PEA AND POWDERY MILDEW: GENETICS OF HOST-PATHOGEN INTERACTION AND IDENTIFICATION OF MOLECULAR MARKERS FOR HOST RESISTANCE

A Thesis Submitted to the Faculty of Graduate Studies,

The University of Manitoba,

by

Khusi Ram Tiwari

In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Graduate Genetics Program

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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KHUSI RAM TIWARI

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

GENERAL ABSTRACT

Tiwari, Khusi Ram Ph.D., The University of Manitoba, June 1998. Pea and Powdery Mildew: Genetics of Host-Pathogen Interaction and Identification of Molecular Markers for Host Resistance.

Pea (*Pisum sativum* L.) suffers significant yield and quality losses because of infection by the parasitic fungus *Erysiphe pisi* Syd., the causal agent of powdery mildew. Resistant cultivars and lines were intercrossed and crossed with susceptible lines to determine the genetic basis of resistance. A high level of resistance in most of the resistant lines, including field pea cultivars grown in Canada (Highlight, AC Tamor and Tara), was conferred by *er-1;* resistance in JI 2480 was conferred by *er-2*. Variability in virulence was examined in naturally occurring populations of *E. pisi* in western Canada and NW USA. Thirty-one single colony isolates were tested on a set of 14 different pea lines, using a detached leaf assay. A low level of variability among the isolates was evident. Ten of the 14 pea lines were evaluated for powdery mildew reaction in Canada, NE USA, SW USA, NW USA, UK and Nepal. Reaction in Nepal differed from that observed in other locations for three of the ten lines. The cultivars/lines Highlight, JI 2480, JI 1559, JI 210, JI 82, Radley and JI 1758 were suggested for use as differential lines for future studies. In a study of winter survival strategies of *E. pisi* in Manitoba, cleistothecia from infected leaves and stems were examined microscopically on a periodic basis throughout the winter of 1996/97. Most ascospores were degraded by spring under field conditions.

In a seed- transmission study, where seeds from severely infected plants were sown in a greenhouse in 1996 and 1997, none of the 4200 plants examined was infected with powdery mildew. Powdery mildew inoculua from other plant species found in the vicinity of pea fields did not infect pea. As molecular markers are useful in gene pyramiding and marker-assisted selection, three random amplified polymorphic DNA (RAPD) markers, OPO-18, OPE-16 and OPL-6 were identified as linked to *er-1* by screening progenies of the cross Highlight/ Radley (susceptible cultivar), using bulked segregant analysis. Five amplified fragment length polymorphism (AFLP) markers linked to *er-2* were identified by screening progenies of the cross JI 2480/Radley using bulked segregant analysis.

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DEDICATION

This thesis is dedicated to my parents

Mr. Nanda Prasad Tiwari

and

Mrs. Shanta Devi Tiwari

FOREWORD

This thesis is written in a paper format. The results are presented in the form of 5 papers. The first paper (Chapter 3) was published in the Canadian Journal of Plant Science, 77: 307-310 (1997). The second paper (Chapter 4) was published in the Canadian Journal of Plant Pathology, 19: 267-271 (1997). The third paper (Chapter 6) is in press in Genome (1998). The fourth paper (Chapter 5) is submitted to the Canadian Journal of Plant Pathology. The fifth paper (Chapter 7) will be submitted to Molecular Breeding. A general introduction and literature review precede the papers. This is followed by a general discussion and conclusion, literature cited and an appendix.

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LIST OF ABBREVIATIONS

AFLP Amplified Fragment Length Polymorphism

ASAP Allele Specific Associated Primer

bp Base pairs

BSA Bulked Segregant Analysis

DNA Deoxyribose Nucleic Acid

dNTP Deoxynucleoside Triphosphate

EDTA Ethylenediamineteteracetic Acid

HVR Hyper Variable Repeats

JI John Innes

JII John Innes Institute

MAS Marker Assisted Selection

NIL Near Isogenic Lines

PCR Polymerase Chain Reaction

RAPD Random Amplified Polymorphic DNA

RFLP Restriction Fragment Length Polymorphism

RNA Ribose Nucleic Acid

SCAR Sequence Characterized Amplified Region

VNTR Variable Number of Tandem Repeats

SSR Simple Sequence Repeat

STR Short Tandem Repeat

TE Tris/EDTA

CHAPTER 1

GENERAL INTRODUCTION

Pea (*Pisum sativum* L.) occupies a central place in the history of genetics as the experimental organism that Mendel used in formulating his celebrated principles of inheritance (Mendel 1866). Pea has continued as an object of genetic study and remains one of the best genetically characterized plant species (Marx 1977). It also occupies a prominent place in the history of agriculture as one of the founder crops of Old World Neolithic agriculture (Zohary and Hopf 1973) and is still one of the most important seed legumes throughout the world (Davies 1993).

Pea was first domesticated in the Mediterranean/Near East area about 8000-9000 years ago (Gritton 1980). The two major types of peas currently in cultivation are the garden pea, harvested when the seeds are immature and used for human consumption (canning, freezing), and the field pea harvested when the seeds are mature and used primarily for feeding livestock (Gritton 1986, Gane 1985). Other uses include dry edible pea, forage and green manure. The pea is a member of the diverse group of cultivated legumes (pulses) belonging to the order *Fabales*, family *Leguminosae (Fabaceae)* and tribe *Viciae* (Marx 1984, Smartt 1990). *Vicia* and *Lathyrus* are the genera that are most closely allied to *Pisum* (Marx 1977). Pea plants fix atmospheric nitrogen, through a symbiotic association with *Rhizobium leguminosarum* (Askin et al. 1985).

The protein content of pea varies from 26% to 33% for wrinkled-seeded and from 23% to 31% for smooth-seeded cultivars (Cousin et al. 1985). The protein content of Canadian field pea cultivars (smooth-seeded) ranges from 27% to 29% (Warkentin et al.

1997). The relatively low protein content in smooth-seeded cultivars is due to a relatively high starch content with a greater proportion of amylopectin. The digestibility of the protein supplied from pea is similar to that of soybean (*Glycine max* L.) meal protein (Marquardt and Bell 1988).

France is the single largest producer of field pea in the world followed by the Ukraine and Canada (Food and Agriculture Organization 1997). France and Canada are the world's largest exporters. Over 80% of Canadian field pea production is exported to about 20 countries in Europe, South America and Asia (Ali-Khan and Zimmer 1989). In Canada, Saskatchewan is the leading producer followed by Alberta and Manitoba and these provinces produce virtually all of the Canadian dry pea (Statistics Canada 1996). Although, a ten-fold increase occurred in field pea cultivation in western Canada in the last ten years, mean yield has not increased (Statistics Canada 1997). Several biotic and abiotic stresses may be the major reason.

Among biotic stresses, diseases are the most important limiting factors in pea production (Bernier et al. 1988, Nene 1988, Ali et al. 1994). Pea is subject to a number of diseases that can reduce yield and quality. Infection can arise from a variety of sources such as seed-borne, air-borne, soil-borne and residue-borne inocula, and from fungal, bacterial and viral pathogens (Simmonds 1979, Hagedorn 1984). *Ascochyta* blight (*Mycosphaerella pinodes*), powdery mildew (*Erysiphe pisi* Syd.), root rot and wilt (*Fusarium spp.*), *Sclerotinia* rot, and bacterial blight (*Pseudomonas pisi*) are economically important diseases in western Canada (Ali-Khan and Zimmer 1989). Genetic resistance is available for many of these diseases (Bernier et al. 1988, Ali et al.

1994) and commercial productions requires cultivars having combined resistance not only to more than one disease, but also to other biotic and abiotic stress factors (Nene 1988). Breeding for disease resistance is one of the major objectives of pea breeding programs in Canada (Tom Warkentin, personal communication 1997).

E. pisi, an obligate fungal parasite which causes powdery mildew of pea, is as widely distributed as the crop (Dixon 1978). It may become severe on field pea crops that mature in late summer. The disease first appears as small diffuse white spots on the upper surface of older leaves (Reiling 1984) and spreads rapidly, giving the leaf a white powdery appearance. Severe infections may result in a 25% to 30% yield reductions (Munjal et al. 1963, Reiling 1984). Studies in Manitoba indicate that the disease usually first appears around July 17-21 (Ali-Khan and Zimmer 1989). Although a number of races have been reported in cereal powdery mildews (Jorgensen 1994), pathogenic variation in *E. pisi* has not been determined. Also, it is not known whether the fungus overwinters in the severe cold winters of western Canada as cleistothecia, in alternate hosts, or is carried in every year through air currents from the USA.

Application of sulphur or sulphur-containing fungicides controls the disease (Bent 1978, Warkentin et al. 1996a). However, application of fungicides is relatively costly and environmentally unfriendly. Genetic resistance is the most economical and desirable means of disease control (Hagedorn 1985, Bernier et al. 1988). Although most pea cultivars grown in western Canada are susceptible to powdery mildew, genetic resistance is available in the cultivars Highlight, AC Tamor and Tara (Warkentin et al.

1996a). However, the identity of their resistance genes and whether they are allelic or not is not known.

Once resistance genes have been identified, the development of molecular markers for resistance genes will facilitate selection. Advances in molecular biology have focussed the attention of plant breeders on DNA markers, such as restriction fragment length polymorphisms (RFLP) (Botstein et al. 1980, Tanskley et al. 1989) and random amplified polymorphic DNA (RAPD) (Williams et al. 1990). RAPD analysis has been successfully used to identify markers for disease resistance genes (for example, Michelmore et al. 1991, Penner et al. 1993a, 1993c) and other agronomic traits (for example, Chalmers et al. 1993, Marshall et al. 1994, Lehner et al. 1995). Recently, a new DNA analysis technique called amplified fragment length polymorphism (AFLP) has been developed which combines the desirable aspects of both RFLP and RAPD. AFLP is based on the selective amplification of a subset of genomic restriction fragments using PCR (Zabeau and Vos 1993, Vos et al. 1995).

Molecular markers are useful tools for marker-assisted selection (MAS) in plant breeding. Development of closely linked molecular markers for powdery mildew resistance genes would facilitate the incorporation of resistance genes in agronomically superior pea cultivars. Therefore, the objectives of this study were to:

1. Elucidate the inheritance of powdery mildew resistance in field pea germplasm grown in Canada,
2. Determine the level of pathogenic variation in *E. pisi*,
3. Study the winter survival strategy of *E. pisi* in Manitoba,

4. Identify RAPD markers for the powdery mildew resistance gene *er-1*, and

5. Identify AFLP markers for the powdery mildew resistance gene *er-2*.

CHAPTER 2

LITERATURE REVIEW

2.1 The genus *Pisum:* Evolution and domestication

Pisum as a wild plant is found in two distinctive growth forms, the vigorous climbing scrambler *P. elatius* Bieb. and the lower growing, less rampant form *P. humile* Boiss. and Noe. (Ben-Ze'ev and Zohary 1973). The wild populations with the closest affinity to the cultigen were the northern Israeli populations of *P. humile* (Ben-Ze'ev and Zohary 1973). The distribution of *P. humile* is confined to the eastern Mediterranean, Turkey and the Fertile Crescent which probably is the area in which domestication occurred (Smartt 1990). This conclusion is supported by the available archaeological evidence: the most ancient finds of pea in archaeological sites are in precisely this area, dating back to 7000-6000 BC. The remains of carbonised seed have been obtained from Jarmo (North Iraq), Cayanu (south-east Turkey) and Jericho (Israel) (Zohary and Hopf 1973). Vavilov (1926) suggested central Asia, the Near East, Abyssinia and the Mediterranean as centres of origin based on genetic diversity. Pea was a companion crop of wheat (*Triticum spp.*) and barley (*Hordeum spp.*) when agriculture began in the Near East (Zohary and Hopf 1973). Wild pea was characterized by rough and granular seed surfaces. The characters of wild pea which created the greatest difficulty in cultivation were seed dormancy and explosive pod dehiscence (Smartt 1990). White flowers and wrinkled seeds may be considered as advanced traits (Marx 1977).

Pisum consists of a broad range of morphologically distinct types spread worldwide, many of which are described as separate species. The wild populations from which the domesticates probably arose were initially described as species in their own right, *P. elatius* Bieb., *P. humile* Boiss & Noe (syn. *P. pumilio* Meikle), *P. syriacum* (Berger Lehm) and *P. fulvum* (Smartt 1990). A distinctive Ethiopian form was recognized as a separate species, *P. abyssinicum* A.Br. The European forms were clearly differentiated into the garden pea (*P. hortens*) and the field pea (*P. arvense*) (Smartt 1990).

A valuable cytogenetic and hybridization study was undertaken by Ben-Ze'ev and Zohary (1973) on the genus *Pisum* to clarify species relationships. *P. elatius*, *P. humile* and *P. sativum* were inter-fertile with normal chromosome complements. Wild *humile* forms had chromosomes that were identical with the standard karyotype of *P. sativum* and are likely the immediate progenitor of the modern pea (*P. sativum*). *P. fulvum* was chromosomally distinct and limited crosses were successful only when *P. fulvum* was used as the male parent (Ben-Ze'ev and Zohary 1973). Palmer et al. (1985) studied the chloroplast DNA variation and evolution in *Pisum* and concluded that *P. fulvum* was quite distinct compared to other *Pisum* taxa and northern *P. humile* was the closest relative of modern *P. sativum*. Similarly, Hoey et al. (1996) conducted phylogenetic analysis of *Pisum* based on morphological characters, allozyme and RAPD markers and supported the findings of Ben-Ze'ev and Zohary (1973). Recent classification systems in pea recognize two main species, 1) *P. sativum* (includes *P. arvense*, *P. elatius* and *P. humile*), and 2) *P. fulvum* (Davis 1970, Ben-Ze'ev and Zohary 1973, Waines 1975,

Palmer et al. 1985. Smartt 1990. Hoey et al. 1996). The pea was called *Pisos* by the Greeks and *Pisum* by the Romans. When the plant was passed on to the English. it became "Peason". then "pease" or "peasse", and finally "pea" (Marx 1977).

2.2 The pea plant

The pea is a cool season. herbaceous. trailing. self-pollinating annual plant (Smartt 1990). The inflorescence is a raceme. germination is hypogeal and the tap root produces a profusion of lateral roots. Stems are slender. angular. glaucous and upright in growth. Two rudimentary leaves are formed immediately above the cotyledonary node. Leaves are alternate and large stipules clasp the stem (Gritton 1986). One to many nodes may bear flowers. The peduncle arises from the axil of the leaf between the stem and petiole. Under field conditions. the number of vegetative nodes before the first inflorescence may vary from four for the earliest to about 25 for the latest (Gritton 1986).

Pea flowers are bilaterally symmetric. Five petals comprise the corolla: a single large banner or standard is flanked by two wings and a keel composed of two fused petals. The pistil is a single carpel. with a style and stigma extending at an angle from its apex. Nine anthers are fused to form a staminal tube that surrounds the pistil. and the tenth stamen is free (Gritton 1986). The number of flowers varies greatly. depending upon the genotype and the environment. Anthesis begins at the lowest floral node and proceeds upward.

2.2.1 Genetics

The modern science of genetics originated when Gregor Mendel's discoveries were brought to light in which he used peas as the experimental organism with seven contrasting characteristics (Mendel 1866). Since then, genetic knowledge has accumulated to extend to Mendelian and non-Mendelian modes of inheritance. Nearly 2000 mutants of *Pisum* have been reported (Marx 1977). Some of the mutants controlled by major genes include foliage mutants (*st, tl, af*); internode length mutants (*le, la, cry*); pigmentation mutants (*a, d*); wax mutants (*wlo, wsp, wel, wp*) and chlorophyll mutants (*alt, py, o*). Linkage maps have been constructed using several recombinant inbred populations, spanning approximately 1700 cM (Ellis et al. 1992, Hall et al. 1997a). The powdery mildew resistance gene *er* (*er-1*) has been placed in linkage group 6; the closest morphological marker is *Gty* (Timmerman et al. 1994, Weeden et al. 1994, Hall et al. 1997b).

Pea has a chromosome number of 2n=2x=14 (Ben-Ze'ev and Zohary 1973, Hall et al. 1997a). Hall et al. (1997a) reported that, of the seven chromosome pairs, five are identifiable on the basis of length and position of the centromere; the other two smaller chromosome pairs (1 and 2) were indistinguishable. Colchicine has been successfully used to induce autotetraploids in pea by seed and seedling treatment with a 0.1 to 0.4% solution (Kaloo 1993). Autotetraploids induced through colchicine treatment had a 11.1 to 21.7% increase in seed protein content compared with the corresponding diploids (Mercykutty et al. 1990). Palmer et al (1985) reported that chloroplast DNA evolved very slowly relative to nuclear and mitochondrial DNAs. Pea has a haploid genome content

of 4.6 picograms (pg) of DNA or 4.5 x 10^9 nucleotide pairs and the majority (85%) of it contains repetitive sequences (Murray et al. 1978).

The bulk of genetic resources available for the improvement of pea reside within the biological species itself. The range of variation found within the cultivated species and wild forms constitutes a notable genetic resource (Smartt 1990). The ease with which all forms of peas can be intercrossed allows plant breeders ready access to the large range of variation that exists in the wild, primitive and cultivated forms. However, *P. fulvum*, *P. humile* and *P. abyssinicum* have numerous undesirable genes which are dominant, and, thus, have limited potential use for breeding purposes (Kaloo 1993). Substantial collections of pea germplasm are held at many centres, the largest being in Italy, Syria, Poland, UK, Sweden, USA, Germany and India (Davies 1993). The work of Gritton and Wierzbicka (1975) suggested the presence of an extensive tertiary gene pool, comprising at least part of the related genera *Vicia* and *Lathyrus*.

2.2.2 Breeding

The pea hybridization procedure is explained in detail by Gritton (1980, 1986). The stigma is receptive a few days before anthesis until one day or more after the flower wilts (Wornock and Hagedorn 1954). Pollen is shed and fertilization occurs about one day before the flowers open (Marx 1977). Pollen on the stigma germinates in about 8-12 hours and fertilization occurs 24-28 hours after pollination (Gritton and Weirzbicka 1975). Layne and Hagedorn (1963) reported that untreated pollen could be stored for up to six days after dehiscence, whereas vacuum drying and storage at -25° C extended

viability to one year. The ovary bears up to 13 ovules alternatively attached to the fused placentas (Gritton 1986).

Thomas Andrew Knight, who hybridized pea as early as 1787, introduced a number of improved cultivars with wrinkled seed (Marx 1977). Exploitation of hybrid vigour may be possible for various traits. Gritton (1975) reported heterosis in plant height, pods per plant, seeds per pod, seeds per plant, seed weight and seed yield per plant. Yield heterosis of F_1 hybrids, based on the mid-parent, averaged 55%. Intergeneric hybrids involving pea have so far been unsuccessful (Gritton 1969, Gritton and Wierzbicka 1975). The extent of natural outcrossing has been estimated as 0% in New York (Yarnell 1962) and up to 60% in Peru (Harland 1948) due to insects. Outcrossing was less than 1% with commercial pea cultivars in the USA (Gritton 1986).

Breeding for disease resistance is one of the most important aspects of many pea breeding programs. Resistance against fungal and viral diseases and insects (Hagedorn 1985), as well as resistance against environmental stresses (Jackson 1985), are important. Among fungal diseases, *Ascochyta* blight, caused by *Mycosphaerella pinodes*, is the most important in North America and efforts are underway to identify and incorporate sources of resistance in adapted cultivars (Porta-Puglia et al. 1994, Warkentin et al. 1996b). Breeding efforts are also underway to incorporate powdery mildew resistance genes into new cultivars (Warkentin et al. 1996a).

Hybridization among cultivars, landraces and primitive forms, followed by pedigree, bulk or backcross methods of selection, have been traditionally used in pea breeding (Gritton 1986, Davies 1993). These procedures provide new combinations of

genes that can lead to progeny superior to either parent. More recently, the single seed descent method, as proposed by Brim (1966) in soybean has been used (Davies 1993). The backcross procedure is well suited to maintain the unique constitution of an adapted cultivar while incorporating one or a few simply inherited improvements (Gritton 1986).

Plant gene technology has catalysed progress in plant breeding, but has not yet been applied to food legume improvement on a large scale (Kahl et al. 1994). Pea is a natural host of *Agrobacterium* and transformation using *Agrobacterium tumefaciens* has been successful (Puonti-Kaerlas et al. 1990, Davies et al. 1993, Schroeder et al. 1993, Grant et al. 1995, Bean et al. 1997). Success in transformation and plant regeneration has made it possible to initiate experiments through *in vitro* approaches to improve genetic resistance against several diseases (Christou et al. 1994).

2.3 The powdery mildews

Powdery mildews produce white superficial hyphae on the aerial parts of living plants, with large one-celled conidia produced terminally on isolated aerial unbranched conidiophores and with haustoria in the epidermal cells of their hosts (Yarwood 1978). The powdery mildew fungi are widely distributed on several plant species which are classified into 20 genera and about 400 species (Hirata 1986). The powdery mildews of various crop plants are grouped into six main genera (Agrios 1988, Hirata 1986), as illustrated in Table 2.1.

Table 2.1 Powdery mildews of major crop species.

Powdery mildews	**Hosts (crop plants)**
Erysiphe graminis	Cereals, grasses
E. polygoni (pisi)	Pea, bean, sugarbeet, cabbage
E. cichoracearum	Begonia, chrysanthemum, flax
Microsphaera alni	Blueberry, sweet pea, elm
M. diffusa	Soybean
Phyllictinia spp	Elm, maple, oak
Podosphaera leucotricha	Apple, pear
P. oxyacanthae	Apricot, cherry, plum
Sphaerotheca fuliginea	Cucurbits
S. macularis	Strawberry
S. pannosa	Peach, rose
Uncinula necator	Grape, horse chestnut

According to Hirata (1986), the host plants of powdery mildew fungi are 9838 angiosperm species dispersed in 1617 genera of which 9176 are dicotyledons and 662 monocotyledons (634 *Gramineae*). Interestingly, powdery mildew has not been found on *Oryza sativa* L., *Zea mays* L., *Digitaria spp.*, *Panicum miliaceum* L., or *Setaria italica* L. (Hirata 1986). The first well-recorded epidemic of powdery mildew was grape powdery mildew (*U. necator*) in Europe from 1847-1851 when the grape harvest was reduced to one fourth of normal (Yarwood, 1978). Powdery mildew is the most important leaf

disease of barley in most European countries and losses average 10% in the United Kingdom (Mathre 1982).

The three ways in which the pathology of powdery mildews differs most from that of other plant pathogens include the speed of spread (Ruppel et al. 1975), the tolerance of the fungi to dryness (Brodie and Neufeld 1942) and the ease of control with sulphur (Bent 1978). Conidia of powdery mildews germinate at low humidity (Brodie and Neufeld 1942, Clayton 1942). The high water content in the spores supply the water necessary for germination, and the high lipid content protects them from rapid desiccation in a dry environment (McKeen 1970).

2.3.1 Genetics of host pathogen interactions

The coexistence of host plants and their pathogens side-by-side in nature indicates that the two have been evolving together. Changes in the virulence of the pathogen must be continually balanced by changes in the resistance of the host, and vice versa (Agrios 1988). Such a step-wise evolution of resistance and virulence can be explained by the gene-for- gene concept, which states that, for each gene in the host that confers resistance, there is a corresponding gene in the pathogen that confers virulence (Flor 1955, 1971).

The capacity of a host to limit the growth of a plant pathogen which is attempting to infect the host is known as resistance. Resistance, which is only expressed toward certain races of a pathogen and not to the other races, is known as race-specific resistance. This type of resistance provides the basis for the gene-for-gene theory and is characterized as vertical, major gene, or oligogenic resistance. Race-nonspecific

resistance is effective against most isolates of the pathogen. This type of resistance is characterized as race-nonspecific, polygenic, adult plant, horizontal, generalized, slow-rusting, rate reducing, partial, or minor gene resistance (Simmonds 1979, Agrios 1988).

Host plants of powdery mildews exhibit both categories of resistance, race-specific and race-non specific. Most analyzed resistance reactions of cereals and other hosts of powdery mildew were specified by dominantly or semidominantly inherited resistance genes, that act race specifically (Kenigsbuch and Cohen 1989, Menzies et al.1989, Chan and Boyd 1992, Jorgensen 1994, Persaud and Lipps, 1995). The *Mlo* resistance of barley powdery mildew acts in a race-nonspecific manner and confers resistance to most isolates of *E. graminis* f. sp. *hordei* (Jorgensen 1977, 1988, 1993; Freialdenhoven et al. 1996).

At the molecular level, several physiological changes have been documented in other host-parasite interactions. These changes include release of antimicrobial phytoalexins, pathogenesis-related proteins, induction of enzymes of the phenylpropanoid pathway, changes in composition of the cell wall and secretion of proteinase inhibitors and lytic enzyme (Van Loon and Van Kammen 1970, Broglie et al. 1991, Hain et al. 1993, Jakobek and Lindgren 1993, Zhu et al. 1994). These responses constitute the defense arsenal of plants to invading pathogens.

2.3.2 Variation in virulence

In Europe and Australia, considerable efforts have been put into maintaining host resistance to cereal powdery mildew and control with fungicide, but the disease remains a

significant problem (McIntosh 1978, Whisson 1996). Concern about crop production costs and pollution of the environment has emphasized the use of genetic resistance (Jorgensen 1993). This is due to the evolution of new pathotypes through recombination, mutation, the development of fungicide resistance, migration and the introduction of new resistance genes (Whisson 1996).

The genetics of host-pathogen interaction has been extensively studied in cereal powdery mildew (*E. graminis*). Powdery mildew of barley (*E. graminis* f. sp. *hordei* on *Hordeum vulgare*) is of greatest relative importance among the cereal powdery mildews in Europe (McIntosh 1978, Mathre 1982, Jorgensen 1993). More than 200 gene symbols assigned to barley powdery mildew resistance genes over time were summarized and revised to 85 gene symbols (Jorgensen 1994). Chan and Boyd (1992) identified 15 virulence genes in *E. graminis* f. sp. *hordei* in Australia. In wheat (*Triticum aestivum*), Wolfe (1967) reported 38 races of *E. graminis* f. sp. *tritici* in the UK. Menzies et al. (1989) identified 8 virulence genes in *E. graminis* f. sp. *tritici* in southern Ontario using 12 differential lines. Similarly, Persaud and Lipps (1995) identified 11 virulence genes in the wheat powdery mildew pathogen against 11 host resistance genes (*Pm*) in wheat. Kenigsbuch and Cohen (1989) reported independent inheritance of resistance to two races of powdery mildew in muskmelon (*Cucumis melo*).

Some evidence of a physiological basis of resistance to *Erysiphe spp.* has been documented. Leaves of trees and shrubs were very susceptible to powdery mildew when they emerged, but rapidly acquired resistance as they unfolded and expanded (Mence and Hilderbrandt 1966). Resistance of cereals to *E. graminis* increased with plant age and,

thus, infection rate and ultimate disease severity were greatest on the lower leaves (Shaner 1973).

2.3.3 Overwintering

The overwintering of cereal powdery mildew occurs in the vegetative stage on the overwintering green plants. Menzies and MacNeill (1989) reported that cleistothecia of *E. graminis* f. sp. *tritici* were first observed in early June and were less abundant after October in Ontario. In other climates, ascospores overwinter in cleistothecia and constitute initial inoculua for the spring-sown crop (Jorgensen 1988). Two sexually compatible strains are necessary for perithecium formation (Smith 1970). Continuous culture in a glasshouse may lead to loss of perithecium formation (Mamluk and Weltzien 1973).

