INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600

DISSERTATION

DEVELOPING SCALD RESISTANT BARLEY GERMPLASM UTILIZING TRADITIONAL AND MARKER SCREENING TECHNIQUES

Submitted by

Jolanta Lucyna Menert

Department of Bioagricultural Sciences

and Pest Management

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 1999

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

COLORADO STATE UNIVERSITY

May 14, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOLANTA MENERT ENTITLED DEVELOPING SCALD RESISTANT BARLEY GERMPLASM UTILIZING TRADITIONAL AND MARKER SCREENING TECHNIQUES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Committee on Graduate Work

Nora Lapitan

Frank B. Peairs

William M. Brown

Co-adviser

Adviser

Thomas O. Holtzer

Department Head/Director

ABSTRACT OF DISSERTATION

DEVELOPING SCALD RESISTANT BARLEY GERMPLASM UTILIZING TRADITIONAL AND MARKER SCREENING TECHNIQUES

Scald, caused by *Rhynchosporium secalis* (Oud) J.J. Davis, is present in most barley growing areas. Annual losses caused by the pathogen average 1 – 10%, but losses of 30 - 40% can occur. It is difficult to breed for disease resistance because of high pathogen genetic variability.

In this study, progenies of four crosses of resistant (SM89010, CDC Silky) and susceptible (Harrington) barley cultivars were evaluated for scald in the F2 to F4 generations with traditional screening methods and marker assisted selection, using Random Amplified Polymorphic DNA. Plants were evaluated using a 0 to 3 scale, with score 0 for a resistant and 3 for a susceptible reaction. Two Canadian isolates of *R. secalis*, 1493 and 1824, were used in the study. Crosses and screening of the plants at all generations were performed in a greenhouse environment.

Distribution of the progeny disease reaction was skewed toward resistance. Progeny reactions were tested using Chi-square analysis to determine the fit to hypothesized ratios. Crosses 1 and 2 fit the hypothesized ratios for the single dominant gene model in F2 and F3 generations. Cross 3 was designed to develop a marker for a resistance gene, which was not successfully mapped. The segregation ratio for cross 3 at the F2 population did not fit the hypothesized

model for single dominant gene or two dominant genes. Progenies from Cross 4 fit the two gene hypothesized model in the F2 generation. Resistance to *R. secalis* was inherited qualitatively.

Jolanta Lucyna Menert
Department of Bioagricultural Sciences
and Pest Management
Colorado State University
Fort Collins, CO 80523
Summer 1999

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Joe Hill and Dr. Bill Brown who advised me through my graduate studies and patiently reviewed multiple drafts of this dissertation. I would also like to thank my committee members, Dr. Frank Peairs and Dr. Nora Lapitan, for their support and suggestions.

I also wish to thank Busch Agricultural Resources, Inc. for the financial support and the use of their facilities during this research project. My graduate career would not be possible without it.

A special thanks goes to my husband, Tadeusz, for being there when I needed him. Finally, I would like to thank the rest of my family and my friends for putting up with me for all these years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth most important crop in the world. While it is used both as feed and human food, it is also a crucial crop for the brewing industry (Mathre, 1997). To be profitable, malting barley must meet stringent quality requirements such as proper germination, kernel plumpness, protein content, and extract. Extract is the portion of the malt soluble in the wort and is as important for the brewer as yield for the farmer (Pollock, 1979).

There are two main areas in the United States where spring malting barley is grown, the Midwest area, where six-row barley is predominant and the Intermountain area, where two-row barley is traditionally grown (Figure 1). The midwestern region includes North Dakota, Minnesota and South Dakota while the intermountain area includes Montana, Idaho and Wyoming.

Grain quality and yield can be adversely affected by barley pathogens. Some pathogens not only lower quality, but make the grain unacceptable for the malting process. This means a loss of income for the farmer and potential loss of malting barley for maltsters. Breeding for disease resistance is therefore crucial to the brewing industry. In the intermountain area only a few foliage pathogens regularly cause annual yield and quality losses. One such disease is scald, caused by *Rhynchosporium secalis* (Oudem.) J. J. Davis.

Figure 1. Barley heads, two-row (left) and six-row (right).

