973). The center of origin for pea is central Asia, the Middle East, Ethiopia and the Mediterranean (Vavilov, 1926). Blixt (1970) considered the Mediterranean as the main center of genetic diversity and Ethiopia, and the Middle East as secondary centers of origin.

Pea is a legume from the Fabaceae family, subdivision Viciae which includes the genus *Pisum* and the basic number of chromosomes n=x=7 (Griton, 1980). Within this genus, White (1917) reported seven species: *P. arvense, P. elatius, P. formosum, P. fulvum, P. humile, P.*

jomardi and *P. sativum*. The differences between *P. arvense* and *P. sativum* are not clear; therefore, *P. arvense* is considered a subspecies of *P. sativum* (Lamprecht, 1974).

There are three main types of peas; green and yellow pea, called field pea or "dry pea" and Austrian winter pea. Pea has a high nutritive value with several nutrients required in the human diet. Pea is also marketed as a dry product for livestock food. Field pea differs from fresh or succulent pea, which is harvested immature and marketed as a fresh or canned vegetable. Winter pea can be planted around September/October and harvested the following summer. The major producing countries of field pea are Canada, China, Russia, India and the United States (FAO 2010). The United States exported approximately 500,000 Mt of pea in 2010 (USDA-ERS, 2011).

The pea crop is susceptible to several diseases, including fungal, bacterial and viral diseases. Some example of pea diseases are: bacterial blight, downy mildew, powdery mildew, Ascochyta blight and pea seed-borne mosaic virus. Ascochyta blight disease is the main disease affecting field pea production. It is a fungal disease causing black spots or lesions on the leaves, stems, flowers, pods and seeds.

Ascochyta blight of pea is caused by three different pathogens *Ascochyta pisi* Lib., *Mycosphaerella pinodes* (Berk. & Bloxam) Vestergr., and *Phoma medicaginis* (L.K. Jones) Boerma. It reduces grain yield up to 40% and causes damage on the leaves, stems, and roots limiting proper plant metabolism and also can reduce grain quality.

High humidity coupled with warm temperatures ranging from 28 to 32°C is optimal conditions for infection. Disease incidence and severity increase with high levels of moisture, especially when the canopy is closed. Control of Ascochyta blight can be accomplished using

fungicides, although these chemicals do not show satisfactory control. Therefore, development of resistant cultivars is desired and it is more sustainable.

Economic losses impacting the pea industry due to Ascochyta blight have focused research on the development and release of cultivars that demonstrate acceptable levels of resistance to Ascochyta blight since chemical and cultural control methods are either expensive or unsuccessful. Studies to identify source of resistant to *A. pisi*, *M. pinodes* and *P. pinodella* in *P. sativum* and other related species revised by Bretag (2004), started in 1926. The major challenge was the lack of uniformity in the testing procedure and methods of disease assessments.

As the resistance to Ascochyta blight is quantitative in nature and disease incidence is impacted by environmental conditions, conventional plant breeding methods to develop resistant varieties are laborious and time consuming. Use of DNA markers linked to important QTL through marker-assisted selection (MAS) are expected to aid development of resistant varieties.

Many preceding studies have identified molecular markers closely linked to major and minor quantitative trait loci (QTL) associated with resistance to Ascochyta blight. One such major QTL is from three different crosses in a study by (Timmerman-Vaughan et al., 2002). This source of resistance has been widely studied and has been used by many breeding programs.

OBJECTIVES

The objective of this study was to identify molecular markers linked to QTL for resistance to Ascochyta blight caused by *Mycosphaerella pinodes*. The following hypotheses are being tested.

1. QTL for resistance to *Mycosphaerella pinodes* identified in previous studies are present in the Lifter/Radley population.
2. New QTL for Ascochyta blight resistance can be identified in the Radley/Lifter population.

LITERATURE REVIEW

Importance of the disease

Ascochyta blight is a worldwide disease affecting field peas. The disease can be found in almost every area where pea is grown and when environmental conditions are favorable the disease can cause damage in all parts of the plant. The yield reduction can range from 3 to 25% (Bretag et al., 1995a). This yield reduction is mainly due to reduction in the number of pods. Ascochyta blight reduces yield and quality, especially when the environment produces high humidity, for example, a closed leaf canopy (Bretag et al., 1995b). All commercial cultivars are susceptible, and considerable yield losses occur. Ascochyta blight is a significant biotic stress in field pea in North Dakota because of its negative economic impact in pea production (NDSU Extension, 2005). Introducing disease resistance into new cultivars will reduce costs to the farmer and will be better for the environment because fewer chemicals will be applied. Knowledge of the disease mechanism is necessary to develop effective methods for crop disease prevention. Careful study of the disease triangle, which includes a susceptible host plant, a pathogen capable of causing disease, and an environment conducive to disease development provides insight into possible control mechanisms. Planting resistant cultivars eliminates one aspect of the disease triangle, because the susceptible host is not provided, making disease development difficult.

Diseases in pea plants can be caused by fungi, virus, or bacteria and production losses can be significant. Management of crop production requires careful attention to the biotic and abiotic problems that will affect production. Field pea is susceptible to many diseases including damping off, seedling blights and root rots, caused by *Pythium, Rhizoctonia*, *Botrytis*, and *Fusarium* spp.; powdery mildew, caused by *Eryisphe pisi* DC. (syn. *E. polygoni* DC.); stem and

pod rot, caused by *Sclerotinia sclerotiorum* (Lib.) De Bary; bacterial blight, caused by *Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye and Wilkie (syn. *P. pisi* Sackett); and Ascochyta blight caused by three fungal species *Ascochyta pisi*, *Mycosphaerella pinodes* and *Phoma medicaginis*.

Ascochyta pisi Lib. causes leaf and pod spot; *Mycosphaerella pinodes* (Berk. & Bloxam) Vestergr., the perfect stage of *A. pinodes*, causes blight; and *A. pinodela* which is designated *Phoma medicaginis* var. *pinodella* (L.K. Jones) Boerema, and causes stem rot. These fungi were first identified by L.K. Jones in 1927 (Nasir and Hoppe, 1991). The most prevalent disease in North Dakota is Mycosphaerella blight caused by *Mycosphaerella pinodes*, and affects roots, leaves, stems, flowers, and pods (NDSU Extension, 2005). This disease is responsible for seed quality losses and yield losses of up to 30% (Beasse et al., 1999).

There are three ways that the fungus species listed above can be identified and distinguished. First, the presence of pseudothecia in *M. pinodes*; second, the size of the conidia (conidia of *P. pinodella* are smaller than those of *A. pisi* or *M. pinodes*); and third, the carrot red spore masses of *A. pisi* compared with the light beige spore masses of *M. pinodes* and *P. pinodella* on oatmeal agar (Jones, 1927). Identification of the pathogens on the basis of microscope observation is difficult because of isolate variation.

Punithalingam and Holliday (1972a), Punithalingam and Gibson (1976) and Punithalingam and Holliday (1972b) describe with more clarity the characteristics and differences among *M. pinodes*, *P. pinodella*, and *A. pisi*, respectively. Boerema et al. (2004) reported greater detail on the taxonomy of *P. pinodela* (L.K. Jones). Madhosing and Wallen (1968) used serological techniques and confirmed that the three species can be distinguished; it was found that a much closer relationship exist between *M. pinodes* and *P. pinodella* than either

of these species with *A. pisi*. Additional techniques to distinguish the three species include isoenzyme analysis and restriction fragment length polymorphisms (RFLPs) (Faris Mokaiesh et al., 1996), random amplified polymorphic DNA (RAPD) assays (Tohamy et al., 1997; Onfroy et al., 1999; Wang et al., 2000) and monoclonal antibodies (Bowen et al., 1996).

Symptoms

Spores can germinate over a wide range of temperatures (5-35°C) and are able to be carried by the wind for 1.6 km or more resulting in widespread distribution in large areas (Bretag, 1989). Symptoms observed by *M. pinodes* appear in 2 to 4 days and *A. pisi* in 6 to 8 days after infection. These fungi are capable of surviving in the soil for more than a year (Bretag, 1989).

Ascochyta blight is widespread and can be found in most pea growing regions of the world, including temperate areas of Europe, North America, Australia, and New Zealand (Beasse et al., 1999). The symptoms caused by *M. pinodes*, *P. pinodella*, and *A. pisi* are different; nevertheless the most common are leaf, stem, and pod lesions plus discoloration of the cotyledon, hypocotyl, and root areas.

Disease cycle

The life cycle of the three pathogens responsible for ascochyta blight disease are very similar, all of them can survive on seed and pea debris. *M. pinodes* and *P. pinodela* also survive in soil. The pathogens can be transmitted through infected seed. Seeds infected with either *M. pinodes* or *P. pinodella* will show the first symptoms where the seed attaches to the root and lower stem. From this point, stem rot can develop, causing damage and even death of young plants. Plants that originate from infected seeds with *A. pisi* do not develop stem rot, although they develop lesions on the first leaves and young plants are often killed. In ideal conditions, the

disease could be spread from infected plants to adjacent healthy plants.Seed infection is most important for *A. pisi* because it is a weak saprophyte and rarely survives over winter on pea trash, while *M. pinodes* and *P. pinodella* are more frequently observed on pea stubble than seed, the disease often spreads from previously infected fields.

Mycosphaerella pinodes and *P. pinodella* can survive as mycelium on infected pea debris, or in the soil as sclerotia and chlamydospores. The chances of pea cultivated in previously infected fields to be attacked by either of these pathogens are high with consequent root damage resulting in the death of young plants. *A. pisi* rarely survives in soil and there is a low chance of survival in pea stubble.

In fields where rotation is not an option and peas have been grown for many years, wind and rain splash are important mechanisms to carry spores from old pea debris into new fields and within the crop. Wet weather often coincides with occurrence of infection. In contrast, pycnidiospores of *M. pinodes*, *P. pinodella* and *A. pisi* are spread by rain splash and infect nearby plants.

Symptoms start to appear 2-4 days after primary infections for *M. pinodes* and *P. pinodella* and in 6-8 days for *A. pisi*. Depending on the environment, if moisture is available, sporocarps develop in new lesions and produce secondary inoculum. In wet weather, all pathogens produce pycnidia from which pycnidiospores are released and splashed onto the lower leaves of nearby plants resulting in secondary infection. In *A. pisi* and *M. pinodes*, pycnidia are produced in abundance while it is less common in *P. pinodella*. *M. pinodes* also forms pseudothecia and produces wind-borne ascospores which allow rapid spread through the field.

Plants can be attacked at any growth stage and all plant parts are susceptible, including the above and below ground parts. If moist conditions occur throughout the growing season, there can be considerable infection of pods resulting in high levels of seed infection.

Epidemiology

Examination of epidemiological factors of Ascochyta blight of pea provides insight into its development over time and area and aids establishment of better control strategies.

Seed-borne inoculum

All three pathogens are seed-borne; consequently, the disease develops from the use of infected seed. Uncontrolled seed movement from infected regions to others is dangerous for the risk of Ascochyta blight transmission (Moore 1946). Studies that have been done in Australia by Ali et al. (1982); Bathgate et al. (1989); Bretag et al. (1995b) and Neergaard (1979) have shown that all three pathogens can be found in and on pea seed from the majority of pea growing areas in the world.

Pea seed infected with Ascochyta spp. often have reduced germination and serve as a source of inoculum for future infection (Wallen et al., 1967). Optimal conditions that benefit disease spread from seed to crop are most likely to occur under humid conditions and should not be as great of a concern in dry regions (Bretag et al., 1995b). The level of contamination among the three pathogens was reported by Wallen (1965); the effect of seed contamination was greatest for *M. pinodes* and least for *A. pisi*. A contrast of opinion is found in some publications, for example Anselme et al. (1970) stated that up to 5% infected seed could be tolerated, while others, such as Cruickshank (1954) have suggested zero tolerance.

Transmission from seed to crop

Disease development is dependent on the primary factors: i) the amount of seed-borne inoculum, ii) the rate of growth and transmission to the seedling, and iii) the increase of disease in the field. Several reports of transmission of *M. pinodes*, *P. pinodella* and *A.pisi* are present in the literature (e.g. Maude, 1973; Xue, 2000); however, there is very little quantitative data about the level of seed infection and its effect on grain yield (Maude 1973; Bretag et al., 1995b; Xue 2000). Comparing the three pathogens, the transmission rate tends to be higher for *M. pinodes* and *P. pinodella* because they attack the hypocotyl soon after germination with little chance of escape, while *A. pisi* infects the plumule of young plants (Jones, 1927; Maude, 1973).

Transmission from seed to crop is known to vary depending on infection conditions (Anselme et al., 1970; Bretag et al., 1995b; Xue, 2000). Among the three fungi, the disease spread was shown to be more effectively on seeds infected by *M. pinodes*; the germination rates were smaller and more disease was present where seeds with *M. pinodes* were used (e.g. Bretag et al., 1995b).

Survival on pea seed

Seed-borne *M. pinodes* can remain in active for several years in and on pea seed (Bretag et al., 1995b). Spores or mycelium are not always carried on the seed coat, but can be protected deep inside seeds (Bretag et al., 1995b). Abd El Rehim et al. (1997) showed that *M. pinodes*, *P. pinodella*, and *A. pisi* inoculum were mostly encountered in the seed coat and embryo infection was uncommon. During storage, the infestation rates are reported to decrease rapidly, if they are stored for seven years, there is a possibility to eliminate the pathogens (Bretag et al., 1995b).

Seed infection

Ascochyta blight development is favored by humid conditions and seeds produced under warm, wet conditions usually become heavily infected with *M. pinodes* while seeds produced in drier conditions are often free of infection (Walker, 1969; Bathgate et al., 1989, Bretag et al., 1995b). Seed infection level is often greater when weather conditions are warm and wet between flowering and maturity (Jones, 1927; Skolko et al., 1954; Bretag et al., 1995) and also in warm, humid conditions during this period cause heavy pod and seed infection (Maude, 1966). Pod infection from either *A. pisi* or *M. pinodes* can result in 50-60% seed infection (Maude, 1966; Bretag, 1995b). One way to minimize disease is to have dry weather between flowering and maturity and the chance of producing pathogen-free seeds is higher (Cruickshank, 1957). Considerable variation between pea lines with regard to resistance to seed infection by *M. pinodes* is well documented (Xue et al., 1996).

Chen et al., (1994) examined pods and seed infection of pea cultivars caused by *M. pinodes* in the field. Fungal mycelium was observed within pod and seed tissues 2 weeks after young pods (7 days after bloom) were inoculated with spore suspensions (10^5 spores/mL). It was observed that the fungus penetrated seeds through the infected pods. Pods at different developmental stages were inoculated and young pods (7 days after bloom) were more susceptible to infection than older pods (14 days after bloom) and resulted in greater seed infection in young pods. Temperature and humidity were also important for *M. pinodes* infection. Favorable conditions for pod infection were 24°C and 100% relative humidity.

Survival in soil and on pea trash

The pathogen can survive in the soil and on infected crop residues; therefore, caution should be taken when peas are planted in the same area year after year due to increased incidence

and severity of Ascochyta blight (Jones, 1927; Davidson and Ramsey, 2000; Bretag and Ramsey, 2001; Zhang et al., 2005). Infected pea trash and soil-borne spores that remain from the previous year constitute a reservoir of primary inoculum that can cause infections (Jones 1927; Hare and Walker, 1944; Walker, 1961; Bretag, 1991). In established pea growing areas, infected pea trash is the main source of primary inoculum (Walker 1961; Bretag, 1991). Survival in soil is more important for *M. pinodes* and *P. pinodella* because they survive well as a saprophyte when buried. In contrast *A. pisi* has poor saprophytic ability and is usually destroyed by other soil microorganisms when buried (Dickinson and Sheridan, 1968; Wallen and Cuddy, 1968). Reports show that the *M. pinodes* and *P. pinodella* pathogens are able to survive in soil previously cultivated with peas for at least one year as dormant mycelium, sclerotia or chlamydospores (Linford and Sprague, 1927; Dickinson and Sheridan, 1968; Wallen and Cuddy, 1968).

The mechanism of spore survival starts when infected pea straw is buried and then pycnidiospores and ascospores contained within mature pycnidia and pseudothecia are transformed into chlamydospores (Carter, 1961; Dickinson and Sheridan, 1968). Carter (1961) reported that *M. pinodes* created vegetative chlamydospores within two weeks of burial and, therefore, could survive in the soil. The survival mechanism of *P. pinodella* is to form larger chlamydospores and it appears to have better performance for survival in soil than *M. pinodes* (Linford and Sprague, 1927; Wallen and Cuddy, 1968). In the drier pea cultivated regions of the United States, conditions are usually unfavorable for the development of the pathogens for foliar infections, although *M. pinodella* is still a threat because of its capability to survive in the soil between successive crops (Lawyer, 1984). Some fungi have been isolated from pea fields up to 17 years after the last pea crop and the survival mechanism was chlamydospores (Wallen, 1974).

Infection from soil-borne inoculum occurs when chlamydospores and sclerotia germinates in moist soil at temperatures of 12 to 28°C (Linford and Sprague, 1927). Infection type varies according to temperature: For example at 28°C in the soil, roots showed more severe damage than when peas are grown at lower temperatures (Linford and Sprague, 1927).

Previous studies have conducted detailed methods on how to detect Ascochyta fungi in the soil. Bretag et al. (2001) successfully estimated populations of soil-borne Ascochyta blight fungi by simply plating a soil-water suspension into a selective agar medium. Wallen et al. (1967) also reported a consistent technique for the isolation of *M. pinodes*, *A. pisi*, and *P. pinodella* from field soil. Heated soil (82°C) was used to eliminate most saprophytic organisms present. Soil was then mixed with water to form a suspension. A dilution series was prepared and then spread evenly over peptone dextrose agar containing rose bengal and chlortetracyline. The fungi were identified after 21 to 28 days. Growth might be restricted on this medium; however, most bacteria and actinomycetes are excluded and isolates can be subcultured onto different medium for further study. Peck et al. (2001) also isolated fungi from previous field cultivated soil using a seedling bioassay to estimate the amount of soil-borne inoculum. Fungi were isolated from lesions that developed on the basal stem and then identified.

Development of pycnidia and pseudothecia

Pycnidia and pseudothecia develop throughout the growing season on infected plants and after harvest on pea debris and infected volunteer plants (Bretag, 1991; Roger and Tivoli, 1996). Moisture is required for the formation of pycnidia and pseudothecia. Dry conditions are not beneficial for them because it delays development and maturation (Jones, 1927; Roger and Tivoli, 1996; Roger et al., 1998b). Within 3 days at 20°C, pycnidia can be formed, although longer wetting periods are required at higher or lower temperatures for pycnidia to form (Stone,

1912; Chung and Wilcoxson, 1971; Roger et al., 1999). In a separate study, Roger et al. (1999) reported that the number of pycnidia formed on leaves increased with temperature from 5 to 20°C and consequently decreased between 20 and 30°C. Other studies based on the development of both pycnidia and pseudothecia of *M. pinodes* on artificial media; found 16°C to be the optimum temperature for the development of pseudothecia (Hare and Walker, 1944; Snyder and Hansen, 1947). Pseudothecia development occurred in 3 weeks at 16°C and in 5 weeks at 2.4°C. However, in field situations where plants were infected with *M. pinodes*, fungal development was shown to be more rapid, and in a few cases mature pseudothecia can be formed on infected plants within 13 to 14 days of initial infection (Jones, 1927; Carter, 1963). The minimum period from infection to formation of mature pseudothecia in the field was 6 days for pycnidia and 13 to 14 days for pseudothecia (Jones, 1927).