In an overwintering study in Alberta, cleistocarps of *Uncinula salicis* on willow *(Salix discolor* Muhl.) and aspen *(Populus tremuloides* Michx.) and *Microsphaera penicillata* on wild sweet pea (*Lathyrus ochroleucus* Hook.) discharged viable ascospores by the time the hosts were in full leaf the following year (Smith 1971). The method of overwintering of *Sphaerotheca spp.* on *Cucumis*, *Erysiphe* on *Phaseolus* and several other powdery mildews is obscure. It is possible that they remain viable throughout the year in the southern USA and are carried north by wind each summer (Yarwood 1978).

The annual nature of the pea crop precludes survival as mycelium on host stems, but perennation in the seed and survival on other hosts are possible alternatives. Though some workers have suggested that *E. polygoni* could perennate as mycelium in seed

(Crawford 1927), it is unlikely that mycelium borne externally on the seed could remain viable (Smith 1969). Uppal et al. (1936) reported that pea powdery mildew was transmitted internally through the seed and germination was reduced drastically in infected seed. In a host range study (Smith 1969), an isolate of *E. polygoni* from pea was distinct from those obtained from other hosts. Smith (1969) stated that it was very unlikely *E. polygoni* overwintered in alternate hosts in England and that the reason for the late appearance of powdery mildew was that conidia had to spread from warmer areas in the south. Yu (1946) reported that *E. pisi* was capable of overwintering in China, both as conidia and cleistothecia.

2.3.4 Control

Early planting (Gritton and Ebert 1975) and hot water seed treatment at 56^0 C for 20 minutes (Crawford 1927) reduced damage caused by pea powdery mildew. However, Srivastava et al. (1973) did not observe any significant effect of hot water treatment on reducing damage by powdery mildew. Rain or sprinkler irrigation inhibited the germination of powdery mildew conidia and mechanically removed the mycelium (Cherewick 1944, Faloon et al. 1991). Soluble silicon (Si) at 100 ppm controlled powdery mildew on cucumber (*Cucumis sativa*), musk melon (*Cucumis melo*), zucchini (*Cucurbita pepo*) and rose (*Rosa spp.*) (Belanger et al. 1995, Menzies et al. 1992). Several fungicides, along with garlic (*Allium sativum* L.) oil and bulb extract, neem (*Azadirachta indica* Juss.) leaf extract, and ginger (*Zingiber officinale* Rosc.) extract reduced disease intensity (Singh et al. 1984).

The application of sulphur to control powdery mildew was recommended in 1802 and is still an effective control method for many powdery mildews (Yarwood 1978). Sulphur dust at the rate of 20 kg/ha or wettable sulphur at the rate of 4 to 8 kg/ha has been recommended in the Pacific Northwest of the USA (Sakr 1989). About 20 different fungicides (contact and systemic) are extensively used to control powdery mildew diseases of various crop plants (Bent 1978). Warkentin et al. (1996a) reported that both sulphur (Kumulus S) and myclobutanil (Nova 40W) were effective in reducing disease severity and that fungicide application was economically beneficial in western Canada under conditions of high disease pressure.

Genetic variation in cereal powdery mildew with respect to fungicide resistance has become an issue of concern in Europe (Wolfe 1984). Although mildews have acquired tolerance to several recently developed fungicides, they have apparently not acquired resistance to sulphur during the past 200 years (Yarwood 1978). However, genetic resistance in the host plant is the most economical and environmentally friendly approach to controlling powdery mildews (Mathre 1982, Reiling 1984, Hagedorn 1985, Bernier et al. 1988).

2.3.5 Taxonomy

Taxonomically, powdery mildews comprise the family *Erysiphaceae* in the order *Erysiphales*, class *Pyrenomycetes*, and subdivision *Ascomycotina* (Harshberger 1917). Classification is based primarily on characters of the sexual stage (cleistothecia or perithecia). These are primarily size; dorsoventrality; attachments to mycelium; surface of

occurrence; transparency; number of layers of the wall; size of wall cells; number, size and shape of asci; number, size and shape of ascospores; size, number, type, position, septation and colour of appendages; seasonal development; and spore discharge (Yarwood 1978). Number of asci per perithecium and type of appendages are universally used in the separation of major genera.

Although classification is primarily based on the characteristics of the sexual stage, perithecia are of limited value in taxonomy of powdery mildew because most collections do not contain them (Yarwood 1978). The presence of two mating types (antheridium and ascogonium) are necessary for the formation of cleistothecia in heterothalic fungi (Smith 1970). Old leaves, a low state of host nutrition, a dry atmosphere and low temperature favour perithecium formation (Singh 1968). Asexual morphological characters of powdery mildew pathogens have been used for identification in cases where the sexual state was not observed (Boesewinkel 1977, 1980; Menzies and Kempler 1991). Boesewinkel (1980) reported that the presence or absence of fibrosin bodies, shape of hyphal appressoria, size and shape of conidiophores and conidia, and the production of conidia in chains or singly were the most important characteristics for identification of the asexual state of powdery mildew fungi.

Pea powdery mildew is confined to the genus *Erysiphe* because it possesses a superficial mycelium, mycelium-like appendages on the cleistothecium and has several asci per ascocarp (Salmon 1900). Eight species were recognized in the genus *Erysiphe* among which *E. polygoni* causes powdery mildew on pea. Blumer (1933) and Homma

(1937) divided *E. polygoni* into additional sub-species, based on the morphology of the cleistothecial appendages, and called the pea pathogen *E. pisi*.

2.3.6 Pea powdery mildew (*Erysiphe pisi* Syd.)

The unique characteristics of *E. pisi* are variable and persistent mycelium, and conidia which are formed singly, rarely on short chains, and are ellipsoid. Cleistothecia are gregarious to scattered, globose, 85-126 microns (μ) diameter; asci 3-10, ovate to broadly ovate, 50-60 x 30-40 μ; and ascospores 3-5, rarely 6, 22-27 x 13-16 μ ((Kapoor 1967). Conidiophores are 62-105 x 7-10 μ. Foot cells are 22-72 μ long and decrease in width from 7.2-8.5 μ at the base to 6.2-7 μ at the top. Conidia are ovoid-cylindric or ellipsoid-cylindrical, 40-47 x 15-17 μ, producing a long or short germ tube on the end (Boesewinkel 1977, 1980).

E. pisi attacks all aerial portions of the pea plant. Usually, it is most prevalent on the upper surface of the lower leaves. Usually late-sown crops and those at the dried pea stage of maturity are most liable to infection (Reiling 1984). Leaves, stems and pods may be infected, causing death of the vine, withering of foliage and occasional plant death (Dixon 1978). Severe pod infection may result in "hollow" peas (Reiling 1984). In some cases this disease is seed-borne (Crawford 1927, Uppal et al. 1936).

Infected pods become brown and produce an objectionable odour. Infected seeds have a gray-brown discolouration. The objectionable odour and brown spots on infected pods make them unacceptable for the pea market (Crawford 1927, Cousin 1965).

2.3.6.1 Host range

The host range of *E. pisi* has been extensively investigated, but the results are still contradictory. Hammarlund (1925) proposed 26 formae species of *E. polygoni* and reported that f. sp. *pisi* infected only *Pisum sativum* and *P. arvense*. However, Hirata (1986) reported that *E. pisi* infected 85 species in the family *Leguminosae*. *Cicer arietinum*, *Cajanus cajan*, *Phaseolus mungo* and *Lens culinaris* were attacked by *E. pisi* (Dixon 1978). Stavely and Hanson (1966) reported that pea powdery mildew was pathogenic to four species of *Lathyrus* and *L. sativus* was as susceptible as pea. Yu (1946) reported that powdery mildew on *Vicia spp.* and *Pisum sativum* was caused by a single physiological race. Reiling (1984) reported that of the three biological forms of *E. pisi* found in *Pisum*, *Medicago*, *Vicia*, *Lupinus* and *Lens*, only the "pea form" infected pea.

Mignucci and Chamberlain (1978) found *Microsphaera diffusa* to severely infect pea. Smith (1969) studied the host range of *E. polygoni* on pea and other hosts and found that only conidia from *Lathyrus odoratus* produced sporulating mildew colonies on greenhouse pea plants. However, the isolate from pea did not sporulate on *L. odoratus* plants.

2.3.6.2 Infection

Following the overwintering phase, primary infection on a susceptible pea plant may occur through ascospores or conidia. The process of infection consists of a number of morphologically identifiable stages including spore germination and formation of

appressoria, penetration peg, haustorium and secondary hyphae. The formation of elongating secondary hyphae, capable of initiating secondary infection, is taken as evidence that the host and pathogen have established a compatible functional relationship (Ellingboe 1972). Hyphae develop from up to five positions on each conidium with two or three hyphae growing from each end (Falloon et al. 1989). Germination of the conidia starts within three hours of landing on a leaf of a susceptible host. About 2% to 3% of the conidia formed circular, lobed appressoria on stomatal cells and penetrated them later, but direct penetration through the cuticle was more common (Singh and Singh 1983). Spores germinate and penetrate the epidermal cells under low humidity conditions (Reiling 1984). The germ tubes of both spore types penetrate and establish haustoria in epidermal cells, and give rise to superficial sporulating colonies. Production of conidiophores and conidia started by 72 hours and 96 hours, respectively, after inoculation (Singh and Singh 1983).

2.3.6.3 Morphology

Falloon et al. (1989) studied the morphological details of conidial germination, hyphae, appressoria, conidiophores and organization within colonies of *E. pisi* on pea leaves. Several hyphae were produced from each conidium and unidirectional growth of individual hyphae occurred. Hyphal cells on leaf surfaces were specialised to produce either appressoria or conidiophores and hyphal branches.

Conidia of *E. pisi* are usually borne singly on conidiophores (Falloon et al. 1989). The resultant conidia are dispersed by wind and induce secondary infection. Conidia

develop on the mycelium giving a powdery appearance to the leaf. The sexual stage results from the fusion of an antheridium and ascogonium and yields a minute, black, fruiting structure called a cleistothecium. Cleistothecia develop in the cottony mycelial growth on older leaves as plants mature or as the fungus and host mature or become environmentally stressed (Yarwood 1978). Low temperatures, together with wetting of the cleistothecia, induce maturation of ascospores (Mathre 1982).

2.3.6.4 Epidemiology

Conidia of *E. polygoni* and certain other powdery mildews are capable of germinating at low relative humidity, even approaching zero (Brodie and Neufeld 1942, Cherewick 1944, Yarwood 1978). General infection occurs during dry weather when nights are cool enough for dew formation (Reiling 1984). Powdery mildew is most prevalent on fall crops or crops that mature in late summer (Dixon 1978). The general increase in inoculua throughout the summer provides an abundant supply of spores by late summer.

Yarwood et al. (1954) reported that the minimum, optimum and maximum temperatures for *E. pisi* were 8-10^0 C, 23^0 C, and 32^0 C, respectively. Yarwood (1949), working on bean and pea powdery mildew, reported that mildew grew more luxuriantly on plants grown at low rather than at high soil moisture and in the shade rather than under full light.

Reports disagree on the effect of rain on mildews. Frequent rains or dews are deleterious to both spore survival and dissemination of spores from the host tissue (Reiling 1984). Consequently, the disease is less destructive in areas of high rainfall or

under overhead irrigation systems. Peries (1962) reported that the internal structure of powdery mildew conidia collapses when the spores are in water, and immersion for as brief as three minutes can kill 50% of the conidia. Similarly, spraying distilled water onto leaves caused collapse of many hyphae and the impact of water droplets caused severe disruption of colonies (Falloon et al. 1991). However, conidia of *E. pisi* on pea germinated normally and retained their ability to grow on leaves after a period of 24 hours on or in water (Sivapalan 1993).

2.3.6.5 Host resistance

Using Peruvian accessions, Harland (1948) showed that resistance to powdery mildew in pea was controlled by a single recessive gene *er* and this finding was supported by Pierce (1948) who found resistance in the cultivar Stratagem. This resistance was later reported to break down under field conditions (Schroeder and Providenti 1965). Cousin (1965) reported that resistance in the cultivars Mexique 4 and Stratagem was conditioned by a recessive gene. Later, Heringa et al. (1969) conducted an extensive study under both field and greenhouse conditions and showed that resistance to powdery mildew in local cultivars was governed by a recessive gene, *er-1*, and resistance in Peruvian accessions (leaf resistance) was conditioned by a second recessive gene, *er-2*. They further suggested that the lines SVP 950 and Mexique 4 carried both resistance genes, *er-1* and *er-2*. Kumar and Singh (1981) crossed 15 susceptible lines with a resistant line and concluded that duplicate resistant genes (*er-1* and *er-2*) were required for field resistance. More recently, Gupta et al. (1995) evaluated 45 F_1s (excluding reciprocals) derived from a diallel cross

and suggested that resistance to powdery mildew was polygenically controlled. Banyal and Tyagi (1997) reported slow mildewing resistance in the pea cultivar DPP-68 in India.

The phytoalexin "pisatin" was present (3 ug/g fresh weight) two days after inoculation of leaves of susceptible pea cultivar with *E. pisi* and then reached 300 ug/g after four days (Oku et al. 1975). When inoculated with a nonpathogenic fungus, *E. graminis hordei*, pisatin was detected within 15 hours after inoculations (Oku et al. 1975). The conidia of *E. pisi* were 13 times more tolerant to pisatin than those of *E. graminis hordei*. A biochemical study on phenolic contents and on the activities of phenol-oxidizing enzymes revealed that the resistant cultivars contained higher levels of phenolics and phenol-oxidizing enzymes than the susceptible cultivars (Kalia and Sharma 1988). Ozone at a concentration of 0.12 ul L^{-1} suppressed growth of *E. pisi*, indicating a protective effect in disease development (Rusch and Laurence 1993).

In a host-parasite interaction study with red clover (*Trifolium pratense* L.) and *E. polygoni*, Smith (1938) stated that the early stages of infection, such as conidia germination, appressoria formation and penetration into epidermal cells, were similar in both resistant and susceptible lines. Falloon et al. (1991) reported that initial growth of *E. pisi* on both resistant and susceptible plants was similar, but further development ceased on the resistant plants, suggesting that resistance in the host may be a response to penetration of the leaves by the pathogen. Similarly, Singh and Singh (1983) observed no differences in spore germination of *E. pisi* on resistant and susceptible host plants.

2.4 Molecular markers

Genetic markers were being used in biology well before it was known that DNA was the hereditary material. Morphological markers, mutations in genes with visible consequences such as dwarfism or eye colour, were used in genetic studies since early in the Twentieth Century (Morgan 1911). Morphological markers are limited in number, influenced by the environment, and may have pleiotropic effects (Eberhard 1994). Markert and Moller (1959) showed genetic differences in enzymes and characterized these variants as isozymes. Isozymes were more abundant than morphological markers, and, thus, were used in plant genome analysis (Mahmoud et al. 1984, Zamir and Ladizinsky 1984). However, isozymes are tissue and development specific; therefore, DNA markers are more attractive and are the current markers of choice (Paterson et al. 1991).

In recent years, molecular biology has provided tools suitable for rapid and detailed genetic analysis of higher organisms, including agricultural species. Perhaps, the most fundamental of these tool is the DNA marker, which simply detects differences in the genetic information carried by two or more individuals (Paterson et al. 1991). Since the advent of DNA-based markers for genetic analysis (Botstein et al. 1980), new techniques and marker systems have developed rapidly.

Restriction fragment length polymorphism (RFLP) markers (Botstein et al. 1980) proved valuable in establishing linkage maps in many crop species (Diers et al. 1992, Dirlewanger et al. 1994) and as markers for traits of agronomic importance (Tanksley et al. 1989, Barzen et al. 1992). RFLPs are codominant markers and are inherited in a

Mendelian fashion. In RFLP analysis, a relatively large quantity of DNA is digested with restriction enzymes followed by gel electrophoresis, Southern transfer and filter hybridization with radioactive probes (Tanksley 1983, Beckmann and Soller 1983). Since these steps are time consuming, labourious, and costly, the use of RFLPs is incompatible with the high analytical throughput required for many applications in plant breeding (Williams et al. 1990, Waugh and Powell 1992, Schondelmaier et al. 1996).

A variation of the RFLP method was developed which uses various suitable probes to detect short tandem repeated sequences with a highly variable number of repeats between flanking restriction sites (Weber 1990). Minisatellites, also called hypervariable repeats (HVR) and/or variable number of tandem repeats (VNTR), are tandem repeat DNA sequences which generally consist of 10-60 bp motifs (Zhou et al. 1997). Most of the minisatellites share a common motif known as the core sequences. Genetic variation in rice (*Oryza sativa*) has been detected using minisatellite probes and primers (Zhou and Gustafson 1995, Zhou et al. 1997). Microsatellites or simple sequence repeats (SSRs) or short tandem repeats (STRs) are composed of tandemly repeated 2-5 nucleotide DNA core sequences such as (CA)n, (AT)n or (AGAT)n. The DNA sequence flanking SSRs are generally conserved within individuals of the same species allowing the selection of polymerase chain reaction (PCR) primers that will amplify the intervening microsatellites in all genotypes. Variation in "n" results in PCR product length differences (Tautz 1989, Rongwen et al. 1995). Microsatellites have been used in fingerprinting and genome mapping (Beyermann et al. 1992, Hellens et al. 1993, Yu et al. 1994, Rongwen et al. 1995). Morgante and Oliveri (1993) reported that PCR amplified

microsatellites in soybean were highly polymorphic, somatically stable and inherited in a codominant Mendelian manner. "AT" repeats were by far the most frequently observed class of dinucleotide microsatellites and "TAT" repeats were common among the more common trinucleotides (Morgante and Oliveri 1993, Wang et al. 1994). Generally, minisatellite and microsatellite analyses are more difficult and expensive than fingerprinting with RAPDs.

Introduction of the PCR-based marker systems (Saiki et al. 1988) has revolutionized many standard molecular biological techniques (Schondelmaier et al. 1996). One such marker system is random amplified polymorphic DNA (RAPD) (Williams et al. 1990). This procedure has the advantage of being technically simple, quick to perform, requires only small amounts of DNA and involves no radioactivity. RAPDs are well suited for use in the high sample number throughput systems required for plant breeding, population genetics and biodiversity studies (Williams et al. 1990, Michelmore et al. 1991, Waugh and Powell 1992, Marshall et al. 1994).

2.4.1 Random amplified polymorphic DNA

PCR with single arbitrary short primers relies on the chance that complementary primer sites occur somewhere in the genome as inverted repeats enclosing a relatively short stretch of DNA (Williams et al. 1990). The DNA between the two opposite primer sites can be amplified (arbitrarily primed PCR, AP-PCR: Welsh and McClelland, 1990; random amplified polymorphic DNAs, RAPDs: Williams et al., 1990; DNA amplification fingerprinting, DAF:Caetano-Anolles et al. 1991). Welsh and McClelland (1990)

suggested comparatively long oligonucleotide primers (typically 20-34 bases), whereas Caetano-Anolles et al. (1991) suggested short oligonucleotide primers (5-8 bases) with polyacrylamide gel electrophoresis and silver staining (Bassam et al. 1991). The RAPD method of Williams et al. (1990), using 10 base-pair (decamer) primers, has been used extensively in many applications. Decamer primers are commercially available from various sources such as Operon Technologies Inc., Almeda, CA and University of British Columbia (UBC), Vancouver, BC. Although the sequence of the RAPD primer is arbitrarily chosen, two basic criteria should be met: a minimum of 40% G+C content (50-80% G+C is generally used) and an absence of palindromic sequences (DNA sequence is identical in a 5' to a 3' direction on both strands) (Williams et al. 1990). Polymorphisms between individuals are detected as differences between the patterns of DNA fragments amplified from different genomic DNA sources using a given primer(s).

Combining the use of RAPDs and near-isogenic lines (NILs) provides a means for quickly identifying markers linked to a trait of interest. NILs have been used to identify RAPD markers (Martin et al. 1991, Paran et al. 1991, Penner et al. 1993a, Johnson et al. 1995) that are linked to disease resistance genes. Martin et al. (1991) developed a formula to estimate the number of primers that would need to be screened on average in order to have a high probability of finding at least one marker within a specified distance from the target gene. Expected minimum distance = $c/2\ (nx + 1)$, where c = genome size in cM, n = number of primers, x = average number of PCR products per primer. The distance at a 95 % confidence level = $(c/2)\ (1-0.05^{1/nx})$. Michelmore et al. (1991) described a bulked segregant analysis (BSA) method that is not dependent on the availability of NILs, to

identify RAPD markers linked to major genes. Since then, this approach has been successfully used to identify RAPD markers in several crop species and in several traits of interest (Chalmers et al. 1993, Penner et al. 1993c, Lehner et al. 1995, Urrea et al. 1996). Paran and Michelmore (1993) demonstrated that sites described by RAPD markers can be sequenced and converted into specific PCR amplicons known as "sequence characterized amplified regions" (SCARs). SCARs are advantageous over RAPD markers as they detect only a single locus and their amplification is less sensitive to reaction conditions (Paran and Michelmore 1993, Penner 1996). Several SCAR markers have been used in marker assisted selection (Adamblondon et al. 1994, Timmerman et al. 1994, Gu et al. 1995, Horvath et al. 1995, Dedryver et al. 1996, Urrea et al. 1996).

2.4.1.1 Applications

RAPD analysis has been used to generate genomic maps of plant species (Reiter et al. 1992, Kiss et al. 1993) and to identify markers for disease resistance genes (Adamblondon et al. 1994, Haley et al. 1994b, Urrea et al. 1996, Young and Kelly 1997) and other agronomic traits (Chalmers et al. 1993, Lehner et al. 1995). Other common uses of RAPDs include cultivar identification (Demeke et al. 1993, Ko et al. 1994, Mackill 1995), genetic relatedness and biodiversity (Castiglione et al. 1993, Gonzalez and Ferrer 1993, Hallden et al. 1994, Jain et al. 1994, Hoey et al. 1996, Karp et al. 1997) and identification of hybrids (Marshall et al. 1994, Grattapaglia et al. 1996). Organ-specific amplifications of RAPD fragments have been reported in soybean (Chen et al. 1997).

2.4.1.2 Reproducibility

RAPD analysis, though extensively used in various laboratories, is not free from criticism. The reproducibility of RAPD analysis both within and among laboratories has been questioned (Devos and Gale 1992, Kleinhofs et al. 1993). Ellsworth et al. (1993) demonstrated that changes in primer to template concentration ratio, the annealing temperature, and the magnesium concentration can qualitatively affect banding patterns produced by arbitrary primers. A problem associated with RAPD analysis was the relatively low reliability (5-10% error rate) of the phenotypes (Weeden et al. 1992). A 5% error intrinsic to the procedure greatly compromises the value of the technique for MAS and virtually precludes its use in seed quality control applications (Gu et al. 1995). The major obstacles with RAPD analysis are its reduced reliability because of the use of short random primers that are not always completely homologous to the binding sites and the relatively low annealing temperature, creating the risk of non-specific amplification (Schondelmaier et al. 1996). Jones et al. (1997) reported that the reproducibility of RAPD was not satisfactory in a comparative study among RAPD, AFLP and SSRs.

Penner et al. (1993b) evaluated the reproducibility of RAPD analyses among six laboratories in North America and results indicated that, if the annealing temperature profiles inside the reaction tubes were identical, then RAPD fragments were likely reproducible. The conversion of RAPD fragments into allele-specific amplicons or SCAR leads to increased reliability of amplification, increased allele specificity and facilitates the multiplexing of primers (Paran and Michelmore 1993, Penner 1996, Penner 1997).

2.4.2 Amplified fragment length polymorphism

Many DNA fingerprinting techniques have been developed in the past few years and are generally based on either classical, hybridization-based fingerprinting (Botstein et al. 1980, Tanksley et al. 1989) or PCR based fingerprinting (Welsh and McClelland 1990, Williams et al. 1990). Amplified fragment length polymorphism (AFLP) technology is a DNA fingerprinting technique that combines both of these strategies. This technique is robust and reliable because stringent reaction conditions are used for primer annealing, and the reliability of the RFLP technique is combined with the power of the PCR technique (Vos et al.1995). AFLP has become the synonym for a powerful new marker technology, based on simultaneous PCR amplifications of many restriction fragments and their detection on sequencing gels (Zabeau and Vos 1993). This technique has the capacity to inspect a much greater number of loci for polymorphism than other currently available PCR-based techniques (Thomas et al. 1995).

2.4.2.1 Principles

In AFLP, DNA is digested with restriction endonucleases, and double-stranded DNA adaptors are ligated to the ends of the DNA fragments to generate template DNA for amplification (Zabeau and Vos 1993). Thus, the sequence of adaptors and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. One to three arbitrary nucleotides serve as selective nucleotides extending beyond the 3' end of the restriction site (Lin and Kuo 1995, Vos et al. 1995). Only those restriction fragments in which the nucleotides flanking the

restriction site match the selective nucleotides will be amplified. The restriction fragments for amplification are generated by two restriction enzymes, a rare cutter (6 base-pairs) and a frequent cutter (4 base-pairs). The AFLP procedure results in predominant amplification of those restriction fragments, which have a rare cutter sequence on one end and a frequent cutter sequence on the other end (Vos et al. 1995). Restriction enzyme combinations for AFLP included *Eco*RI, *Hin*dIII, *Pst*I, *Bgl*II, *Xba*I, *Sse*83871 in combination with either *Mse*I or *Taq*I (Vos et al., 1995). The subset of amplified fragments are then analysed by denaturing polyacrylamide gel electrophoresis to generate a fingerprint. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence including mutations, insertions and deletions.

2.4.2.2 Applications

DNA polymorphisms identified using AFLP are typically inherited in a Mendelian fashion and may, therefore, be used for fingerprinting, identification of molecular markers for agronomically important traits and mapping of genetic loci (Vos et al. 1995). AFLP analysis has been used successfully to integrate AFLP markers into a linkage map of sugar beet (*Beta vulgaris*) (Schondelmaier et al. 1996) and rice (Cho et al. 1996). AFLP techniques have been used to study genetic relatedness and gene pool similarities of wild bean (*Phaseolus spp.*) (Tohme et al. 1996), sunflower (*Helianthus annus*) (Hongtrakul et al. 1997), *Arabidopsis thaliana* (Lin and Kuo 1995) and potato cyst nematode

populations (Folkertsma et al. 1996). Thomas et al. (1995) identified markers for the disease resistance gene *Cf-9* in tomato (*Lycopersicon esculentum*).

AFLP patterns were not affected by the amount of genomic DNA (100 ng to 5 ug), but they were complicated by partially digested genomic DNA (Lin and Kuo 1995). On average, 50 to 100 DNA bands were produced per lane. Mackill et al. (1996) compared levels of polymorphism for AFLP, RAPD, and microsatellite markers in rice cultivars and concluded that, while all marker types generated similar classifications, the frequency of polymorphic bands was much higher for AFLP. Jones et al. (1997) reported a high level of reproducibility for this technique among European laboratories. Donini et al. (1997) reported high reproducibility for the AFLP technique, although AFLP pattern differences were revealed between template DNA extracted from different plant organs (leaf, seed and root). The silver staining detection method was preferred to labelling with ^{32}P for AFLP analysis because it avoids the use of radioactivity and provides greater resolution (Cho et al. 1996).