Breeding for scald resistance has been carried out for many years, however, breeding for resistance to the pathogen is a complicated endeavor because of its highly variable nature (Brown, 1985, 1990; Ceoloni, 1980; Hansen and Magnus, 1973; Lyngs Jorgensen and Smedegaard-Petersen, 1994; Tekauz, 1991).

This fungal disease occurs in regions where cool temperatures and wet conditions prevail. Initial symptoms of the disease are characterized by distinctive lesions on leaves. In severe infections lesions can also occur on the stem and the head. Lesions, at first gray or bluish, later appear to be water soaked. The shape of the lesions can vary from ovate to irregular scald-like blotches with brown, sharp margins (Mathre, 1997).

Average yield losses caused by scald usually range from 1% to 10 %. However, losses as high as 30 - 40% have also been reported under conditions favorable for disease development (Nelson, 1977; Tekauz, 1991). Barley infected with *R. secalis* has fewer and less plump kernels. When the disease occurs on heads, kernels are also discolored. Affected barley can have higher protein content and therefore may not be suitable for malting.

A study by Biffen in 1905 opened a new chapter on the inheritance of disease resistance to plant pathogens (Ellingboe, 1981). Subsequently the genetic variability within a pathogenic species was demonstrated, along with the necessity of incorporating new genes into a host species to protect it against new emerging pathogen races (Flor, 1946, 1947). "The pathogenic characters of physiologic

races obtained by selfing and hybridizing could be accounted for by Mendelian segregation and recombination of pathogenic characters inherent in the parent races" (Flor, 1942).

In case of the barley scald, there is clear evidence of pathogenic races within this fungus population. Pathogenic variability has been reported from most of the geographic areas where the fungus is present. Historically, the earliest reports about the existence of the pathogenic races of *R. secalis* from the United States appeared in 1960's (Harrabi, 1996).

The very first reports of *Rhynchosporium* resistance genes date back to 1961 as a result of work by Dyck and Schaller (Hahn et al., 1993). The reported genes are limited to four chromosomes and it remains questionable, at least in some cases, which genes are responsible for conditioning resistance and on which chromosome they are located. The barley cultivar LaMesita (CI 7565) is such an example. This cultivar was found, in one study, to contain two dominant resistance genes, Rh4 and Rh10. However, a later study conducted in 1982 with *R. secalis* isolates from Morocco found these two genes in LaMesita to be recessive (Dyck and Schaller, 1961; Harrabi, 1996).

Although the list of scald resistance genes is a result of extensive work of many researchers, still more data are needed to confirm some of the conclusions. The tests conducted usually included large number of plants screened to determine segregation ratios and to establish if a genes in question were recessive or dominant. Most of these studies also included several isolates of the pathogen

due to its high variability. Unfortunately, since in most studies the sets of differentials varied, comparing results among the diverse sets of data is extremely difficult. This points to a very important aspect of the study of barley scald, and other diseases as well, a lack of uniformity in differential sets. To create a uniform database, a set of international differentials should be established and certain races should always be included in the screening process.

In recent years new technologies for screening have been developed or improved, with molecular tools being used more extensively in breeding programs (Abbot, 1992; Borovkova et al., 1995). The use of molecular markers is especially useful in diseases caused by obligate parasites, or highly variable pathogens such as *R. secalis*, where screening with the conventional methods is often complicated. Conventional methods are too cumbersome to keep up with changes in the pathogen population. Most of these newer techniques, however, require expensive equipment, supplies and extensive staff training.

Efforts by barley researchers in the early 1990's resulted in an extensive map of Restriction Fragment Length Polymorphism (RFLP) for barley (Graner et al., 1991; Kleinhofs et al., 1994). Markers are being added to the existing gene map as they are discovered. In 1994, two additional scald resistance genes were added. Sources of those genes were accessions of a wild barley *Hordeum vulgare* ssp. *spontaneum* from Israel, Turkey and Iran. Resistance gene Rh12 is located on chromosome 1 while resistant gene Rh13 is located on chromosome 6 (Abbott et al., 1992, 1995).