Another study showed the formation of large numbers of *M. pinodes* pseudothecia on wheat (*Triticum aestivum*) straw that was placed in an agar medium (Snyder and Hansen 1947). The ability of *M. pinodes* to form pseudothecia on wheat stubble under field conditions has not been reported.

Sporulation

Moisture is important for the release of both pycnidiospores and ascospores (Carter 1963; Bretag 1991; Zhang et al., 2005); therefore, little disease is found in dry years. Kerling (1949) demonstrated a close correlation between rainfall and spore release which was later confirmed by Carter and Moller (1961), Bretag (1991) and Thomas et al. (2000). Moisture from dew was enough for the ascospores to be released. However, the greatest spore release was observed soon after rainfall events. Hourly analysis suggested a regular diurnal rhythm with maximum output of spores occurring in the afternoon. Light was also a factor that stimulated sporulation (Hare and

Walker, 1944; Leach, 1959). Spore release by *A. pisi* was optimum at 20°C on artificial media (Amit et al., 2002). However, temperature is not critical for A. pisi, which can survive in a range of 0 to 37°C (Hare and Walker 1944).

Ascospores can be carried as far as 1.6 km by the wind, affecting large areas where peas have not been previously planted (Lawyer, 1984). It was estimated that up to 80% of the ascospores carried from pea debris developed from pseudothecia formed in the newly affected crop. In this case it is very important to control pea trash before spores can be produced and dispersed by wind and rain.

Spore germination and infection

The optimum temperature range for spore germination is 20 to 25°C (Sattar, 1934), although most spores are able to germinate from 5 to 35°C (Roger et al., 1998). Comparing humidity and temperature, Bretag (1991) found that with at least 12 hours of leaf wetness a temperature of 10°C is necessary for infection, but if the wetness is present only 6 hours 20°C is necessary. An increase in the wetness period from 6h to 12h at 20°C resulted in a large increase in disease severity symptoms and leaves were more susceptible to infection than stems. Brewer (1960) concluded that temperature after infection also influences disease development. At 5 to 10°C lesions were often larger and more numerous than 15 to 25°C.

Even though humidity is the perfect environment for spores to develop, Roger et al. (1999) showed that the opposite is also possible. In his study, pycnidiospores survived in dry periods up to 21 days after inoculation. This fact explains that the effect of wet-dry-wet depended on when the dry period occurred during the infection period. *M. pinodes* can survive dry days after overnight dews and maintain the ability to infect when favorable moisture is present. No disease was observed during the dry period; however, infection levels in different

parts of the plants vary depending upon the microclimate created and inoculum concentration. The lower leaves and those that were in the shade usually were wet for longer periods providing better conditions for infection than the top part of the plant. An important conclusion can be taken from this study: irrigated conditions have this exact microclimate and the irrigation method can influence disease development. Gent (1988) observed that *A. pisi* infection was more severe on peas watered by overhead sprinklers and the leaves were kept wet for long periods while using flood irrigation resulted in the foliage being wet for shorter time periods.

Inoculum concentration

Bretag (1991) showed that an increase in disease symptoms was correlated with increased inoculum concentration and that leaves had more symptoms than stems with a spore concentration of 10^3 spores/mL. Zimmer and Sabourin (1986) noticed that disease symptoms were more severe in older leaves which led to a conclusion that Ascochyta blight affects the plants as they mature. Studies in Canada showed that leaf damage promotes infection by foliar pathogens (Banniza and Vandenberg 2003).

Host-pathogen interactions

Spores of *M. pinodes* propagate on the leaf surface and generate germ tubes that are able to directly penetrate the cuticle or enter through stomata (Wroth, 1998). *M. pinodes* can form either limited or spreading necrotic lesions, which often grow beyond the necrotic area (Heath and Wood, 1969). Jones and Vaughan (1921) showed that appressoria are formed before the spores penetrate the epidermis. In a controversial study, Brewer and MacNeill (1953) reported that the infection could also occur without the formation of appressoria. Using resistant and susceptible varieties, Clulow et al. (1992) observed the different reactions of plants against the fungus development. In the susceptible cultivars, abundant appressoria formed and penetration

occurred after a short period of hyphal growth on the epicotyls. In the resistant varieties, hyphae grew extensively on the epicotyls but rarely formed appressoria, and if appressoria did form, they did not penetrate the cuticle. A hypersensitive reaction was also observed where 2 to 6 epicotyl cells died quickly around the infection site. Leaves only expressed disease after penetration, involving localization of the fungus by a mechanism that delays leaf cell death. Clulow et al. (1991) provided more detail regarding how the pathogen penetration into cuticle with infection pins formed under appressoria. The infection pins grew through the outside wall of the epidermis, sporadically penetrating the cells without causing damage. This stage of the disease, which is probably biotrophic, had a typical necrotrophic stage as a next step, followed by progressive necrosis; the resulting macroscopic lesions often encircled the epicotyl.

Important enzymes

All three fungi are able to produce cell wall degrading enzymes (Heath and Wood, 1969, 1971; Anderson and Powelson, 1979). *A. pisi* and *M. pinodes* produce amylase, aminopeptidase and invertase. *M. pinodes* has greater cellulose and pectinase activity than *A. pisi*, which is related to production of larger lesions. Heath and Wood (1971) studied different lesion levels on pea leaves. It was found that the spreading lesions contained more cell wall degrading enzymes. *A. pisi* has a different method of infection; the fungus can produce polygalacturonases, which have the ability to destroy leaf cells. However, some pea varieties demonstrated an ability to resist this enzyme, which is important for resistant material studies (Hoffman and Turner 1982, 1984). Rattan (1974) showed that all three species produce enzymes to digest cellulose and recommended that this is important in pathogenicity and survival as saprophytes. The fungi have an easy carbon source that allows them to digest cellulose even when some of the host tissues or the entire host plant is dead. Kaur and Deshpande (1980) isolated a heat stable compound from

A. pisi, ascochitine, and showed that it could cause ascochyta blight in peas. Another report showed that *A. pisi* and *M. pinodes* cultured on sterilized wheat produced metabolites that were toxic to peas (Evidente et al., 1993a; 1993b).

Host range

Ascochyta blight is present in other crops as well. *M. pinodes* and *P. pinodella* also infect many legume species, including *Pisum*, *Lathyrus*, *Vicia*, *Vigna*, *Medicago*, *Melilotus*, *Lens*, *Trifolium*, *Lupinus*, *Cicer*, and *Phaseolus*. *A. pisi* has been reported in the same species described above except *Vigna* and *Melilotus* (Brettag, 2004).

Effect on yield components and grain yield

Climatic conditions are the main factors that determine the severity of disease and the level of yield loss (Skolko et al., 1954). Using the same amount of inoculum, the yield losses related to these three fungi are: *M. pinodes* 45%, *A. pisi* 11% and *P. pinodella* 25% (Wallen, 1965). Lawyer (1984) also reported that *M. pinodes* is the most dangerous of the three with yield losses up to 75%. Study comparison of fungicide control and non-fungicide control in a pea field contaminated with Ascochyta blight resulted that the field where the control was applied had 15 to 75% greater yield (Morral et al., 1976; Bidle, 1989; Bretag et al., 1995a; Nasir and Hope, 1998). Bretag et al. (1995a) observed a close correlation between disease severity and yield loss with most varieties showing a 5 to 6% reduction in grain yield for every 10% of stem area affected by disease (first 10 internodes on the main branch). Le May et al. (2001) observed that yield loss differed with varying canopy structure. Yield loss was between 7 to 23% across different cultivars and those with a closed canopy maintained higher humidity, benefiting the pathogens and had more disease. Decrease in radiation use efficiency (RUE) caused 80% of

reported yield losses and among all fungi *M. pinodes* was the one that most affected pea growth. Leaf lesions caused by *M. pinodes* were one of the factors that reduced radiation use efficiency (Tivoli et al., 1998; Le May et al., 2005). Porta Puglia et al. (2000) conducted a morphological study observing yield loss differences between cultivars. However, a similar study (Bretag and Brouwer, 1995) did not find significant differences comparing pea lines and effects in their phenotypes regarding disease severity of Ascochyta blight.

Ascochyta blight reduces seed yield by infection on leaves reducing the green photosynthesizing area, consequently, photosynthetic efficiency is reduced. Seed number and individual seed weight is also reduced. *M. pinodes* also influences translocation of carbohydrates and nitrogenous compounds from the leaf and body to the seed (Tivoli et al., 1996; Xue et al., 1997; Garry et al., 1998; Beasse et al., 1999).

Disease control

Ascochyta blight can be controlled in many ways including cultural practices, chemical control, foliar sprays, biological control and breeding for resistance. Of these, chemical control and breeding for resistance are the most important, although cultural practices are also useful.

Several cultural management practices have been recommended to prevent losses (Hernandez-Bello et al., 2006). These practices include the rapid destruction of crop residues following harvesting, use of a 3 to 4 year rotation, growth of non-host crops between pea crops, selection of an appropriate sowing density and sowing date for the local climate conditions. While chemical treatments can reduce disease severity and preserve pea yield, multiple sprays are often needed during the growing season. Development of resistant cultivars is an important

component in an integrated strategy to control *M. pinodes*, decrease production costs, and preserve the environment.

Cultural practices

The best method to control the disease using cultural practices is to reduce the amount of primary inoculum, i.e. infected seed, infected pea trash, and soil-borne inoculum. Gadd (1950) demonstrated a technique to reduce the level of seed-borne inoculum without damaging seed where seeds were immersed in hot water for a short period of time. Often cultivated pea areas have variations in the air-borne inoculum amount. The recommendation to avoid this issue is to change the planting time because the period of highest air-borne spores will be missed with consequent infection reduction. Soil-borne inoculums can be reduced through crop rotation with non-legumes. The period of rotation should be 3 to 6 years between successive pea crops to minimize losses from Ascochyta blight and also root rot (Walker, 1961; Bretag et al., 2001).

Chemical control

There are many fungicides developed to control seed and soil-borne pathogens (Ogle, 1997). These fungicides are mainly copper and mercury based compounds (Walker, 1961). Other inorganic and organic compounds that are less toxic are also used and they replace organo-mercury compounds. Some of these fungicides are Captan and Thiram. One problem of using a chemical treatment is the seed coat may be impenetrable to controlling deep seated infections (Maude, 1966). Another problem of using these chemicals is that they affect the rhizobia and can reduce the nodulation of grain legumes (Rennie et al., 1985).

Breeding for resistance

Genetic resistance to each of the three pathogens causing Ascochyta blight in peas has been studied extensively worldwide. However, few sources of resistance have been reported (Bretag, 2004). Good level of resistance to *A. pisi* and *P. pinodella* have been reported, but few for *M. pinodes*. Kraft et al. (1998) tested several accessions trying to find resistance; however, no pea accession with major gene resistance to *M. pinodes* was found. Xue and Warkentin (2001) tested 335 pea lines from 30 countries and were able to find 7 lines that were partially resistant to *M. pinodes*. Breeding for resistance is difficult due to the quantitative and partially resistant nature of resistance (Prioul et al., 2004). The resistance mechanism in the plant was studied by Clulow et al. (1991, 1992) and Wroth (1996, 1998). They compared host-pathogen interactions between pea lines that were either partially resistant or susceptible to *M. pinodes*. The conclusion was that resistance is attributed to failure of the fungus to penetrate the host tissue and/or a hypersensitive reaction that restricts the fungi within the host. Nasir et al. (1992) also discovered that the development and spread of the fungus was limited in resistant plants.

The majority of studies that were done to identify sources of resistance to *A. pisi*, *M. pinodes* and *P. pinodella* in pea lack uniformity of testing procedures and methods of disease assessment. The lack of uniformity makes it difficult to obtain an accurate comparison of the relative susceptibilities of the varieties tested. Most studies showed a range of reactions, from highly susceptible to moderately resistant. Some good *A. pisi* resistant sources have been found in landraces pea plants contributing in the variety development. In spite of this, resistance to *M. pinodes* or *P. pinodella* that have been found only provide moderate levels of resistance in conventional pea types. The best source of resistance that has been reported was found by

Gurung et al. (2002) in primitive *Pisum* and *Lathyrus* species; although the attempts to transfer this resistant material into conventional pea types have been unsuccessful.

Inheritance of resistance to *A. pisi* is still unclear because the number of genes involved has not been determined (Bretag, 2004). However, it is known that the mechanism of resistance against each pathogen is under separate genetic control. This applies to all parts of plants involved in the disease: stem, foliar, seed infections, and root disease. Three genes, *Rap-1, Rap-3* and *Rap-4* were identified by Brittain (1987). *Rap-1* had general resistance and *Rap-3* and *Rap-4* were race specific. A single dominant gene controls resistance to *A. pisi* according to Csizmadia (1995). Using genetic analyses of stem and foliar resistance, a series of four single dominant genes were identified for stem resistance and also four genes for foliar resistance against *M. pinodes* (Clulow et al., 1991).

The first report of physiological speciation in *A. pisi* was reported by Jones (1927) and many later studies have shown that there are different physiological races of *A. pisi, M. pinodes* and *P. pinodella.* The isolates have the same morphology, but different ways to cause disease on different hosts (Bretag, 2004). It is hard to compare studies because of the differences in testing conditions, methods of disease assessments and the cultivars used. Molecular methods to distinguish the different pathotypes are still undefined. Potential RAPD (bands of random amplified polymorphic DNA) markers for the identification and differentiation of Ascochyta blight pathogens were reported by Wang et al. (2000), although they were unable to establish a relationship between RAPD genotype and pathogenic variation in *A. pisi* isolates.

Good sources of resistance to *A. pisi* have been reported for leaf and pod spot in Canada where this disease is rarely seen (Wallen and Cuddy, 1968). Contrary to this, the progress in breeding for resistance to both *M. pinodes* and *P. pinodella* has not been successful. The pea lines

that reportedly have resistance to either *M. pinodes* or *P. pinodella* have shown only partial resistance and it was inefficient in field conditions. *M. pinodes* resistance is inherited as a quantitative trait, and it is possible for progeny lines to show transgressive segregation (Wroth, 1999).

State of molecular markers

The advance of molecular biology has improved the use of molecular markers and biotechnology for plant breeders. Plant breeders are able to get quicker results related to the development of resistant varieties and desirable traits. Use of molecular techniques has made it possible to select genes of interest with greater efficiency (Mohan et al., 1997).

DNA-based molecular markers have been used as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, and others. Microsatellites are an important tool to develop genetic maps suitable for QTL detection studies and marker-assisted selection research.

DNA based molecular markers have become an important tool in many plant breeding programs worldwide. Molecular markers have several different applications in plant breeding including construction of linkage maps, germplasm characterization, genetic diagnostics, and characterization of transformants, study of genome organization, phylogenetic analysis, and fingerprinting for crop variety protection (Baird et al., 1997; Winter and Kahl, 1995 Smith and Smith, 1992). Tanksley et al. (1996) used molecular markers to map and introduce useful traits from wild tomato (*Solanum habrochaites*) species into adapted germplasm in an advanced backcross breeding scheme. Molecular markers have been used for crop improvement in many crops including rice (*Oryza sativa*) (Mackill et al., 1999; McCouch and Doerge, 1995), wheat

(Eagles et al., 2001; Koebner and Summers, 2003), barley (*Hordeum vulgare* L.) (Thomas, 2003; Williams, 2003), tuber crops (Barone, 2004; Fregene et al., 2001; Gebhardt and Valkonen, 2001), pulses (Kelly et al., 2003; Muehlbauer et al., 1994; Svetleva et al., 2003; Weeden et al., 1994), oilseeds (Snowdon and Friedt, 2004), horticultural crop species (Baird et al., 1996, 1997; Mehlenbacher, 1995) and pasture species (Jahufer et al., 2002). Research done by Kasha (1999) and Ortiz (1998) suggested that DNA markers will have a great contribution to enhancing global food production by improving the efficiency of traditional and/or conventional breeding programs. A limitation, to the use of markers is the cost, availability of lower prices supplies and ability to tag QTL (Bernatzky and Tanksley, 1989).

The type of molecular marker used to select desirable characters include restriction fragment length polymorphisms (RFLPs) and PCR-based DNA markers such as random-amplified polymorphic DNAs (RAPDs), microsatellites, sequence characterized amplified regions (SCAR), sequence-tagged sites (STS), inter-simple sequence repeat amplification (ISSR), and amplified fragment length polymorphic DNAs (AFLP).

Genetic populations have included F_2 and backcross populations, near-isogenic lines, doubled haploids and recombinant inbred lines (RILs). Molecular marker studies using RILs have accelerated the mapping of many genes in different plant species (Mohan et al., 1997). Moreover, markers can be used across different environments and obtain the same results; therefore, markers can be applied independently of the stage of the plant growth.

In order to evaluate the polymorphism of microsatellite markers in pea, Loridon et al. (2005) used three segregating populations to build a composite genetic map. They were RILs derived by single seed descent from three crosses. Four hundred thirty-four microsatellite primer

pairs were tested and optimal PCR conditions were established for three hundred forty of these markers.

The challenges in breeding for resistance of Ascochyta blight are due to the contribution of three different pathogens and that each has a different mechanism of resistance and different genes for resistance (Skolko et al., 1954). A second reason would be the physiological specialization within each pathogen. Genetic studies suggest that there are different races of each pathogen and specific genes for resistance to each race (Matthews, 1989). Therefore, more studies regarding the pathogenic variability of these fungi are needed (Zhang et al., 2003). The third difficulty is the broad methodology for screening fungi. The method with which the individual plants are scored can influence the results whether it is resistant or susceptible (Sakar et al., 1982). A study performed *in vitro* by Gretenkort and Helsper (1993) and a study done in the field by Hillstrand and Auld (1982) showed distinct results. A test using plantlets for resistance to *P. pinodella in vitro* proved that showing resistance is possible under laboratory conditions. On the other hand, in the field and greenhouse where the conditions are generally more severe, the resistance level is different. Within these two environments, plants in the greenhouse show more susceptibility, because in the field there are chances that the fungus was not spreading. Under greenhouse conditions, the inoculation and incubation processes have to be very meticulous, because the age of the plant and the plant tissue influence incidence and severity of disease. Some varieties are more susceptible in the stems and others in the pods. A variety may be considered resistant or susceptible depending on the tissue evaluation (Sakar et al., 1982).