2.5 Marker-assisted selection

Marker-assisted selection (MAS) has been integrated into several plant breeding programs to select traits of agronomic importance. Isozymes were initially sought for this purpose, but their use was hindered by the low variability detected between closely related genotypes (Paterson et al. 1991, Marshall et al. 1994). Molecular markers are especially advantageous for agronomic traits that are otherwise difficult to tag such as resistance to pathogens, insects, nematodes, tolerance to abiotic stresses, quality

parameters and quantitative traits (Dudley 1993, Mohan et al. 1997). Conventional screening techniques for disease and pest resistance are often time consuming and expensive. Furthermore, the pathogens or pests must be maintained either on the host or on alternate hosts, if they are obligate parasites. Screening of plants with several different pathogens and their pathotypes, or pests and their biotypes, simultaneously or even sequentially is difficult. Once molecular markers are identified, MAS can be performed in early segregating populations and at early stages of plant development. Thus, with MAS, it is possible to conduct many rounds of selection in a year (Mohan et al. 1997).

Based on visual scoring of the host-parasite interaction, it is often not possible to determine the presence of additional resistance genes. With MAS, the segregation of new resistance genes can be followed, even in the presence of the existing resistance genes, and, thus, resistance genes from diverse sources can be incorporated in a single genotype for durable resistance. Pyramiding of the bacterial blight resistance genes *Xa1*, *Xa3*, *Xa4*, *Xa5*, and *Xa10* in different combinations has been done in rice using molecular markers (Yoshimura et al. 1995). MAS can be successfully exploited in hybrid breeding programs. Several studies on maize (*Zea mays*) inbred lines in USA and Europe have established the utility of molecular markers in quantifying relatedness among the inbreds, assigning inbreds to heterotic groups and predicting the subsequent performance of hybrids (Messmer et al. 1992, Mumm and Dudley 1994).

The application of MAS to plant breeding is constrained by the cost of the technology employed and throughput capacity (Penner 1997). Gu et al. (1995) reported on large-scale, cost-effective screening of PCR products in MAS. McDonald et al. (1994)

developed a fast and simple DNA extraction procedure from dry seeds. Penner et al. (1996)developed a dot blot hybridization technique particularly suitable for large scale MAS. Molecular marker technology is now integrated into several plant breeding programmes and allows researchers to access, transfer and combine genes at a rate and with a precision not previously possible (Mohan et al. 1997).

CHAPTER 3

INHERITANCE OF POWDERY MILDEW RESISTANCE IN PEA

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3.1 Abstract

Pea, an important grain legume crop, suffers significant yield and quality losses because of infection by the parasitic fungus *Erysiphe pisi* Syd., the causal agent of powdery mildew. Most pea cultivars in western Canada are susceptible to this fungus. The genetic basis of resistance in certain Canadian cultivars is unclear. Resistant cultivars and lines were intercrossed with each other and with susceptible lines to determine the genetic basis of resistance. Resistance in the cultivars Highlight, AC Tamor, Tara, Mexique 4, Stratagem and lines JI 210, JI 1951, JI 1210 was conferred by a single recessive gene, *er-1*. The resistance in line JI 2480 was conferred by a different recessive gene, *er-2*. Resistance provided by *er-1* was durable under both field and growth cabinet conditions. However, the resistance provided by *er-2* was broken under controlled growth conditions. Combining *er-1* and *er-2* in a cultivar could increase the durability of resistance.

Key words: pea, *Pisum sativum*, powdery mildew, *Erysiphe pisi*, inheritance, resistance.

3.2 Introduction

Grain legumes are known for their high protein content and quality and for their ability to fix nitrogen. Pea (*Pisum sativum L.*) is an important grain legume used for human food, animal feed, forage and green manure. Dry edible pea, often considered a separate crop, constitutes the bulk of world pea production. Pea is an ideal field crop to include in crop rotations to break cereal disease cycles and to improve soil nitrogen status. A ten-fold increase in the cultivation of field pea in the last ten years in western Canada (Statistics Canada 1995) indicates the increasing importance of this legume in the cropping system of the prairie provinces.

Powdery mildew caused by the ascomycete *Erysiphe pisi* Syd (syn. *E. polygoni* DC) can cause severe damage to pea, often becoming epidemic in nature. *Erysiphe pisi* is an obligate parasite which obtains nutrients from the plant through haustoria in epidermal cells (Agrios 1988). Severe infection may result in 25-50% yield reduction (Munjal *et al.* 1963, Kumar and Singh 1981, Reiling 1984) along with a deterioration of seed quality. Out of 56 recommended field pea cultivars in western Canada only three (Highlight, AC Tamor and Tara) are resistant to the western Canadian population of *E. pisi* (Warkentin et al. 1996a).

Resistance to powdery mildew in pea has been reported to be controlled by a recessive gene, *er-1* (Harland 1948, Heringa *et al.* 1969). Leaf resistance of Peruvian lines may be under the control of a second recessive gene, *er-2* (Heringa *et al.* 1969). Kumar and Singh (1981) reported that duplicate recessive genes (*er-1, er-2*) were required for field resistance to their population of *E. pisi*. Gupta et al. (1995) suggested that resistance

to powdery mildew was polygenically inherited. Since the genetic basis of the powdery mildew resistance in Canadian cultivars is unknown, we have designed experiments aimed at clarifying the genetic basis of powdery mildew resistance in Canadian field pea cultivars.

3.3 Materials and methods

Powdery mildew resistant field pea cultivars Highlight, AC Tamor and Tara, were crossed to each other and with the susceptible cultivar Radley. Additional pea accessions reported to possess powdery mildew resistance genes (Figure 3.1a), originating from diverse geographical regions (Table 3.1), were obtained from Dr. Mike Ambrose, John Innes Institute (JII), Norwich, UK. These accessions were crossed to each other (Figure 3.1b) and with Highlight in a growth cabinet. Growth conditions were, day/night temperature of 20/15^0 C, 80 % relative humidity, light intensity of 380 umol m^{-2} s^{-1}, and a 16 h light/8 h dark. Emasculation of female parents was done before the anthers dehisced and pollinations were performed with freshly dehisced anthers the following day. Reciprocal crosses were made and evaluated in the F_1 progeny of the crosses JI2302/Highlight, Highlight/JI 2480 and Radley/JI 1559. A fraction of the F_1 seed was grown out in a growth cabinet and harvested in bulk to produce F_2 seed. In the summer of 1994, 1995 and 1996, parents, F_1 and F_2 progeny were evaluated under field conditions at the Agriculture and Agri-Food Canada, Morden Research Centre, Morden, Manitoba (Figure 3.1c). A random selection of susceptible and resistant individual plants of some of the crosses was grown as F_3 progeny under field conditions in 1995. The number of

plants of parental lines and F_1 progeny ranged from 10 to 35. Field experiments were planted in the last week of May and harvested in the first week of September. Rows were 5 m long with inter-row and intra-row spacing of 1 m and 30 cm, respectively. Natural infection by *E.pisi* occurred in all three years. Disease incidence was more severe in 1994 and 1996 than in 1995. Severity of the disease was estimated visually on individual plants using a 0 to 9 scale based on the percentage of foliage area mildewed, where 0=no infection, 1=<1%, 2=1-5%, 3=5-10%, 4=10-20%, 5=20-40%, 6=40-60%,7=60-80%, 8=80-90%, 9=>90% mildewed (Warkentin et al. 1996a). Plants were at the podding stage when the disease was first detected on the upper surface of the lower leaves. Disease scoring was done after the susceptible plants were heavily infected and near maturity. Scores of 0 to 4 were classified as "resistant", and 5 to 9 as "susceptible". In general, no visible colonies developed on resistant plants; however, under some conditions a few small colonies which were slow to develop, appeared on the lower leaves of resistant plants. Chi-squared values were calculated to confirm segregation ratios. For the purpose of testing homogeneity of the data, Yates correction factor was not used even though only one degree of freedom was involved in each calculation (Strickberger 1985).

To confirm the segregation ratios of some crosses, F_2 progeny were re-evaluated in a growth cabinet in the presence of bulk field inoculum of the fungus. A susceptible cultivar, Trump, was planted every two weeks in the growth cabinet to maintain inoculum. Growth conditions were maintained as described above. Pea plants for screening were transferred into the growth cabinet 2 weeks after planting and were individually inoculated by dusting powdery mildew conidia onto the leaves using heavily

Table 3.1 Pea genotypes used in the evaluation of the inheritance of powdery mildew resistance.

Genotypes	Source[x]	PM[y]	RG[z]
1. Highlight	Sweden	R	
2. AC Tamor	Canada	R	
3. Tara	Canada	R	
4. JI 2480	UK	R	*er-2*
5. JI 1559 (Mexique 4)	Mexico	R	
6. JI 1758	Nepal	S	
7. JI 210	India	R	
8. JI 1951	China	R	
9. JI 1648	Ethiopia	S	
10.JI 82	Afghanistan	R	
11.JI 1210	USA	R	
12.JI 2302 (Stratagem)	USA	R	*er-1*
13.Radley	UK	S	*Er*

[x]Source country.

[y]Reaction to powdery mildew under field conditions in Morden.

R=Resistant, S=susceptible

[z]Reported genotypes.

Figure 3.1 (a) Powdery mildew of pea under field conditions in Morden, Manitoba (1996), resistant (centre plot) and susceptible (side plots) pea lines, (b) Crosses made in the greenhouse and (c) Segregation for powdery mildew reaction in the F_2 progeny.

infested susceptible plants. Individual plants were then scored for powdery mildew reaction in the seedling (7-8 days after inoculation) and adult plant stages.

3.4 Results

Among the Canadian pea cultivars, the F_1 of resistant/susceptible crosses were susceptible, and F_1 of resistant/resistant crosses were resistant, indicating resistance was recessive (Table 3.2). The F_2 of resistant/susceptible crosses segregated in a 3 susceptible:1 resistant ratio (Table 3.2) indicating monogenic inheritance. No segregation occurred in resistant/resistant crosses. These results indicated that in all three Canadian pea cultivars Highlight, AC Tamor, and Tara, resistance to powdery mildew was imparted by the same recessive gene.

Other powdery mildew resistant accessions included JI 2302, a source of *er-1* (Heringa et al. 1969) and JI 2480, a putative source of a second resistance gene, *er-2* (Ali et al. 1994). JI 2480 was susceptible to powdery mildew under controlled growth conditions with greater disease development on stems than leaves (data not shown). However, under field conditions, JI 2480 was resistant to powdery mildew in both 1995 and 1996. Lines JI 1758 and JI 1648 (Mike Ambrose, personal communication, 1995) and Slow (Timmerman et al. 1994) were previously reported resistant but were susceptible under both controlled growth conditions and under field conditions in our tests.

In 1995, analyses of the F_1 indicated that resistance was recessive in all resistant/susceptible crosses (Table 3.2). Other crosses evaluated in the F_1 but not advanced to the F_2, included: JI 1758/JI 1559, JI 1648/JI 1559, JI 1758/JI210,

Table 3.2 Reaction of F_1 and F_2 populations of pea to powdery mildew under field conditions in Morden. Manitoba.

Crosses	F_1 reaction[x]	F_2 plants[y] S	R	Exp[z]	X^2(P)
1994					
Resistant/Susceptible					
1. Highlight/Radley	S	78	23	3:1	0.27(0.5-0.7)
2. AC Tamor/Radley	S	41	17	3:1	0.57(0.3-0.5)
3. Tara/Radley	S	41	11	3:1	0.41(0.5-0.7)
Resistant/Resistant					
4. Highlight/AC Tamor	R	0	115	0:all	0.00(1.0)
5. Highlight/Tara	R	0	105	0:all	0.00(1.0)
6. AC Tamor/Tara	R	0	85	0:all	0.00(1.0)
1995:					
Resistant/Susceptible					
1. Radley/JI 1559	S	154	51	3:1	0.00(0.9-1.0)
2. Radley/JI 2480	S	140	51	3:1	0.29(0.5-0.7)
3. JI 1758/JI 2302	S	87	27	3:1	0.11(0.7-0.8)
4.JI 1951/JI 1648	S	96	26	3:1	1.00(0.5-0.7)
5.JI 82/JI 1648	S	102	30	3:1	0.36(0.5-0.7)
Resistant/Resistant					
6. Highlight/JI 2302	R	0	236	0:all	0.00(1.0)
7. Highlight/JI 1559	R	0	72	0:all	0.00(1.0)
8. Highlight/JI 1210	R	0	44	0:all	0.00(1.0)
9. JI 210/JI 2302	R	0	70	0:all	0.00(1.0)
10.Highlight/JI 2480	S	79	71	9:7	0.78(0.3-0.5)
11.JI 2480/JI 1559	S	33	32	9:7	0.78(0.3-0.5)
Susceptible/Susceptible					
12.JI 1758/JI 1648	S	143	0	all:0	0.00(1.0)
Homogeneity for 3:1 segregation:					
Total summed		739	236		3.01 (8df)
Expected		731	244		0.33 (1df)
Homogeneity					2.68 (7df)>0.9
Homogeneity for 9:7 segregation					
Total summed		112	103		1.56 (2df)
Expected		121	94		1.49 (1df)
Homogeneity					0.07 (1df)>0.9

[x]Reaction of F_1 plants.
[y]Number of F_2 plants observed, R=resistant, S=susceptible, df=degrees of freedom.
[z]Expected ratio.

JI 1758/JI 1951, JI 1758/JI 82, JI 1758/JI 1210, JI 1648/JI 210, JI 210/JI 1559, JI 1951/JI 1559, and JI 1210/JI 1559. Reciprocal crosses were made to evaluate the possibility of cytoplasmic influence on susceptibility, however, no such effect was detected. The F_1 of JI 2480/Highlight and JI 2480/JI 1559 were susceptible, indicating that the resistance of line JI 2480 was governed by a different gene (*er-2*) than the gene present in Highlight and JI 1559. Resistant sources from diverse origins were crossed and screened in the F_1 in an attempt to identify other resistance genes. Susceptibility was not observed in the F_1 of any of the resistant/resistant crosses (except crosses involving JI 2480)..

The F_2 of all resistant/susceptible crosses segregated in a 3 susceptible to 1 resistant ratio. None of the resistant/resistant crosses segregated for susceptibility, except for crosses involving JI 2480. These results indicate that resistance to powdery mildew in lines JI 1559, JI 210, JI 1951, JI 1210, JI 2302 and the Canadian resistant cultivars Highlight, AC Tamor, and Tara is governed by the single recessive gene *er-1* (Table 3.2). JI 2480, as expected, carries a different resistance gene (*er-2*). F_2 progeny of the cross JI 2480/Radley segregated in a 3 susceptible to 1 resistant ratio confirming *er-2* as a recessive gene (Table 3.2). F_2 from crosses between JI 2480 and other resistant accessions (Highlight and JI 1559) segregated in a 9 susceptible : 7 resistant ratio (Table 3.2), as expected in a digenic model of inheritance with complementary gene action. The resistance genes *er-1* or *er-2* provided full resistance under field conditions when present in the homozygous condition. However, under growth room conditions, and in the detached leaf assay (Warkentin et al. 1995) resistance in JI 2480 was broken (data not shown). No segregation for resistance occurred when two susceptible lines were crossed.

To confirm the field results, F_2 of selected crosses were evaluated in a growth cabinet. Similar results were found to those observed under field conditions for all crosses, except for crosses involving line JI 2480. All resistant/susceptible crosses segregated in a 3 susceptible to 1 resistant ratio (Table 3.3). The F_2 of Highlight/JI 2480 segregated in a 3 susceptible: 1 resistant ratio, as expected, since accession JI 2480 was susceptible under controlled growth conditions. All the F_2 of JI 2480/Radley were susceptible (Table 3.3). The F_3 families, derived from susceptible F_2 in all crosses, segregated into two segregating: to one nonsegregating families, as expected. Progeny of the resistant/resistant and resistant progeny of the resistant/susceptible crosses did not segregate, thus, confirming single gene inheritance (Table 3.4).

3.5 Discussion

Powdery mildew is an economically important disease of pea in western Canada, significantly affecting quality and quantity of pea production. Although fungicidal control is available as an alternative (Warkentin et al. 1996a), genetic resistance is preferred, because it is a more sustainable means of controlling disease. In the present investigation, the genetic basis of mildew resistance was confirmed and an attempt was made to identify other resistance genes by inter-crossing resistant accessions from diverse geographical regions. In all resistant/susceptible crosses, resistance was recessive, but none of the F_1 of resistant/resistant crosses was susceptible except crosses involving JI 2480, hence, only JI 2480 had a different resistance gene.

Table 3.3 Reaction of selected F_2 progeny of pea to powdery mildew in a growth cabinet.

Crosses	F_2 plants[x]			
	S	R	Exp[y]	X^2(P)
Resistant/Susceptible				
1. Radley/JI 1559	51	21	3:1	0.67(0.3-0.5)
2. Highlight/JI 2480[z]	49	14	3:1	0.25(0.5-0.7)
3. JI 1648/JI 1559	64	25	3:1	0.44(0.5-0.7)
4. JI 1758/JI 1559	57	18	3:1	1.32(0.1-0.3)
Susceptible/Susceptible				
5. Radley/JI 2480	58	0	all:0	0.00(1.00)
Homogeneity for 3:1 segregation:				
Total summed	221	78		2.68(4df)
Expected	224	75		0.19(1df)
Homogeneity				2.49(3df)(>0.30)

[x]Number of F_2 plants. S=Susceptible, R=Resistant.

[y]Expected ratio.

[z]Susceptible in growth cabinet.

df=degrees of freedom.

Table 3.4 Reaction of F_3 families of pea to powdery mildew in Morden, Manitoba.

Crosses	Total[x]	NSS[y]	NSR[z]	X^2(P)
Susceptible F_2 plants				
1. Highlight/Radley	73	26	0	0.2(0.5-0.7)
2. AC Tamor/Radley	34	13	0	0.4(0.5-0.7)
3. Tara/Radley	30	11	0	0.2(0.5-0.7)
4. JI 1559/Radley	42	16	0	0.4(0.5-0.7)
5. JI 2480/Radley	122	48	0	1.5(0.1-0.5)
Resistant F_2 plants				
5. Highlight/Radley	22	0	22	0(1.0)
6. AC Tamor/Radley	23	0	23	0(1.0)
7. Tara/Radley	18	0	18	0(1.0)
8. Highlight/AC Tamor	20	0	20	0(1.0)
9. AC Tamor/Tara	20	0	20	0(1.0)
10.Highlight/Tara	20	0	20	0(1.0)
11.JI 1559/Radley	12	0	12	0(1.0)

[x]Total families.

[y]Non-segregating susceptible.

[z]Non-segregating resistant.

Previous studies of the inheritance of powdery mildew resistance are somewhat contradictory. Resistance is controlled by one to many genes (Heringa et al. 1969, Kumar and Singh 1981, Gupta et al. 1995). Stratagem (JI 2302), a source of *er-1*, was included in the present study and it carries the same resistance gene as the Canadian cultivars. Heringa et al. (1969) also concluded that Stratagem carries *er-1*. However, they reported line JI 1559 (Mexique 4) to contain both resistant genes *er-1* and *er-2*. In the present investigation, JI 1559 was crossed with the susceptible cultivar Radley, the resistant cultivar Highlight (*er-1*) and accession JI 2480 (*er-2*) and conclusively shown to carry only one gene for resistance (*er-1*). However, this accession displayed a complete resistance reaction. The presence of two genes for resistance, as indicated by Kumar and Singh (1981), was not confirmed since the F_2 in all resistant/susceptible crosses in the present investigation consistently segregated in a 3 susceptible : 1 resistant ratio. Polygenic inheritance, as reported by Gupta et al. (1995), was not observed in lines carrying *er-1* since all segregating F_2 and F_3 progeny were qualitatively distinguished as resistant and susceptible plants. These differences in the interpretation of the number of genes involved in powdery mildew resistance could be due to the diversity of genotypes studied and possibly differences in the race structure of natural populations of *E. pisi*. Similar to our results, Mishra and Shukla (1984) and Narsinghani (1979) reported powdery mildew resistance to be inherited monogenically in Indian cultivars.

Accession JI 2480 (*er-2*) was resistant to powdery mildew under field conditions in Morden in 1995 and 1996. Powdery mildew infection appeared two weeks later than the expected normal date (third week of July) in 1995 and the infection was moderate.

Both leaves and stems of JI 2480 were free from infection. The F_2 of the cross JI 2480/Radley segregated in a three susceptible : one resistant ratio. However, this population had a wider distribution of disease scores than progeny segregating for *er-1*, and in the growth cabinet studies, this accession was susceptible. Stems were more heavily infected than leaves (data not shown). This discrepancy in reaction could possibly be due to differences in environmental conditions and/or higher disease inoculum present in the growth cabinet. This may imply that the resistance gene *er-2* provides partial resistance and may succumb under high disease pressure. This is in agreement with the observations of Heringa et al. (1969), that resistance of genotypes carrying *er-2* was confined to leaves. Marx (1986) also reported heavy stem infection on plants carrying *er-2*. Another line Peru II, which may carry *er-2* (N.F. Weeden, Cornell University, personal communication 1997) exhibited a similar reaction to JI 2480 under field conditions and in the growth cabinet. Races of *E. pisi* are not reported in the pathogen population. A low level of variability in reaction has been detected among single colony isolates from western Canada and western USA (Chapter 4).

The resistance gene *er-1* may be present in many resistant lines from around the world. A recent genetic study demonstrated that *er-1* resides on linkage group 6 (Timmerman et al. 1994). No indication of the chromosomal location of *er-2* is available. None of the tested lines contained both resistance genes (*er-1*, *er-2*). Incorporation of both of these genes in a cultivar could increase the durability of resistance. This process would be greatly simplified with DNA markers for resistance genes *er-1* and *er-2*.

CHAPTER 4

PATHOGENIC VARIATION IN *ERYSIPHE PISI*, THE CAUSAL ORGANISM OF POWDERY MILDEW OF PEA

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4.1 Abstract

Erysiphe pisi is the causal organism of powdery mildew of pea (*Pisum sativum*), an economically important disease in western Canada. This study was conducted to examine the variability for virulence in naturally occurring populations of *E. pisi*. In 1995, 31 single- colony isolates of *E. pisi* were isolated and tested on a set of 14 different pea lines, using a detached leaf assay. Some variability was evident, as isolates PUI-2 and LAI-1 were slightly virulent on the resistant lines Highlight and JI 82, respectively. Other isolates caused similar reactions in all the tested lines. Ten of the 14 pea lines were evaluated in Manitoba, Canada; New York, California and Washington, USA; Norwich, UK; and Kathmandu, Nepal. Disease reaction of the tested lines in Nepal exhibited some differences compared to other test locations, indicating variability in virulence of the pathogen. Seven pea cultivars/lines, Highlight, JI 2480, JI 1559, JI 210, JI 82, Radley, and JI 1758 are suggested for use as differential lines for future studies.

Key words: Host parasite interaction, *Pisum sativum*, race(s).

4.2 Introduction

Erysiphe pisi Syd. (syn. *E. polygoni* DC.) is an ectophytic, obligate parasite which causes powdery mildew on pea (*Pisum sativum* L.) wherever pea is grown (Dixon 1978). This disease adversely affects total biomass yield, number of pods per plant, number of seeds per pod, plant height and number of nodes (Gritton and Ebert 1975). Severe infection may result in 25 to 50% yield reduction (Munjal et al. 1963, Reiling 1984). In western Canada, powdery mildew is a pea disease of economic importance because of reductions in yield and quality. Reports from recent disease surveys in western Canada have shown that 33% to 69% of pea fields were infected with powdery mildew (Berkenkamp and Kirkham 1991, Orr and Burnett 1993, Xue et al. 1995).

Conidia and ascospores of *E. pisi* germinate on susceptible pea leaves and produce large-lobed primary appressoria which develop several hyphae radiating out across the host epidermis (Falloon et al. 1989). Subsequent mycelial growth depends on nutrients obtained through haustoria from epidermal cells. The first symptoms are small, diffuse, light-coloured spots on the upper surface of the lowest and oldest leaves. These lesions become covered by white, powdery fungal colonies (Reiling 1984). Mycelial hyphae produce short conidiophores on the plant surface. Conidia are usually borne singly on conidiophores (Falloon et al. 1989) and are disseminated mainly by wind to cause secondary infections. The incidence and severity of this disease can be controlled through the use of resistant cultivars.

Any powdery mildew management program that includes the use of host resistance will require information on the virulence genes that exist in the pathogen

population of interest and the effective resistance genes in the host germplasm (Persaud and Lipps 1995). The authors are unaware of reports on physiological races of *E. pisi*. Resistance to powdery mildew is controlled by the recessive genes *er-1* or *er-2* (Heringa et al. 1969). The objective of this study was to examine the variability in virulence within naturally occurring populations of *E. pisi* in western Canada and in several internationally diverse geographical regions.

4.3 Materials and methods

Detached leaf assay

Infected leaf samples from powdery mildew infected pea plants were collected in 1995 from Morden and Plum Coulee, Manitoba; Melfort and Indian Head Saskatchewan; Lacombe, Alberta; and Pullman, Washington. Disease-free leaves from the highly susceptible cultivar Trump were inoculated with these samples individually by dusting conidia onto detached leaves in petri dishes as described by Warkentin et al. (1995). Briefly, two to four stipules from the second or third node below the apex of plants of the cultivar Trump were excised with a scalpel then placed immediately on a sheet of filter paper in petri dishes containing 6 mL of a 5% sucrose solution. The stipules were oriented with the adaxial side up. The source of inoculum was young leaflets that were 80 to 100% covered with powdery mildew. Conidia were dusted onto the stipules using a small brush. Petri dishes were then wrapped with parafilm and placed in a growth chamber at 22^0 C with a 16 h photoperiod (high-intensity fluorescent light, 40 umol $m^{-2}s^{-2}$) (Figure 4.1a). The development of powdery mildew hyphae on the stipules

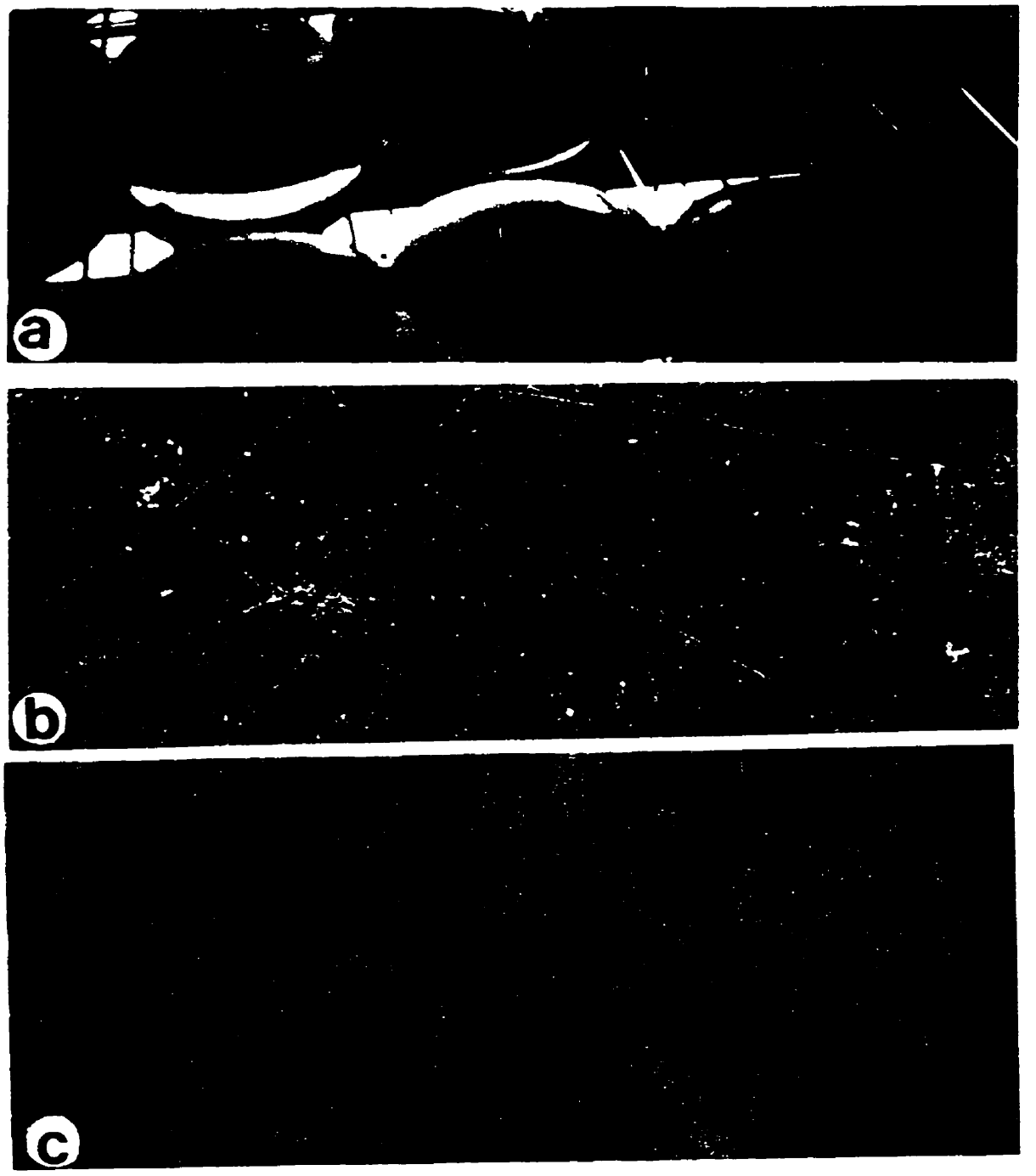

Figure 4.1 Detached leaf assay: (a) incubation of detached pea leaves, (b) limited growth of fungal hyphae on the resistant cultivar, Highlight, and (c) abundant growth of fungal hyphae, conidiophore and conidia on the susceptible cultivar, Trump.

was assessed by observation under a dissecting microscope 5 to 7 days after inoculation using a 0 to 9 scale (0 = highly resistant, 9 = highly susceptible). Inoculation of individual leaves was done in varying concentrations in such a way that separation of individual colonies was possible. After 3 to 4 days, fungal colonies were examined under a dissecting microscope and individual colonies from these leaves were isolated and multiplied on detached leaves of the cultivar Trump.