In the early 1990's, studies were initiated at the Winnipeg Research Centre to identify molecular markers for fungal pathogens of small grains. RFLP markers were converted to the Randomly Amplified Polymorphic DNA (RAPD) markers and reactions to five *R. secalis* isolates were evaluated for a group of western Canadian barley cultivars to determine how many genes were involved in controlling scald resistance. These studies concluded the presence of two different genes in western Canadian barley cultivars (Penner et al., 1996).

Breeding for scald resistance is complicated also because of the highly variable nature of this pathogen. A single gene for resistance does not provide sufficiently durable control of this disease. To obtain a highly resistant cultivar, gene pyramiding may be the best solution.

This research focuses on the following objectives:

1. Incorporation of resistance genes into the commercial malting cultivar Harrington;
2. Screen for scald resistance with traditional methods;
3. Screen for the presence of resistance gene(s) with molecular markers, using the Polymerase Chain Reaction method (PCR); and
4. Evaluate and compare the traditional and non traditional (PCR) screening techniques.

LITERATURE REVIEW

Barley (*Hordeum vulgare* L.), belonging to the grass family Poaceae, tribe Triticeae, is one of the oldest cultivated cereal grains. Barley contributes to the food supply not only as human food, but also as a malt product and livestock feed. It is also used as an important experimental and model plant species for numerous studies in malting and brewing, plant breeding, genetics and plant pathology.

The origin of barley is not clear. Evidence of its cultivation is reported about 17,000 years ago in the Nile River Valley of Egypt (Foster, 1987). Barley culture most likely extended later to other countries and continents, as the remains of barley kernels were also found during archeological excavations in Ethiopia, Tibet, Afghanistan, and the Near East (Foster, 1987). The most immediate ancestor of barley is the two-rowed *Hordeum vulgare* spp. *spontaneum*. Six row cultivated barley is considered a result of mutations and hybridization.

Barley is adapted to grow well in a broad range of environmental conditions, e.g. at high altitudes, below the sea level near Dead Sea, in the desert and under semi-arid conditions. *H. vulgare* also is impressively salt and alkali tolerant. However the optimum conditions for best yields are well-drained, fertile loam soils, moderate temperatures (15-30° C) and moderate seasonal rainfall (500-1,000 mm).

Barley has been intensively cultivated for centuries but remains relatively susceptible to a number of plant pathogens. Most of the regions where barley is grown also are known to harbor numerous plant pathogens such those that cause scald, net blotch, kernel blight and various rusts. In the intermountain region of the United States and Canada, where two-row malting barley is predominantly grown, barley scald, caused by *Rhynchosporium secalis*, is one of the most damaging diseases (Brown, 1985).

The genus *Rhynchosporium* has been placed in the class *Deuteromycetes*, because the perfect (sexual) state of this fungus is not known. Like most fungi in this class, *Rhynchosporium secalis* is very likely an *Ascomycetes* with no sexual stage found to date. *R. secalis* reproduces by means of abundant conidia. If water is present for at least 4 hours, *R. secalis* conidia begin to germinate. Appressoria are formed by the germ tubes of conidia. Penetration has also been observed in the absence of the appressoria. Penetration usually occurs directly, rather than through stomates, and takes place among the epidermal, the guard or the subsidiary cells. Initially, the cuticle is penetrated with the help of enzymes that degrade the host cell wall. Subcuticullar mycelium then develops intracellulary, between the cuticle and the epidermal cells. Mycelium varies in thickness from a single to several layers. As the growing hyphae penetrate the epidermal cell layer, host cell walls thicken due to the presence of mycelium (Shipton, 1974). Five to six days after initial infection, the mycelium invades the mesophyl layer. Scald symptoms are not visible until approximately ten days from the infection.

Afterwards symptoms develop rapidly, in some cases causing the collapse of the entire leaf. At this point, the entire mesophyl cells are colonized and broken down by the fungus.

In recent years work on barley scald has been conducted in North and South America, Asia, Africa, Europe and Australia (Ali et al., 1976; Ceoloni, 1980; Fukuyama and Takeda, 1992; Goodwin et al., 1990; Hansen and Magnus, 1973; Khan, 1986; Lyngs Jorgensen and Smedegaard-Petersen, 1994; Metcalfe, 1978; Penner et al., 1996; Welty, 1996). This in itself confirms that barley scald is an important disease in most areas where barley is grown. *R. secalis* also infects rye and several grass weeds (Welty, 1996).