Moreover, a standard testing procedure with all the plants receiving the same treatment has to be developed. Prioul et al. (2003) compared how different genotypes of pea would react

regarding a delay in the Ascochyta blight development caused by the fungus *M. pinodes* at the seedling stage in a controlled environment. Leaflets, stipules, and stems were assessed separately and genotypes were compared on the basis of four variables related to resistance, appearance of disease symptoms, incubation period, degree of disease progress level and area under disease progress curve (AUDPC). The conclusion of this study was that lines with partial resistance could be identified by observing differences between genotypes in the appearance of disease symptoms. However, taking into consideration the AUDPC for different pea lines showed a more accurate measure of disease resistance. Another example of difficulty found in studies when screening pea varieties for disease resistance was the variation of individual plants. It is hard to tell if the variation is due to environment or genetic heterogeneity within varieties (Skolko et al., 1954).

Genetic maps

Genetic mapping of major genes and QTL for many agronomically important traits makes marker assisted selection (MAS) easier by developing tools to make gene mapping faster, cheaper, and more practical. Genetic mapping is used to inform the gene function, gene regulation and their expression; the molecular markers have the function to identify and tag genes of the study interest, for example disease resistance, plant height, flower color, and others. The chromosome where the genes associated to the disease are present can also be identified. One of the first studies done in the development of techniques of genetic mapping and linkage analysis was in 1911 by Morgan and his student Sturtevant (Anderson, 1925). The principle of genetic mapping is the occurrence of “crossing-over” of chromosomes during meiosis; this event is that homologous chromosomes exchange sections of their linkage and distance on the linkage

map. Several investigations have been done since then. The most commonly used are Haldane (1919) and Kosambi (1944) corrections for undetected crossover and interference.

When the genetic linkage map of a chromosome has been developed, QTL related with traits of interest and gene positions that have been mapped in a comparable manner can be integrated into the map. Therefore, the genes/QTL can be evaluated for linkage with the closest markers on the map and indirect selection can be implemented using the markers. Historically, Linkage maps were constructed based on morphological traits with mutant phenotypes (Crow, 1990). One way to avoid the necessity of mutant phenotypes was the introduction of DNA based molecular markers, because they can be found in large numbers and do not affect the progeny viability. DNA based molecular markers result from different mutations on DNA such as substitutions (point mutations), rearrangements (insertions or deletions), or errors in replication of tandemly repeated DNA (Paterson, 1996). The selection of these markers is neutral because they are usually located in non-coding regions of DNA. Different than morphological and biochemical markers, DNA markers are not affected by environmental factors and/or the developmental stage of the plant; plus they are unlimited in number (Winter and Kahl, 1995).

The first DNA-based molecular marker technology used in plants for MAS was RFLPs (Restriction fragment length polymorphism). A linkage map developed by RFLP is available for different crop species such as wheat, rice, barley, oat (*Avena sativa*), maize (*Zea mays* L.), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*) (Van Deynze et al., 1998), tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) (Tanksley et al., 1992). There are also other types of molecular markers that have been included in the linkage maps; such as AFLPs (Becker et al., 1995), microsatellites (Roder et al., 1995; 1998), and sequence tagged sites (STSs) (Mano et al., 1999).

A genetic linkage map of pea was published using morphological, biochemical and molecular markers (Loridon et al., 2005). A similar map was published by Ellis et al. (1992) using highly polymorphic microsatellite markers that are randomly distributed across the linkage groups in pea.

Quantitative trait loci (QTL) analysis

Quantitative traits represent some of the most important traits in the plant. Traits such as yield (grain number, grain weight), plant height and days to flowering are controlled by several genes. Many QTLs have been identified using DNA markers in many different crop plants, such as maize (Edwards et al., 1992) and barley (Hayes et al., 1993).

QTL mapping and association studies have been utilized recently to study economically important traits in plant breeding. Intending to facilitate and understand the nature of disease resistance, QTL mapping of disease resistance loci has been studied, and it will eventually lead to the cloning of fundamental genes (Timmerman-Vaughan et al., 2002). Characterizing the genetics of resistance and identification of molecular markers that can be used in plant breeding, QTLs relating to Ascochyta blight resistance were mapped by Timmerman-Vaughan *et al.*, (2002). Thirteen QTLs for Ascochyta blight across seven linkage groups were identified. However, more than one report was found relating QTLs to Ascochyta blight and flowering date and plant height (Timmerman-Vaughan et al., 2004). Prioul et al. (2004) also found that QTLs for flowering date and plant height were correlated for resistance to *M. pinodes*. Other QTLs for lodging resistance, plant height were correlated with partial resistance to Ascochyta blight in field pea (Tar'an et al., 2003). These studies show that the QTLs for resistance and maturity are

linked or that the Ascochyta blight resistance QTLs detected results from genes influencing multiple traits of the plant-maturity genetic loci.

Timmerman-Vaughan et al. (2002) used a green pea breeding line (3148-A88 [A88]) with resistance to Ascochyta blight (Crop and Food Research, Lincoln, New Zealand), and 'Rovar', a susceptible green pea cultivar (Cebeco, Lelystadt, the Netherlands), to produce a population of 225 F_2 lines. The $F_{2:3}$ seeds were produced from individual F_2 plants grown in the field at Lincoln, New Zealand. The $F_{2:4}$ seeds were produced by bulking seeds grown from at least five F_3 plants either in the greenhouse or in the field. DNA was extracted from leaves bulked from at least five F_3 descendants. They were able to identify three polymorphic SCAR loci in the 'A88' / 'Rovar' population that were previously not described. A genetic linkage map of the A88 / Rovar cross containing RFLP, RAPD, AFLP, and SCAR markers was constructed (MAPMAKER/EXP ver. 3.0.) for the 133 F_3 progenies that were field trialed during three years. Thirteen QTL associated with Ascochyta blight resistance were located on seven linkage groups. The location and number of QTL on linkage group III was still uncertain. Eight of these QTL were expressed in different environments and one QTL was detected for plant developmental stage. Seven significant disease resistance QTL were found across the environments. Five of them (Asc 1.1, Asc 2.1, Asc 4.3, Asc 5.2 and the linkage group V QTL linked to sAFP2P2c) were detected whether the traits were evaluated in a single environment as means across environments, but the location and number of QTL on linkage group III was still uncertain.

Timmerman-Vaughan et al. (2004) conducted an experiment using two crosses: '3147-A26' (A26, partially resistant) / Rovar (susceptible) and 3148-A88 (A88, partially resistant) / Rovar, to validate previous research. Field trials were conducted in Western Australia and New Zealand to develop and increase the knowledge of the genetic resistance and identify linked

molecular markers. The linkage map was constructed using 99 loci and covered 930 cM of the pea genome on 13 linkage groups. DNA marker methods previously described within a population of 148 F_2 progeny were used (Timmerman-Vaughan et al., 2002). Eleven hypothetical QTL for Ascochyta blight resistance on linkage groups II, III, IV, V, VI and VII were detected based on the A26 / Rovar population.

MATERIAL AND METHODS

Material

A population with 394 RILs developed from the cross 'Lifter'/'Radley' was used to map resistance to Ascochyta blight. Lifter is resistant to powdery mildew, Fusarium wilt race 1 and Pea Enation Mosaic Virus (PEMV), but is susceptible to Ascochyta blight. Lifter is a semi-dwarf plant with normal leaves and was developed by USDA-ARS in cooperation with the Washington Agricultural Research Center, Pullman, WA, the Idaho Agricultural Experiment Station, Moscow, ID, and released in 2001 (McPhee and Muehlbauer, 2002). Radley is resistant to Fusarium wilt race 2 and has partial resistance to Ascochyta blight. Radley was developed in Canada and also has semi-dwarf plant stature with semi-leafless morphology. The single seed descent method was used to advance this population to the F_7. A combination of field nurseries and a greenhouse were used to advance this population. DNA of each plant was extracted for the molecular tests and each plant was also inoculated in the greenhouse for phenotypic data collection.

Field experiment

In spring 2009, the 397 RILs were planted at the North Central Research Extension Center, located in Minot, ND. Minot is located at 48.180° N latitude and -101.293° W longitude, with an elevation of 542 meters above sea level and a total average precipitation of 420 mm annually. Soil at Minot is classified as Williams Loam Gently Undulating, with a 5.5 pH. The previous crop planted in the area was hard red spring wheat. The seed bed condition at planting was fair and pea was the first legume on the field site. The population was planted as individual

microplots with a size of 1.5 m by 0.6 m. A three row planter was used. The purpose of the experiment was to increase seed for the next generation.

DNA extraction

Parents and RILs were grown in the greenhouse for DNA isolation. Prior to flowering, approximately 1g of fresh tissue was harvested from plants and frozen in liquid nitrogen. The frozen tissue was freeze dried and later ground into fine powder. The powder was transferred to a 50 mL polypropylene tube and stored at -20° C. Extraction buffer (0.5 M NaCl, 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 0.84% w/v SDS, 3.8 g sodium bisulfate/L, and pH was adjusted to 8.0 with 0.8 M NaCl) was heated to 65°C for 30 min. Tubes were placed on a stand and allowed to cool to room temperature. After cooling, 15 mL of 24:1 chloroform: isoamyl alcohol (CI) was added and mixed by inversion. After centrifugation at 3500 rpm for 15 min, the supernatant was transferred to a new 50 mL tube. One µg/ml of RNase (~10 µl of 10mg/mL) was added and the solution was incubated at 37°C for 30 min. After incubation, 15 mL of CI was added, mixed by inversion and the samples were centrifuged at 3500 rpm for 15 min. The top aqueous portion was transferred to a new 50 mL tube and 3 volumes of cold 95% ethanol were added. After mixing, the tube was incubated at 4°C for 20 min, followed by centrifugation at 3500 rpm for 15 min. The supernatant was discarded and the pellet washed once in 5 ml 70% ethanol and dried for 30 min. The dry pellet was resuspended in 600µl-1mL of Tris-EDTA buffer (TE) and then quantified with a spectrophotometer.

Marker analyses

SSR markers developed through a consortium by the Agrogene Company were used to screen the parents for polymorphism and develop the genetic map. Polymerase chain reaction

(PCR) amplification of genomic or DNA was done in a 15 µL reaction volume containing 50 ng template DNA, 0.2 µM dNTPs, 2.5 mM $MgCl_2$, 1x PCR buffer and 0.5 U Taq DNA polymerase. The amplification profile was one cycle of 3 min at 94°C, followed by 40 cycles of 20 sec at 94°C, 50 sec at annealing temperature (51-55°C), and 50 sec at 72°C. The last cycle was followed by a final extension step of 7 min at 72°C. The PCR products were separated by electrophoresis on 8% polyacrylamide gel or 2% agarose.

Prior to fragment analysis, each reaction was mixed with 8 µL of loading solution. The gels were then loaded and run at constant voltage, (120V for agarose and 200V for polyacrylamide) for 2 to 6 hours. DNA fragments were then visualized using ethidium bromide.

Disease screening methods

Pathogen culture

Five isolates were acquired from the North Dakota State University Plant Pathology Department. They were tested before in previous research and showed Ascochyta blight symptoms. The isolates were A1, A8, A10, A13 and A17. They were all tested in a detached leaf assay to determine virulence on the parents of the mapping population used in this research.

Media preparation

Five culture media including, V-8, oatmeal, 2% malt extract, pea media and potato dextrose agar were tested for optimal conidial growth and sporulation of *M. pinodes*. Approximately half of the volume of a petri dish was dispensed into sterile disposable petri dishes. V-8 media was composed of 200 ml diluted V-8 juice and 20 g l^{-1} agar (manufactured by Becton Dickinson Becton Drive Franklin Lakes, NJ 07417). The V-8 juice was cleared by centrifugation for 10 min at 3000 RPM. The supernatant was filtered through a Buchner funnel

using a vacuum. Two hundred milliliters of cleared V-8 juice and 20 g agar were added to 1 L distilled water and autoclaved for 30 min prior to pouring into petri plates.

The oatmeal agar was composed of 20 g of oatmeal added to 1L of distilled water and boiled for 20 min. After the solution was filtered, 18 g of agar was added. Since water was lost in the boiling process, distilled water was added to bring the solution to 1L again. In order to sterilize the solution, it was autoclaved for 30 min.Twenty grams of malt extract agar was added in 1L of distilled water and autoclaved for 30 min to make the 2% malt agar solution.Pea media was made up of 150 g of pea mixed with 15 g of bacto agar. 1L of water was boiled with the 150 g of pea for 40 min. The solution was filtered and mixed with the 15 g of bacto agar with consequently autoclaved for 30 min. Potato dextrose agar was made up of 40 g of PDA mixed in 1L of distilled water and autoclaved for 30 min.

Inoculum preparation

Single-spore isolates of *M. pinodes* were obtained from leaf samples of field-grown *P. sativum* collected by researchers in the North Dakota State University Plant Pathology Department. Two of the most aggressive isolates were selected based on leaf assay and used to screen all genotypes throughout the experiments. Small pieces (4 mm^2) of leaf tissue infected with Mycosphaerella blight were sterilized in 1% NaOCl for 90s, rinsed in sterile distilled water and put on sterile filter paper to remove excess moisture. Leaf segments were planted on potato dextrose agar (PDA) for 14 days. The development of lesions was observed daily and lesion diameter was measured fourteen days after inoculation. After 14 days, spores were collected by removing the conidia and spreading them on oatmeal agar (OMA) (1.5% water agar plus 1% oat powder). Colonies were grown on OMA for 4 weeks, and then flooded with sterile water and scrubbed with a sterile metal knife to release conidia. The incubation temperature was 20° C with

a 14 hour photoperiod with fluorescent lamps. After filtration through cotton, the spore concentration was determined using a haemocytometer. The spore suspension was diluted in water to 100 conidia/μL and the suspension was adjusted to 10^5 conidia/ml for inoculation (Hwang et al., 2006).

Detached-leaf assay

Three plants of each of the parents, Radley and Lifter, were planted in 6 inch pots filled with LC1 sunshine mix (manufactured by Sun Gro Horticulture distribution Inc. 15831 N.E. 8th St. Suite 100 Bellevue, Washington, USA, 98008). Two weeks after planting or when the plants reached the five node stage, plant material from the fourth node were collected. The plant material used was stipules for Radley and Stirling and leaflets for Lifter. Moist chambers were created by placing two moistened, autoclaved paper towels on the bottom of a 10x100 mm plastic petri dish (manufactured by VWR, Building One, Suite 200, 100 Matsonford Road, P.O. Box 6660 Radnor, PA, 19087) and covering them with a hard plastic mesh. The intact plant material attached was removed from the stem just below the fourth node and placed on the agar solution. An agar solution supplies better moisture and nutrition to the leaves. On some leaves the presence of roots was observed after a few days. The agar solution was made of 5 g of glucose or dextrose plus 15 g of bacto agar in 1L of distilled water.

The detached leaf assay was performed using 5 *M. pinodes* strains, A1, A8, A10, A13 and A17. The inoculations were done at three different dates and measurements were done after 15 days. The first inoculation was done February 3, 2011, second March 8, 2011 and third on March 30, 2011. Stock cultures for each strain were kept at -80°C in one ml tubes. Concentrations of conidial suspensions for each strain were equalized prior to inoculation. One ml of spore solution

was mixed with a calculated volume of water plus 0.05% Tween 20 (polyoxyethylene sorbitan). The calculated volumes of solution were used after suspensions were equalized according to the formula C_1 x V_1 = C_2 x V_2. After all volumes were adjusted to 10^5 conidia/ml, the leaves and stipules were inoculated with a 10 µL droplet of fresh conidial suspension at four sites on each leaflet. After this period, disease development and severity was observed and could be differentiated between the resistant and susceptible parents after incubation at 22 ± 1°C for 7 days, with a 16:8 hour, light: dark photoperiod. Length and width of lesions were measured and the area calculated using the equation:

Area of lesion = ((length × width) × 3.14)/4.

Disease severity was rated 14 days after inoculation on a 0 to 4 scale based on lesion size, where 0 = no lesion development, 1 = 0 to 33 mm^2, 2 = 34 to 66 mm^2, 3 = 67 to 100 mm^2, and 4 > 100 mm^2 (Hwang et al., 2006). Lesion size values were averaged for 20 plants total in a combination of 4 replicates with 5 leaves of each parent in petri dishes for each isolate. The total results were obtained after 3 repetitions that were done on 3 different days with 5 different isolates.

Analysis of variance was conducted to determine the genotypic differences in partial resistance for each of the 5 isolates and treatment means were separated using the Least Significant Difference (LSD) test at P=0.05 probability using Statistical Analyses System 9.2 (SAS Institute, 2004).

Greenhouse material

The experiment was conducted subsequently rather than simultaneously because of limitation of space in the greenhouse. A total of two replications, one at a time were plated with staring dates from June 2011to September 2011. Because of time limitation and to avoid a large

number of plants to be scored in the same day, the RIL were divided in 4 sets with different planting dates within each replication, i.e. four sets of flats for the first repetition and four sets of flats for the second repetition. The resistant and susceptible parent plus a susceptible check were included in all set for comparison.

Genotypes were planted in planting trays with fifty cell each and each genotype was repeated 5 times with ten different RILs planted in each flat. The 394 plants were divided into four sets having respectively 111, 117, 114 and 51 lines, respectively, and were planted on four different dates. The first set was planted June 12, 2011, the second set was planted on June 26, 2011, the third set was planted July 12, 2011 and the fourth set on July 28, 2011. A second replication of each set was planted on the following dates: first set, July 29, 2011, second set August 23, 2011, third set September 3, 2011, and the fourth set September 9, 2011. The parents 'Radley', 'Lifter' and 'Stirling' were included with each set. The plants were inoculated fifteen days after planting and placed in humidity chambers for two days to create an optimal environment for disease development. The humidity chambers were set to release mist for thirty seconds each 30 minutes. The plants received light on a 16:8 h, light: dark photoperiod since *Mycosphaerella pinodes* has melanin biosynthesis and; therefore, requires a dark period. The temperature in the humidity chamber was maintained at approximately 23°C at all times. After two days in the humidity chambers the plants were returned to the greenhouse and left for a period of seven days.

Disease symptoms were observed on the most susceptible plants on the second day. The first score was given seven days after inoculation. The remaining four scores were given at three day intervals. Disease severity scores were assigned according to the following scale: 0= when no disease present, 1= 5 to 7 spots on the plant material, 2= few necrotic spots but plant material

still healthy and green, 3= plant material with 50% of the green area affected and color starting changing to yellow, 4= almost the entire plant material yellow and lots of brown spots, and 5= plants were dead. Plants in which the four bottom leaves were dead also received a score of 5 even though the plant top was green. This happened because when the plants were inoculated, they had 4 leaves, the disease develops more slowly than the plants grow, for example the top of the plants were green by the time the fifth score was given, and even though that plant had the bottom dead and the top would eventually die. Disease severity scores were based only on leaf symptoms despite the stems also showing disease symptoms.

The area under the disease progress curve (AUDPC) was calculated in order to observe disease development over time, general repeated measurements were converted in a single number (Jeger, 2004). This area was calculated by breaking up the disease curve, within a graphic, into small trapezoids and then calculating their areas. AUDPC values were generated using the same data and measurements days, the first measurement day being 21 days after planting, the second measurement day 24 days after planting, third day 28 days after planting, fourth day 31 days and the fifth measurement day 34 days after planting.