Eleven single colonies were individually isolated from powdery mildew collections from Morden, MB.; four from Plum Coulee, MB.; three from Melfort, SK.; four from Indian Head, SK.; four from Lacombe, AB.; and five from Pullman, WA. The conidia from each single-colony isolate were dusted onto disease-free leaves of the cultivar Trump using a small brush and multiplied on detached leaves as described above. When enough inoculum was obtained, disease-free leaves of 14 pea lines (Table 4.1) at the 4- to 8-node stage were detached, placed in petri dishes, inoculated and incubated (Figure 4.1a) as described above, to determine the disease reaction (Figure 4.1b, 4.1c). Isolations and leaf inoculations of all isolates were done in a laminar flow hood to avoid cross contamination. Control petri dishes with noninoculated leaves were assessed every time to confirm lack of cross contamination. The 14 pea lines originated from diverse geographical regions and represented the known powdery mildew reaction genotypes (Table 4.2). Each *E. pisi* isolate was tested at least twice. Stipules were used instead of leaflets in semileafless lines. Three to four leaves from the second or third node below the apex of the plant were used. Leaves were oriented adaxial side up. The development of powdery mildew hyphae on the leaves was assessed by observation under a dissecting

microscope 5-7 days after inoculation and scored using a 0 to 9 scale based on the percentage of foliar area affected (Warkentin et al. 1995). Scores of 0 to 4 were classified as "resistant" and 5 to 9 as "susceptible".

Field experiments

Ten of the 14 lines were evaluated for reaction to natural populations of *E. pisi* in the field at the Agriculture and Agri-Food Canada, Morden Research Centre, Morden, Manitoba, Canada; John Innes Institute, Norwich, UK; Nepal Agricultural Research Council. Kathmandu, Nepal; Washington State University, Pullman, Washington, and USDA/ARS research station, Brawley, California, USA (Table 4.2). The lines were also evaluated for their reaction to bulk field isolates of *E. pisi* in a greenhouse trial at Cornell University, NY, USA . In addition, 34 pea lines reported resistant to natural populations of *E. pisi* (Mike Ambrose, John Innes Institute, Norwich, UK, personal communication, 1995) in the UK, were screened for powdery mildew reaction in Morden, Manitoba in 1995. Field trials were seeded in June and harvested in September in Manitoba in 1995 and 1996; seeded in October 1996 and harvested in March 1997 in California; seeded in May and harvested in August in Norwich in 1996; seeded in November 1995 and harvested in April 1996 in Kathmandu; and seeded in May and harvested in August in Washington in 1997. In all field experiments, plot size was one row, 5 m long, with plots 1 m apart. No fungicides were sprayed in the experimental plots. Fertilizer was applied according to the recommendation of the specific locations. Number of plants per plot ranged from 15 to 35. Under field conditions, disease developed naturally in all test

locations. Individual plants were visually scored using the 0 to 9 scale described above after the plants were severely infected or near maturity. A clear, standard rating scale and instructions on how to interpret observations were provided to all evaluators by the senior author. Scores of 0 to 4 were classified as "resistant" and 5 to 9 as "susceptible".

4.4 Results

Detached leaf assay

Disease reaction of individual isolates to the 14 pea lines indicated that variability among the isolates in virulence pattern was low. Generally, many known resistant lines remained resistant and susceptible lines remained susceptible to many of the tested isolates (Table 4.1). However, isolates PUI-2 and LAI-1 exhibited slight virulence (score 5, 20-40% leaf area affected) on the resistant cultivar Highlight and line JI 82, respectively, in the detached leaf assay (Table 4.1). Whole plants of these lines were tested with the same isolates in a growth cabinet; on whole plants, isolates, PUI-2 and LAI-1, were scored up to 4 (10-20% leaf area infected) on Highlight and JI 82, respectively (data not shown). This slight discrepancy between detached leaf assay and whole plant scores could have been due to differences in environmental conditions and inoculum levels between the two types of tests. Line JI 1559 consistently exhibited a high level of resistance to all isolates throughout the experiment, with <1% leaf area infected. AC Tamor, Tara, JI 2302, JI 210, JI 1210, and JI 1951 exhibited consistent resistance reactions with <5% of leaf area affected. Highlight also showed <5% leaf area infection with isolates other than PUI-2. JI 82 exhibited moderate resistance, with <20% leaf area affected except with LAI-1. JI

Table 4.1 Reaction of 14 pea lines to single colony isolates of *E. pisi* in a detached leaf assay.

Isolates[b]	Pea lines[a]													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Disease score[c]													
MOI-1	0	7	0	9	0	1	9	4	1	1	1	1	9	9
MOI-2	1	7	1	9	1	1	9	4	2	1	2	2	9	9
MOI-3	1	7	0	9	1	1	9	4	2	0	2	2	9	9
MOI-4	2	7	0	9	2	2	9	1	2	0	2	2	9	9
MOI-5	2	7	1	9	2	1	9	1	2	2	2	2	9	9
MOI-6	2	7	1	9	1	1	9	2	0	1	1	2	9	9
MOI-7	1	7	0	9	1	1	9	2	0	1	1	1	9	9
MOI-8	1	7	0	9	1	2	9	2	1	1	1	1	9	9
MOI-9	2	7	1	9	2	2	9	4	2	2	2	1	9	9
MOI-10	2	7	1	9	2	2	9	4	2	2	2	2	9	9
MOI-11	2	7	1	9	2	2	9	2	2	2	1	1	9	9
PUI-1	2	5	1	9	2	1	9	4	0	0	1	2	9	9
PUI-2	5	7	0	9	2	2	9	4	2	0	1	2	9	9
PUI-3	2	7	0	9	1	0	9	1	2	2	2	2	9	9
PUI-4	2	7	1	9	1	0	9	1	2	2	2	1	9	9
MFI-1	2	7	1	9	1	1	9	2	1	1	1	2	9	9
MFI-2	2	7	1	9	0	1	9	3	1	1	1	2	9	9
MFI-3	2	7	0	9	1	0	9	3	0	1	0	2	9	9
IHI-1	1	7	1	9	0	0	9	4	1	1	1	1	9	9
IHI-2	1	7	0	9	1	1	9	4	1	1	0	1	9	9
IHI-3	2	7	1	9	2	2	9	4	2	2	1	1	9	9
IHI-4	0	7	1	9	2	1	9	2	1	2	1	2	9	9
LAI-1	2	7	1	9	2	1	9	5	2	2	0	2	9	9
LAI-2	1	7	1	9	2	2	9	2	2	2	2	2	9	9
LAI-3	2	7	0	9	2	2	9	4	2	1	0	1	9	9
LAI-4	2	7	1	9	2	2	9	2	1	2	2	1	9	9
WAI-1	1	7	0	-	-	-	-	-	-	-	-	-	-	9
WAI-2	2	7	0	-	-	-	-	-	-	-	-	-	-	9
WAI-3	1	7	0	-	-	-	-	-	-	-	-	-	-	9
WAI-4	1	7	0	-	-	-	-	-	-	-	-	-	-	9
WAI-5	2	7	0	-	-	-	-	-	-	-	-	-	-	9

[a]1. Highlight 2. JI 2480 3. JI 1559 4. JI 1758 5. JI 210
6. JI 1951 7. JI 1648 8. JI 82 9. JI 1210 10.JI 2302
11. AC Tamor12.Tara 13.Radley 14.Trump

[b]MOI=Morden isolate, PUI=Plum Coulee isolate, MFI=Melfort isolate, IHI=Indian Head isolate, LAI=Lacombe isolate, WAI=Washington isolate.

[c]0=no infection, 1=<1%, 2=1-5%, 3=5-10%, 4=10-20%, 5=20-40%, 6=40-60%, 7=60-80%, 8=80-90%, 9=>90% of area affected. -=Not tested.

Table 4.2 Reaction of pea lines to *E. pisi* in diverse environments.

		MB[a]		NY	KA	NW	CA	WA	GT
Pea lines	Origin	1995	1996	1995	95/96	1996	96/97	1997	
1. Highlight	Sweden	R(1)[b]	R(0)	R(1)	R(3)	R(0)	R(0)	R	*er-1*
2. JI 2480	UK	R(1)	R(3)	S(6)	R(3)	S(9)	R(0)	R	*er-2*
3. JI 1559	Mexico	R(0)	R(0)	R(1)	R(0)	R(0)	R(0)	R	*er-1*
4. JI 1758	Nepal	S(8)	S(8)	S(9)	S(5)	S(9)	S(9)	-	*Er*
5. JI 210	India	R(1)	R(1)	R(2)	S(5)	R(O)	R(0)	R	*er-1*
6. JI 1951	China	R(2)	R(1)	R(1)	R(3)	R(O)	R(0)	R	*er-1*
7. JI 1648	Ethiopia	S(7)	S(7)	S(7)	R(3)	S(9)	S(9)	S	*Er*
8. JI 82	Afghanistan	R(2)	R(3)	S(7)	R(3)	R(0)	R(0)	R	*er-1*
9. JI 1210	USA	R(1)	R(1)	R(2)	R(3)	R(0)	R(0)	R	*er-1*
10.Radley	UK	S(9)	S(9)	S(9)	R(3)	S(9)	S(9)	S	*Er*

[a]MB=Manitoba, Canada; NY=New York, USA; KA=Kathmandu, Nepal; NW=Norwich, UK; CA=California, USA; WA=Washington, USA, GT=Genotypes inferred, -=Data not available. R=resistant (0 to 4), S=susceptible (5 to 9)

[b]Values in parenthesis are disease scores. 0=no infection, 1=<1%, 2=1-5%, 3=5-10%, 4=10-20%, 5=20-40%, 6=40-60%, 7=60-80%, 8=80-90%, 9=>90% of area affected.

2480 was scored susceptible, with >70% of leaf area affected. JI 1758, JI 1648, Radley, and Trump were completely susceptible to all the isolates, with >90% leaf area affected (Table 4.1). All of the noninoculated leaves in the control petri dishes stayed free from powdery mildew hypha, indicating lack of cross contamination in the experiment.

Field experiments

These experiments were designed to evaluate the reaction of pea lines to *E. pisi* under diverse geographical and climatic conditions. Disease reactions of all the tested lines were similar in 1995 and 1996 in Manitoba (Table 4.2). Highlight, JI 2480, JI 1559, JI 82, JI 210, JI 1210, and JI 1951 exhibited a resistance response both years. Radley, JI 1648, and JI 1758 were fully susceptible both years. In California, Washington and in the UK, disease reactions were very similar to Manitoba except for the reaction of JI 2480 which was completely susceptible in the UK. Radley, JI 1758, and JI 1648 exhibited susceptible reactions and all other lines were resistant. In Washington, JI 1758 matured before the onset of powdery mildew. In New York, under greenhouse conditions, JI 82 and JI 2480 exhibited a susceptible reaction. Reaction of pea lines to powdery mildew in Nepal was different from that in North America and the UK. In Nepal two lines, JI 1758 and JI 210, exhibited a susceptible reaction, and all other lines, Highlight, JI 2480, JI 1559, JI 1951 JI 1648, JI 82, JI 1210, and Radley exhibited a resistant reaction (Table 2). Line JI 1559 exhibited a high level of resistance, as in other test locations.

Of 34 pea lines reported resistant in the UK were screened in Morden, Manitoba. Five (JI 105, JI 1648, JI 1696, JI 1758, and JI 1870) exhibited susceptible reactions while

the other 29 lines (JI 26, JI 40, JI 48, JI 73, JI 82, JI 92, JI 95, JI 96, JI 100, JI 102, JI 143, JI 713, JI 803, JI 1056, JI 1059, JI 1064, JI 1069, JI 1128, JI 1213, JI 1399, JI 1401, JI 1412, JI 1702, JI 1748, JI 1752, JI 1783, JI 1951, JI 2072, and JI 2217) were resistant.

4.5 Discussion

The genetics of host-parasite interaction in cereal powdery mildew (*E. graminis*) has been studied extensively. A number of host resistance genes and pathogen races have been reported (Wolfe 1972, Jorgensen 1988, Menzies et al. 1989). More than 40 host resistance genes and 40 pathogen virulence genes have been reported in barley (Chan and Boyd 1992). Major genes for powdery mildew resistance have been described at 17 different loci in wheat (Persaud and Lipps 1995). Six resistance genes and three pathogen races have been reported in powdery mildew of muskmelon (Kenigsbuch and Cohen 1989). Although we did not find a highly variable population of powdery mildew on pea, a low level of variability in virulence was evident among the isolates tested. *Erysiphe pisi* is a widely distributed pathogen around the world and evidence of physiological specialization (Schroeder and Provvidenti 1965) has been reported. Schroeder and Provvidenti (1965) reported that resistance to powdery mildew in pea conferred by the *er-1* genotype was overcome by an isolate of the fungus obtained from naturally infected pea plants. Our results with isolates PUI-2 and LAI-1 confirm this possibility. In the present investigation, the collection of pathogen isolates was from western Canada and northwestern USA. Although this covers a large agricultural area, major differences in

reaction would perhaps be found if samples were collected from a wider geographical area and/or if the number of single colony isolates was increased.

Powdery mildew reaction in Nepal differed from that of North America and the UK for several lines. Radley, a susceptible cultivar in western Canada, and JI 1648, a susceptible line in North America and the UK, exhibited resistance reactions in Nepal, indicating that these lines may contain some other resistance gene(s). JI 210, a resistant line in North America and the UK carrying *er-1* (chapter 3) exhibited a susceptible reaction in Nepal, whereas the other lines reputed to carry *er-1* did not. These observations suggest the presence of different virulences of *E. pisi* in Nepal. Highlight, JI 2480, JI 82, and JI 1210 exhibited similar resistance reactions with 5-10% of foliar area infected (Table 4.2). These lines may carry additional genes (other than *er-1*, *er-2*) for resistance to pathotypes in Nepal. Similarly, pathogen genotypes present in North America and the UK are virulent on Radley and JI 1648. Interestingly, the two lines, JI 210 and JI 1758, which exhibited a susceptible reaction in Nepal, originated from India and Nepal, respectively (Table 4.2). Divergence of the virulence pattern of *E. pisi* in Nepal and North America could be caused by wider geographical separation, different environmental conditions or the presence of different host genotypes. Similarly, Harland (1948) reported that six pea cultivars which were immune to powdery mildew in Peru were susceptible in Australia.

JI 1559 consistently expressed a high level of resistance in detached leaf assays, in greenhouse studies, and under field conditions in Canada, USA, the UK, and Nepal. Heringa et al. (1969) reported that JI 1559 (Mexique 4) carried the *er-1* and *er-2* genes for

resistance. However, we found that (Chapter 3), JI 1559 carried only one gene, *er-1*, for resistance. The high level of resistance in this line, as compared to other lines carrying *er-1*, could be due to the presence of other modifier genes in JI 1559.

Differences in the reaction of a specific genotype in different test locations could be due to either the presence of different pathotypes in the test locations or the effect of environment on the expression of a resistance gene(s). In the present investigation, five pea lines (JI 105, JI 1648, JI 1696, JI 1758 and JI 1870) exhibited a susceptible reaction in Manitoba as opposed to a resistant reaction in the UK. Similarly, JI 2480 exhibited a susceptible reaction in New York, the UK and in the detached leaf assay, but was resistant in Manitoba, California and Kathmandu (Tables 4.1, 4.2). Perhaps, the resistance of JI 2480 is dependent on the level of inoculum present and environmental conditions, such as temperature and humidity. Our results (Chapter 3) indicated that JI 2480 carried a gene (*er-2*) which could become ineffective under high disease pressure.

Presently no differential pea lines are available to differentiate virulences in *E. pisi*. Due to the lack of near-isogenic lines, seven pea lines, Highlight, JI 2480, JI 1559, JI 210, JI 82, Radley, and JI 1758 are suggested for use as host differential lines for future work. This suggestion is based on the specific reaction of pea lines on the detached leaf assay and from field data. Highlight and JI 2480 serve as standard resistant sources for *er-1* and *er-2*, respectively (Chapter 3). Although JI 1559 shares the same resistant gene, *er-1*, as Highlight, it is included in this set because it expresses a high level of resistance under diverse conditions as compared to other lines carrying *er-1* (Tables 4.1, 4.2). Line JI 210, though resistant in all other test locations, was susceptible in Nepal. Resistance of

JI 82 seems to be more influenced by the environment than the common *er-1* gene. Radley, a susceptible cultivar in western Canada, was resistant in Nepal. JI 1758 exhibited a susceptible reaction across all test locations and may serve as the standard susceptible source (*Er*).

CHAPTER 5

STUDIES ON THE WINTER SURVIVAL STRATEGIES OF *ERYSIPHE PISI* IN MANITOBA

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5.1 Abstract

Erysiphe pisi, the causal agent of powdery mildew is an important disease of field pea in western Canada, but very little information is available on whether or how it survives the winter in the prairie provinces. Observations were made of cleistothecia on pea stubble and of the possibilities of seed transmission. Survival on other plant species acting as alternate host(s) were examined. Observations on heavily infected plants in 1996 and 1997 indicated that cleistothecia were abundantly formed in late August to September under field conditions. Microscopic observation of ascospores during the winter of 1996/97 indicated that by May 1997, more than 95% of the cleistothecia had degenerated under field conditions, whereas in samples stored at room temperature, 50% of the cleistothecia contained healthy appearing ascospores. When seeds from plants heavily infected with powdery mildew were grown in a greenhouse during the winter of 1996/97 and 1997/98, none of the 4200 plants examined developed powdery mildew symptoms, suggesting that the possibility of *E. pisi* transmission through infected seed is very remote. When isolates of powdery mildew originating on weed species found in the vicinity of pea fields were inoculated onto peas, no infection occurred. None of four legume crop species (chickpea, lentil, field bean and faba bean) inoculated with *E. pisi* became infected. Wind dispersed conidia from northern USA could possibly be the source of primary inoculum of pea powdery mildew in western Canada.

Key words: Cleistothecia, *Erysiphe pisi*, pea, overwintering.

5.2 Introduction

Field pea (*Pisum sativum* L.) is an important grain legume crop grown worldwide (Dixon 1978). Canada ranks third in world field pea production after France and the Ukraine, and France and Canada are the world's largest exporters (Food and agriculture organization 97). Saskatchewan is the leading field pea producing province in Canada followed by Alberta and Manitoba (Statistics Canada 1996). Diseases are among the most important field pea production constraints in western Canada. Pea powdery mildew, caused by the obligate parasite *Erysiphe pisi* Syd (syn. *E. polygoni* DC.) reduces yield and quality of pea in Canada (Martens et al. 1988). In the pacific northwest USA, yield reductions of up to 46% have been reported (Sakr 1989).

Normally under field conditions in Manitoba, colonies of *E. pisi* first appear in field pea in mid to late July (Martens et al. 1988, Ali-Khan and Zimmer 1989). The pathogen spreads rapidly on susceptible cultivars and colonizes the entire surface of leaves and stems. In mid to late August, minute black cleistothecia are found within the mycelial mats as host tissues begin to senesce. The cleistothecia are considered the overwintering structure of *E. pisi* in Canada (Martens et al. 1988), but little is known about the survival of the fungus under natural conditions.

The overwintering strategies of other *Erysiphe* species have been studied to a greater extent than *E. pisi*. Several researchers studied the role of cleistothecia in the disease cycle of *Erysiphe graminis* DC. ex. Merat f. sp. *tritici* Em. Marchal and concluded that the cleistothecia are not important as overwintering structures (Cherewick 1944, Smith and Blair 1950, Turner 1956, Leijerstrom 1962, Menzies and MacNeill

1989). Cherewick (1944) concluded that *E. graminis* f. sp. *tritici* overwinters as mycelial mats on dead straw and as mycelial infections on overwintering hosts. Smith and Wheeler (1969) studied the overwintering mechanisms of *E. polygoni* DC. on pea and other host species in the UK. However, the role of cleistothecia in winter survival of pea powdery mildew could not be clearly demonstrated.

The objectives of this study were to: 1) monitor the development of ascospores in cleistothecia of *E. pisi* during the winter, 2) investigate the possibility of seed transmission of the pathogen and 3) investigate if other crop species act as alternative host (s).

5.3 Materials and methods

Cleistothecial development

In September 1996, straw of the highly susceptible field pea cultivar Trump with abundant cleistothecia was collected from the Agriculture and Agri-Food Canada, Morden Research Centre, Morden, Manitoba. The straw was bagged in nylon net bags and placed under natural conditions on the surface of a field or stored at room temperature. During the winter of 1996/97, the percentage of cleistothecia containing ascospores was assessed microscopically every month by sampling straw with about 500 to 800 cleistothecia from each of the two storage environments. The pea straw was randomly removed from the nylon bags and soaked in water for 20 to 30 minutes. Wet cleistothecia were gently scraped off the straw with a scalpel. The cleistothecia were immersed in a drop of distilled water and mounted in lactophenol onto a glass slide. The

cleistothecia were split open to reveal their contents by applying gentle pressure to a cover slip placed over them. The number of cleistothecia, asci per cleistothecia and ascospores per ascus were recorded. Also, pea straw was collected from the same field in May 1997 and cleistothecial development was examined from May to July as described above.

Attempts were made to determine the viability of ascospores using three different techniques. In all techniques, leaves and stems with abundant cleistothecia of cultivar Trump were soaked in water at 4^0 C for 3 days to promote ascospore maturation (Cherewick 1944) and tested on greenhouse-grown, disease-free Trump leaves. Using the first technique, leaves and stems containing cleistothecia were hung over greenhouse-grown plants (flowering stage) for a week. The second technique consisted of attaching leaves and stems with cleistothecia to a petri dish lid suspended over leaves in petri dishes in a detached leaf assay (Warkentin et al. 1995). Thirdly, cleistothecia were crushed in a mortar and pestle in water and the ascospore suspension was sprayed on plants grown in a greenhouse. In all experiments growth conditions were maintained at 20^0 C, 16 / 8 h light / dark periods, and approximately 80% relative humidity.

Seed transmission of *E. pisi*

Pea seeds were harvested from plants which were heavily infected with powdery mildew and planted in a greenhouse. Approximately 250 plants each of powdery mildew susceptible cultivar Radley, cultivar Trump and line JI 1758 were planted from October 1996 to January 1997 at monthly intervals. In November 1997, approximately 1200

seeds from heavily infected plants of Radley were planted in a greenhouse. Individual plants were examined for powdery mildew symptoms at the flowering stage. Because of the endemic nature of powdery mildew of pea, these investigations were carried out in the greenhouses of the Cereal Research Centre, Winnipeg, Manitoba, where other pea plants were not present.

Alternate host(s)

Disease-free leaves of greenhouse-grown field pea plants were inoculated with powdery mildew from six plant species found in the vicinity of field pea fields in Manitoba. Similarly, disease free leaves of four legume crops (*Cicer arietinum* L., *Lens culinaris* Medikus, *Phaseolus vulgaris* L. and *Vicia faba* L.) were inoculated with field pea powdery mildew inoculum. Inoculation was done by dusting heavily infected leaves onto healthy leaves. The inoculation studies were conducted in a detached leaf assay (Warkentin et al. 1995). Briefly, detached leaves were placed on a sheet of filter paper in petri dishes containing 5% sucrose solution. Powdery mildew conidia were dusted onto healthy leaves, incubated at 20^0 C for 5 to 7 days and assessed for disease development using a 0 to 9 scale (0 = highly resistant and 9 = highly susceptible). Resistant and susceptible checks were utilized in each experiment and experiments were repeated at least twice.

5.4 Results

Cleistothecial development

Abundant cleistothecia were observed on leaves, stems and pods of naturally infected field pea plants in August 1996 and 1997 (Figures 5.1a, 5.1b). Initially, cleistothecia were white to brown; later they turned dark brown to black (Figure 5.1c). About 20 field pea lines susceptible to powdery mildew supported the development of cleistothecia under field conditions in Manitoba.

Microscopic examination of samples stored outside under field conditions revealed that asci and ascospores developed in early October (Figure 5.1d). Development of ascospores did not occur until November on samples stored at room temperature. Two to six asci were normally observed per cleistothecium with an average of four. Each ascus contained one to five ascospores with an average of three (Figure 5.2a, 5.2b). One or few vacuoles were observed (Figure 5.2c) in ascospores after December 1996 under both environmental conditions. By May 1997, most of the ascospores had degraded (Figure 5.2d) under field conditions.

The number of cleistothecia with apparently mature ascospores was fairly constant from December to February under both environmental conditions (Figure 5.3). After February, the number of cleistothecia with ascospores decreased rapidly under field conditions and slowly at room temperature. Approximately 4% of the cleistothecia contained apparently healthy ascospores in samples stored under field conditions, whereas approximately 50% contained apparently healthy ascospores in samples stored at room temperature in May (Figure 5.3).

Abundant cleistothecia were observed on overwintered field pea stubble collected from the surface of a cultivated field in spring 1997 (Figure 1b). Microscopic examination of these cleistothecia revealed that approximately 3% of the cleistothecia contained apparently healthy ascospores. Few dehisced (burst) cleistothecia were observed. Cleistothecia were not observed on field pea straw which had been incorporated under the soil surface.

Measurements of sexual reproductive structures and conidia of *E. pisi* are presented in Table 5.1. The mean diameter of cleistothecia was 101.4 microns (μ). The mean length and width of asci was 63.3 μ and 38.3 μ, respectively. Conidia were slightly larger than ascospores (Table 5.1). These values are in agreement with previously reported values (Stavely and Hanson 1966, Kapoor 1967, Singh 1968).