However, until now, most of the work has been difficult to compare between different breeding programs mainly due to lack of a uniform nomenclature in studying scald by the researchers from different countries. In the early 1990's, a group of U.S. barley workers proposed adopting a standard nomenclature for scald pathotypes and that a standard set of differentials that included most of the common differential cultivars be used. To be effective, the new nomenclature for *Rhynchosporium secalis* pathotypes should meet several requirements and assure the inclusiveness of all important *R. secalis* resistance genes along with complete information on race pathogenicity (Goodwin et al., 1990; Webster, 1980). Hopefully, in the near future, efforts to create a universal, international nomenclature will succeed and a universal ability to share data and compare the results of the various studies will materialize.

Identification of genes governing desirable characteristics in barley such as high yield, disease resistance or exceptional malting qualities is a significant factor in the process of developing resistant cultivars. The complex task of combining several genes controlling important traits into one cultivar is also a limiting factor in achieving acceptable results (Barua, 1993). Most agronomically important traits, such as grain yield and protein content, are governed by several genes and therefore are difficult to manipulate in a breeding program. In most instances of qualitative inheritance, one or a small number of genes control the trait (Stoskopf et al., 1993). An example of a simply inherited trait in barley is two-rowed versus six-rowed spike type. Recent advances in the area of the biotechnology has accelerated the process of identifying and manipulating genes. Some of the remaining questions regarding presence or absence of resistance genes should soon be answered and applied in the development of new barley cultivars.

Since the early 1970's research programs have been attempting to incorporate various genes for resistance to scald into acceptable barley cultivars. So far, thirteen such genes regulating resistance to *R. secalis* have been reported (Bockelman et al., 1977; Briggs and Johal; 1994, Brown, 1990; Abbot et al., 1995; Baum et al., 1996).

Table 1. *Rhynchosporium secalis* resistance genes in barley reported in literature

Cultivar/Accessions	Gene	Chromosome
Hudson (CI 8067)	Rh	3L
Bey (CI 5581); CI 8162	Rh3	3
Forrajera, Osiris	Rh4	3
Atlas (CI 4118)	Rh2 rh or Rh2	1S
CI 3940	Rhrh6	
La Mesita	Rh10rh6rh7 or Rh4Rh10	3
Atlas 46 (CI 7323)	Rh2Rh3rh5 or Rh2Rh3	
Turk (CI 14400)	Rh3Rh5rh6 or Rh3Rh5	
Jet (CI 967)	rh6rh7	3, 4
Nigrinudum	rh8	
Kitchin, Abyssinian (CI 668)	Rh9	4
Osiris (CI 1622)	Rh10 or Rh4	
CI 4364	rh11	
Trebi	Rh4, Rhrh6	
CI 2376	Rh4Rh9	
Modoc (CI 7566)	Rh2rh6 or Rh4rh?	
E224/3	Rh4Rh10	3
Hordeum vulgare ssp. *Spontaneum*	Rh12	1
Hordeum vulgare ssp. *Spontaneum*	Rh13	6H

Cultivars/accessions listed in Table 1 as a source of the resistance genes do not represent cultivars commercially grown for feed or malting purposes (Abbott et al., 1995; Goodwin et al., 1990; Webster, 1980). These cultivars/accessions are known as sources of resistance, but most of them have not been used in breeding programs. The majority of currently grown commercial cultivars remain susceptible or moderately susceptible to *R. secalis*. A classic malting cultivar example is Klages, grown until 1981, and later replaced by Harrington. Both cultivars are susceptible to most isolates of *R. secalis*. Harrington still dominates the malting barley market with about 70% of the acreage. Clearly, there is a need for a new two-row commercial malting cultivar resistant to scald that would result in higher yield and grain with improved quality. With the advances in molecular biology, breeding programs may implement molecular markers into developing quality germplasm.

The first markers used by researchers were Restriction Fragment Length Polymorphism (RFLP) markers that distinguished differences in lengths of fragments made by cutting the deoxyribonucleic acid (DNA) with restriction enzymes. After having been cleaved into fragments of different sizes with restriction enzymes, the DNA is later electrophoretically separated and identified by hybridization with an homologous probe sequence. RFLPs usually require the use of radioactive materials (Laurie et al., 1992). Subsequent research produced safer and more efficient techniques like Amplified Length Fragment Polymorphism (ALFP) or Sequence Tagged Site (STS).