Greenhouse inoculation procedure

Each line was inoculated with a mixture of two strains (A8 and A10) of *Mycosphaerella pinodes* on the 4^{th} node approximately 15 days after planting. . One ml of spore solution with $1x10^7$ conidia per milliliter of each strain was mixed in 200 ml of water containing one drop of Tween 20 to facilitate the spores sticking on the leaf. The water solution with spores was then kept on ice until the inoculation process started. A paint brush adapted to spray spores was used

for the inoculation process for more uniform spore distribution. The whole plant was inoculated, and the plants were sprayed until little drops formed on the leaves.

Statistical analysis

The scoring system used in the experiment was in an ordinal scale, which means given values were used to interpret disease grade, for example any number used has a proper value. It was just assumed that the value 0 is equal to a completely healthy plant and the value 5 is equal to a dead plant. The experiment was divided into four sets, and each set had twelve fifty-cell flats. Sets of RILs were planted on different dates and the checks were included in all of them. An augmented design (Federer et al., 2001) was used to analyze sets. Kruskal-Wallis test (Conover, 1998), a nonparametric version of a one-way ANOVA, was conducted for each scoring date using SAS Enterprise Guide (v. 4.3, SAS Institute 2010, Cary NC). Median scores were calculated for each line on each scoring date and ranked to determine which lines were most resistant and which lines were most susceptible. In the augmented design, means of experimental entries are adjusted based on a repeated check within sets. Data analyses, least square (LS) and test for significance of the experimental lines, were calculated using PROC MIXED in SAS 9.2 (SAS institute Inc., Cary, NC 2004). A mixed model was used and dates were considered random and checks were considered fixed effects.

The area under the disease progress curve was calculated with the formula

$$AUDPC = \sum_{j=1}^{n_j - 1} \left(\frac{y_j + y_{j+1}}{2} \right) \left(t_{j+1} - t_j \right)$$

Where *j* was the order index for time (and *nj* is the number of times); *y* is the disease incidence and *t* is time, number of days.

Linkage analysis and genetic map construction

The linkage analyses were conducted using the software JoinMap version 4.0 (Kyazma B.V., Wageningen, The Netherlands). The map distances were calculated in centimorgan units (cM) and the Kosambi function was used (Kosambi, 1944) to create maps at minimum lod 3.

QTL analysis

QTL Cartographer was used to identify QTL. Simple interval mapping (SIM) and simplified composite interval mapping (sCIM) were ised and a walking distance of 1 cM and a type I error of 5% were used. For SIM, the calculation was done with threshold values of 1000 permutations. A QTL can be considered acceptable when both the SIM and sCIM show significant peak presence. Background markers had a distance of 25 cM along the length of the chromosome.

RESULTS AND DISCUSSION

Ascochyta blight greenhouse studies

Parental screening

This study on "Molecular mapping of Ascochyta blight resistance in pea (*Pisum sativum* L.)" had three major purposes: i) construction of a genetic linkage map ii) detection of QTL regions responsible for Ascochyta blight resistance against *Mycosphaerella pinodes* isolates and iii) validation of previous reported QTL with QTLs detected in Lifter/Radley population.

Parents were tested to verify differential (resistant or susceptible) reaction of the parents against the *Mycosphaerella pinodes* isolates. Isolates concentrations are shown on Table 1 and parent reaction (Table 2).

Table 1. Concentration of *Mycosphaerella pinodes* isolates used to evaluate virulence among parents of recombinant inbred line populations.

ISOLATES	CONCENTRATION
A1	3.25×10^7
A8	4.10×10^7
A10	3.30×10^7
A13	12.00×10^7
A17	14.00×10^7

The two most aggressive isolates (A8 and A10) based on lesion area were selected for all future experiments. The isolates A8 and A10 showed similar results and bigger lesions compared with the other three isolates on both parents. A mixture of these isolates was used to inoclulate the plants.

Table 2. Mean lesion area and range for Lifter, Radley and Stirling challenged with isolates A1, A8, A10, A13, and A17.

Isolates	Area mean ----------mm2----------			Range ----------mm2----------		
	Radley	Lifter	Stirling	Radley	Lifter	Stirling
A10	13	39	90	12-26	15-92	8-110
A8	12	61	111	4-56	4-113	5-172
A13	8	30	70	4-20	8-85	11-99
A17	5	35	60	4-15	9-62	8-62
A1	8	24	36	4-18	12-82	29-51

Greenhouse inoculation

The two replicates showed similar results, the first replicate was done in the beginning of the year started in June while the second replicate started in August. Because of the summer time, plants from replication 1 showed lower scores on in the 4^{th} and 5^{th} dates. This was likely due to the temperatures being too warm for the pathogen in the greenhouse. For example, mean lesion area for Radley, the resistant donor parent, was 3.12 on the 19^{th} day after inoculation of replication one while for replicate 2 the mean lesion area was 3.44. The replicates were combined since there was no significance difference between replicates for each of the five scores (Appendix A).

In both replicates, few lines had lower disease score than Radley, 28 in replicate 1 and 42 in replicate 2 and 14 in the combined analysis. Transgressive segregation for resistance was observed. Transgressive segregation is important for the breeder because it allows selection of offspring with more favorable genes than the parents. A more detailed study with more environments and plants response would be needed to justify if the transgressive segregation was an allelic effect was a random effect or experimental error.

The reaction to Ascochyta blight shows no significant difference among replications and lines with the exception of set 3 from the replicate 1. Higher temperatures and low humidity in the greenhouse were the cause of fewer disease symptoms during this season.

The frequency distribution of the combined recombinant inbred lines with the exception of the first score for set 3 is shown in Figure 1. The first measurement date, 7 days after inoculation, did not show many differences among all lines, probably because of the short time of interaction between plant and pathogen. The minimum observed score was 0.5 with 63 lines better than Radley and the maximum was 4. The overall mean score for the RILs on the 7^{th} day after inoculation was 2.

The second date of measurement, 10 days after inoculation, showed some difference among lines although the plants were still very similar (Figure 2). The minimum observed scored value was 1.0 with 14 lines showing less disease than Radley and the maximum was 5 again only for line 241. The overall mean score for the RILs on the 10^{th} day after inoculation was 3.

Third scoring date was 13 days after inoculation and it was the first day when the difference between susceptible and resistant plants was more evident and could be observed by looking at the two parents (Figure 3). The minimum observed scored value was 1.5 with 44 lines showed less disease than Radley. The maximum was 5 and the overall mean score for the RILs on the 13^{th} day after inoculation was 3.

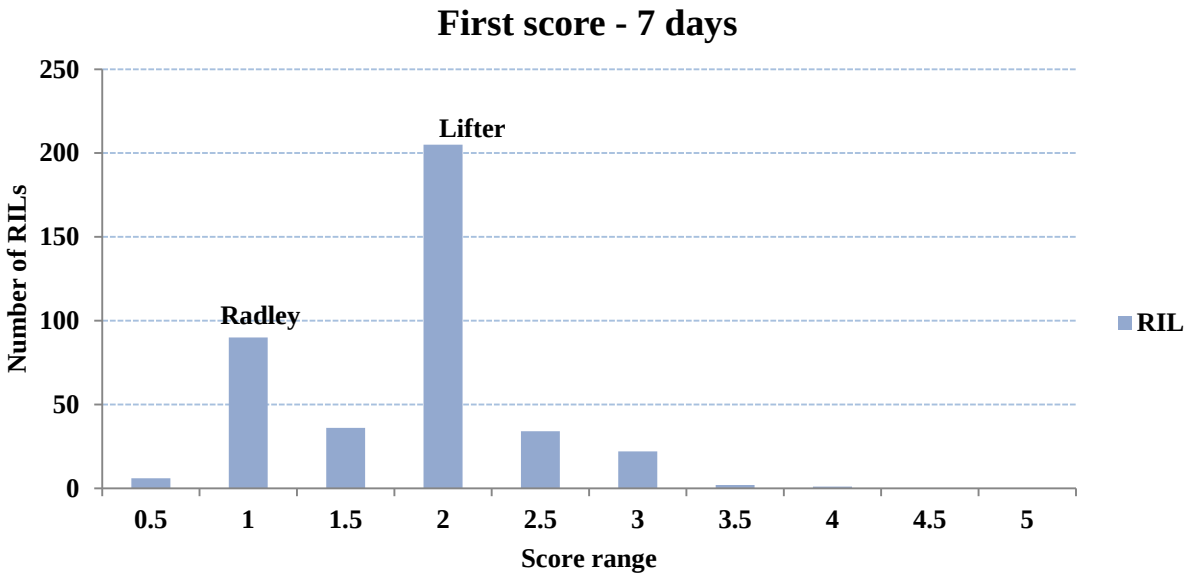

Figure 1. Frequency distribution of 393 RILs and their parents for disease score ratio means after 7 days of inoculation (Parental means are marked with names).

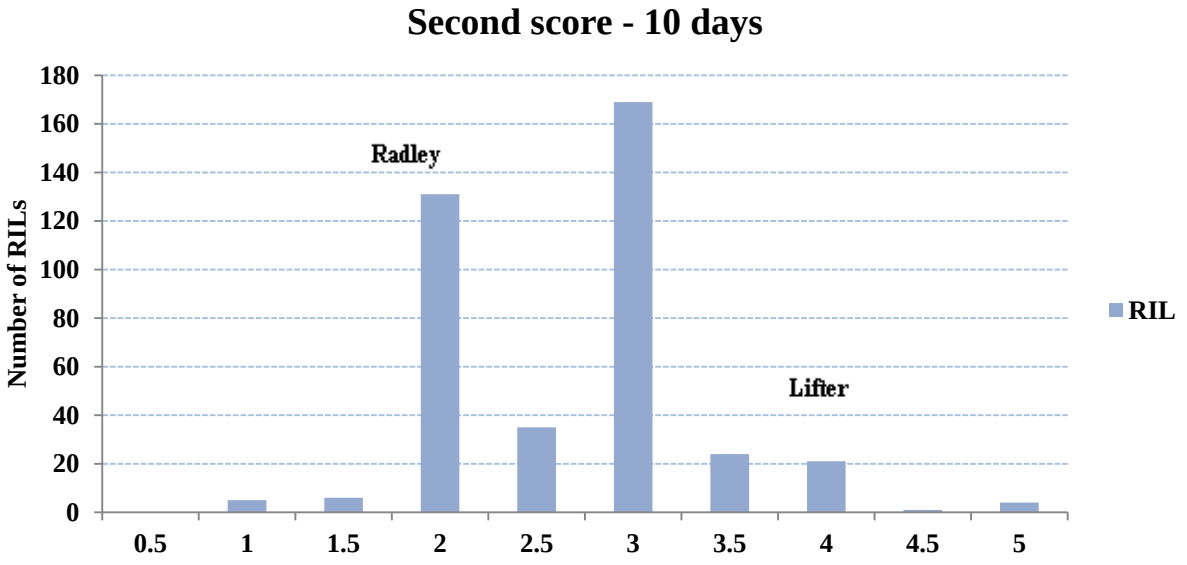

Figure 2. Frequency distribution of 393 RIL and their parents for disease score ratio means after 10 days of inoculation (Parental means are marked with names).

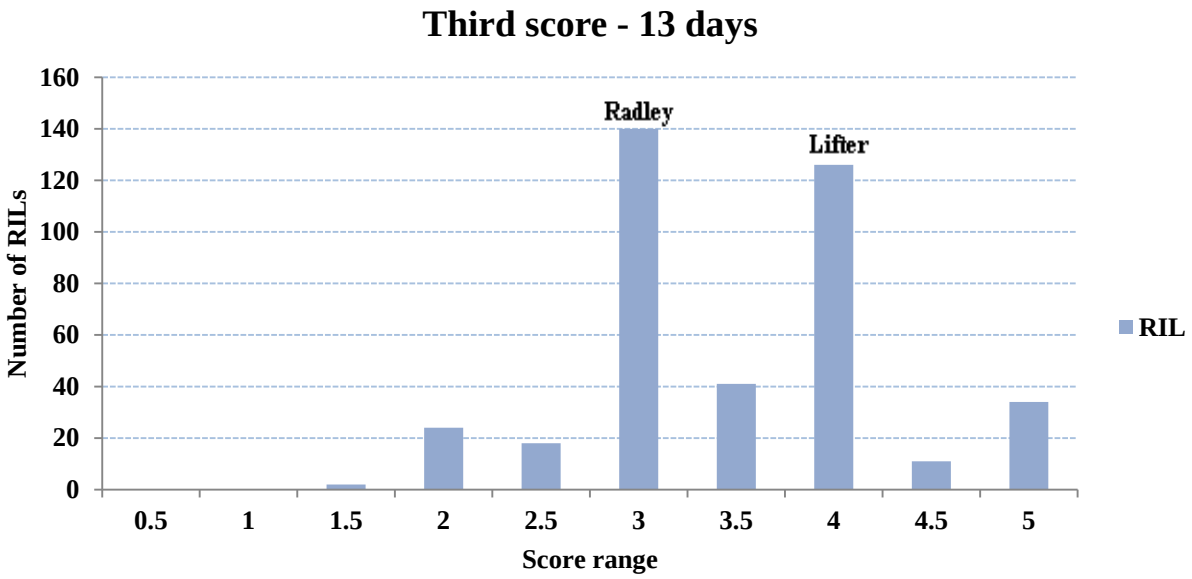

Figure 3. Frequency distribution of 393 RILs and their parents for disease score ratio means after 13 days of inoculation (Parental means are marked with names).

Fourth scoring date was 16 days of inoculation. The difference between resistant and susceptible lines was very clear, although the greenhouse environment also impacted the score; some plants were dying with a combination of heat and humidity plus lesions of the pathogens. Differentiating plant symptoms for scoring was difficult. The minimum observed scored value was 2 for line 30 and 9 lines showed less disease than Radley. The maximum value was 5 and the overall mean score for the RILs on the 16th day after inoculation was 4 (Figure 4).

Day 5 scores were recorded 19 days after inoculation (Figure 5). The plants were dying not only because of the pathogen but also because of the greenhouse environment. This fact made the population mean score higher. The minimum observed value was 2.5 for line 369, the only line showing better results than Radley, and 25 lines had the same score as Radley. The maximum value was 5 and the overall mean score for the RILs on the 19th day after inoculation was 5.

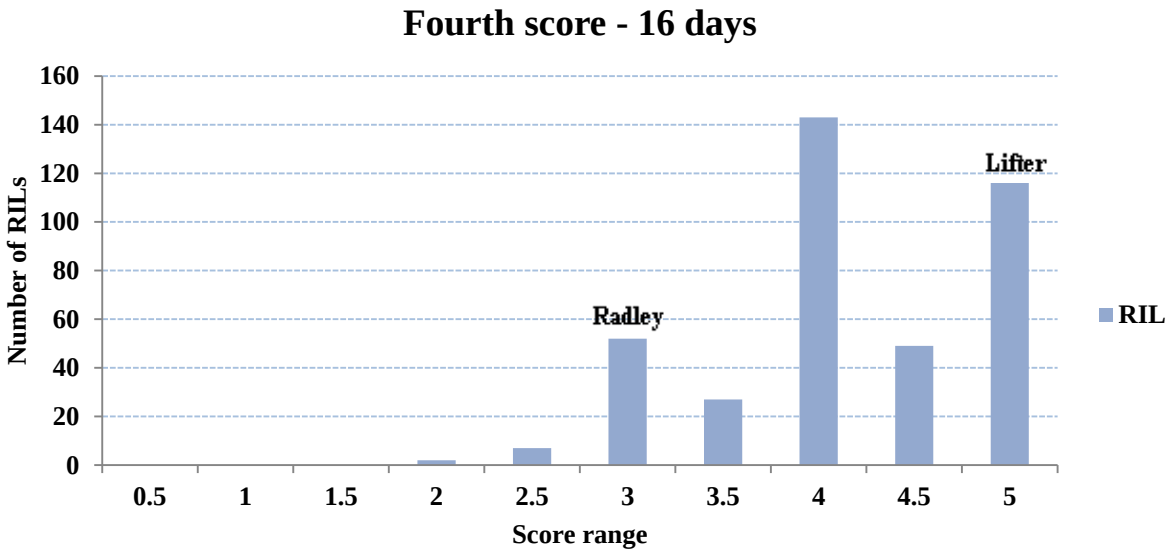

Figure 4. Frequency distribution of 393 RILs and their parents for disease score ratio means after 16 days of inoculation (Parental means are marked with names).

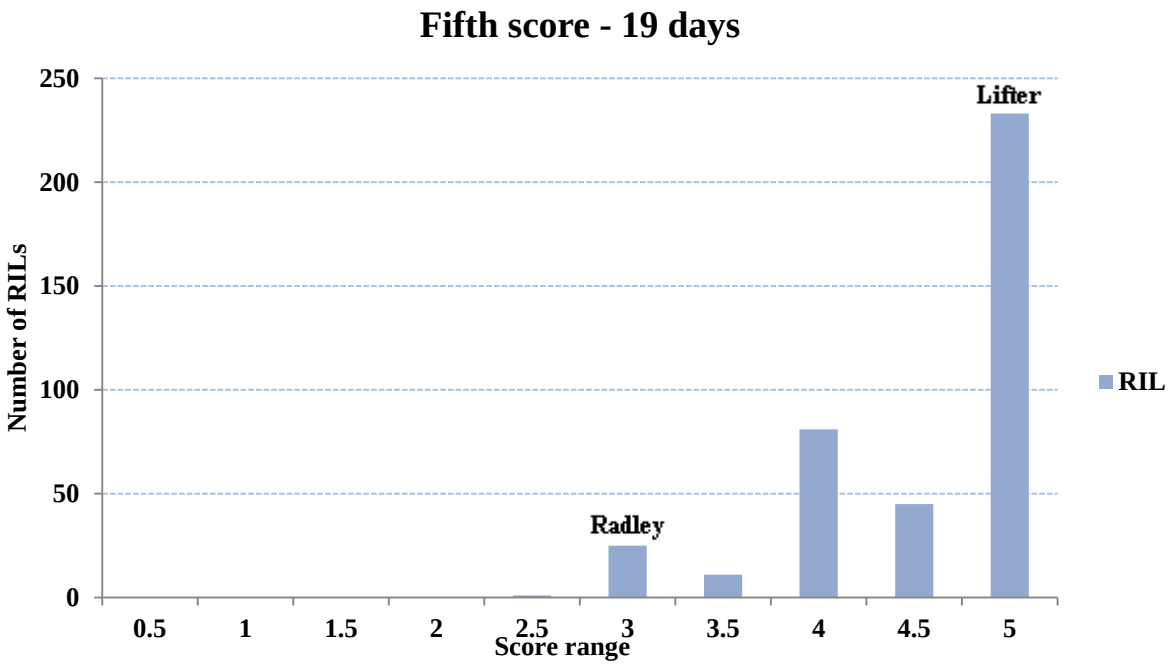

Figure 5. Frequency distribution of 393 RILs and their parents for disease score ratio means after 19 days of inoculation (Parental means are marked with names).