Despite the different methods tested in these experiments, we were unable to infect field pea leaves with ascospores in detached leaf assays or on whole plants. In the first two methods where cleistothecia were hung over greenhouse-grown plants and on petri dish lids, cleistothecia were not dehisced.

Seed transmission of *E. pisi*

The seeds harvested from plants heavily infected with powdery mildew were planted in a greenhouse in 1996 and 1997. A total of approximately 4200 plants of three susceptible cultivars/lines, Radley, Trump, and JI 1758, were evaluated for the development of powdery mildew symptoms. Upon examination of individual plants, none of the plants were observed with symptoms.

Alternate host(s)

A number of dicot weed species and herbs were found naturally infected with powdery mildew in the vicinity of field pea fields in southern Manitoba and Winnipeg (Table 5.2). These weed and herb species were highly infected under natural conditions in August/September 1996 and 1997. Disease-free field pea leaves of a susceptible cultivar, Trump, were inoculated using powdery mildew inoculua from white clover (*Trifolium repens* L.), sweet pea (*Lathyrus odoratus* L.), dandelion (*Taraxacum officinale* Weber.), pineapple weed (*Matricaria matricarioides* (Less.) Porter, broad leaved plantain (*Plantago major* L.) and prostate knot weed (*Polygonum aviculare* L.) using a detached leaf assay (Warkentin et al. 1995). None of the tested inoculua infected field pea. None of chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medikus), field bean (*Phaseolus vulgaris* L.) and faba bean (*Vicia faba* L.) were infected with the inoculua of field pea powdery mildew. Control plates with susceptible field pea leaves were severely infected.

Sweet clover (*Melilotus alba*) plants were heavily infected with powdery mildew in the vicinity of Winnipeg in early June 1997. Mycelia may have survived on the plant due to the perennial nature of the species. Attempts were made to infect pea with conidia from these plants in a detached leaf assay, but the pea leaves were not infected. Microscopic examination of the conidia revealed that the conidia were borne in a chain of 4-8 in a conidiophore as opposed to a single conidium in a conidiophore of *E. pisi* on pea (Faloon et al. 1989).

Table 5.1 Measurements of *E. pisi* reproductive structures.

Reproductive Structures	**N[a]**	**Mean(μ)**	**SD[b]**	**CV%[c]**	**Range (μ)**
Cleistothecia (diameter)	63	101.4	8.9	8.7	86.4 to 135.0
Asci (length)	63	63.3	2.8	4.4	59.4 to 70.2
Asci (width)	63	38.3	2.7	7	32.4 to 43.2
Ascospores (length)	63	21.8	2.5	11.4	16.2 to 27.0
Ascospores (width)	63	11.5	1.7	14.7	8.1 to 13.5
Conidia (length)	63	36.2	4.8	13.2	27.0 to 45.9
Conidia (width)	63	14.8	1.4	10	13.5 to 18.9

[a] = Number of observations, [b] = Standard deviation, [c] = Coefficient of variation.

Table 5.2 Dicot plant species found infected with powdery mildew in the fall of 1996 and 1997 in Winnipeg and vicinity.

Common name	Botanical name	Family
Perennial sow thistle	*Sonchus arvensis* L.	*Asteraceae*
Pumpkin and squash	*Cucurbita spp.*	*Cucurbitaceae*
Cucumber	*Cucumis sativus* L.	*Cucurbitaceae*
Dandelion	*Taraxacum officinale* Weber.	*Compositae*
Pineapple weed	*Matricaria matricarioides*(Less.) P.	*Compositae*
Canada fleabane	*Erigeron canadensis* L .	*Compositae*
Alfalfa	*Medicago sativa* L.	*Leguminosae*
Sweetpea	*Lathyrus odoratus* L.	*Leguminosae*
White clover	*Trifolium repens* L.	*Leguminosae*
Sweet clover	*Melilotus spp.*	*Leguminosae*
Hemp nettle	*Galeopsis tetrahit* L.	*Labiatae*
Broad leaved plantain	*Plantago major* L.	*Plantaginaceae*
Prostrate knot weed	*Polygonum aviculare* L.	*Polygonaceae*
Striate knot weed	*P. achoreum* Blake	*Polygonaceae*
Rose	*Rosa spp.*	*Rosaceae*

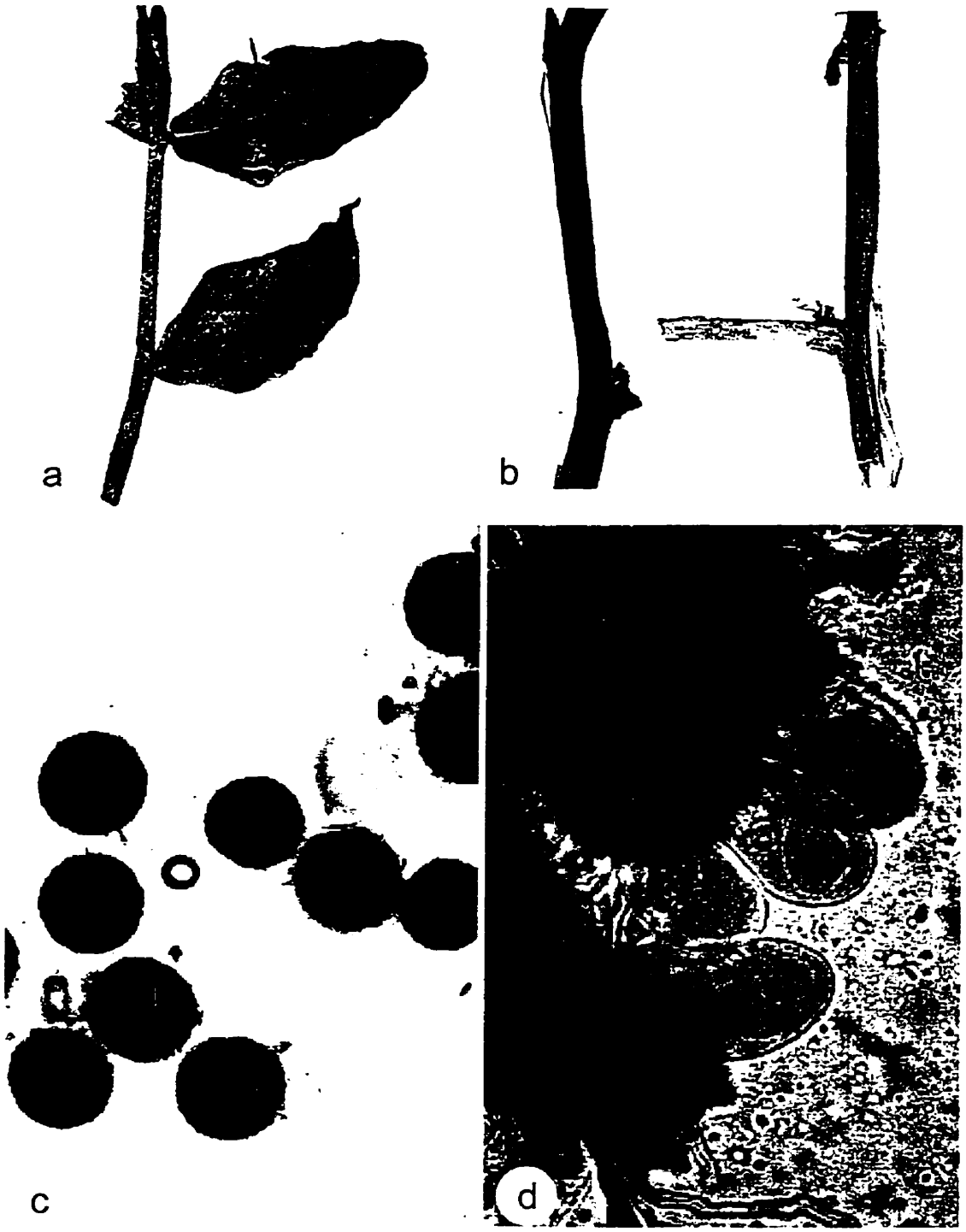

Figure 5.1 *E. pisi* (a) Cleistothecia on pea leaflets and petiole, (b) Cleistothecia overwintered on field debris, (c) Cleistothecia under microscope x 780 and (d) Developing ascospores and ascus in cleistothecia x 1875.

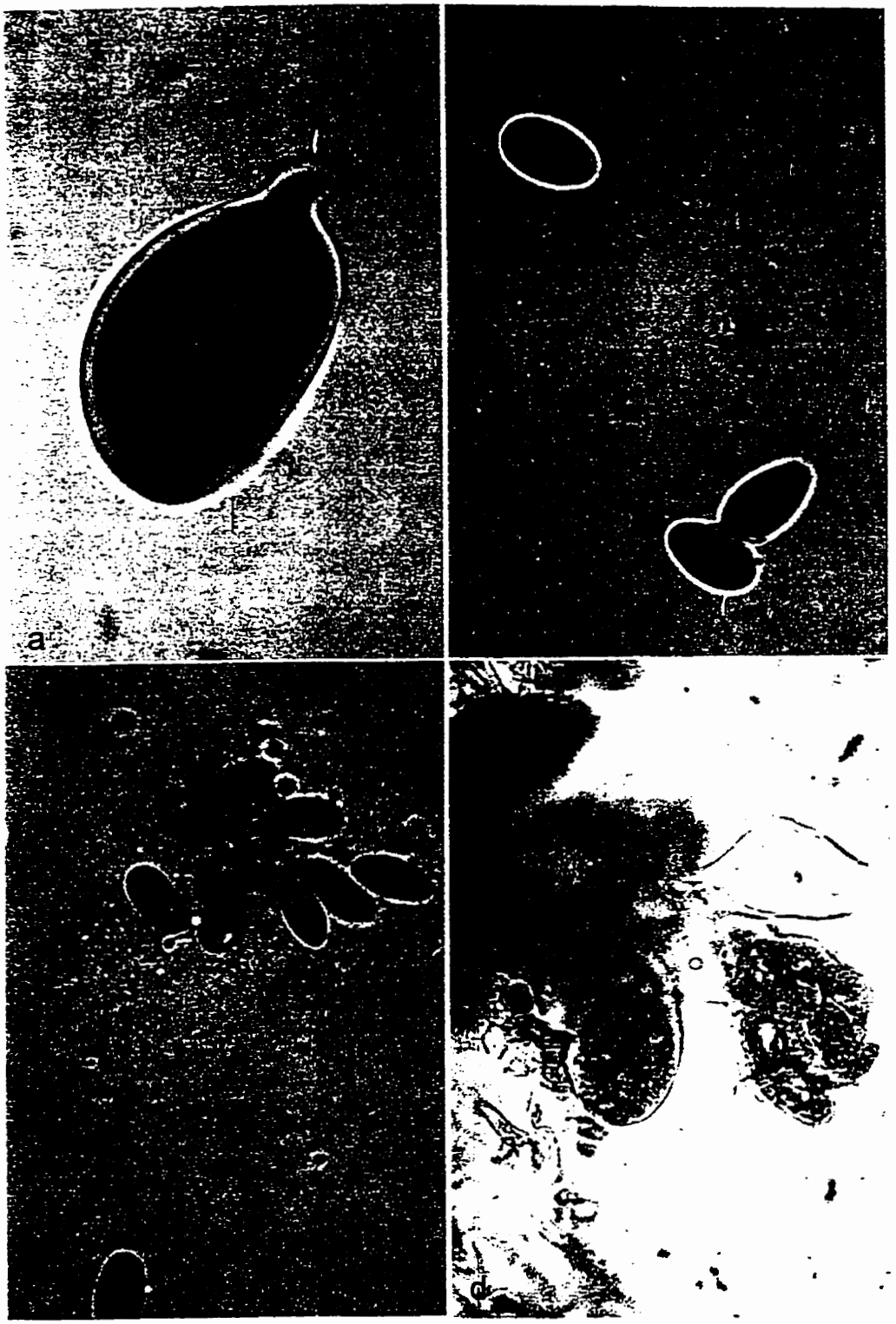

Figure 5.2 *E. pisi* (a) Ascospores in an intact ascus x 3125, (b) Ascospores released from an ascus x 3125, (c) Vacuolated ascospores x 1875 and (d) degrading ascospores in an ascus x 1875.

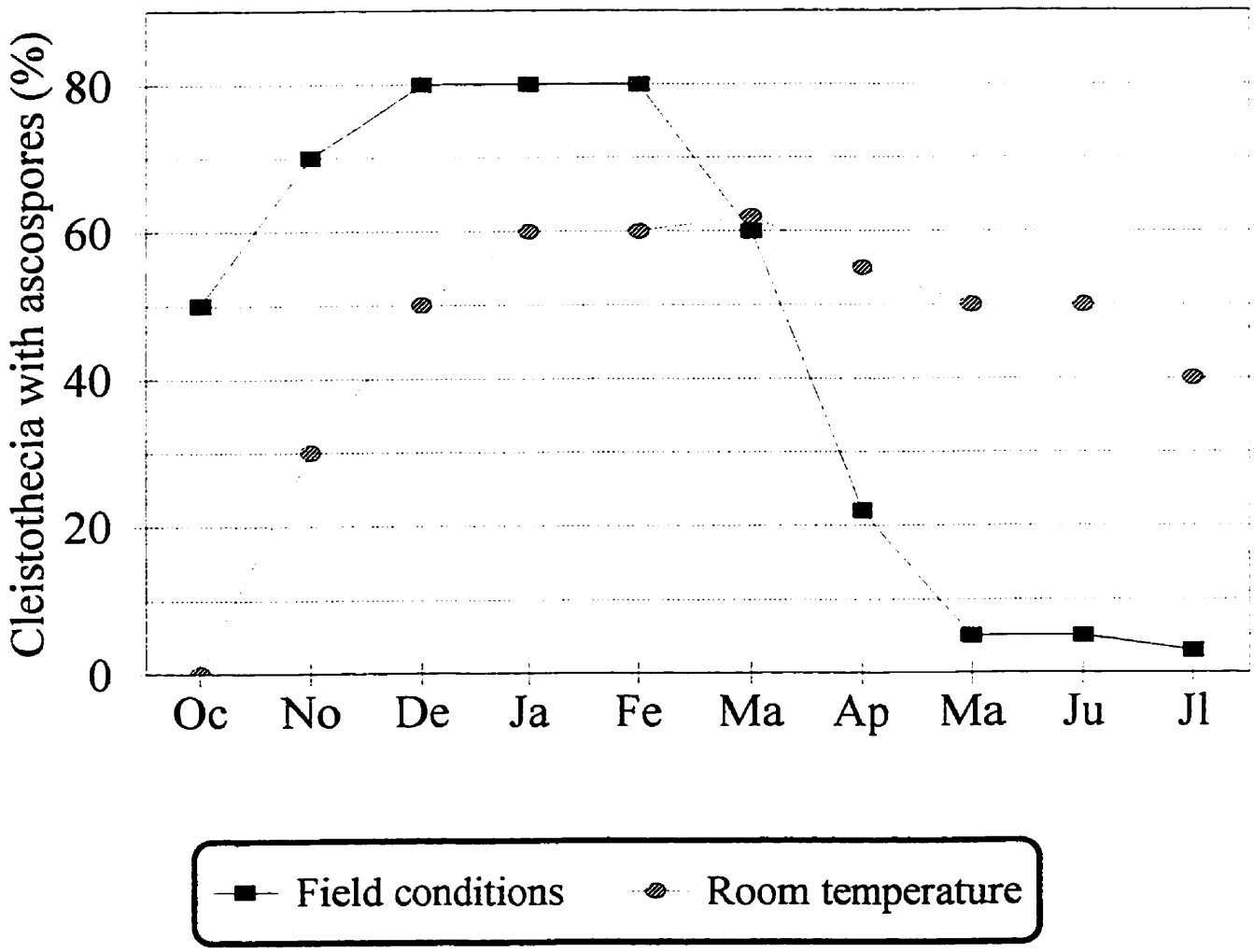

Figure 5.3 Observations on *E. pisi* ascospore development under field conditions in Manitoba and at room temperature during the winter of 1996/97. Oc=October, No=November, De=December, Ja=January, Fe=February, Ma=March, Ap=April, My=May, Ju=June, Jl=July.

Figure 5.4 *E. pisi* (a) Conidia x 1250, (b) Germinated conidia x 1250 (4 h after inoculation) and (c) Germinated conidia x 1250 (24 h after inoculation).

5.5 Discussion

The microscopic examination of cleistothecia during the winter of 1996/97 revealed that ascospores present in cleistothecia on field pea stubble may have the potential to serve as the primary source of inoculum for initiation of the disease in the spring. The fact that the leaves closest to the base of the plants are initially infected (Reiling 1984) may suggest that infection by ascospores from cleistothecia on the soil surface in the immediate vicinity may occur, rather than the initial infection arriving from conidia from some more distant source. However, if the inoculum is present in the close vicinity of a fully susceptible crop, it is interesting to note that powdery mildew does not appear before mid-July in Manitoba.

Several authors have indicated that cool and alternating temperatures, low host nutrition and senescing leaves are necessary for the development of cleistothecia (Cherewick 1944, Pierce 1970, Agrios 1988). However, Smith (1970) reported that in addition to a favourable environment, the presence of two mating types (antheridium and ascogonium) were necessary for formation of cleistothecia because of the heterothallic nature of *E. pisi*. Thus, it appears that the two mating types are common in the Manitoba population of *E. pisi*.

Smith and Wheeler (1969) were unable to infect field pea with ascospores because of undehisced cleistothecia. In the present investigation, infection did not occur of field pea leaves with ascospores as well. In one of our inoculation techniques, cleistothecia were crushed in water and sprayed onto field pea plants without successful infection occurring. The ascospores may have degenerated due to immersion in water. Peries

(1962) reported that immersion of powdery mildew conidia in water for as brief as 3 minutes can kill 50% of conidia.

One or few vacuoles of differing sizes were observed (Figure 5.2c) in ascospores after December 1996. The number and size of the vacuoles increased with time. Significance of vacuole development on ascospores is not known, although vacuoles on mycelium and conidia have been reported (Yarwood 1978). Vacuole formation may be a pre-degradation symptom of the ascospores.

The annual nature of the field pea crop precludes survival as mycelium on host stems, but perennation in the seed and survival on perennial host are possible alternatives. Observations on seed transmission revealed that it is very unlikely that *E. pisi* is transmitted through the seed. Although, some workers have suggested that *Erysiphe* species can perennate as mycelium in seed such as with pea (Crawford 1927, Uppal et al. 1936), or on dead straw such as with wheat and barley (Cherewick 1944), their statements were not supported by microscopic examination of straw or seed or by macroscopic observation of young seedlings grown under controlled conditions from supposedly infected seed. It was also unclear how powdery mildew mycelium on or in the seed could give rise to infection on the leaves. It seems unlikely that mycelium borne externally on the stem or seed could remain viable. To assume that the mycelium is borne inside the seed coat presupposes a growth habit unproven for powdery mildews (Smith 1969). The infection of pea seed within a pod also appears to presuppose a growth habit unproven for *E. pisi*.

Although successful infection of different legume hosts by *E. pisi* has been reported (Dixon 1978, Hirata 1986), none of the inoculua from the weed species that were tried infected field pea and none of the tested legume crops were infected with a powdery mildew that could infect field pea in the present investigation. This observation suggests that powdery mildew found on these weed species is not *E. pisi*. Similarly, Reiling (1984) stated that only the "pea form" of *E. pisi* infected pea out of three biological forms reported in other legume species. Smith (1969) studied cross-inoculation of *E. polygoni* on pea and other hosts and reported that conidia from *Lathyrus odoratus* produced sporulating mildew colonies on pea. However, conidia from pea did not produce sporulating colonies on *L. odoratus*. On microscopic examination, it was observed that cleistothecial appendages of powdery mildew of *Lathyrus odoratus* were quite abundant and different than cleistothecia of powdery mildew of pea.

One of the interesting features of *E. pisi* on pea is the late appearance of the disease in western Canada. Studies in Manitoba indicated that the disease first appears around July 17-21 (Ali-Khan and Zimmer 1989). If cleistothecia or an alternate host were responsible for the overwintering, then an earlier development of symptoms in the field would be expected. Ruppel et al. (1975) reported the sequential occurrence of sugar beet powdery mildew (*E. polygoni* DC.) from southern to northern USA. Movements of conidia of the cereal powdery mildews over comparatively long distances are an important feature in the epidemiology of the disease (Yarwood 1944, Harmansen 1964, Yarwood 1978). Thus, a possible explanation of the late appearance of powdery mildew in Manitoba is that conidia may have spread from warmer areas, i.e. from the northern

USA. Conidia of powdery mildew are quite hardy and germinate even at low relative humidity (Brodie and Neufeld 1942, Yarwood 1978) and, was observed on a glass slide at room temperature (Figure 5.4).

CHAPTER 6

IDENTIFICATION OF COUPLING AND REPULSION PHASE RAPD MARKERS FOR THE POWDERY MILDEW RESISTANCE GENE *er-1* IN PEA

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6.1 Abstract

Powdery mildew is a serious disease of pea caused by the obligate parasite *Erysiphe pisi* Syd. Random amplified polymorphic DNA (RAPD) analysis has emerged as a cost effective and efficient marker system. The objective of this study was to identify RAPD markers for the powdery mildew resistance gene *er-1*. The resistant cultivar Highlight (carrying *er-1*) and the susceptible cultivar Radley were crossed and F_3 plants were screened with Operon (OP) and University of British Columbia (UBC) primers, using bulked segregant analysis. A total of 416 primers were screened of which amplicons of three Operon primers, OPO-18, OPE-16 and OPL-6 were linked to *er-1*. OPO-18_{1200} was linked in coupling (*trans* to *er-1*) and no recombinants were found. OPE-16_{1600} (4 ± 2 cM) and OPL-6_{1900} (2 ± 2 cM) were linked in repulsion (*cis* to *er-1*). The fragments OPO-18_{1200} and OPE-16_{1600} were sequenced and specific primers designed. The specific primer pair Sc-OPO-18_{1200} will be useful in identifying homozygous resistant individuals in F_2 and subsequent segregating generations. Sc-OPE-16_{1600} will have greatest utility in selecting heterozygous BC_nF_1 individuals in backcross breeding programs.

Key words: Bulked segregant analysis, *E. pisi*, pea, RAPD.

6.2 Introduction

Molecular markers are useful tools for marker-assisted selection (MAS) in crop improvement. Restriction fragment length polymorphism (RFLP), though commonly used for plant genome analysis in the past (Tanksley et al. 1989), has limited use because of the cost involved and the use of radioisotopes. Random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990, Williams et. al. 1990) analysis involves the amplification of random segments of genomic DNA using polymerase chain reaction (PCR) methodology (Saiki et al. 1988). RAPD analysis is an efficient marker detection system for disease resistance genes and plant breeding programs (Michelmore et al. 1991, Penner et al. 1993a, 1993b). Within six years of its inception, RAPD analysis has become the dominant technology in many laboratories.

Pea (*Pisum sativum* L.) is an important grain legume crop grown worldwide for human food, animal feed, forage and green manure (Marx 1984). The area in pea cultivation in western Canada has increased from 74,000 ha in 1985 to 800,000 ha in 1995 (Statistics Canada, 1995). Of the diseases that infect pea in western Canada, powdery mildew, caused by the obligate parasite *Erysiphe pisi* Syd. (Syn. *E. polygoni* DC.), may cause severe damage to late-seeded crops or when hot, dry conditions occur in July. Most of the pea production area in western Canada is planted with cultivars susceptible to powdery mildew (Warkentin et al. 1996a). Severe infection may result in 25 to 30% yield reduction (Munjal et al. 1963) along with a deterioration of seed quality. Resistance to this pathogen is controlled by the recessive genes *er-1* and/or *er-2* (Heringa

et al. 1969). All of the resistant Canadian cultivars (Highlight, AC Tamor and Tara) carry only *er-1* (Chapter 3).

Combining both resistance genes, *er-1* and *er-2*, in a cultivar should increase the durability of resistance. The identification of molecular markers for *er-1* and *er-2* would greatly facilitate the incorporation of both genes into a cultivar. Although Timmerman et al. (1994) developed a repulsion-phase RAPD marker for the powdery mildew resistance gene *er-1*. this marker was not applicable to Canadian germplasm. Therefore. the objective of this study was to develop user-friendly DNA-based markers linked to *er-1* for use in Canadian pea breeding programs. In this paper. we report the development of three RAPD markers closely linked to *er-1*, of which one is in coupling and two are in repulsion phase.

6.3 Materials and methods

Plant materials and DNA extraction

Parents, F_1 and the F_2 progeny of a cross between the resistant cultivar Highlight (*er-1*) and the susceptible cultivar Radley were screened under field conditions at the Agriculture and Agri-Food Canada. Morden Research Centre. Morden. Manitoba in 1994 to determine the disease reaction of individual plants. F_2-derived F_3 families were grown under field conditions in 1995. Powdery mildew infection occurred naturally in both years. Analysis of disease reaction in F_3 families was used to identify homozygous susceptible F_2 plants. A total of 22 homozygous resistant and 35 homozygous susceptible plants were used to screen decamer primers with GC contents of 60 to 70%. Genomic

DNA was extracted from freeze-dried leaflets and stipules, harvested from 2-to 3-week-old seedlings using the cetyltrimethylammonium bromide (CTAB) method (Kleinhofs et al. 1993). Lyophilized leaflets were carefully ground in a morter and pestle to a fine powder with sterile grinding sand and stored at -20^0 C until the next step. Twenty mL of pre-warmed (65^0 C) buffer S (110 mM Tris, pH 8.0; 55 mM EDTA, pH 8.0; 1.54 M NaCl; 1.1 % CTAB) was added, followed by 15 micro liter (μL) of fresh Proteinase K solution (20 mg/mL in cold 1x TE (10 mM Tris, 1 mM EDTA) and immediately vortexed. Then, 2.2 mL of 20% SDS was added and gently mixed. Then the samples were incubated in a 65^0 C water bath for 2 h with inversion every 30 minutes.

After removing the samples from the water bath, 10 ml of phenol (200 ml 1x TE, pH 8.0, added to 500 g solid phenol) and 10 mL of chloroform : isoamoyl alcohol (IAA) (24:1) were added. Individual samples were mixed thoroughly for 15 to 20 minutes and centrifuged for 15 to 20 minutes at 2000 to 3000 rpm. The top phase was carefully removed and transferred to a fresh 50 mL Corning tube. DNA was precipitated with 95% cold ethanol (2 to 2.5 volumes) or isopropanol (0.6 volume).

The precipitated DNA was removed with a glass hook, and briefly rinsed with 70 % ethanol; lightly touched on to a clean, sterile Kim-wipe to blot off remaining ethanol and transferred to a fresh tube containing 2 ml of 1x TE. After the DNA was dissolved, RNAaseA (1 ul of a 10 mg / mL RNAaseA, for each mL of TE) was added and incubated for an hour at room temperature or overnight at 4^0 C. Working solutions were quantified and stored at 4^0 C. The stock solution was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of 95% ethanol and stored at -20^0 C. (Note: If samples

were not lyophilized, grinding was done in a chilled mortar and pestle with 20 to 30 mL of liquid nitrogen. For 100 mg of lyophilized leaf samples, half of the given amount of buffers and solutions was used for DNA extraction without affecting the quality and quantity of DNA).