The scores ranged from 0 to 5 and scores lower than 3 on the 19th day post inoculation were considered highly resistant. Stirling was used as a check and Radley and Lifter, the population parents, were repeated in the sets. The population, parents and check means are shown in the Table 3.

Table 3. Mean disease ratings for the Lifter, Radley, Stirling and the population for each of the five days ratings were recorded.

Day	Mean			
	Radley	Lifter	Stirling	Population
1	1	2	2	2
2	2	4	3	3
3	3	4	5	3.5
4	3	5	5	4.5
5	3	5	5	4.5

The majority of the RILs were susceptible to Ascochyta blight. RIL 369 was identified as having a high level of resistance (mean score lower than Radley), another 25 lines had similar ratings to Radley on the 19th day after inoculation of measurement. The population mean always lied between the parents as expected. Stirling was the most susceptible line and was used as the check to ensure that the spores were functional. Scores on 7th and 10th days after inoculation were clearly lower than those on day 3, 4 and 5. After day 3, the pathogen reaction was more evident on the plants.

A nonparametric statistic was used for this study; there were no parameters such as mean or variance from distribution to determine the effects and hypothesis (Shah and Madden, 2004). Although the data was collected at five time points during the experiment, data from the fifth time point were used to identify the susceptible and resistant reactions (Table 4). The median disease ratings ranged from 2.5 to 5; therefore, treatment effects values from 0.33 to 0.70 were generated. The null hypothesis being tested in this experiment considers an expected value to be

the same for all treatments. The treatment effect is a quantified number estimated by the differences of the expected values.

The differences observed in the treatment effect value are used to compare the resistant and susceptible lines. The lines that were used to determine resistance or susceptibility were chosen from the 19th day after inoculation. Two hundred thirty-three lines had a mean value of 5, the same value as the susceptible parent Lifter. Twenty-five lines had a mean value of 3 similar to Radley, the resistant parent. Line 369 showed a median value of 2.5 being the only line with a mean value less than Radley.

The relative treatment effect was calculated by SAS (SAS Institute, Cary, NC) and it is estimation by replacement of distribution functions by their corresponding empirical distribution. Relative treatment effect can also be determined directly from observations midranks (for example: if there are three plants with the same values, they would all have the same rank).

Table 4. Median, mean rank and estimated relative treatment effect for the severity of symptoms on pea caused by *Mycosphaerella pinodes*.

Lines	Median disease rating	Relative effect	Confidence interval (95%) for relative treatment effect	
			Lower limit	Upper limit
RIL-369	2.5	0.38	0.33	0.45
Radley	3	0.45	0.40	0.48
RIL-388	3	0.45	0.40	0.48
Stirling	5	0.69	0.67	0.70
Lifter	5	0.69	0.67	0.70
RIL-33	5	0.69	0.67	0.70

Area under disease progression curve

Disease development was measured using the area under the disease progression curve (AUDPC) calculation. A combined area under disease progression curve was generated excluding the third set of replication 1. It was observed that clear differences in AUDPC exisited between highly resistant and susceptible lines. AUDPC values ranged from 0 to 100.5 among the 393 RILs. AUDPC values for the parents, Lifter and Radley, and the check Stirling showed clear differences between the resistant and susceptible reaction (Figure 6). AUDPC calculations revealed that 39 lines were more resistant than Radley, the best two lines were lines 30 and 38 with AUDPC values of 24.6 and 26.5, respectively. The two lines showing the most susceptible reaction were 240 and 241 with AUDPC values of 90.9 and 100.5, respectively. Radley had an AUDPC value of 42.9, Lifter, 68.3, and Stirling 73.6. Transgressive segregation at both ends of the distributions present as expected.

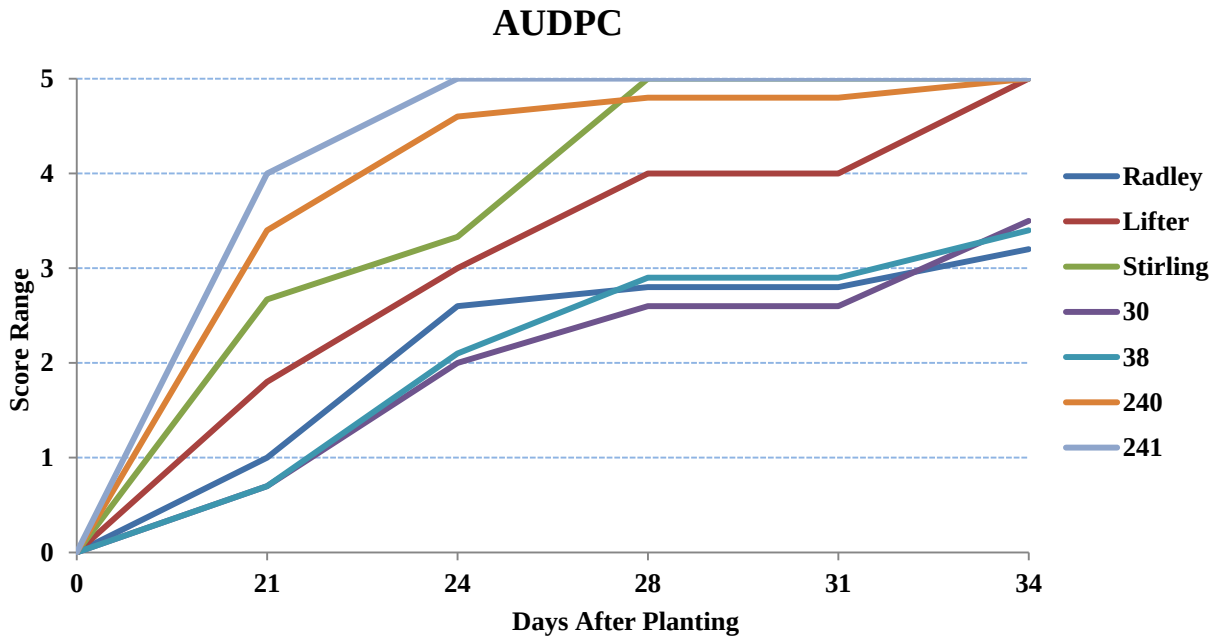

Figure 6. Ascochyta blight symptom developed over time. The selected lines were: Radley, Lifter, Stirling and the two best and worst RILs.

QTL mapping

SSR markers and RAPDs that were previously reported in other studies for resistance to Ascochyta blight were tested in this population. The DNA fragment size that was amplified in this study ranged from 150 and 1200 bp. Polymorphic bands for Lifter and Radley were used as the reference for markers screened in the mapping population. Figure 7 shows a typical banding pattern of amplified DNA fragments viewed in a polyacrylamide gel. The first picture shows the population screen having the parents as the first two bands indicated with the arrow. Lifter is the first band and Radley is the second band. The rest of the bands are the inheritance coming from Lifter (upper bands) and Radley (lowers bands). The second picture shows the parental screening for different markers. Indicated by the arrows is the difference from Lifter and Radley for the marker PSMPSAC74.

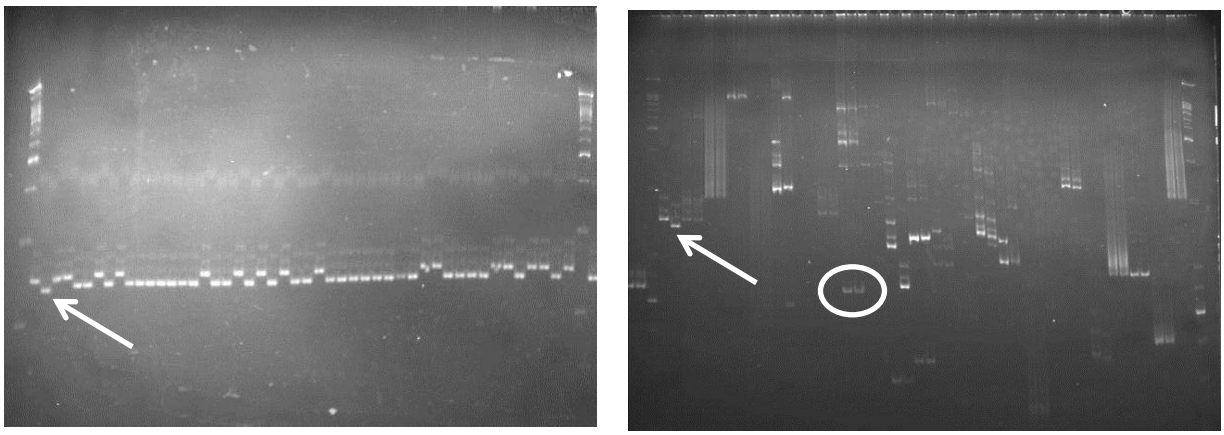

Figure 7. Polymorphism for marker PSMPSAC74 among RILs (a) and in a gel for polymorphism among parents of various mapping populations (b). Arrows indicate polymorphism and the circle indicates a monomorphic band for Lifter and Radley.

Four hundred sixteen SSR and 100 RAPD primers were screened against the two parents to identify polymorphism. Ninety-one primers were polymorphic, and were used for mapping. Among these markers, 81 were SSR and 10 were RAPD. A total of 115 markers were scored in a population of 385 RILs across all the 7 chromosomes where 16 of them had multiple scoring

loci (Table 4). Eighteen percent of RAPD and SSRs primers were polymorphic. One hundred two markers showed the expected segregation ratio of 1:1 and 13 deviated from the expected ratio based on the Chi-square test.

Table 5. Summary of molecular markers used to create a linkage map for the cross Lifter x Radley.

	Primers		
Type of Marker	Tested	Polymorphic	Total Bands
	----------Number of markers----------		
SSR	416	81	99
RAPD	100	10	16
Total	516	91	115

Linkage map

There were 115 loci used to generate the linkage map. Ninety-nine markers showed linkage in 9 linkage groups (I, II, III, IV-a, IV-b, IV-c, V, VI, VII) and 16 markers were not able to be linked. The total map length was 615.9 cM corresponding to 45% of the map obtained by Loridon et al. (2005). The mean distance between markers was 6.2 cM. Most of the markers were present in the group VI-a, which corresponds to the linkage group IV in the pea consensus map. The LG I and III had the least number of markers (4 markers each). Among the 115 markers, 30 were used as anchor markers.

The linkage analyses of the marker data had a minimum of LOD score of 2.5 and a maximum distance of 5.1 cM was used to establish the linkage groups. The linked group interval in the QTL identification was 28 cM, according to Lee (1995) if the value is in between 15 to 20cM or little bit higher values the intervals are good for QTL identification.

The anchor markers that were used to identify and ensure which group relates to the chromosome in the pea genome based on the Loridon et al. (2005) map were AA121 and AA19-476 in the LG-I. The markers average density distribution was 3.5 cM. LG-II contained 11

markers with an average distance of 4.87 cM and 6 markers, AA480, AA504, AD134_298, AA205, AA189, AD134_200 and AB40-402, were used as anchor markers. LG-III had 4 markers with an average map distance of 10.25 cM and marker AD57 served as the anchor marker. LG-IV comprised 39 markers and was broken into 3 smaller linkage groups, LGIV-a, LGIV-b and LGIV-c. LGIV-a had 32 markers with and average map distance of 5.1 cM and AA174 and AB31 served as anchor markers. Group IV-b had 5 markers with an average distance of 10.22 cM and all of them were anchor markers, AA349, AD126, AA122, AA255, and AA94. The group IVc had 2 markers with a linkage distance of 7.55 cM and marker AA219 was the anchor. LG-V had 17 markers with an average distance of 5.94 cM and markers AB23 and AB47 served as the anchor markers. LG-VI had 7 markers with an average marker distance of 9.8 cM and markers AB20_1060, AC76a, AB20_409, and AC76b served as anchors. LG-VII had 17 markers with an average distance of 6.1 cM and 8 anchor markers; AB133, AA57, AA19_347, AD56, AB65, AA317, AB136, AA387, and AA19_926.

Using the Loridon et al. (2005) map as a reference each of the seven linkage groups in the current map represent only small portion of the total genetic map. LG-I covered 14.4 cM and represented only 9% at the top of the reference linkage group. LG-II covered 53.6 cM of the pea chromosome and represented 22% at the distal end of the reference linkage group. LG-III covered 41.7 cM of the pea genome, however only 4 markers were identified, and this region correspond of 14% of the chromosome III and it is located on the middle top part. LG IV was the one with the better representation, covering 80% of the chromosome IV and it is located LG-IVc at the top, LGIV-a in the middle right above IV-b. LG-V covered 101.8 cM of the pea genome, this region represents 50% of the chromosome V and it is located at the top of the chromosome region. LG-VI covered 68.9 cM of the pea genome and this region represents 34%

of the chromosome VI and it is located at the bottom of the chromosome region. LG-VII covered 103.8 cM of the pea genome with the region representing 46% of the chromosome VI and it is located in the middle bottom part. The map in this study shared 30 markers with the reference map of Loridon et al. (2005). These markers show transferability across populations.

The genetic map created in this study with the 9 linkage groups, markers names, and distances is shown in Figure 8.

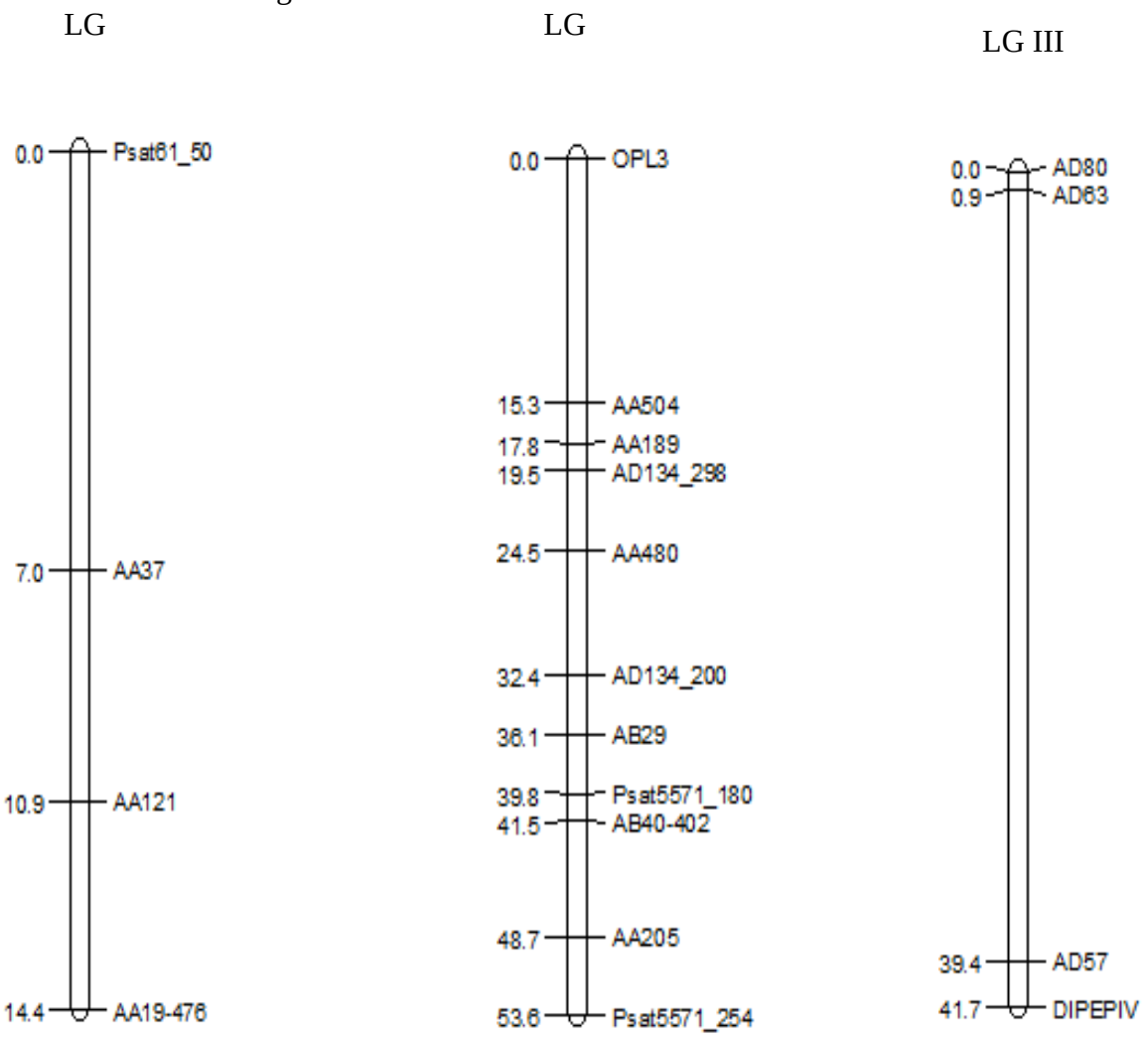

Figure 8. Linkage map of the pea population derived from a cross between Lifter and Radley. The scale represents centimorgan (Kosambi units).

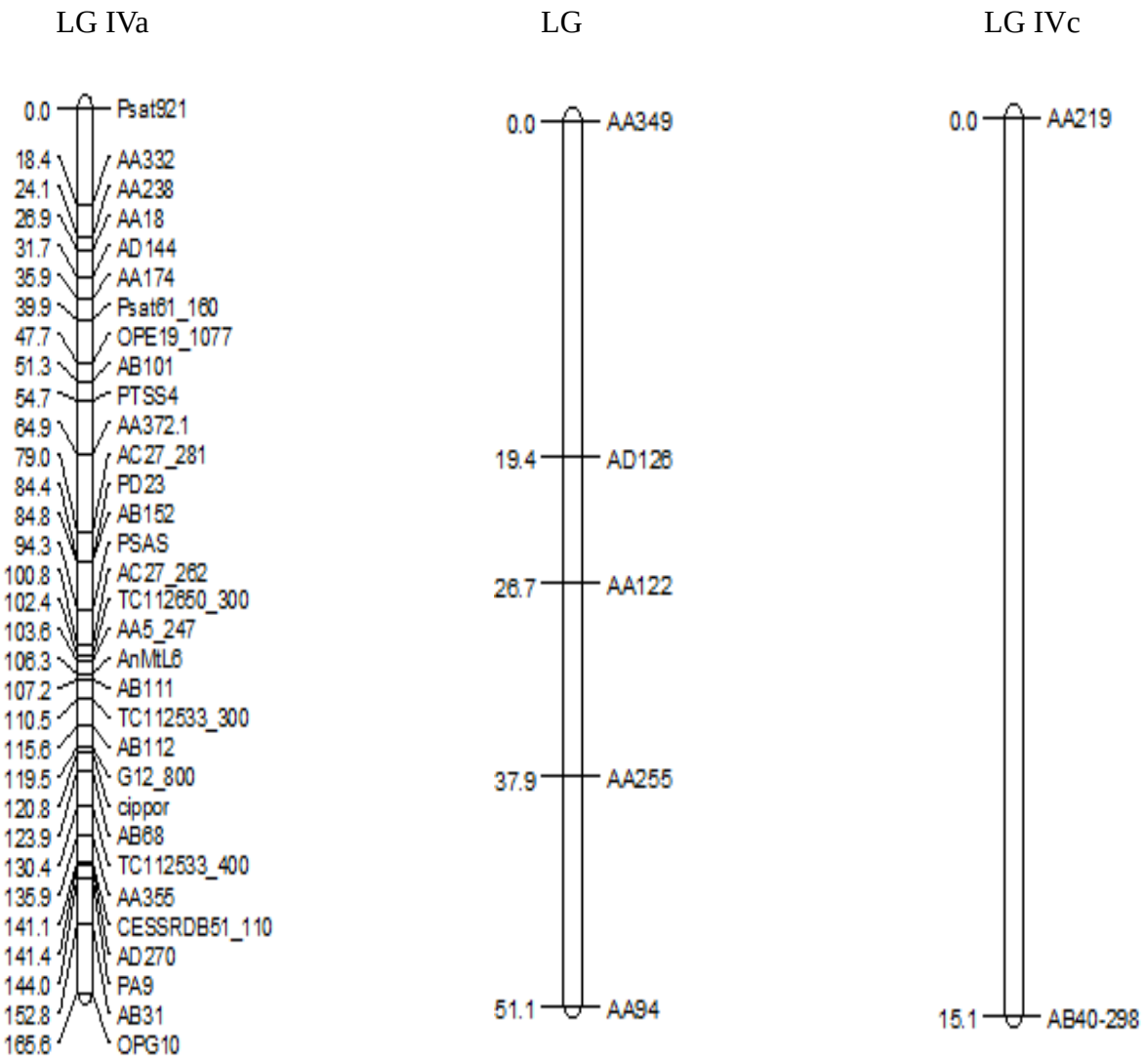

Figure 8. Linkage map of the pea population derived from a cross between Lifter and Radley. The scale represents centimorgan (Kosambi units)(continued).

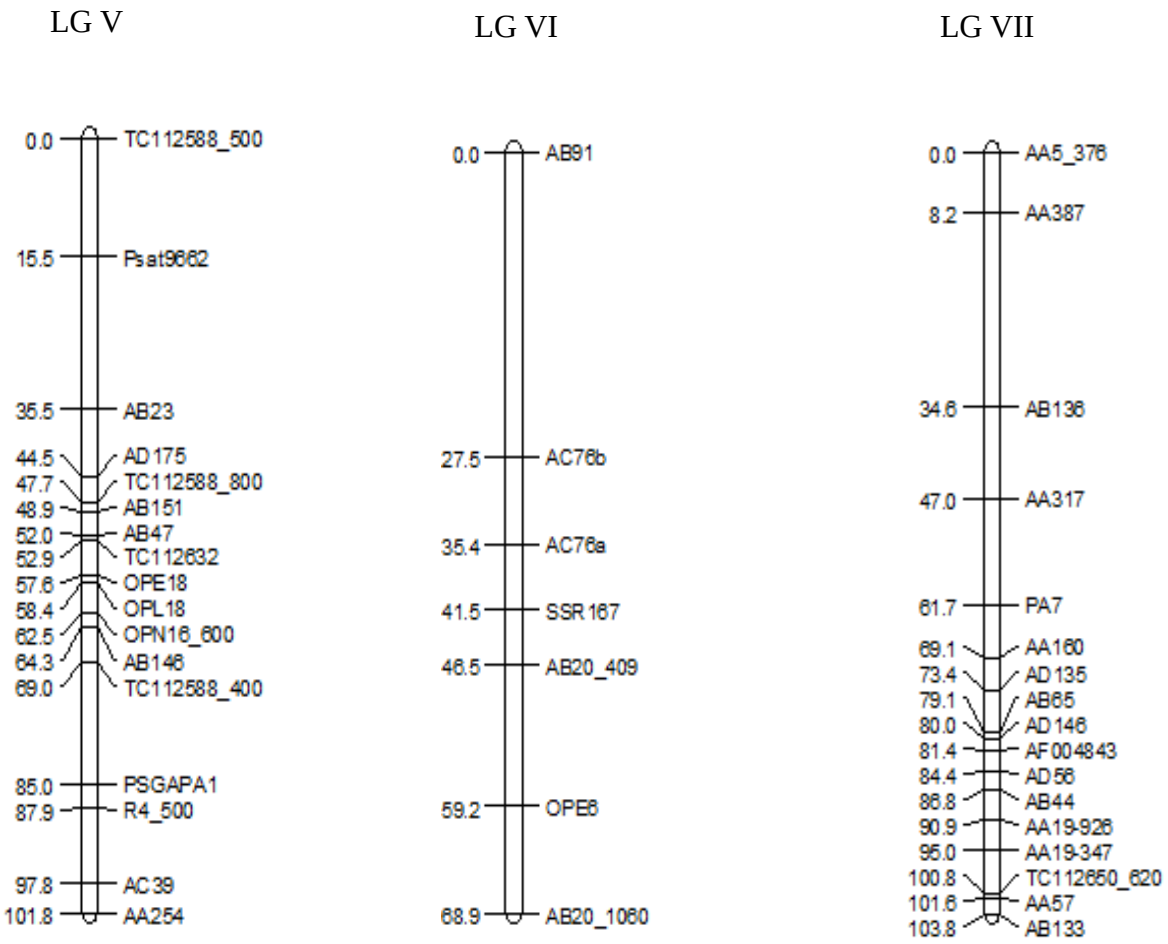

Figure 8. Linkage map of the pea population derived from a cross between Lifter and Radley. The scale represents centimorgan (Kosambi units)(continued).

Ungrouped markers

Sixteen markers remained ungrouped and showed deviation from the expected Mendelian segregation ratio of 1:1. The reason for the deviation is excessive missing data due to poor gel quality which inhibited accurate scoring.

Identification of Ascochyta blight resistance QTLs

There were 18 traits analyzed to detect QTLs. The first 6 traits came from the first repetition in the greenhouse with 385 lines scored on 5 different days. Each day represented one trait and the AUDPC based on data from the first repetition. The next 6 traits came from the second repetition with the same 5 days of measurement plus AUDPC. The last 6 traits came from a combination of the two first reps with the 5 days of measurement plus the AUDPC.

QTL analysis was performed by QTL Cartographer v. 2.5 on all linkage groups using composite interval mapping (CIM) for QTL main effects. The chromosome walk pace used was 2 cM and experiment-wise type I error rate was of 5% with 500 permutations to calculate the significance threshold. Significant peaks for QTL main effects were observed for CIM at 5 of the 9 linkage groups. The first significant peak was detected on LG I with a LOD score of 2.7 based on combined data collected 16 days post inoculation. More markers are needed for a better idea of where the peak is significant, for the identification of the QTL in that region. A QTL on LG II with a LOD score of 2.5 was detected based on data collected in the first replication 19 days post inoculation. An environmental influence could be observed in this step of the project, the second rep was planted in June, the greenhouse was warmer and the plants had different results; therefore for some data, only rep one was significant and some only rep two was significant even though statistically, they were not significant different. The QTL on LG II near marker AA205 is in the same region as a QTL detected by Prioul et al. (2003) (Figure 10). Favorable alleles for this QTL were contributed by Radley. Two significant QTL were detected on LG III. The first had a LOD score of 2.6 and was based on data collected 10 days post inoculation in the second replication. The second QTL on LG III also with a LOD score of 2.6 and based on data collected 10 days post inoculation. Marker AD57 was most

closely linked to the QTL and no QTL has been previously reported in this region (Figure 11). The linkage group IV-a showed Four significant QTL were detected on LG IV-a and were based on data collected 13 and 19 days post inoculation in the second replication and combined data for scores recorded 10 and 13 days post inoculation. The LOD scores for these four QTL were 3.2, 3.4, 2.7, and 2.5, respectively.

The interesting fact of this LG IV was a presence of two different peaks; one for the 13th day after inoculation and another peak for the 19th day after inoculation, the markers associated with the QTL on LG IV-a for data collected 13 and 19 days post inoculation were Psat61_160 and AB101, respectively. The region on LG IV-a where QTL were detected in this study has not had QTL detected previously (Figure 12). Three QTL were detected on LG VI. These QTL were based on combined data collected 10 and 13 days post inoculation and data collected 16 days post inoculation in replication 2. The LOD scores for these QTL were 5, 4.7, 2.8, respectively (Figure 13). Although significant QTL were detected on LG VI, additional markers are needed to validate the QTL. A QTL in this region near AB91 has been previously reported (Timmerman-Vaughan et al. 2002). Data collected on 10 and 13 days post inoculation resulted in detection of the most QTL. The greatest separation among the RILs for reaction to *M. pinodes* was observed on these two dates allowing a more accurate detection of QTL. Ten days post inoculation plants were still healthy while 13 dpi they started showing evidence of the first susceptible plants. All QTL identified in this study explain from 4 to 6% of the variation for Ascochyta blight resistance. The low percentage is due to the method of scoring. In retrospect it would have been better to base the scores on the whole plant including the stems and leaves.

No QTL were detected based on the AUDPC values. A possible explanation for this is that data collected 16 and 19 dpi artificially inflated the average ratio and overshadowed the

differences observed on the earlier scoring dates. A significant increase in mean disease ratings was observed between 13 and 16 dpi. One solution for it would be an environment where the Greater control of the humidity and temperature during the experiments may have allowed a more gradual increase in disease progression and allowed the AUDPC calculations to be more informative.

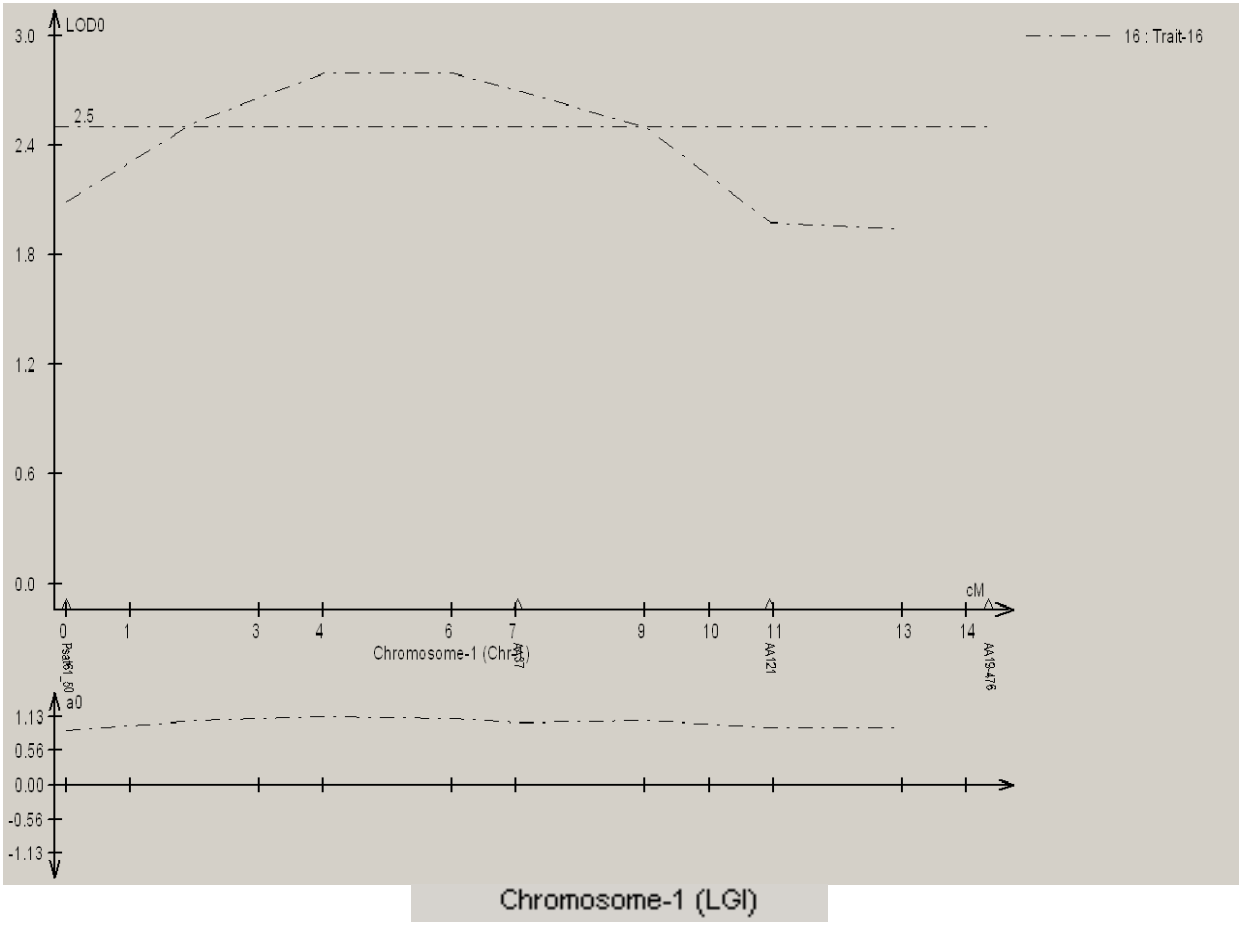

Figure 9. QTL for Ascochyta blight resistance on LG I (Chr-1 corresponds to LG I) based on data collected 16 days post inoculation.

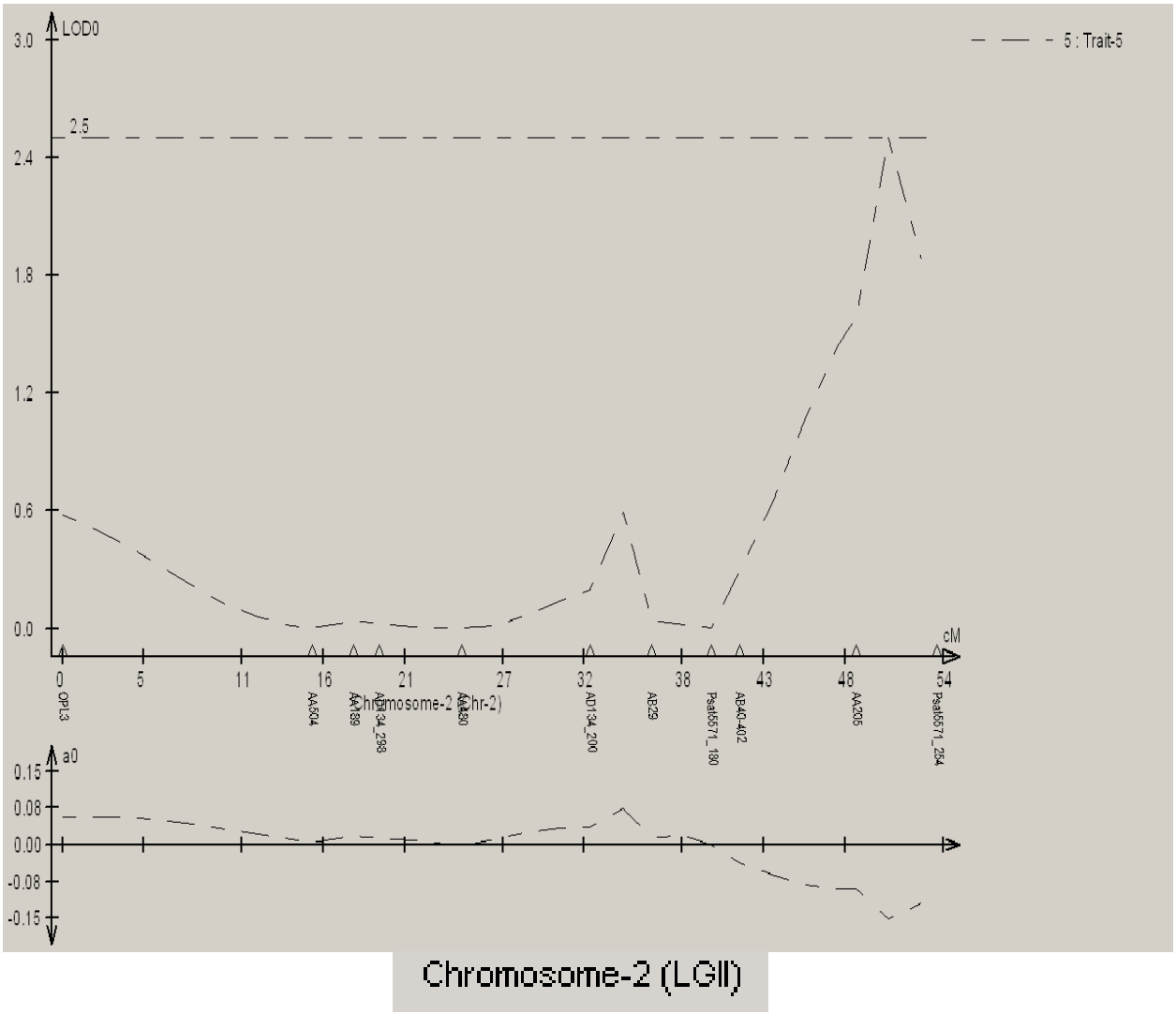

Figure 10. QTL for Ascochyta blight resistance on LG II (Chr-2 corresponds to LG II) based on data collected 19 days post inoculation.