RAPD analysis

Two separate DNA pools were prepared from 10 homozygous resistant plants and 10 homozygous susceptible plants respectively. Each pool contained an equal amount of DNA from each individual plant. Operon (Operon Technologies, Inc. CA.) primers, OPA to OPQ (each series containing 20 primers) and University of British Columbia (UBC) primers, UBC 101 to 200, were screened between the pools. PCR volumes were 25 ul, overlayed with 15 ul of light mineral oil (Fisher). Each reaction consisted of 1x Promega Biotech Taq activity buffer, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase, 800 uM total dNTPs, 20 pMoles of primer and 40 ng of genomic DNA. Substrate DNA in PCR reactions was denatured at 94^0 C for 7 minutes and amplified for 35 cycles (94^0 C 5 sec., 36^0 C 30 sec., 72^0 C 60 sec.) in a Perkin-Elmer Cetus thermal cycler. Following the final cycle, all strands were completed with a 10 min. 72^0 C segment followed by storage at 4^0 C. If the samples could not be electrophoresed within 12 h of the PCR run, they were stored at -20^0 C. Electrophoresis was performed in 1.6% agarose with a 1x Tris/acetate/EDTA (TAE) buffer for 3.5 to 4 hours at 72 V (constant voltage). Ethidium bromide-stained gels were visualized on an ultraviolet light transilluminator and photographed.

Cloning RAPD products

Fragment Preparation: Genomic DNA was amplified with appropriate primer(s) and electrophoresed on an agarose gel to separate the fragments. The fragment to be cloned was excised from the gel using a weak UV light and reamplified. Fragments were phosphorylated either using kinased primers or were kinased after the amplification (Sambrook et al. 1989). The kinase reaction was performed as follow: 1x forward reaction buffer, 15 to 20 µL primer (20-25 pmol/µL) or the fragment DNA, 1mM ATP, 5 units (U) T4 Kinase to a total volume of 20 to 30 µL. The samples were incubated at 37^0 C for 30 minutes followed by 65^0 C for 10 minutes.

The re-amplified fragments were excised (approximately 15 to 20 fragments / clone) using a weak UV light. The fragments were placed in a 1.5 mL Eppendorf tube and stored at -20^0 C overnight. The freeze and squeeze method (Tautz and Renz 1983) was used to recover DNA fragments. With the gloved thumb, firm pressure was applied to a chunk of agarose containing the diagnostic fragment on parafilm. The extruded liquid was pipetted into a fresh Eppendorf tube and extracted with phenol: chloroform (1:1). DNA was precipitated with 95% ethanol and resuspended with sterile water. A one uL aliquot of DNA was run on an agarose gel along with a known marker and T-tailed plasmid to estimate quantity of DNA and plasmid for ligation.

Restriction/digestion and T-tailing of plasmid: Restriction and T-tailing of pUC 19 was performed as follows: 1 microgram (µg) pUC 19, 3 U *Sma*I, 1x buffer A, and sterile water was mixed to a total volume of 20 µL. Samples were incubated at 25^0 C for 2 hours

and enzymes were inactivated at 65^0 C for 10 minutes. Restricted DNA was electrophoresed on an agarose gel to verify restriction and to estimate the quantity of DNA. Linearized plasmid fragments were recovered from the agarose gel with phenol chloroform as described above. For T-tailing reaction of the restricted plasmid, 0.2 mM dTTP, 1x PCR buffer, 1.5 mM $MgCl_2$, 1 U *Taq*, and 1 µg pUC 19 was added to a total volume of 25 µL. Samples were incubated at 72^0 C for 2 hours. DNA was precipitated with 95% ethanol and resuspended in sterile water (10 to 20 µL). One µL was run on agarose gel along with re-amplified fragment to determine concentrations for ligation reaction (equimolar ratio of insert to plasmid). Quantity of insert (nanogram (ng)) required was determined by: Quantity of plasmid (ng) x (# bp of insert / # bp of plasmid).

Ligation and transformation: Equimolar amounts of T-tailed plasmid DNA and insert DNA were mixed with 1x ligase buffer, 5 U T4 DNA ligase and sterile water was added to a total volume of 20 µL (or 10 µL) for ligation reaction. Contents were mixed, quickly spun and incubated overnight at 15^0 C.

Competent *E.coli* cells were thawed on ice and transferred to a sterile microfuge tube using chilled pipette tips. Approximately 30 to 50 ng of ligation mix (plasmids with inserts) was added and gently mixed. Tubes with reaction mix were stored on ice for 30 minutes and were heat shocked at 42^0 C for 90 seconds. Tubes were immediately returned to ice and chilled for 1 to 2 minutes. The reaction mix with *E. coli* cells were added to 800 µL of pre-warmed LB (Luria Bretani) media and incubated at 37^0 C for 45 minutes with gentle shaking. Recovered cells were plated on LB plates (100, 200

and 400 μL) and incubated overnight at 37^0 C. Ampicillin was used as selection media on LB plates.

Individual *E.coli* colonies were picked with a sterile tip and heated to 90^0 C for 10 minutes in 10 ul of double distilled water to lyse cell contents. A one uL aliquot of the supernatant was used as substrate for PCR analysis after a quick spin. Fragment insertion of the correct size was confirmed by PCR analysis using M13. -40 forward and reverse primers (Figure 6.1). Positive colonies were grown in LB media overnight and plasmid DNA was extracted by the alkali-lysis method (Sambrook et al. 1989). DNA was sequenced by the dideoxy chain termination method (Sanger et al. 1977) using M13. -40 forward and reverse primers by the National Research Council, Plant Biotechnology Institute (PBI) Saskatoon, Saskatchewan. Allele specific primers were designed using the program Oligo 4.

Linkage analysis

Maximum-likelihood estimation was used to calculate recombination frequency (r = R/N), where r = recombination frequency, R = number of recombinants and N = total number of progeny tested. The maximum-likelihood estimate of the standard error of r was $SE_r = \sqrt{r(1-r)/N}$ (Adams and Joly 1980).

6.4 Results

The cultivar Highlight was highly resistant to powdery mildew and Radley was fully susceptible, while all F_1 individuals were susceptible, indicating that resistance was a

recessive trait. The F_2 population consisted of 78 susceptible and 23 resistant plants. These results are in agreement with a 3:1 segregation ratio with monogenic inheritance (Chi-squared = 0.27, P=0.5 to 0.7). Out of 73 susceptible F_2-derived F_3 families screened under field conditions, 26 were nonsegregating susceptible as expected (Chi-squared = 0.20, P=0.5 to 0.7) (Chapter 3).

A total of 416 Operon and UBC primers were screened on resistant and susceptible bulks. The number of bands amplified per lane per primer ranged from one to nine with an average of four. Initially, more than 10 polymorphic fragments were identified between the pools. However, in repeated experiments, only three Operon primers OPO-18, OPE-16, and OPL-6 amplified polymorphic fragments in one pool only.

OPO-18 (5'-CTCGCTATCC-3') amplified a fragment of approximately 1200 base pairs (bp) in the susceptible parent Radley. The polymorphic fragment cosegregated with susceptibility in the segregating F_3 population (Figure 6.2). This was a coupling-phase linkage as susceptibility is a dominant trait. The polymorphic fragment was cloned and sequenced from both ends. Specific primers were designed using the program Oligo-4, with a forward sequence of 5'-CCCTCTCGCTATCCAATCC-3' and a reverse sequence of 5'-CCTCTCGCTATCCGGTGTG -3'. This primer pair was designated as Sc-OPO-18_{1200}. Sc-OPO-18_{1200} amplified a fragment of appropriate size in Radley and the susceptible progeny at an annealing temperature of 66^0 C. This fragment was absent in the resistant cultivar Highlight and the resistant progeny (Figure 6.3). Sc-OPO-18_{1200} was tested on 57 segregating individuals (22 resistant and 35 susceptible) and no recombinants were found.

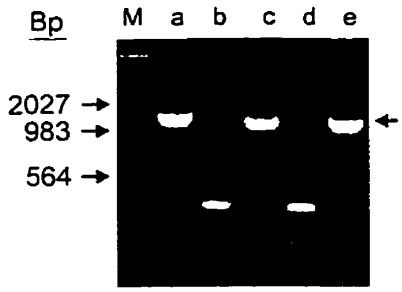

Figure 6.1 PCR analysis of positive *E. coli* colonies using M13, -40 forward and reverse primers, showing presence of inserts in lanes a, c and e and absence of inserts in lanes b and d in pUC19. M=molecular weight markers (Lambda DNA digested with *Eco*RI and *Hin*dIII). The arrow on the right indicates the polymorphic fragment.

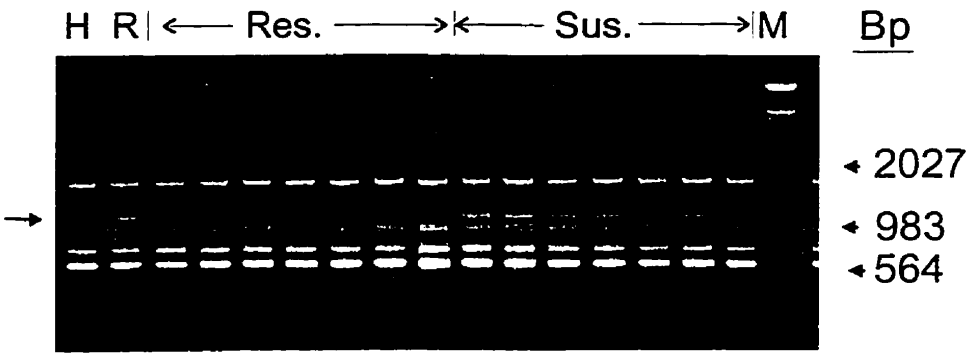

Figure 6.2 Polymorphic RAPD fragment amplified in the susceptible parent Radley pea and susceptible progeny with OPO-18. H=Highlight, R=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny, M=Molecular weight marker (Lambda DNA digested with *Eco*RI and *Hin*dIII). The arrow on the left indicates the polymorphic fragment.

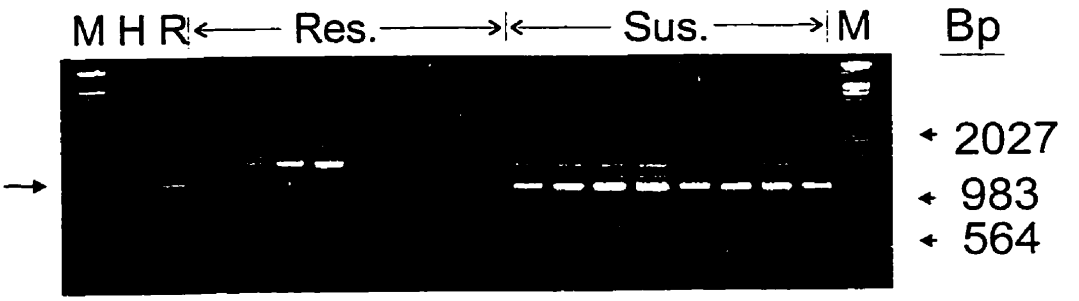

Figure 6.3 Polymorphic amplicon amplified by the specific primer Sc-OPO-18_{1200} in Radley pea and susceptible progeny. H=Highlight, R=Radley, Res.=Resistant progeny, Sus.= Susceptible progeny, M=Molecular weight markers (Lambda DNA digested with *Eco*RI and *Hin*dIII). The arrow on the left indicates the polymorphic fragment.

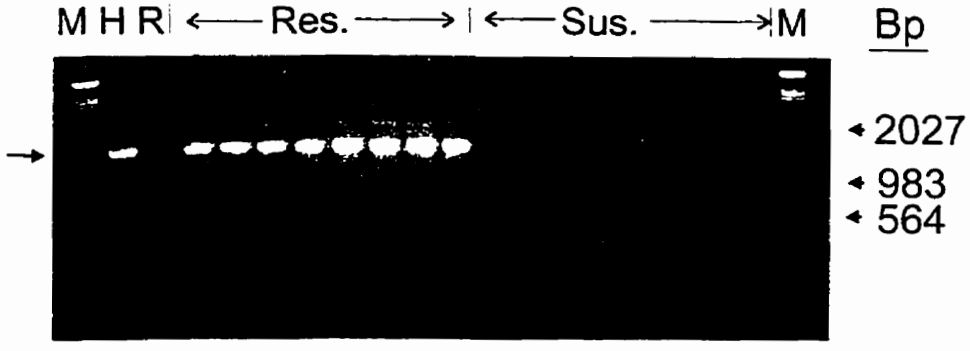

Figure 6.4 Polymorphic fragment amplified by the specific primer pair Sc-OPE-16_{1600} in Highlight pea and resistant progeny. H=Highlight, R=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny, M=Molecular weight markers (Lambda DNA digested with *Eco*RI and *Hin*dIII). The arrow on the left indicates the polymorphic fragment.

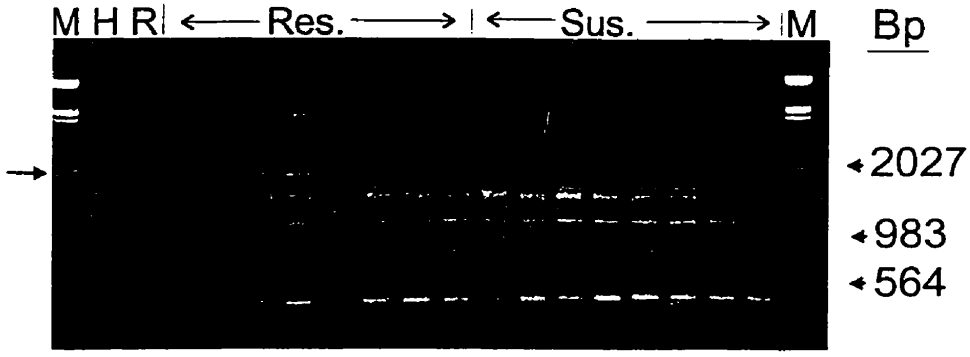

Figure 6.5 Polymorphic RAPD fragment amplified by the primer OPL-6, in Highlight pea and resistant progeny. H=Highlight, R=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny, M=Molecular weight markers (Lambda DNA digested with *Eco*RI and *Hin*dIII). The arrow on the left indicates the polymorphic fragment.

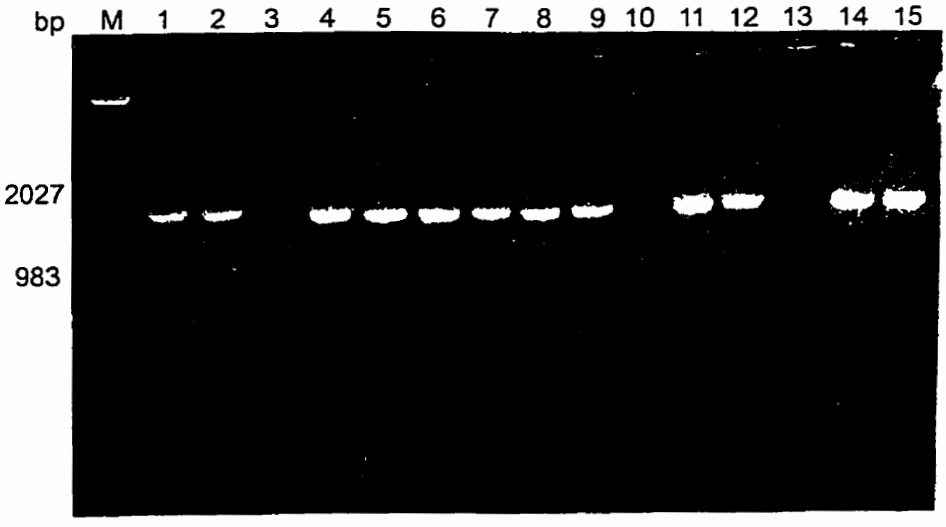

Figure 6.6 Polymorphic fragment amplified by the specific primer pair Sc-OPE-16_{1600} in different pea lines. 1=Highlight, 2=AC Tamor , 3=Tara , 4=JI 2302, 5=JI 1758, 6=JI 1210, 7=JI 1951, 8=JI 1648, 9=JI 82, 10=JI 210, 11=JI 2480, 12=JI 1559, 13=Radley, 14=Trump, 15=Montana. M=Molecular weight markers (Lambda DNA digested with *Eco*RI and *Hin*dIII).

Primer OPE-16 (5'-GGTGACTGTG-3') amplified a polymorphic fragment of approximately 1600 bp in the resistant parent Highlight and the resistant progeny. This was a repulsion-phase linkage, as resistance is recessive. The fragment was present in all of the resistant progeny and two of the susceptible progeny tested, indicating a linkage distance of 4 ± 2 cM to *er-1*. Specific primers were designed with a forward sequence of 5'-GGTGACTGTGGAATGACAAA-3' and a reverse sequence of 5'-GGTGACTGTGA CAATTCCAG-3'. This primer pair was designated as Sc-OPE-16_{1600}. Sc-OPE-16_{1600} amplified the specific amplicon at an annealing temperature of 67^0 C in Highlight and resistant individuals, whereas the fragment was completely absent from Radley and susceptible individuals (Figure 6.4).

Similarly, primer OPL-6 (5'-GAGGGAAGAG-3') amplified a polymorphic fragment of approximately 1900 bp in Highlight and resistant individuals (Figure 6.5). The fragment was present in Highlight and all resistant individuals tested and absent in Radley and all susceptible individuals except one, indicating a linkage distance of 2 ± 2 cM to *er-1*. This was a repulsion-phase marker. Specific primers were not developed, since we have already developed Sc-OPO-16_{1600}, as a specific primer pair for repulsion-phase linkage.

6.5 Discussion

Timmerman et al. (1994) reported a sequence characterized amplified region (SCAR) marker (Paran and Michelmore 1993) $PD10_{650}$ for the powdery mildew resistance gene *er-1* present in the pea line, "Slow". We synthesized the specific primer pair $PD10_{650}$ (5'-

GGTCTACACCTCATATCTTGATGA-3' and 5'-GGTCTACACCTAAACAGTGTCC-GT-3') and attempted to use this marker in Canadian pea cultivars. A total of 15 pea lines were evaluated with this primer pair including four susceptible lines. Primer pair $PD10_{650}$ amplified an appropriate size amplicon in all tested lines (except JI 1758) including Highlight and susceptible lines such as Radley, Trump and JI 1648. The amplicons from Highlight and Radley were sequenced and compared; sequence differences were not detected. Further, "Slow" was susceptible to powdery mildew in our tests under field conditions and in the greenhouse. Thus, $PD10_{650}$ was not useful for MAS in our breeding program and we proceeded to identify other RAPD markers.

For introgression purposes, and in the absence of selection for powdery mildew resistance, the recessive nature of *er-1* requires a generation of selfing after every odd numbered backcross to obtain homozygous resistant BC_nF_2 parents for the next backcross cycle. Marker-assisted selection provides an ideal strategy for transferring *er-1* into agronomically superior pea cultivars. We have identified 3 RAPD markers for *er-1*, of which one is in coupling-phase and two are in repulsion-phase. Haley et al. (1994b) reported that molecular markers are effective in MAS if present in repulsion-phase. However, availability of both coupling- and repulsion-phase markers would be more useful in breeding programs (Johnson et al. 1995).

In the present investigation, 15 pea lines were evaluated including four susceptible lines, for polymorphisms. The polymorphic amplicon from the coupling-phase specific primer Sc-OPO-18_{1200} was present in the susceptible cultivars/lines, Radley, JI 1758, and JI 1648 and absent in the resistant cultivars/lines Highlight, Tara and JI 2302. The

polymorphic amplicon from repulsion phase primer Sc-OPE-16_{1600} was present in the resistant cultivars/ lines Highlight, AC Tamor, JI 2302, JI 1210, JI 1951, JI 82, JI 2480, JI 1559 and absent in the susceptible cultivar Radley (Figure 6.6). The primer Sc-OPE-16_{1600} will be most useful in MAS of heterozygous BC_n F_1 individuals for Highlight-derived resistance during backcross breeding. The primer Sc-OPO-18_{1200} will have greatest utility in identifying homozygous resistant individuals in F_2 and subsequent segregating generations. A further advantage of Sc-OPE-16_{1600} is that it can be used to identify *er-1* genotypes without electrophoresis (Gu et al. 1995). Amplified products from SCAR primers can be visualized qualitatively through the analysis of ethidium bromide fluorescence.

The obligate parasitic nature of *Erysiphe pisi* makes it impossible to maintain the pathogen in culture and difficult to apply for use in screening segregating progeny in controlled growth conditions. Occurrence of disease under field conditions is dependent upon the occurrence of appropriate environmental conditions. However, selection of pea genotypes containing both *er-1* and *er-2* on the basis of visual scoring is very difficult, since *er-1* alone provides a high level of resistance (Chapter 3). Therefore, the development of reliable and user-friendly specific primers closely linked to *er-1* is useful for breeders in gene pyramiding. The pyramiding of *er-1* and *er-2* in a cultivar would increase the durability of resistance.

CHAPTER 7

IDENTIFICATION OF AFLP MARKERS FOR THE POWDERY MILDEW RESISTANCE GENE *er-2* IN PEA

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7.1 Abstract

Powdery mildew of pea, caused by *Erysiphe pisi*, is a serious disease which may cause severe yield and quality losses. Resistance to this disease is conditioned by the recessive genes *er-1* and/or *er-2*. Line JI 2480 has been previously shown to carry *er-2*. Homozygous F_4 progeny of the cross JI 2480/Radley were used to identify markers linked to *er-2*. A total of 128 amplified restriction fragment polymorphism (AFLP) primer combinations (8 *Eco*RI and 16 *Mse*I primers) were screened of which three primer combinations were linked in coupling phase (*trans* to *er-2*) and one primer combination was linked in repulsion phase (*cis* to *er-2*). Among these primer combinations, one was tightly linked (5 ± 2 cM) to *er-2*. These markers will be useful to identify JI 2480-based resistance to powdery mildew of pea. AFLP analysis will offer an efficient means for genetic analysis of pea.

Key words: AFLP, *E. pisi*, *er-2*, pea, powdery mildew.

7.2 Introduction

With the introduction of polymerase chain reaction (PCR) based DNA markers (Saiki et al. 1988), novel marker technologies such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and microsatellites (Morgante and Olivieri 1993) became available. RAPDs have particularly generated interest as a cost effective and efficient marker system (Penner et al. 1996, Ko et al. 1994). A relatively new marker system, amplified restriction fragment polymorphism (AFLP), has been developed (Zabeau and Vos 1993, Vos et al. 1995). In this method, the reliability of RFLP (Tanksley 1983) is combined with the power of the PCR technique (Vos et al. 1995). This technique has been successfully used to generate genetic maps (Mackill et al. 1996, Schondelmaier et al. 1996), fingerprints (Lin and Kuo 1995, Folkertsma et al. 1996) and to identify markers for disease resistance genes (Thomas et al. 1995).

Powdery mildew, caused by the obligate parasite *Erysiphe pisi* Syd. (Syn. *E. polygoni* DC.), is a serious disease of pea (*Pisum sativum* L) which may result in 25% to 30% yield reduction (Munjal *et al.* 1963). Resistance to this pathogen is controlled by the recessive gene(s) *er-1* and/or *er-2* (Heringa *et al.* 1969, Chapter 3). RAPD markers closely linked to *er-1* have been identified (Timmerman et al. 1994, Chapter 6). Pea line JI 2480 has been previously shown to carry *er-2* (Chapter 3). Combining both resistance genes (*er-1* and *er-2*) in a cultivar should increase the durability of resistance. Identification of molecular markers for *er-2* would facilitate the introgression of this gene in lines carrying *er-1*. Therefore, the objective of this study was to identify AFLP markers linked to *er-2*.

7.3 Materials and methods

Plant materials

Crosses were made between the resistant line JI 2480 *(er-2)* and the susceptible cultivar Radley in 1994. A fraction of the F_1 seed was grown out in a greenhouse to produce F_2 seed. Parents, F_1 and the F_2 population were screened under field conditions at the Agriculture and Agri-Food Canada, Morden Research Centre, Morden, Manitoba in 1995 to determine the disease reaction of individual plants. The F_2-derived F_3 families were grown under field conditions in 1996. Infection of powdery mildew occurred naturally in both years. Disease reaction exhibited by the F_3 families was used to determine homozygous susceptible lines. A total of 42 homozygous resistant and 39 homozygous susceptible lines was used to screen AFLP primers. Genomic DNA was extracted from freeze-dried leaflets or stipules of two-to three-week-old seedlings by the cetyltrimethylammonium bromide (CTAB) method as described in Chapter 6 and quantified using a Spectronic Genesys 5 (Milton Roy) spectrophotometer.

AFLP analysis

Restriction of genomic DNA and ligation of adaptors: The AFLP procedure was performed following the protocol of Vos et al. (1995) with minor modifications. Genomic DNA from each sample was restricted with *Eco*RI and *Mse*I as follows: 1 µg genomic DNA, 1x appropriate restriction buffers for both enxymes, 5 units (U) each of *Eco*RI (Boehringer Manheim) and *Mse*I (BRL) and sterile double distilled water were mixed to a total volume of 20 to 25 µl. Contents were mixed and incubated at 37°C for 2 h and

temperature was raised to 70^0 C for 15 minutes. The smear of restricted DNA was visible in agarose gel indicating that DNA samples were digestible by these enzymes (Figure 7.1).

A 20 μM solution of single-stranded *Mse*I oligo (adaptor) and a 2 μM solution of each single-stranded *Eco*RI oligo were prepared and annealed at 65^0 C for 10 minutes, 37^0 C for 10 minutes and 25^0 C for 10 minutes. Sequence information of adaptors is presented in Table 7.1. Ligation of restricted DNA fragments and adaptors was carried out as follows: 20 to 25 μL of restricted DNA (from above), 1 μL (20 μM) *Mse*I adaptor, 1 μL (2 uM) *Eco*RI adaptor, 1 μL of (10 mM) ATP, 2 μL of 5x reaction buffer, 1 U T4 DNA Ligase, and 4 to 9 μL sterile water to a total volume of 35 μL. Contents were mixed and incubated at room temperature (20° ± 2° C) for 2 hours. A portion of the reaction mix was diluted to 5 ng/μL with $TE_{0.1}$ (10 mM Tris-HCl, 0.1 mM EDTA (pH 7.5) and the stock solution was stored at -20^0 C.

Amplifications: The first (pre-amplification) PCR was performed with one selective nucleotide (*Eco*RI +A, *Mse*I + C) (Table 7.1). Each reaction consisted of 1x Promega Biotech Taq activity buffer, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 800 uM total dNTPs, 30 ng of *Eco*RI primer and 30 ng of *Mse*I primer with 5 ng of genomic DNA. PCR volumes were 50 μL and amplified for 20 cycles at 94^0 C for 30 sec., 56^0 C for 60 sec., 72^0 C for 60 sec. in a MJ research DNA engine. Pre-amplified solutions were diluted 10 fold in $TE_{0.1}$ and using the protocol given above in pre-amplification PCR, selective amplification was performed on the pre-amplified DNA with the *Eco*RI primer + A+ 2 selective nucleotides and *Mse*I primer + C+ 2 selective nucleotides in a total volume of 20

µl. AFLPs were generated using a touchdown-PCR, one cycle of 94^{0}C denaturation for 60 sec., 65^0 C annealing for 60 sec. and 72^0 C extension for 90 sec. followed by 10 cycles with the annealing temperature lowered by 1^{0}C each cycle to 56^0 C. Another 23 cycles were conducted as described above for the pre-amplification, but 30 sec. at 56^0 C. Samples were held at 4^0 C until either frozen to 20^0 C or loaded onto a gel.

Two separate DNA pools were prepared from eight homozygous resistant plants and eight homozygous susceptible plants respectively. Each pool contained an equal amount of pre-amplified DNA from each of the individual plants. All possible primer combinations between 8 *Eco*RI (AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG) and 16 *Mse*I (CAA, CAC, CAG, CAT, CCA, CCC, CCG, CCT, CGA, CGC, CGG, CGT, CTA, CTC, CTG, CTT) primers were screened between the pools with a total of 128 primer combinations. Following amplification, reaction products were mixed with an equal volume (20 ul) stop solution (98% formamide dye, 10 mM EDTA (pH 8.0), and bromo phenol blue and xylene cyanol as tracking dyes). The resulting mixtures were denatured at 94^0 C for 5 minutes and placed immediately on ice until ready to load.