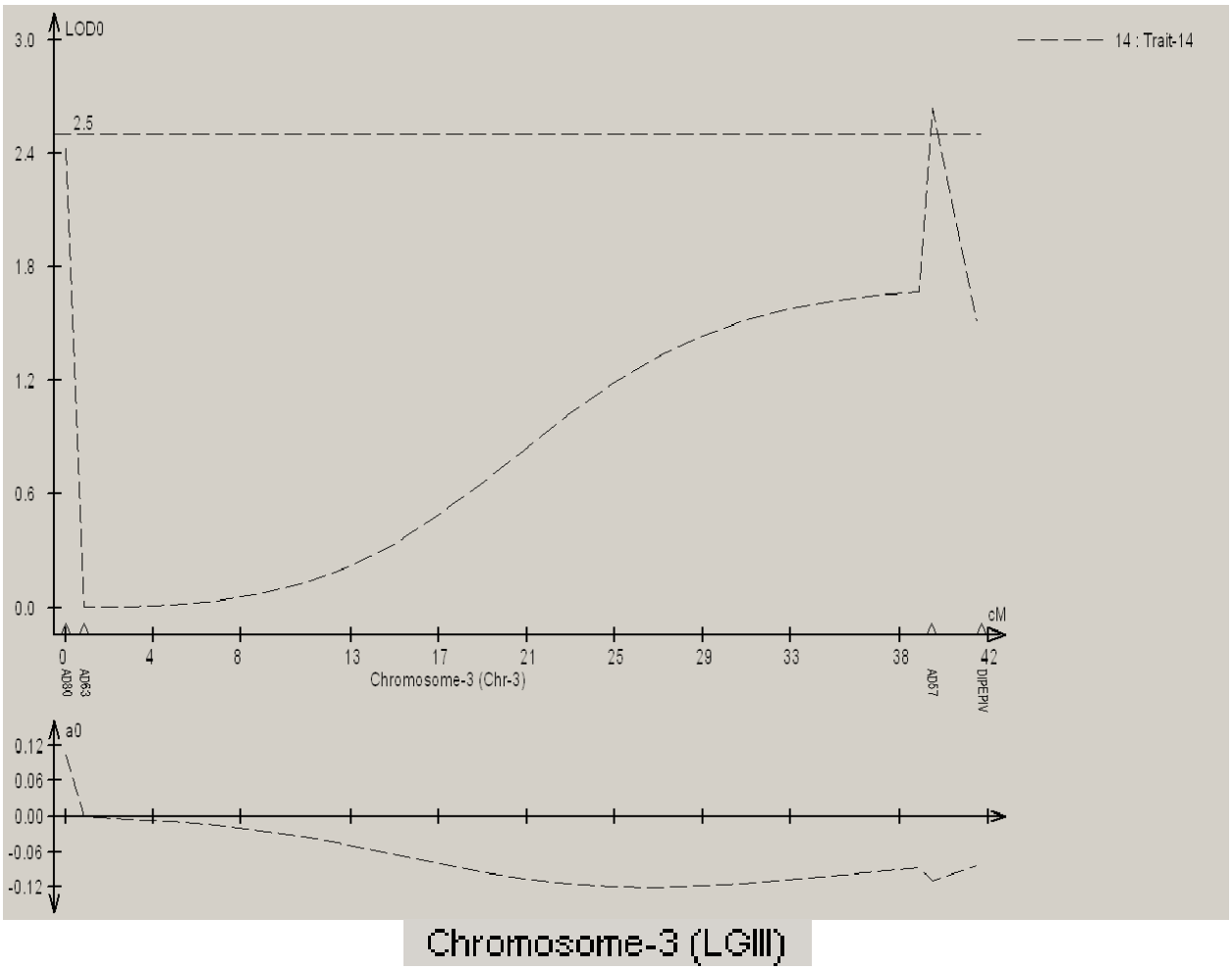

Figure 11. QTL for Asochyta blight resistance on LG III (Chr-3 corresponds to LG III) based on data collected 10 days post inoculation.

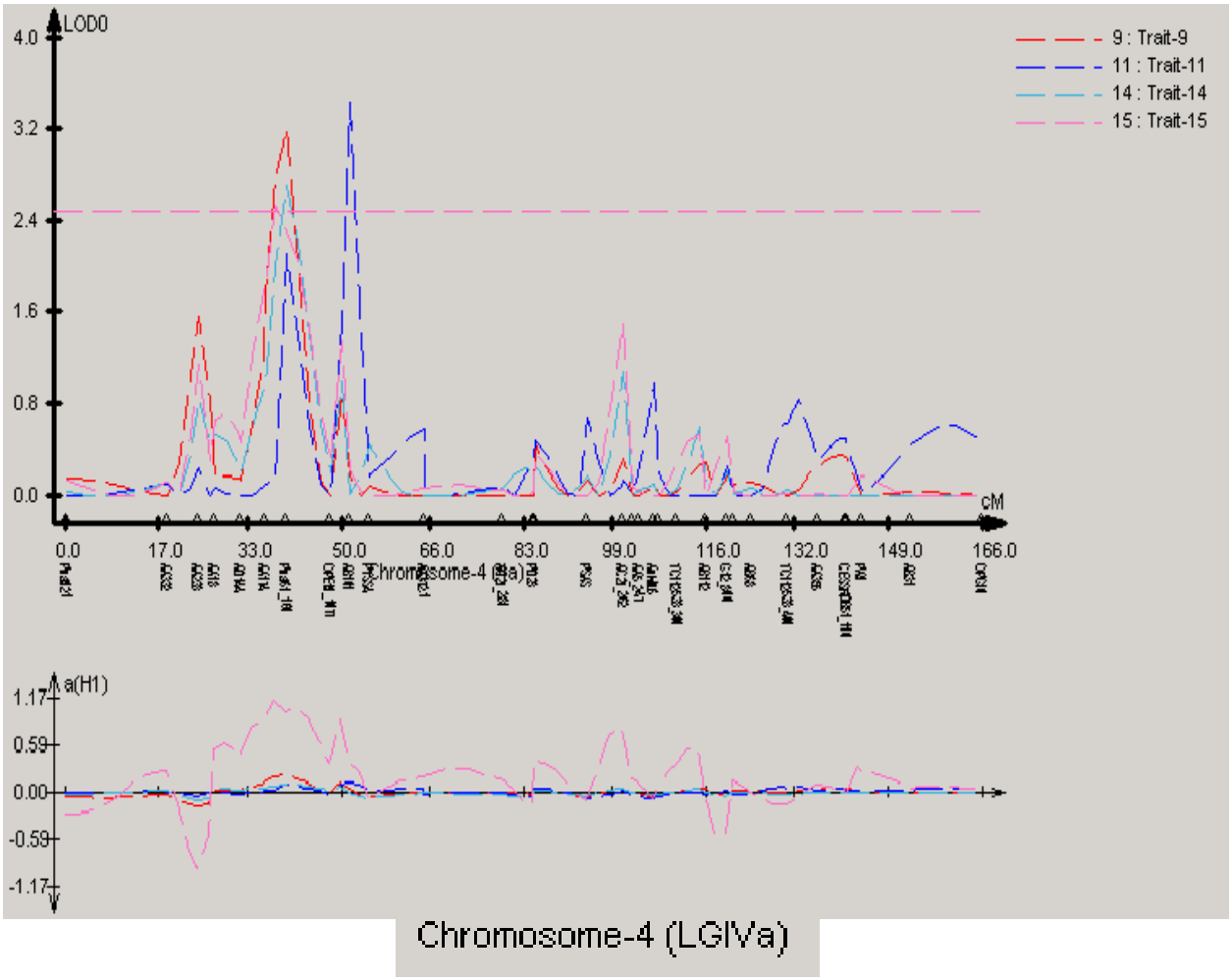

Figure 12. QTL for Ascochyta blight resistance on LG IV (Chr-4a corresponds to LG IV) based on data collected 13 and 16 days post inoculation.

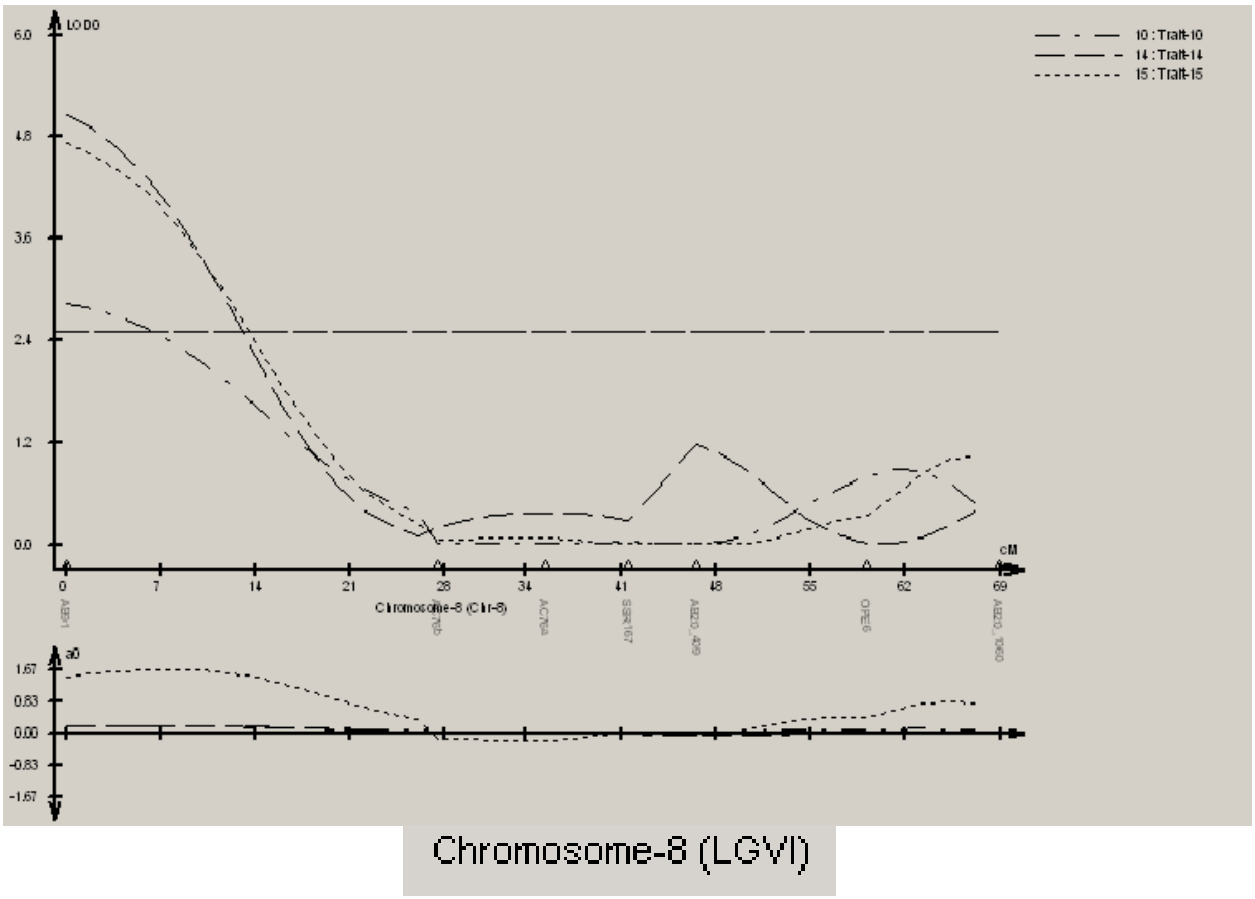

Figure 13. QTL for Ascochyta blight resistance on LG IV (Chr-8 corresponds to LG IV) based on data collected 10, 13 and 16 days post inoculation.

Table 6. Summary of QTLs detected in pea, with location, name and closet single marker or marker interval with associated LOD scores and percent of variation explained.

QTL	Population	Location	QTL identification			Marker		References
			Name	LG	Effect (%)	Name	LOD	
Resistance to Mycosphaerella blight	Carneval x MP1401	11 locations in Canada	1	II	5.0	ccta2	2.9	Ta'ran et al. 2003
			2	IV	19.1	cccc1	3.3	
			3	VI	16.8	acct1	3.1	
Resistance to Mycosphaerella pinodes	DP x JI 296	GH and 1 field	mpII-1	II	6 (field, stipule)	AD12-800	3.1	Prioul et al. 2004
			mpII-2	II	9 (field, stipule)	a	4.6	
					9 (field, stem)		4.3	
			mp-1	III	18 (CC, stipule)	E08-980	13	
					20 (CC, stipule)		13.9	
					26 (field, stipule)		12.5	
					42 (field, stem)	V03-1200	18	
			mpIII-2	III	7 (CC, stipule)	PSSGP	4.8	
					9 (CC, stems)	V03-1000	6.1	
			mpIII-3	III	6 (CC, stems)	V03-1000	4.9	
					7 (field, stipule)	PSMPSAA175	3.9	
					6 (field, stem)	F09-1900	3.2	

Table 6. Summary of QTLs detected in pea, with location, name and closet single marker or marker interval with associated LOD scores and percent of variation explained (continued).

Resistance to Mycosphaerella pinodes	DP x JI 296	GH and 1 field	mpIII-4	III	29	(field, stem)	PSMPSAA374a 6.8	
			mpIII-5	III	11	(field, stem)	PSMPSAA163.2	5.8
			mpVa-1	V	10	(CC, stipule)	PSMPSAA163.2	7.2
					8	(CC, stems)	PSMPSAA163.2	5.9
					7	(field, stems)	T14-2200	3.1
			mpVa-2	V	16	(field, stems)	G04-950	4.4
			mpVI-1	VI	15	(CC, stipules)	G04-950	9.3
					9	(CC, stems)	PSPMPSAA399	12.3
			mpVII-1	VII	5	(CC, stipules)	PSPMPSAA399	3.2
					6	(CC, stems)	PSPMPSAA399	3.3
					9	(field,stipules)	Z17-550	3.0
			mpVII-2	VII	8	(field,stipules)		4.3

Table 7. QTLs obtained in this study.

QTL	Population	Location	QTL identification			Marker	
			Trait (day)	LG	Effect (%)	Name	LOD
Resistance to *Mycosphaerella pinodes*	Lifter/Radley	Greenhouse	4	I	4	AA37	2.8
			5	II	5	Psat5571_254	2.5
			2	III	4	AD57	2.6
			2	IV	4	Psat61_160	2.7
			3	IV	4	Psat61_160	3.2
			4	IV	4	Psat61_160	2.6
			5	IV	4	AB101	3.4
			2	VI	6	AB91	5.1
			3	VI	6	AB91	4.7
			4	VI	4	AB91	2.8

QTLs found in this study correspond with previous QTLs on LG II, III, and IV described by Tar'an et al. (2003) and Prioul et al. (2004). The Differences in the percent of variation explained is explained by differences in scoring procedure, pathogen isolate and genetic population. In the present study, 9 linkage groups were identified covering 615.9 cM of the pea genome. Four of these LGs were aligned with previous pea maps, with the exception of LG I. Six QTL were identified for resistance to Mycosphaerella blight and accounted for 27 % of the total phenotypic variation.

A previous map by Tar'an et al. (2003) identified three QTLs for Mycosphaerella blight located on linkage group II, IV and VI and explained 35.9% of the phenotypic variation. The population used for this study was composed of 88 RIL and they were tested in 11 different environments. The LG presented in the map covered 1274 cM of the pea genome with an average of linkage distance between pairs of marker of 6.2 cM. The difference in coverage distance in the map could be explained by population size and marker density. Tar'an observed in his study that lodging is associated with Mycosphaerella blightresistance.

QTL for Mycosphaerella blight resistance were identified by Prioul et al. (2004) using a population of 135 RILs derived from a cross between DP (partially resistant) and JI296 (susceptible). The map in this study is based on SSR markers while the Prioul et al. (2004) map was based on 122 RAPDs, 71 SSRs and ten STSs and three morphological genes distributed over eight linkage groups and covered 1061 cM. Sixteen QTLs were identified on linkage groups: III, V, VI and VII. In the field experiment 10 QTLs were identified, although just three of them were common for both stipule and stems. The same situation happened with Xue and Warkentin (2001); in that study it was found 13 QTLs, although, only two QTLs were valid for both stems and leaves.

CONCLUSION

Lifter and Radley showed clear resistant and susceptible reaction to *Mycosphaerella pinodes* infection. Consequently, the RILS showed variation for Ascochyta blight disease reaction. Data collected at each of the five time points showed a normal distribution with the disease score shifting towards susceptibility by the fifth time point (19 dpi). A more controlled environment would be suggested for Future studies should be conducted in more precisely controlled conditions since the disease symptoms increased significantly after the third time point.

Effective sources of resistance to *Mycosphaerella pinodes* were not identified. However, at the end of the 19th day after inoculation measurement, 1 RIL showed less disease than Radley, the resistant parent. Transgressive segregation was observed; after the 19th day of inoculation, 1 line, RIL 369 had lower scores than the resistant parent and 25 others had the same score.

Although only one line was observed to lower disease than the resistant parent, the results should be viewed with caution since the experiment was conducted in the greenhouse where it may be difficult for the breeder to make selections. An important tool for the breeder is the marker-assisted selection. As demonstrated in this study, some QTLs could not be identified because closely linked markers were not present. Tightly linked markers were not available for this study; the maximum effect explained by a QTL was 6% of resistance of Ascochyta blight in the population, even though one QTL found in this study was also present in the same region in previous studies.

A genetic map for Ascochyta blight resistance in pea with 615.9 cM was generated using SSRs, RAPD and CAPS. This result is based on 115 loci covering seven linkage groups with a

mean distance of 6.2 cM and represented 40% of the previous pea map that was developed by Loridon et al. (2005).

Three significant QTLs were found on LG II, III and VI; the identified QTL on LG II was in the same region as described previous studies. The most informative data for detection of QTL was collected 10 days post inoculation. Two QTLs were identified based on data collected 10 dpi and could be due to this date corresponding with the start of disease development. Thirteen days post inoculation plants showed the impact of the environment while at 10 dpi they were still looking healthy. This study differs from previous studies due to few factors; density of the linkage map, high density linkage map would have high accuracy in detecting and mapping QTLs. The size of the population, this study used 385 RILs; with larger population more recombination will occur with consequently more chance to detect QTLs. It was reported before that for major QTLs, a small population is enough for it detection, although, for minor effects, a larger population would be necessary. Accurate phenotypic scoring directly influences QTL detection, for example, if only leaves are scored, the whole plant could be susceptible, and not detected in the leaf.

Future studies are needed for mapping and identification of QTL for Ascochyta blight resistance. Higher genotypic variation is needed in order to confirm if the QTL are really efficient. The possibilities to achieve this objective could be using different screening methods, locations and markers.

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APPENDIX A. COMBINED SETS ANOVA TABLES

Table A1. Day 1.

Type 3 Tests of Fixed Effects									
					ANOVA F				
Effect	Num DF	Den DF	F Value	Pr> F	Num DF	Den DF	Value	Pr>F (DDF)	Pr>F(infty)
Entry	395	0	.81	.	352	1	.79	.7395	.9988

Table A2. Day 2.

Type 3 Tests of Fixed Effects									
					ANOVA F				
Effect	Num DF	Den DF	F Value	Pr> F	Num DF	Den DF	Value	Pr>F (DDF)	Pr>F(infty)
Entry	395	0	.95	.	328	55.8	.93	.6663	.8299

Table A3. Day 3.

Type 3 Tests of Fixed Effects									
					ANOVA F				
Effect	Num DF	Den DF	F Value	Pr> F	Num DF	Den DF	Value	Pr>F (DDF)	Pr>F(infty)
Entry	326	0	1.35	.	298	281	1.28	0.0174	.0007

Table A4. Day 4.

Type 3 Tests of Fixed Effects									
					ANOVA F				
Effect	Num DF	Den DF	F Value	Pr> F	Num DF	Den DF	Value	Pr>F (DDF)	Pr>F(infty)
Entry	395	0	.95	.	352	1	.94	.6975	.7934

Table A5. Day 5.

Type 3 Tests of Fixed Effects									
					ANOVA F				
Effect	Num DF	Den DF	F Value	Pr> F	Num DF	Den DF	Value	Pr>F (DDF)	Pr>F(infty)
Entry	395	0	1.13	.	299	1	1.10	.6599	.1234

APPENDIX B. RESULTS

Table B1. Measurements day data.