Electrophoresis in polyacrylamide denaturing gels and silver staining: A 5% sequencing gel (19:1 acrylamide:bis-acrylamide, 7 M urea, 1x TBE) was prepared (12.5 mL 40% acrylamide:bis-acrylamide, 42 g urea, 10 mL TBE (Tris, boric acid and EDTA) buffer dissolved in 26 mL double distilled water and final volume to 100 mL). Immediately before pouring the gel, 100 uL of N, N, N', N'-Tetramethylethylenediamine (TEMED) and 100 uL of ammonium persulfate (APS) solution (60 mg APS in 250 µL

doub'e distilled water) was added and gently mixed. After the gel was poured. it was polymerized at room temperature for an hour to overnight. The gel was then pre-run at constant voltage (55 watts for small plates (50 x 21 cm), 80 watts for large plates (50 x 38 cm)) for 45 minutes. Samples (3 μL each) were loaded and electrophoresed for 2 to 2.5 hours.

Preparation of the sequencing plates and gel

Long glass plate preparation: A scrupulously clean glass plate was wiped with a KimWipe tissue saturated with 2 mL of freshly prepared binding solution (3 μL bind silane to 1 mL of ethanol and 0.5% glacial acetic acid). After 4 to 5 minutes. approximately 2 mL of 95% ethanol was applied to the plate and wiped with a paper tissue in one direction and then perpendicular to the first direction using gentle pressure. This wash was repeated three times using a fresh paper towel each time.

Short glass plate preparation: A scrupulously clean plate was wiped using a tissue saturated with Sigma Cote solution (0.5 mL). After 5 to 10 minutes, excess Sigma Cote was removed by wiping the plate with a Kim Wipe tissue. The gel frame was then set and gel solution was poured.

Preparation of solutions

Fix/stop solution (10% glacial acetic acid): 200 mL of glacial acetic acid was added into 1800 mL of double distilled water.

Staining solution: 2 g of silver nitrate and 3 mL of 37% formaldehyde were dissolved in 2 L of ultrapure water.

Developing solution: 120 g of sodium carbonate was dissolved in 4 L of ultrapure water and chilled to 10^0 C. Immediately before using, 6 mL of 37% formaldehyde and 800 uL of sodium thiosulfate (10 mg/ml) was added to the developing solution.

After electrophoresis, plates were carefully separated. The gel was affixed to the long glass plate. The gel, along with long glass plate, was placed in a shallow plastic tray, with 2 L of stop solution and agitated for 30 minutes (or stored overnight, without shaking). The gel was then rinsed three times (two minutes each) with ultrapure water using agitation (stop solution was saved to terminate the developing reaction). The gel was then stained with gentle shaking in a staining solution for 30 minutes.

After removing the gel from the staining solution, it was briefly dipped in ultrapure water, drained, and placed immediately (5 to 7 seconds, including dipping) into the tray of chilled developing solution (2 L). The gel was then agitated until the template bands started to appear and immediately transferred to the next plastic tray with the remaining 2 L of chilled developing solution. Agitation was continued for an additional 2 to 3 minutes or until all bands were visible. Two liters of stop solution (saved from previous use) was directly added to the gel in the developing solution to terminate the developing reaction and incubated for 2 to 3 minutes with gentle shaking. The gel was then rinsed three times with ultrapure water (2 minutes each). Finally, the gel was air dried at room temperature and the image was stored by scanning.

Cloning, sequencing and linkage analysis were performed as described in Chapter 6.

7.4 Results

The line JI 2480 was resistant to powdery mildew and the cultivar Radley was fully susceptible under field conditions in Morden, Manitoba. All F_1 plants exhibited a susceptible reaction. indicating that resistance was recessive. The F_2 population segregated in a three susceptible: one resistant ratio, suggesting monogenic inheritance (Chi- squared 0.29. P= 0.5 to 0.7) (Chapter 3).

A total of 40 to 80 DNA bands per lane was evident in AFLP denaturing polyacrylamide gels (Figure 7.2), as compared to two to eight bands in RAPD analysis followed by agarose gel electrophoresis. Initially, 15 primer combinations were identified polymorphic between the bulks. When these primers were screened among the individuals which constituted the bulk, ten primer combinations showed a 0% to 20% recombination. The entire population of 81 individual lines was screened with these ten primer combinations, and four primer combinations were found useful.

*Eco*RI primer 5'- GACTGCGTACCAATTC-3' (E) + three selective nucleotides and *Mse*I primer 5'-GATGAGTCCTGAGTAA-3' (M) + three selective nucleotides were used to screen the entire population of 81 individuals from the cross JI 2480/Radley. The primer combination E+ACT (selective nucleotides) / M+CGC amplified a polymorphic fragment of approximately 1000 base pairs (bp) the susceptible parent Radley and in the susceptible progeny (Figure 7.3). Out of 81 progeny, the fragment was present in eight of the resistant lines (8/81) and absent in three of the susceptible lines (3/81), indicating a linkage distance of 11/81 = 14 ± 4 cM. The primer combination E+ACG/M+CCC amplified a polymorphic fragment of approximately 460 bp in the susceptible parent Radley and the susceptible

progeny (Figure 7.4). Upon screening the entire population, the fragment was present in seven of the resistant progeny and absent in three of the susceptible progeny indicating a linkage distance of 12 ± 4 cM. Both of these primer combinations were linked in the *trans* position to *er-2*. Similarly, the primer combination E+AGG/M+CTA was linked in the *trans* position to *er-2* with a linkage distance of 5 ± 2 cM. The amplicon was present in two of the resistant progeny and absent in two of the susceptible progeny. This primer combination amplified a polymorphic amplicon of 241 bp in the susceptible parent and the susceptible progeny (Figure 7.5).

The primer combination E+AGG/M+CTG amplified two polymorphic fragments in the *cis* position to *er-2* in the resistant parent JI 2480 and the resistant progeny. One of the fragments was approximately 600 bp (Figure 7.6). The fragment was absent in two of the resistant progeny and present in six of the susceptible progeny, indicating a linkage distance of 10 ± 3 cM. The second amplicon of this primer combination was 123 bp and the fragment was absent in three of the resistant progeny and present in five of the susceptible progeny, indicating a linkage distance of 10 ± 3 cM.

In order to design allele specific primers, AFLP amplicons of two primer combinations E+AGG/M+CTA (241 bp) and E+AGG/M+CTG (123 bp) were cloned into pUC 19 and sequenced. On the basis of sequence information, longer primers were designed to amplify allele specific amplicons. However, both primer combinations amplified locus specific amplicons (Figure 7.7). Moreover, the alternate alleles were

Table 7.1 Sequence of primers and adapters used in the pea experiments[1].

Name	Sequence
EcoRI adapter	5-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5
EcoRI primer	5-GACTGCGTACC AATTC
EcoRI primer + A (Pre-amplificatiion)	5-GACTGCGTACC AATTC A
EcoRI primer + A +2 selective amplification	5-GACTGCGTACC AATTC A NN[2]
MseI adapter	5-GACGATGAGTCCTGAG TACTCAGGACTCAT-5
MseI primer	5-GATGAGTCCTGAG TAA
MseI primer +C (pre-amplification)	5-GATGAGTCCTGAG TAA C
MseI primer +C +2 selective amplification	5-GATGAGTCCTGAG TAA C NN

[1]Adapter information after Vos et al. (1995). Recognition sequence of *Eco*RI: G/AATTC and *Mse*I: T/TAA.

[2]Selective nucleotides.

Table 7.2 Sequence of specific primers designed on the basis of sequence differences between the two parental lines of pea.

Name	Sequence (5' to 3')	Specificity
E+AGG/M+CTA		
E+AGG	AGCGAGTAGCTAATTCCA**ATGA**[1]	Radley
E+AGG	AGCGAGTAGCTAATTCCA**TATG**	JI 2480
E+AGG	TCAGGAGCGAGATGGAC**AT**	JI 2480
M+CTA	CTACGTCAAGTATTCTCA	Radley/JI 2480
E+AGG/M+CTG		
E+AGG	CAAATCAAGGGATTCAA**C**	JI 2480
M+CTG	TAACTGAGCAAAGCTACT	Radley/JI 2480

[1]Nucleotides in bold cases are polymorphic to the specific parent.

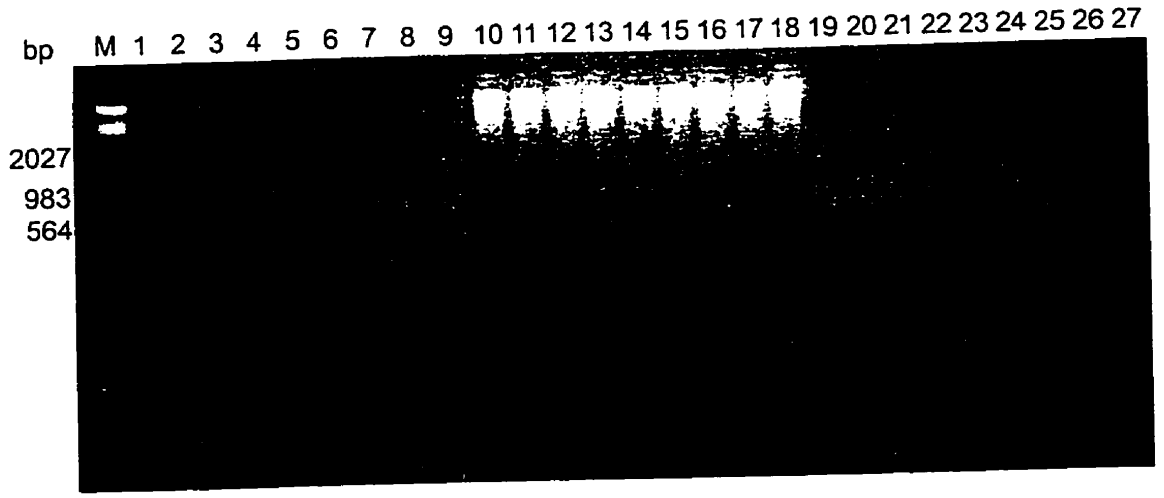

Figure 7.1 Restriction digestion of pea DNA with *Mse*I (lanes 1 to 9), *Eco*RI (lanes 10 to 18) and with *Mse*I+*Eco*RI (lanes 19 to 27).

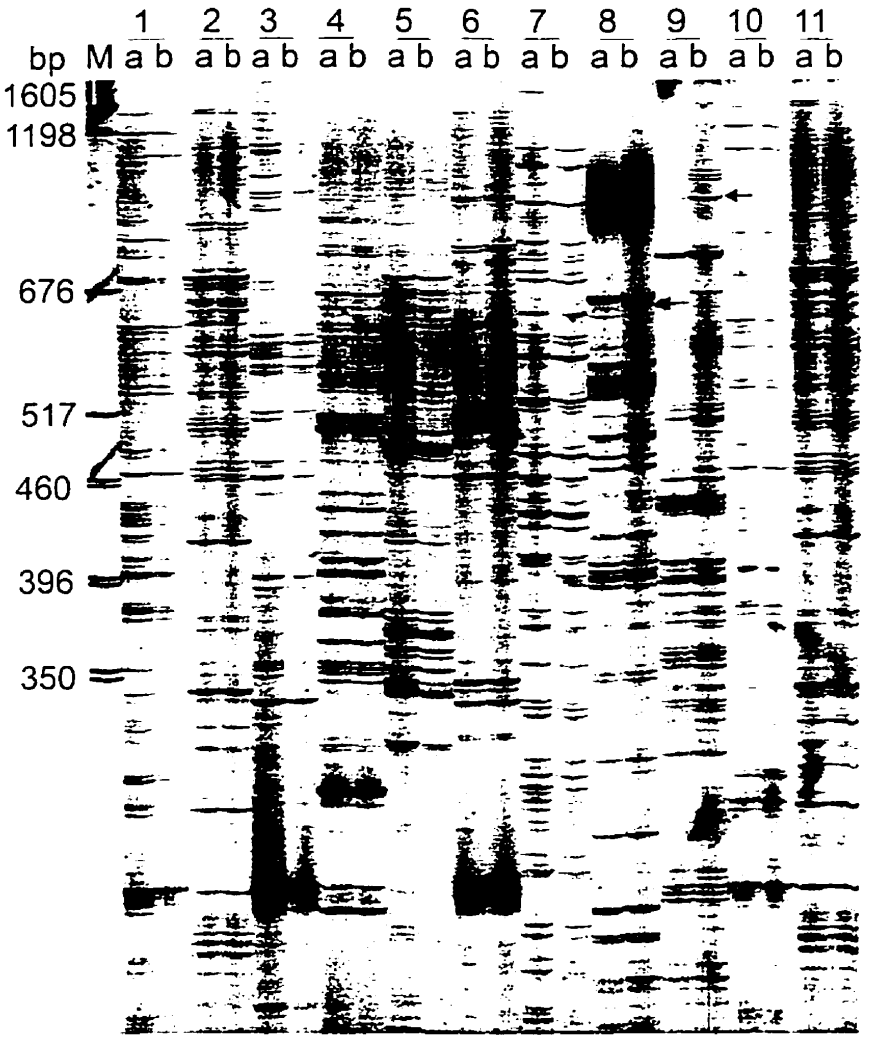

Figure 7.2 Silver stained AFLP polyacrylamide gel with bulked segregant analysis of pea. 1 to 11=different combination of *Eco*RI and *Mse*I primers. a=resistant bulk. b=susceptible bulk and M=Molecular weight marker. Arrows indicate the polymorphic bands.

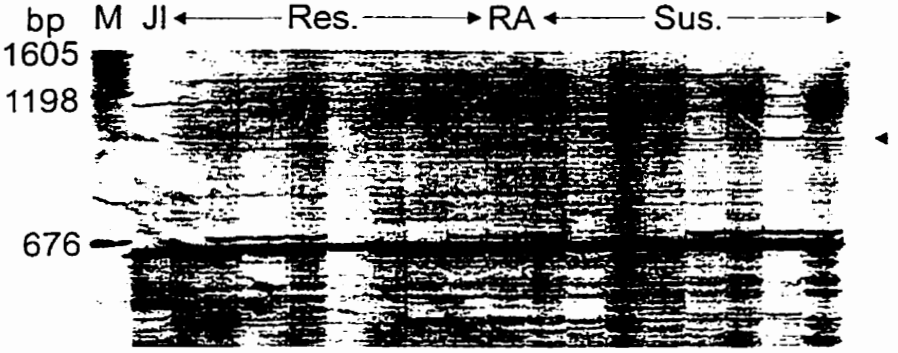

Figure 7.3 Polymorphic amplicon (~1000 bp) amplified by primer combination 5-GACTGCGTACCAATTCACT-3' / 5-GATGAGTCCTGAGTAACGC-3'. JI=JI 2480, RA=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny and M=Molecular weight marker. The arrow on the right indicates the polymorphic band.

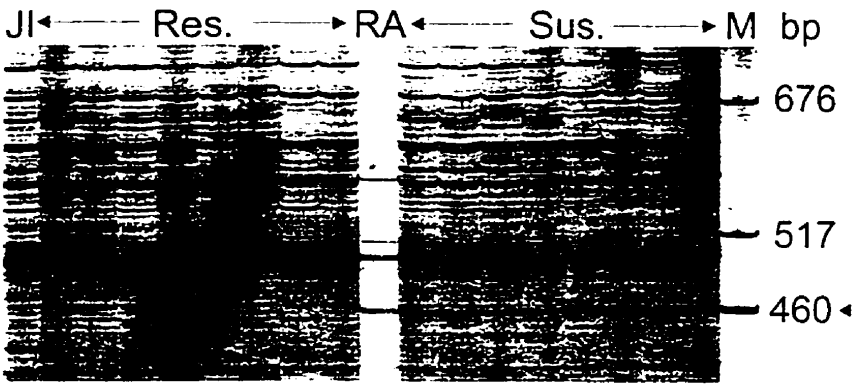

Figure 7.4 Polymorphic amplicon (~460 bp) amplified by primer combination 5-GACTGCGTACCAATTCACG-3' / 5-GATGAGTCCTGAGTAACCC-3'. JI=JI 2480, RA=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny and M=Molecular weight marker. The arrow on the right indicates the polymorphic band.

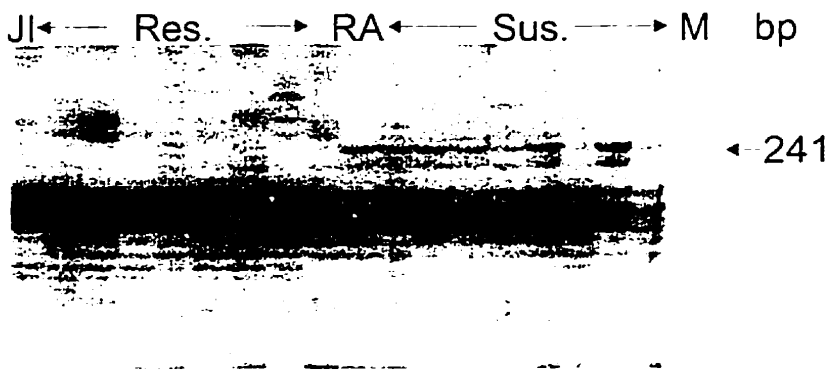

Figure 7.5 Polymorphic amplicon (241 bp) amplified by primer combination 5-GACTGCGTACCAATTCAGG-3' / 5-GATGAGTCCTGAGTAACTA-3'. JI=JI 2480. RA=Radley. Res.=Resistant progeny, Sus.=Susceptible progeny and M=Molecular weight marker. The arrow on the right indicates the polymorphic band.

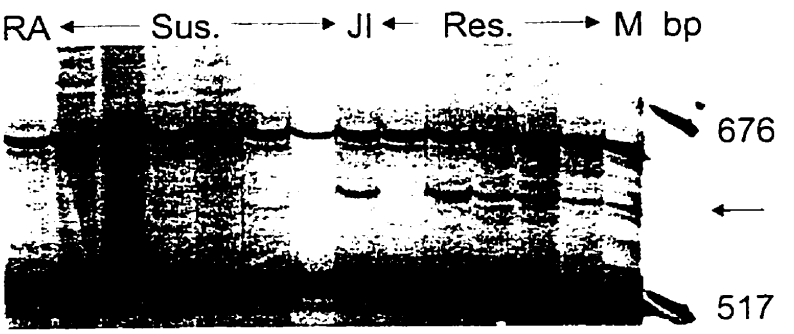

Figure 7.6 Polymorphic amplicon (~600 bp) amplified by primer combination 5-GACTGCGTACCAATTCAGG-3' / 5-GATGAGTCCTGAGTAACTG-3'. JI=JI 2480, RA=Radley, Res.=Resistant progeny, Sus.=Susceptible progeny and M=Molecular weight marker. The arrow on the right indicates the polymorphic band.

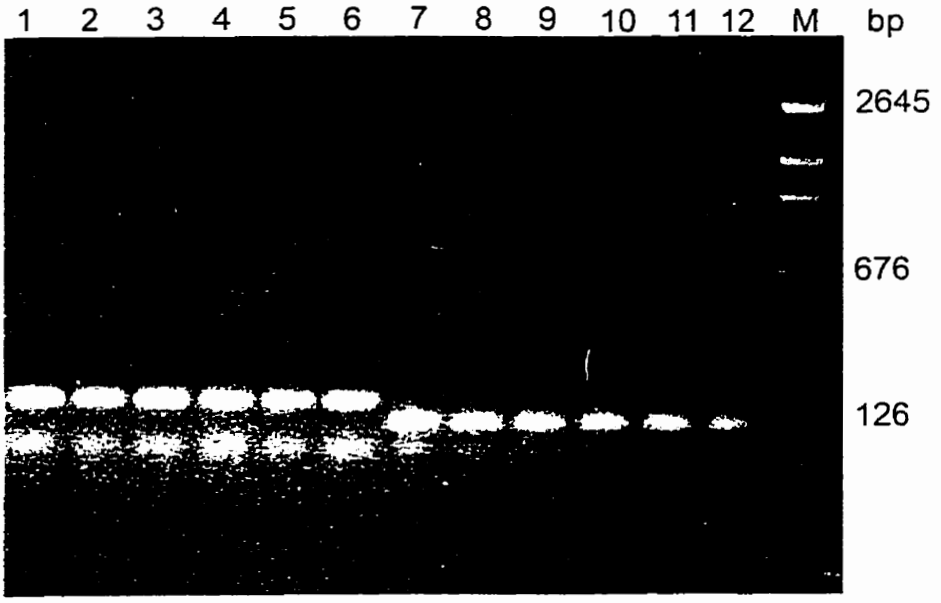

Figure 7.7 Locus specific amplicons amplified by the primer pairs 5'-AGGAGCGAGT-AGCTAATT-3'/5'-CTACGTCAAGTATTCTCA-3' (lanes 1-6) and 5'-AGGTGCAAAT-CAAGGGAT-3'/5'-CTGAGCAAAGCTACTCTG-3' (lanes 7-12) in pea lines JI 2480 (lanes 1-3 and 7-9) and Radley (lanes 4-6 and 10-12). M=Molecular weight marker.

sequenced and sequence data were compared. Sequence differences between the allele were detected and the primers were designed accordingly (Table 7.2). Further amplification with these specific primers amplified only locus specific fragments. Repeated alteration of forward and reverse primers, magnesium ion concentration (1.2 mM to 2.0 mM) and annealing temperature (55^0 C to 65^0 C) did not provide allele specificity. Increasing the annealing temperature higher than 66^0 C resulted in reduced amplification in both the resistant and the susceptible lines.

7.5 Discussion

To the best of our knowledge, this is the first report of AFLP analysis of pea. AFLP analysis is be promising for genetic studies of pea. Forty to 80 amplicons were amplified in a size range of 50 bp to 2500 bp / primer combination. In the present investigation, we used a combination of restriction endonucleases *Eco*RI and *Mse*I, however, other enzyme combinations such as *Hin*dIII, *Pst*I, *Bgl*II, *Xba*I and *Sse*8387I in combinations with either *Mse*I or *Taq*I have been reported in other plant species (Vos et al. 1995). The combination of *Pst*I and *Mse*I has been successfully used in the analysis of cereal genomes in our laboratory.

Although AFLPs are dominant markers as are RAPDs, AFLPs have several advantages over both RFLPs and RAPDs. In contrast to RFLP, AFLP has a virtually unlimited number of DNA fragments. As compared to RAPD, AFLP uses stringent reaction conditions; hence, better reproducibility (Thomas et al. 1995, Folkertsma 1996). Lin et al. (1996) reported that AFLP was the most efficient technique in detecting

polymorphism in soybean lines among RFLP, RAPD and AFLP. Mackill et al. (1996) showed that AFLP produced the highest number of polymorphic bands as compared to RAPD and microsatellites in rice. The banding patterns were not affected by the amounts of genomic DNA (100 ng to 5 ug) in AFLP, but were complicated by partially digested DNA (Lin and Kuo 1995). Jones et al. (1997) reported a high level of reproducibility of AFLP bands among European laboratories, as compared to RAPD.

The literature is confusing regarding the use of the terms "coupling" and "repulsion" phase. The classical definition of the term coupling is " when both nonallelic mutants are present on one homologue and the other homologous chromosome carries the plus alleles (ab / ++). The repulsion configuration refers to a situation in which each homologue contains a mutant and a wild-type gene (a+ / +b)" (King and Stansfield 1990). Wild type is referred to as dominant and a mutant is referred to as a recessive phenotype. RAPDs are dominant markers because a marker is either present or absent (Williams et al. 1990). When a dominant RAPD marker is associated with a trait that is recessive, then the configuration would be referred to as a repulsion (Timmerman et al. 1994). However, in the literature the terms coupling and repulsion have been used irrespective of the dominant / recessive nature of the trait (Haley et al. 1994b). The terms "*cis*" and "*trans*" have been used to describe "coupling" and "repulsion" configuration, respectively, in recent literature (Thomas et al. 1995, Johnson et al. 1997).

The resistance gene *er-1* provided a high level of resistance to powdery mildew in pea, whereas *er-2* provided partial resistance (Chapter 3). Pathogen isolates have been detected which were slightly virulent in lines carrying *er-1* (Chapter 4). Incorporation of

both genes (*er-1* and *er-2*) in a cultivar should increase the durability of resistance to powdery mildew. However, selection of lines carrying both *er-1* and *er-2* on the basis of visual scoring would be extremely difficult since *er-1* alone provides a high level of resistance. Identification of molecular markers for *er-1* and *er-2* would facilitate this process. Molecular markers offer the opportunity to pyramid major resistance genes into a cultivar, since they bypass the constraints usually encountered by conventional selection procedures (Tanksley 1983, Young and Kelly 1997).

For the purpose of gene pyramiding, identification of reliable and user friendly markers closely linked to *er-2* is important because 1) a high level of resistance is provided by *er-1* alone, 2) the obligate parasitic nature of *Erysiphe pisi* makes it difficult to maintain the pathogen in culture, and 3) disease occurrence is uncertain under field conditions. Molecular markers are effective in MAS, if markers are present both in the coupling and repulsion phases (Johnson et al. 1995). We have identified AFLP markers linked to *er-2*, three in coupling and two in repulsion phase. The repulsion phase primer combination E+AGG/M+CTG will be useful in MAS of heterozygous $BC_n F_1$ individuals for JI 2480-derived resistance. The coupling phase primer combinations E+ACT/M+CGC, E+ACG/M+CCC, and E+AGG/M+CTA will be useful in identifying homozygous resistant individuals.

In the present investigation, our attempt to convert AFLP amplicons to allele specific amplicons or SCARs was not successful. Similarly, Mayer et al. (1997) and Johnson et al. (1997) lost the allele specificity when they attempted to develop ASAPs and SCARs for disease resistance genes in chickpea and bean, respectively.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

Field pea production has increased rapidly in western Canada since 1985 with the opening of the European feed pea market. In 1996, Canada produced 1.2 million tonnes of field peas as compared to 168,000 tonnes in 1985 (Statistics Canada, 1996). The major reason for this increased production was due to the concentrated effort of public research institutions, such as Universities, Agriculture and Agri-Food Canada, pulse growers associations, provincial departments of agriculture and private industries (Slinkard et al. 1994). Other factors contributing to increased production were increased emphasis on crop diversification, crop rotation, value added processing, new industries in rural areas, and increased attention to the sustainability of agriculture. Because of the nitrogen and non-nitrogen benefits of pea and pulses in a cereal crop rotation (Jansen and Haahr 1990, Stevenson and van Kessel 1996, Beckie and Brandt 1997), expansion of pea acreage in western Canada will likely continue.

Although the area and production of field pea in western Canada has dramatically increased in the last 12 years, average yield has remained static (Statistics Canada 1996). Over the years, many high yielding cultivars have been registered and improved agronomic practices have been adopted. Biotic stresses such as *Ascochyta* blight, powdery mildew and *Fusarium* wilt are a major reason for static yields. Although *Ascochyta* blight is the most important pea disease in western Canada, powdery mildew is the second most damaging disease, causing yield and quality losses in most years. Powdery mildew is a

severe problem in late planted and late maturing pea cultivars and adversely affects pods/plant, seeds/pod, and seed weight (Sakr 1989). Most of the field pea cultivars currently grown in western Canada are susceptible to powdery mildew due to the fact that the majority of the cultivars originated from Europe where powdery mildew is not a major concern (T.D. Warkentin, Personal communication 1997). Unprotected plots in Wisconsin yielded only 44% to 71% as much as plots protected from powdery mildew with a fungicide (Gritton and Ebert 1975). Yield reductions due to powdery mildew have been estimated in the range of 7.5% to 75% (Munjal et al. 1963, Laxman et al. 1978, Reiling 1984, Sakr 1989). A conservative estimate of 10% yield reduction cost Canadian farmers 38 million (CAD $ 308 per tonne, Statistics Canada 1996) annually, not counting the losses due to quality reduction. This money could be saved by incorporating genetic resistance into agronomically superior cultivars. Genetic resistance was as effective as fungicide applications (Sakr 1989).

The powdery mildew resistance gene *er-1* has provided a high level of resistance to the common isolates found in North America over the last 50 years (Harland 1948). We have shown that the Canadian cultivars Highlight, AC Tamor, and Tara carry *er-1* for resistance (Chapter 3). Although *er-1* has provided durable resistance, resistance of *er-1* (Stratagem) was overcome by some virulent isolates in New York (Schroeder and Provvidenti 1965). Results presented by Stavely and Hanson (1966) and Sakr (1989) suggest the presence of physiological races of *E. pisi*. We have shown that isolates LAI-1 and PUI-2 were virulent on JI 82 (*er-1*) and Highlight (*er-1*), respectively, and

pathotypes found in Nepal were virulent on JI 210 (*er-1*) (Chapter 4). Therefore, identification and utilization of other resistance genes could be important.

A number of powdery mildew resistance genes have been identified in wheat (Wolfe 1967) and barley (Mathre 1982, Jorgensen 1993). Although we attempted to identify other powdery mildew resistance genes in pea (Chapter 3), we were not successful with the limited number of resistat lines available for screening. Screening of a larger germplasm base and intercrossing the resistant lines and crossing with susceptible lines would be helpful to identify additional resistance genes.

Resistance found in line Mexique 4 (JI 1559) was not overcome by any of the tested isolates on the detached leaf assay and under field conditions in North America, Europe or Nepal (Chapter 4). Although Heringa et al. (1969) reported that Mexique 4 carried both resistance genes (*er-1* and *er-2*), our results showed that Mexique 4 carried only one gene for resistance (*er-1*). Mexique 4 was crossed to Highlight (*er-1*), JI 2480 (*er-2*) and Radley (susceptible) and the progeny were evaluated for segregation. All progeny of the cross Mexique 4/Highlight were resistant and with no segregation for susceptibility. The F_2 progeny of the cross Mexique 4/JI 2480 segregated in a 9:7 ratio as expected in a digenic model of inheritance as did the progeny of the cross Highlight/JI 2480. The F_2 progeny of the cross Mexique 4/Radley segregated in a 3:1 ratio confirming a monogenic inheritance due to *er-1*. However, the high level of resistance of Mexique 4 could be due to the presence of some other modifier genes or a different allele.

Use of Mexique 4 as a source of powdery mildew resistance is recommended where complete resistance is sought. However, where partial resistance is desired, use of JI 2480

(*er-2*) or DPP-68 (Banyal and Tyagi 1997) may be recommended. Resistance provided by JI 2480 was broken down in a growth cabinet and also in a detached leaf assay. In 1995, when the disease incidence was moderate under field conditions, line JI 2480 was completely free from powdery mildew. However in 1996, when the disease pressure was higher, JI 2480 was slightly infected under field conditions. Similar to the observation of Heringa et al. (1969), stems were more susceptible than the leaves.

Thirty-one single colony isolates of powdery mildew were evaluated from the three prairie provinces of western Canada and from NW USA to evaluate variability in virulence. A low level of variability among isolates was detected (Chapter 4). Although, the sampled area covered a wide geographical area, it might have represented similar environmental (agroclimatic) conditions. Collection of samples from more diverse regions and evaluation of more isolates may have revealed wider variability. Of the ten genotypes tested for reaction to powdery mildew in diverse locations (Manitoba, New York, California, Washington, Norwich and Kathmandu), two susceptible lines in North America (Radley and JI 1648) exhibited a resistant reaction and one resistant line in North America (JI 210) exhibited a susceptible reaction in Nepal. However, experiments were conducted in only one site (Kathmandu) and the observation was based on 25 to 35 plants (five m long row) with no replications. For further investigation, a replicated trial with multi-location testing is recommended. Similar to our findings, Sakr (1989) reported site differential reaction of powdery mildew of pea, suggesting the possibility of different races of the pathogen in Morocco and Washington.

To investigate the variability of virulence, near-isogenic lines are used in other crops (Briggle 1969). Since we have identified seven pea lines as a differential set, it would be possible to develop near-isogenic lines by repeated backcrossing with a universal susceptible line using resistant lines as recurrent donor parents. Inclusion of powdery mildew isolates from Nepal or testing the backcross progeny in Nepal would be useful to identify the resistance found in Radley and JI 1648.

Powdery mildew usually appears in mid to late July in western Canada (Ali-Khan and Zimmer 1989). Although, cleistothecia are reported to serve as the overwintering and oversummering structure of powdery mildew in warm areas (Singh 1968, Agrios 1988), our results suggest that the extreme cold winter weather in Manitoba had a negative effect on the survival of ascospores in cleistothecia as compared to samples stored at room temperature. Most of the ascospores were degraded over the winter under field conditions. Although *E. pisi* is reported to infect plant species other than pea (Stavely and Hansen 1966, Smith 1969, Hirata 1986), our attempts to infect other legume crops were unsuccessful. Despite a few early reports of seed transmission of *E. pisi* (Crawford 1927, Uppal et al. 1936), we did not find any infection in the greenhouse when seeds from heavily infected plants were planted. Moreover, no reports are known of transmission of the powdery mildew fungus through seed in other plant species. In the absence of convincing results for winter survival of *E. pisi* in Manitoba, wind dispersal of conidia from warmer areas (northern USA), as reported in other crop species (Harmansen 1964, Ruppel et al. 1975, Yarwood 1978), could possibly serve as primary inoculum in western Canada. Pea is used as a kitchen garden vegetable by most farm families in North

America, and thus could provide for movement of wind blown conidia from one location to another.

Although the host range of *E. pisi* has been extensively studied, results are quite contradictory. Blumer (1933) divided *E. polygoni* into 15 species and narrowed the host range of *E. pisi* to five host genera: *Pisum*, *Dorycnium*, *Medicago*, *Lupinus* and *Vicia*. Hirata (1986) reported that *E. pisi* infected 85 species in the family *Leguminosae*. Stavely and Hanson (1966) found that *E. pisi* was pathogenic in four species of *Lathyrus* including *Lathyrus sativus*. Yu (1946) reported that powdery mildew of broad bean and pea was caused by the same physiological race. In addition to the lack of cross infection in the detached leaf assay in the present investigation, a number of breeding lines of *Lathyrus sativus*, chickpea, lentil, and faba bean were completely free from powdery mildew infection under field conditions in Manitoba, whereas pea plants in the nearby plots/fields were severely infected. Similar to our observations, several authors have reported a lack of cross infection of *E. pisi* on other plant species (Searle 1920, Hammarlund 1925, Smith 1969, Reiling 1984).

Introgression of both powdery mildew resistance genes into a pea cultivar may increase the durability of resistance. Conventional selection methodology, based on visual scoring of phenotypes, is time consuming, costly and dependent upon environmental conditions. Since *er-1* alone provides a high level of resistance to powdery mildew of pea, identification of lines carrying both *er-1* and *er-2* in a line is difficult. Identification of molecular markers for both resistance genes would facilitate the introgression of these genes by MAS.

For molecular markers to be useful in MAS, they should be reliable, simple to perform, and capable of processing a large number of samples per unit time. RAPD analysis meets these requirements as compared to other classes of DNA markers. The ability of RAPD analysis to rapidly and cost-effectively screen hundreds of samples makes molecular marker technology a feasible selection tool in a plant breeder's arsenal (Penner et al. 1993a). Bulked segregant analysis (Michelmore et al. 1991) eliminated the requirement for development of near-isogenic lines (Martin et al. 1991) and has been successfully used to identify markers for monogenic pest resistance genes (Penner et al. 1993a, Timmerman et al. 1994, Urrea et al. 1996, Young and Kelly 1997). Development of specific ASAP and SCAR markers have increased reliability and cost-effectiveness of marker technology (Paran and Michelmore 1993, Gu et al. 1995, Penner 1996).

Although Timmerman et al. (1994) reported a SCAR marker for *er-1*, this marker was not useful for Canadian germplasm. The specific coupling marker (*trans* to *er-1*) Sc-OPO-18_{1200} will be effective in identifying homozygous resistant individuals. Eleven resistant and four susceptible pea lines were evaluated with this primer pair. The polymorphic amplicon was also present in the susceptible cultivars/lines, Radley, JI 1758, and JI 1648 and absent in the resistant cultivars/lines Highlight, Tara and JI 2302. However, the amplicon was present in the resistant lines/cultivars AC Tamor, JI 1210, JI 1951, JI 82, JI 210, JI 2480, and JI 1559.

The specific marker Sc-OPE-16_{1600} will enable the selection of heterozygous lines which would be useful for selecting lines carrying *er-1* while back crossing. Moreover, this marker amplified only a single band on the resistant parent and therefore the genotype

could be identified without electrophoresis (Gu et al. 1995). Out of the 15 lines evaluated, the polymorphic amplicon was present in the resistant cultivars/lines Highlight, AC Tamor, JI 2302, JI 1210, JI 1951, JI 82, JI 2480, JI 1559 and absent in the susceptible cultivar Radley. However, the amplicon was also present in the susceptible cultivars/lines Trump, JI 1758, and JI 1648 and absent in the resistant cultivar/line Tara and JI 210 (Figure 6.6).

Molecular markers would be more useful in MAS if the markers were applicable in a wide range of genetic backgrounds. Several gene pool non-specific (Schachermayr et al. 1994, Urrea et al. 1996, Melotto et al. 1996) and gene pool or cultivar specific (Haley et al. 1993, Miklas et al. 1993, Horvath et al. 1995, Mayer et al. 1997, Johnson 1997) RAPD markers for disease resistance genes have been reported. Miklas et al. (1993) identified a RAPD marker for a rust resistance gene (*Up2)* in bean (*Phaseolus vulgaris* L.) cultivars of Mesoamerican descent which amplified a monomorphic amplicon both on resistant and susceptible cultivars of Andean descent. The polymorphic amplicon of marker, Sc-OPE-16_{1600} was present in eight of the ten resistant lines tested, indicating wide applicability of this marker in MAS.

We used AFLP analysis to identify molecular markers for *er-2*. As compared to RAPD, AFLP was more powerful in assaying the genome. Amplification of a large number of bands (40 to 80) on sequencing gels was the major advantage of AFLP as compared to one to nine bands in RAPD on agarose gels (Chapter 6). AFLP seems suitable where the level of polymorphism between the individuals is low, and for developing high density genomic maps (Schondelmaier et al. 1996, Hongtrakul et al. 1997). Since, DNA

must be completely restricted with restriction endonucleases, the quality of extracted DNA and the method of extraction could affect the profile of AFLP (Vos et al. 1995, Jones et al. 1997). As in RAPDs, AFLP fragments of a specific length do not necessarily represent specific loci, although, they are scored as the same (Vos et al. 1995). The AFLP procedure is technically more demanding than RAPD analysis and conversion of AFLP fragments to allele specific primers (SCARs, ASAPs) may prove difficult. In the present investigation, the conversion of the polymorphic amplicon amplified by the primer combination E+AGG/M+CTA and E+AGG/M+CTG into an allele specific primer (SCAR) was not successful. Although, sequence differences between the alleles were detected, amplification was always locus specific (Figure 7.7). Similarly, loss of allele specificity was encountered by Mayer et al. (1997) and Johnson et al. (1997) when longer primers were designed from RAPD fragments.

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10 APPENDIX

Table 10.1 Reaction of pea leaves (L) and stems (S) to field isolate of *E. pisi* in growth cabinet.

	Days After Seeding[a]				Presumed
	27	35	45	55	genotypes
Pea lines	L/S	L/S	L/S	L/S	
1. Highlight	0/0	0/0	1/0	1/0	*er-1*
2. JI 2480	3/3	3/5	5/7	5/9	*er-2*
3. JI 1559	0/0	0/0	0/0	0/0	*er-1*
4. JI 1758	5/7	7/7	9/9	9/9	*Er*
5. JI 210	0/0	0/0	1/0	1/0	*er-1*
6. JI 1951	0/0	0/0	1/0	1/0	*er-1*
7. JI 1648	5/5	7/7	9/9	9/9	*Er*
8. JI 82	0/0	2/0	2/0	3/0	*er-1*
9. JI 1210	0/0	0/0	1/0	1/0	*er-1*
10.JI 2302	0/0	0/0	1/0	1/0	*er-1*
11.AC Tamor	0/0	1/0	1/0	1/0	*er-1*
12.Tara	1/0	1/0	1/0	1/0	*er-1*
13.Radley	7/7	7/7	9/9	9/9	*Er*
14.Trump	7/7	7/7	9/9	9/9	*Er*

[a]Inoculation was conducted 20 days after seeding.

Table 10.2 Operon and UBC primers screened to identify RAPD markers for powdery mildew resistance gene *er-1* in pea.

Primer series	Total # tested	Primers not-available	Non-amplifying primers
OPA 1-20	20		
OPB 1-20	20		
OPC 1-20	20		
OPD 1-20	19	3	1,9,14,17,18
OPE 1-20	18	3, 4	10, 13
OPF 1-20	19	15	4,19
OPG 1-20	20	20	
OPH 1-20	18	3,15	
OPI 1-20	17	4,7,15	8
OPJ 1-20	18	1,20	2
OPK 1-20	18	9,12	5
OPL 1-20	20		
OPM 1-20	19	19	8
OPN 1-20	14	3,6,8,9,10,18	
OPO 1-20	18	2,11	17
OPP 1-20	20		
OPQ 1-20	20		
UBC 101-200	98	102,127	107,113,117,118
TOTAL #	416	25	19

Table 10.3 Polymorphic amplicons amplified by the specific primer pairs (SCARs), linked in coupling phase (Sc-OPO-18_{1200}) and repulsion phase (Sc-OPE-16_{1600}) to *er-1* in pea lines.

Cultivars	**PM reaction**	**Sc-OPO-18_{1200}**	**Sc-OPE-16_{1600}**
1. Highlight	R	A	P
2. AC Tamor	R	P	P
3. Tara	R	A	A
4. Л 2302	R	A	P
5. Л 1758	S	P	P
6. Л 1210	R	P	P
7. Л 1951	R	P	P
8. Л 1648	S	P	P
9. Л 82	R	P	P
10. Л 210	R	P	A
11. Л 2480	R	P	P
12. Л 1559	R	P	P
13. Radley	S	P	A
14. Trump	S	P	P
15. Montana	S	P	P

PM=Powdery mildew, R=Resistant to powdery mildew, S=Susceptible to powdery mildew, P=Presence of the band and A=Absence of the band.

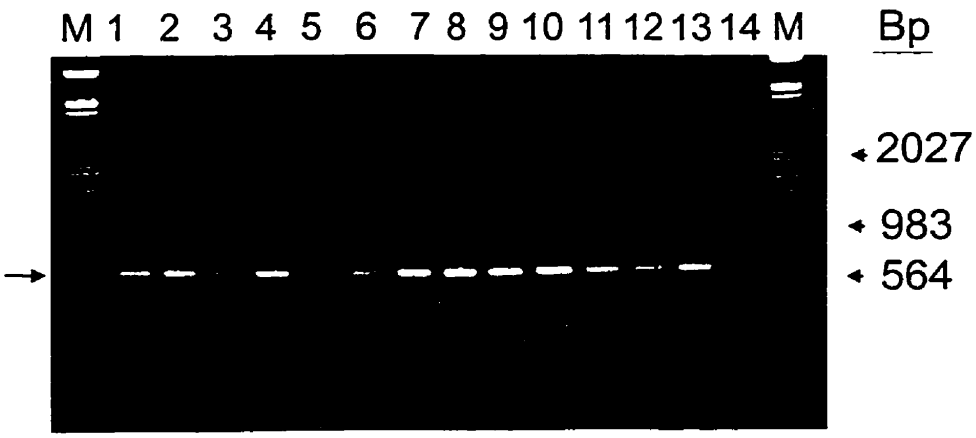


Figure 10.1 PCR amplicons amplified by the SCAR primer pair $PD10_{650}$ (5'-GGTCTAC-ACCTCATATCTTGATGA-3'/5'-GGTCTACACCTAAACAGTGTCCGT-3' (Timmerman et al. 1994). 1=Highlight, 2=AC Tamor, 3=Tara, 4=JI 2302, 5=JI 1758, 6=JI 1210, 7=JI 1951, 8=JI 1648, 9=JI 82, 10=JI 210, 11=JI 2480, 12=JI 1559, 13=Radley, 14=Blank and M=Molecular weight marker. The arrow on the left indicates the polymorphic fragment.

Sequence alignment (A)

```
                    *          *          *          *          *
Radley= AATTCAGGAGCGAGTAGCTAATTCCAATGAGGGATTAGTGAAGCGTTACT
JI248 = AATTCAGGAGCGAGTAGCTAATTCCAtatgGGaATcgGTGAAGtGTTACc

                    *          *          *          *          *
          TATGGTGATAGTAACGATTT-GGTGCACTCTGGTCCTATAGTGGGGATTT
          TATGGTGATAGTAACatTTTtGGTGtgCTCTaGTCCTATgGTGGGGATTc

                    *          *          *          *          *

          AGGAGCGAGATGGACTCGTGTTGCCCATGAATGATACCACATGCATAATG
          AGGAGCGAGATGGACatGTGTTatCCATGAATGgTACCgCATGCATAATG

                    *          *          *          *          *
          AGTCAGTTGTGCAGTTCATTGCATACATATTTGCATATGTAATTGATTGT
          AGTCAGTTGcGgAGTatATTtCATACATgTTTGCATATGTAAaTGATTGT

                    *          *          *          *
          TCATGATGTTGTTGATTGGTTGAGAATACTTGACGTAGTTA
          TtgTGATaTTGTTGATTGGTTGAGAATACTTGACGTAGTTA
                               ACTCTTATGAACTGCATC-5'
```

Sequence alignment (B)

```
                    *          *          *          *          *
Radley= AATTCAGGTGCAAATCAAGGGATTCAACAAAGTCAACAAGCTCATATCCA
JI248 = AATTCAGGTGCAAATCAAGGGATTCAAaAAAtTCAACAAGCTCATATCCA

                    *          *          *          *          *
          AAGGAAACCTTCTCAACTGAAAGAGACCCTGAAAAACTTCATTCGAGCTA
          AAGGAgACtgTCTCAACTGgAAGAGACCCTGcAAAACTTCATcCaAGCaA

                    *          *
          CTCAGAGTAGCTTTGCTCAGTTA
          CTCAGAGTAGCTTTGCTCAGTTA
               TCATCGAAACGAGTCAAT-5'
```

Figure 10.2 DNA sequence comparison of alleles linked to *er-2* in pea cultivars/lines Radley and JI 2480. Sequence differences are shown in lower case. Primer sequences are indicated by bold letters. (A) sequence data amplified by the primer pair E-AGG/M-CTA specific (241 bp). (B) sequence data amplified by the primer pair (2) E-AGG/M-CTG specific (123 bp).

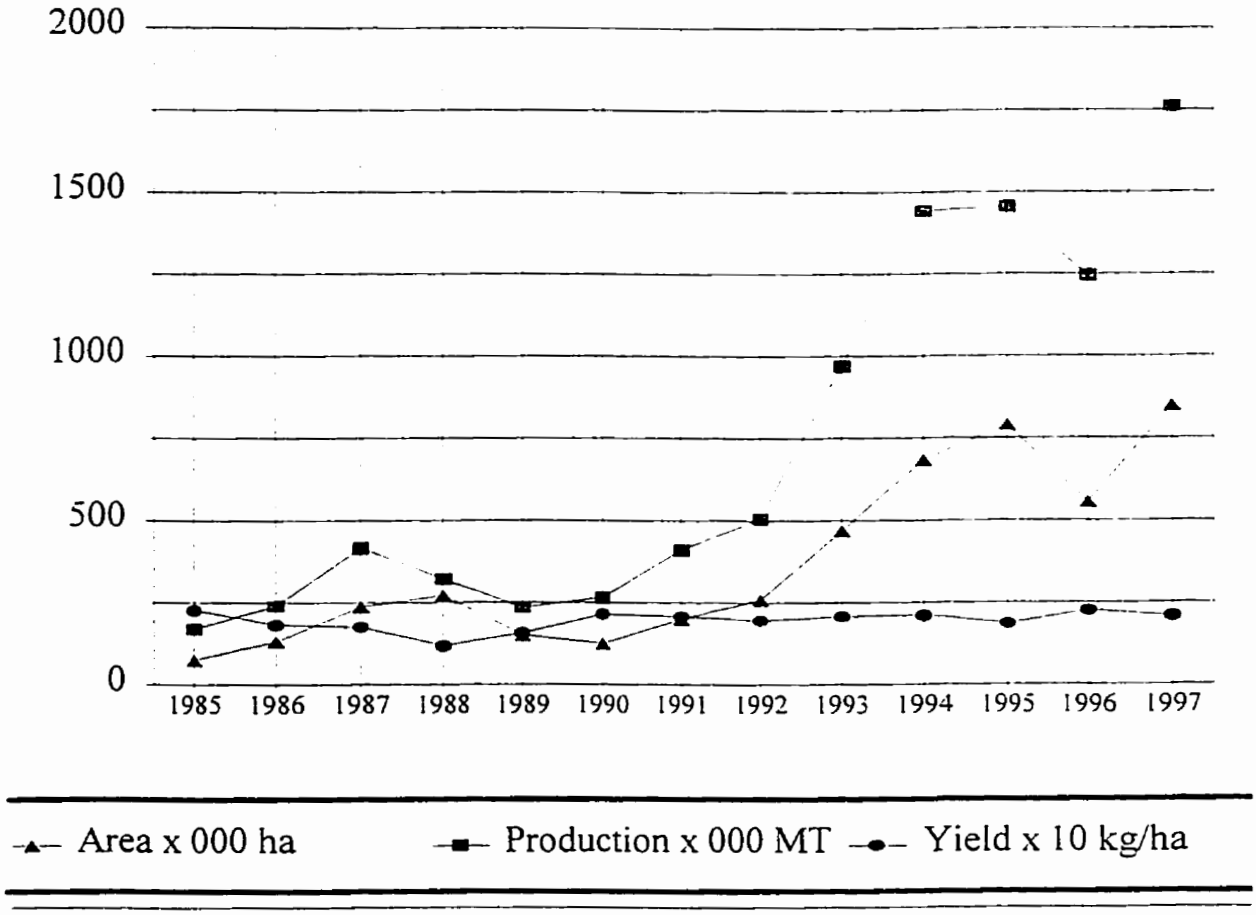
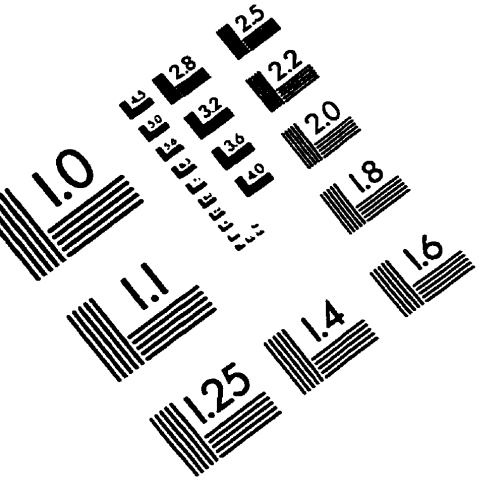
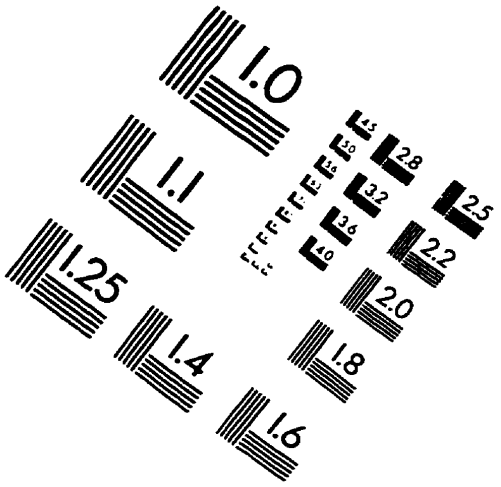
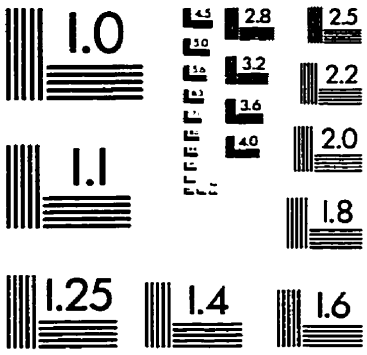


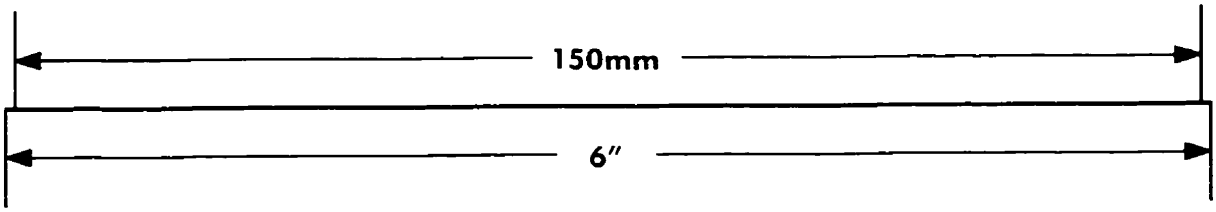
Figure 10.3 Area, production and productivity (yield) of field pea in western Canada (Source: Statistics Canada 1997).

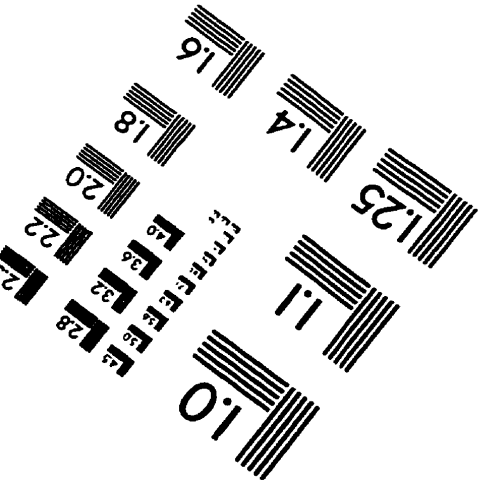
IMAGE EVALUATION
TEST TARGET (QA-3)

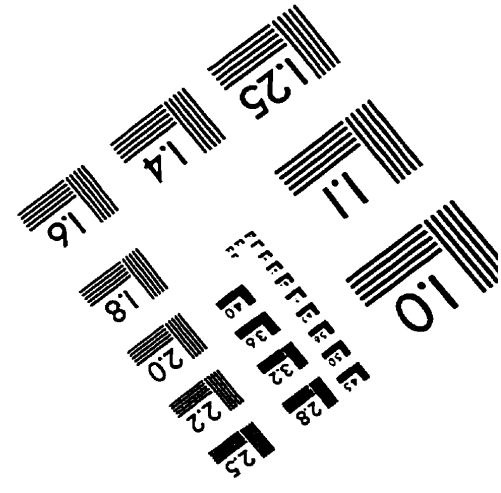












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