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
Lifter	2	4	4	5	5
Radley	1	2	3	3	3
Stirling	2	3	5	5	5
1	1	3	4	4	4.5
2	1	2	2.5	3	4.5
3	1	3	4	5	5
4	1	3	4	4	5
5	3	4	4	5	5
6	1.5	2.5	3	3	4
7	1	2	2	3	4
8	1	2	2.5	3.5	4
9	1.5	2.5	3.5	3.5	4.5
10	1.5	2.5	3.5	4	4.5
11	2.5	4.5	5	5	5
12	2	3.5	4	4	5
13	1	3	3.5	4.5	5
14	1.5	3	4	4.5	4.5
15	2	4	5	5	5
16	2	3	3.5	4	4
17	2	3	4	4	5
18	1	2	3	4	4.5
19	1	3	3	4	4
20	1	2	2	3	3
21	1	2	3	4	5
22	2	3.5	3.5	4.5	5
23	2	3	3	4	4.5
24	1	3	3	4	4.5
25	1	2	3.5	4	5
26	1	2	3	4.5	5
27	1	1.5	2	3	3
28	1	1	2	2.5	4
29	1	2	3	3.5	4
30	0.505	1	2	2	3
31	1	2	2	2.5	4.5
32	1	2	2	3	4
33	1.5	3	4	5	5
34	2	3	4.5	5	5
35	2	3	3	5	5

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
37	1.5	3	3	5	5
38	0.505	1	1.5	2	3.5
39	1	2	2	3	3
40	0.505	2	2	4	4
41	1	2	3	4	5
42	1	2.5	2.5	4.5	5
43	1.5	3	3	5	4.5
44	1	2.5	3	5	5
45	0.505	1	2	2.5	3.5
46	1.5	2	3	4	4
47	2	2.5	3	3	4
48	1.5	2	2.5	3	4
49	1.5	3	3	3	4
50	2	3	3.5	4	5
51	1.5	3.5	3.5	4.5	4.5
52	2	3.5	4	5	5
53	2	2.5	4	4.5	5
54	1.5	2	3	3.5	3.5
55	2	2.5	3	4	4
56	3	4	4	5	5
57	2	3.5	4	4	5
58	2	2	3	4	5
59	2	3	4	4	5
60	2	3	4	5	5
61	2	3	3	5	5
62	2	3	3.5	5	5
63	2	3	4	4	5
64	2	2.5	3	4.5	4.5
66	2	3	3	5	5
67	2	3	4	5	5
68	2	3	3.5	4	5
69	2	2.5	3	4	4.5
70	2	3	3	4	5
71	2	2	3	3.5	4
72	2	2	3	3.5	5
73	1	2	3	4	4
74	2	3	4	4	5
75	2	2	2.5	4	5
76	2.5	3	3.5	4.5	5
77	1.5	3	3	4	5
78	2	2	3	4	4.5

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
79	2	2	3	4	3.5
80	2	2	3	4	5
81	2	2	2.5	3.5	4
82	3	3	4	4.5	5
83	1.5	2	2.5	3	3.5
84	2.5	3	3.5	3.5	5
85	2	3	4	5	5
86	2	3	4	5	5
87	3	3	4	5	5
88	2.5	3	4.5	5	5
89	2.5	3	3.5	5	5
90	2	2	3	5	5
91	3	3	4	5	5
92	3	3.5	4	4.5	5
93	2.5	3	4	4.5	4.5
94	2	3	4	5	5
95	2	3	4.5	5	5
96	1	2	2.5	4.5	4.5
97	2	2.5	3.5	4.5	4.5
98	2	3	4.5	4.5	4.5
99	2	2.5	3.5	4	5
100	2	3	4	5	5
101	2	2.5	4	5	5
102	1	2	3.5	4.5	4.5
103	2	2	3	4.5	4.5
104	2.5	3	4.5	5	5
105	2	3	4	5	5
106	2	2.5	4	5	5
107	2	2	4	5	5
108	2	3	4	4.5	4.5
109	2	3	4	5	5
110	2	3	4	4.5	5
111	2	2	3.5	4	4.5
112	1.5	2	3	4	5
113	2.5	3.5	3.5	4.5	5
114	2.5	3.5	4	4.5	5
115	2	3	3	4.5	5
116	2	2	3	4	4.5
117	2	2	3	4	4
118	2.5	3	3	4.5	5
119	2.5	3	3.5	4.5	5

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
120	2	3	4	4	4
121	2	3	3	4	4
122	2	3	3	3.5	5
123	2	2	3	3	4
124	2	2	3.5	4	5
125	1	2	3	3	4
126	2	2.5	4	4	5
127	2	2	4	4	5
128	2	2.5	4	4	5
129	2	3	3	4	4
130	1.5	2	3	3	3.5
131	1	2	3	4	4.5
132	1.5	2	3	3.5	3.5
133	2	3	3	3.5	5
134	2.5	2.5	3	4	4
135	2	3	3.5	5	4
136	2	2.5	3	4	5
137	2	2	2	4	5
138	2.5	2	3.5	4.5	4.5
139	2	2	3	3.5	4
141	1	1.5	2	3	3
142	1	2	3	3	3
143	2	2	2	3	3
144	2	2	3	3.5	4
145	1.5	2	3	3	3
146	1.5	2	3	4	4
147	1	2	3	3	3
148	1	2	3	3	3.5
149	1	2.5	3	5	5
150	2	2	3	3.5	4
151	2	2	3	4.5	5
152	2	3	4	4	5
153	2	3	3	4	4.5
154	2	3	3	3.5	4.5
155	2.5	3	3.5	4.5	4.5
156	3	3	4	5	5
157	2	2	3	3	5
158	2	2	3	4	4.5
159	1	2	2.5	4	4.5
160	2	2	3	4	4.5
161	2	2	3	4	4

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
162	2	2	4	4.5	4.5
163	2	3	4	4	5
164	2	2.5	3	3.5	4.5
165	2	2.5	3	4.5	5
166	2	2.5	3	4	4
167	2	2	2.5	3	4.5
168	2	2.5	3.5	5	5
169	2	3.5	3.5	4	5
170	2	3	3.5	4.5	5
171	2	2.5	3.5	5	5
172	1.5	3	3	5	5
173	1.5	2	2.5	3.5	4.5
174	2	2	3	5	5
175	2	3	3	4.5	5
176	1	2	3	4	4.5
177	1	2	3	4	4
178	2	3	3.5	4	4.5
179	2.5	3.5	4	5	5
180	2.5	2.5	3.5	5	5
181	2	2.5	3	3	5
182	1.5	2.5	3	4.5	5
183	2.5	3.5	3	4.5	5
184	2	3	4	5	5
185	1.5	3.5	4	5	5
186	2	4	4.5	5	5
187	2	2.5	3.5	3.5	4
188	2.5	3.5	3.5	4	5
189	2	3	3.5	4	4.5
190	3.5	3.5	4.5	4	4
191	3.5	3.5	4.5	5	5
192	2.5	3	4	5	5
193	2	3	4	4	5
194	2	3	4	5	5
195	2	2.5	3.5	5	5
196	2.5	3.5	4	5	5
197	2	2.5	3.5	5	5
198	3	3	4.5	5	5
199	2	3	4	5	5
200	2	2	3	4.5	5
201	2.5	3	3.5	5	5
202	2	3	3.5	5	5

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
203	2	2	2.5	5	5
204	2	2.5	3	4.5	4.5
205	2.5	3.5	4	5	5
206	2.5	3.5	4	5	5
207	2.5	3.5	4	5	5
208	2	3	3.5	4	5
209	3	3	3.5	5	5
210	2	2.5	4	4	5
211	2.5	2.5	4	4	5
212	2	2.5	4	4	5
213	2.5	3.5	4	5	5
214	2	3	3.5	4	5
215	2.5	3	4	4.5	5
216	2.5	3.5	4	4.5	4.5
217	2	3	4	4	4.5
218	2	2.5	3	3.5	4.5
219	2	3	2.5	4	5
220	1.5	2	3.5	4.5	5
221	2	3	4	4.5	5
222	2	3.5	4	4.5	5
223	2.5	3	4	4.5	5
224	2.5	3	3.5	4	5
225	2	1.5	2.5	3.5	4
226	2.5	3	4.5	4.5	5
227	3	4	4.5	4.5	5
228	2.5	3	4	4.5	5
229	2	4	4	5	5
230	3	3	4	4	5
231	2	4	4	5	5
232	2	3	3	4	4
233	2	4	5	5	5
234	1	3	5	5	5
235	3	4	5	5	5
236	2.5	4	5	5	5
237	3	4	5	5	5
238	3	5	5	5	5
239	3	5	5	5	5
240	3	5	5	5	5
241	4	5	5	5	5
242	1	2	4	4	4
243	1	2	4	4	4

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
244	2	3	5	4	5
245	1	2	4	3	4
246	2	3	5	4	5
247	2	3	5	4	5
248	1	2	4	4	4
249	1	2	4	3	4
250	1	2	3	3	3
251	1	2	3	3	3
252	1	2	3	3	3
253	3	3	5	5	5
254	3	4	5	5	5
255	3	4	5	4	5
256	2	3	5	4	5
257	2	3	5	4	5
258	3	3	5	4	5
259	3	4	5	5	5
260	2	3	5	5	5
261	2	3	5	5	5
262	2	2	3	3	3
263	1	2	3	3	3
264	1	2	3	3	3
265	1	2	3	3	3
266	1	2	4	3	4
267	1	2	4	3	4
268	2	3	4	4	4
269	1	2	3	3	3
270	1	2	3	3	3
271	1	2	3	3	3
272	1	1	4	3	4
273	1	2	4	4	4
274	1	2	4	3	4
275	2	3	5	4	5
276	1	2	4	4	4
277	2	3	5	5	5
278	1	3	5	5	5
279	2	3	3	4	5
280	2	3	4	4	5
281	2	2	3	5	5
282	2	2	2	4	5
283	2	2	2	4	5
284	2	2	3	4	5

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
285	2	2	4	4	5
286	2	3	4	4	4
287	2	2	3	5	5
288	2	3	3	4	5
289	2	3	4	5	5
290	2	3	4	5	5
291	2	3	3	5	5
292	2	2	2	4	5
293	2	2	3	4	5
294	2	2	3	3	5
295	2	2	3	4	4
296	2	3	3	4	5
297	2	2	3	4	5
298	2	2	2	3	4
299	2	3	3	4	5
300	2	3	3	5	5
301	2	3	4	5	5
302	2	2	3	4	5
303	2	2	3	5	5
304	1.5	2	3	4	4
305	2	2	3	4	4
306	2	3	4	4	5
307	2	2	4	4	5
308	2	3	4	4	5
309	2	3	5	4	5
310	2	3	4	5	5
311	1	3	4	4	5
312	1	3	4	4	4
313	2	3	4	4	4
314	1	3	3	4	4
315	2	3	4	4	4
316	1	2	3	3	4
317	1	3	3	3	4
318	1	3	4	4	5
319	2	2	3	3	4
320	1	2	3	3	4
321	2	3	4	4	4
322	2	2	3	4	4
323	1	2	3	4	5
324	1	3	3	4	4
325	1	2	2	4	4

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
326	2	3	4	5	5
327	2	3	4	4	4
328	2	4	5	5	5
329	2	3	5	5	5
330	2	3	4	4	4
331	2	3	4	4	5
333	2	3	4	4	4
334	2	3	4	4	4
335	1	2	3	4	5
336	2	3	3	5	5
337	2	2	4	5	5
338	2	3	3	5	5
339	2	3	4	5	5
340	2	3	3	5	5
341	2	2	3	5	5
342	2	2	3	5	5
343	2	3	4	4	5
344	2	3	3	4	4
345	2	2	3	4	4
346	2	3	4	4.5	5
347	1.5	3	4	5	5
348	2	3	4	4	4
349	1.5	4	4	5	5
350	2.5	4	5	5	5
351	2	3	4	4.5	5
352	3	3	4	4	4
353	2	3	4	4	4
354	1	2	2.5	3.5	4
355	2	3	4	5	5
356	2	4	5	5	5
357	1	3	4	4.5	5
358	1.5	3	4	4	5
359	1.5	2	2.5	3.5	4.5
360	1.5	2	3	4	5
361	1	3	3	4	5
362	1	3	3	4	4
363	1	2	2.5	3	5
364	1	2	2	3	3
365	1.5	3	4	4	5
366	1.5	3	3	4.5	5
367	1.5	3	4	4	4

Table B1. Measurements day data (continued).

RIL/Parent	Day 1	Day 2	Day 3	Day 4	Day 5
368	1.5	2	3	3.5	3
369	1	2	2.5	2.5	2.5
370	1	2	3	3.5	3.5
371	1.5	2	3	3.5	4
372	1	2	2	3.5	4.5
373	1.5	3	3	3	5
374	2	3	4	4	5
375	2	3	4	5	5
376	2	4	5	5	5
377	2	4	5	5	5
378	1	3	4	5	5
379	1	3.5	4	5	5
380	1	3	3	4	4
381	1	3	3	5	5
382	1	3	3	5	5
383	2	3.5	4	5	5
384	2	3	3	5	5
385	1	3	3	4	5
386	1	3	3	3	3.5
387	1	2	2	3	3
388	1	2	2	3	3
389	1	3	3	3.5	4.5
390	0.505	1.5	2	2.5	3
391	1	1.5	1.5	2.5	4
392	0.505	1.5	2	2.5	3.5
393	2	3	4	5	5
395	2	3	4	4	5
396	2	3	4	4.5	5
397	2	2	3	4	4

Table B2. AUDPC data.

RIL/Parent	MEAN
Radley	42.91
Lifter	68.27
Stirling	73.55
1	54.55
2	46.40
3	63.25
4	54.05
5	81.65
6	50.90
7	45.31
8	42.05
9	60.40
10	61.95
11	76.45
12	64.70
13	57.20
14	56.80
15	75.80
16	60.75
17	59.35
18	49.90
19	50.30
20	38.15
21	45.65
22	74.28
23	62.50
24	54.35
25	51.70
26	54.95
27	34.80
28	36.25
29	39.05
30	24.60
31	39.05
32	41.85
33	61.70
34	66.50
35	59.70
36	69.25
37	58.55

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
38	26.50
39	40.60
40	36.65
41	47.65
42	53.50
43	59.15
44	58.45
45	32.85
46	46.70
47	53.15
48	44.35
49	51.75
50	64.35
51	61.45
52	65.85
53	61.45
54	47.10
55	56.55
56	78.45
57	72.85
58	59.80
59	66.85
60	73.70
61	69.20
62	74.70
63	63.30
64	51.95
66	67.99
67	71.30
68	63.15
69	52.95
70	68.65
71	45.95
72	52.80
73	47.40
74	65.04
75	54.30
76	71.25
77	58.95
78	52.70
79	58.55

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
81	49.00
82	72.70
83	41.60
84	70.65
85	70.15
86	74.20
87	79.40
88	75.60
89	71.75
90	60.93
91	79.40
92	73.00
93	69.45
94	71.98
95	72.80
96	44.15
97	67.55
98	63.75
99	63.30
100	69.80
101	62.00
102	51.85
103	60.00
104	76.80
105	69.55
106	65.25
107	66.80
108	69.40
109	71.25
110	67.20
111	59.75
112	53.30
113	70.35
114	73.25
115	67.45
116	59.15
117	58.20
118	68.35
119	72.25
120	58.91
121	60.49

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
123	49.20
124	58.40
125	49.40
126	58.80
127	60.05
128	62.90
129	58.99
130	49.65
131	52.70
132	48.00
133	55.55
134	63.25
135	64.50
136	60.70
137	65.50
138	62.05
139	53.80
141	39.90
142	43.65
143	48.80
144	53.85
145	49.35
146	52.15
147	44.20
148	40.70
149	56.60
150	56.75
151	59.30
152	61.90
153	56.96
154	55.95
155	57.85
156	55.56
157	66.80
158	56.45
159	42.85
160	54.40
161	57.35
162	63.50
163	63.65
164	56.60

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
166	57.90
167	47.45
168	69.75
169	63.70
170	66.45
171	67.65
172	61.65
173	52.45
174	60.40
175	62.05
176	51.65
177	49.78
178	66.20
179	72.20
180	65.75
181	59.15
182	57.00
183	66.75
184	64.55
185	66.65
186	77.90
187	63.40
188	70.90
189	66.90
190	79.25
191	84.80
192	74.50
193	60.10
194	69.45
195	71.30
196	78.70
197	68.90
198	80.20
199	63.55
200	65.15
201	68.30
202	68.30
203	56.50
204	56.90
205	70.10
206	71.40

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
208	68.10
209	73.83
210	64.40
211	66.80
212	64.25
213	75.45
214	63.95
215	73.80
216	74.05
217	64.45
218	58.60
219	56.10
220	56.70
221	60.10
222	67.40
223	71.65
224	63.45
225	57.75
226	73.45
227	81.95
228	73.60
229	70.25
230	75.00
231	66.30
232	59.70
233	66.00
234	58.50
235	83.10
236	75.75
237	85.50
238	87.30
239	86.70
240	90.90
241	100.50
242	48.60
243	44.70
244	58.20
245	44.40
246	58.20
247	63.90
248	42.90

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
250	44.40
251	40.20
252	39.30
253	81.90
254	84.30
255	70.50
256	61.50
257	64.50
258	73.20
259	83.70
260	69.00
261	65.70
262	54.30
263	40.80
264	38.40
265	40.50
266	39.00
267	41.40
268	57.90
269	39.30
270	38.40
271	39.90
272	39.60
273	46.80
274	42.90
275	56.10
276	49.20
277	66.30
278	53.10
279	60.30
280	63.30
281	62.10
282	57.30
283	56.40
284	60.90
285	60.30
286	62.10
287	61.80
288	62.10
289	60.90
290	66.90

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
292	53.10
293	58.50
294	54.30
295	54.00
296	62.70
297	57.90
298	52.20
299	62.70
300	64.50
301	65.10
302	56.10
303	61.50
304	53.63
305	55.80
306	63.90
307	60.30
308	64.20
309	64.20
310	62.70
311	49.80
312	52.80
313	68.40
314	53.10
315	63.30
316	42.00
317	49.20
318	50.10
319	51.00
320	41.40
321	58.80
322	54.90
323	50.10
324	48.60
325	42.00
326	66.60
327	64.20
328	70.80
329	68.10
330	61.20
331	67.50
333	64.80

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
335	50.10
336	57.60
337	60.60
338	63.90
339	67.50
340	65.10
341	59.40
342	57.00
343	62.40
344	54.90
345	53.70
346	63.40
347	62.40
348	58.84
349	65.75
350	81.05
351	63.45
352	74.65
353	59.70
354	45.55
355	66.00
356	70.70
357	57.65
358	65.00
359	49.15
360	55.55
361	49.15
362	47.90
363	43.95
364	40.25
365	60.05
366	60.35
367	57.30
368	49.25
369	34.60
370	47.50
371	48.95
372	46.20
373	53.45
374	66.05
375	67.99

Table B2. AUDPC data (continued).

RIL/Parent	MEAN
377	73.65
378	59.68
379	62.60
380	52.25
381	55.35
382	52.35
383	71.00
384	58.50
385	49.20
386	46.75
387	44.45
388	36.50
389	51.70
390	27.55
391	30.80
392	29.30
393	65.90
395	62.20
396	65.40
397	53.80