Another type of marker, Randomly Amplified Polymorphic DNA (RAPD), can be obtained by direct amplification of oligonucleotides, short fragments of DNA (random or specific 10 base-pair (bp)) using polymerase chain reaction (PCR) with genomic DNA as a template. Both RFLP and RAPD type markers provide the means of identifying DNA variations. Molecular markers offer a significant advantage in the detection of polymorphisms in such organisms as barley where coding sequences and their control regions account for only a small component of the genome since sequence variation can sometimes produce RFLP or RAPD without generating any phenotypic variation (Laurie et al., 1992).

Several types of markers have been converted to more efficient types or developed in recent years for *R. secalis*. An example of this is the cooperative work by German and Canadian researchers that resulted in converting the cosegregating RFLP marker cMWG680 into the codominat STS marker used in confirming the Rh resistance gene in the cultivar Triton (Barua et al. 1993; Graner, 1996; Schweizer et al. 1995; Abbot et al., 1995) (Table 2).

Table 2. Scald marker examples

Gene	Type of Marker	Chromosome	Marker
Rh	RAPD	3L	SC10-65-H400
Rh	RFLP	3L	cMWG680
Rh2	RFLP	1S	CDO545
Rh13	RFLP	6H	Cxp3, ABG458

In the early 1990's extensive work was performed by a research group in Canada with three main goals: (1) to compare the scald resistance present in Canadian cultivars; (2) to trace the pedigrees for potential sources of resistance; and (3) to test genetic hypotheses through intercrossing and application of molecular markers. As a result, tested cultivars were grouped into six clusters of resistance phenotypes. Two types, Hudson and Atlas, dominated in the 22 commonly grown Canadian cultivars. Two modern cultivars represent each of the groups, the Hudson type is represented by CDC Silky and the Atlas type is represented by a cultivar Falcon. Moreover, a RADP marker linked to a *R. secalis* resistance gene located on chromosome 3 was converted to the locus specific amplicon SM1000. It was demonstrated, using this amplicon, that resistance in CDC Silky was due to a single dominant gene, which resides within Rh/Rh3/Rh4 cluster (Penner et al., 1996).

Researchers working under the auspices of the North American Barley Genome Mapping Project (NABGMP) have also been adding phenotype and genotype data to the barley chromosome map. They have "focused on generating linkage maps in elite germplasm in order to facilitate the direct application of these maps to plant breeding via QTL (quantitative trait locus) analysis" (Hayes et al., 1996). A number of mapping populations and QTL validation stocks have been developed.

The 7th International Barley Genetics Symposium was held at the University of Saskatchewan, Saskatoon, Canada, on August 5, 1996. To improve

the communication between barley researchers working on gene mapping and breeding the following resolution, regarding the designation of the barley chromosomes and their arms, was passed:

> " 1. Each of the seven barley chromosomes is designated by a figure from 1 to 7 according to its homoeologous relationships with chromosomes of other Triticeae species. The figure is followed by the letter H; e.g., 2H.
>
> 2. The genomes of *Hordeum vulgare* and *H. bulbosum* are symbolized by the letter H.
>
> 3. The chromosome arms are designated by the letters S or L.
>
> 4.The barley genome present in the variety "Betzes" becomes the reference genome in the Triticeae to which definitions of translocations, short arm/long arm reversals, etc. are standardized in all species." (Anonymous, 1997)

Recent advances in computer technology now allows almost instant access to new developments and research data concerning both barley and its pathogens. Search engines list numerous internet sites providing detailed information on newly mapped barley genes and markers. This also greatly improved the communication between researchers all over the world working on similar projects. The results can be compared and discussed without necessity to travel. The amount of information available is now enormous, but there is still a lot to discover.

COMPARISON OF THE TRADITIONAL SCREENING TECHNIQUES AND SCREENING WITH MOLECULAR MARKERS

Rhynchosporium secalis is a highly variable pathogen with new isolates appearing frequently in the fungus population (Ali, 1976). Traditional breeding for resistance to the pathogen is therefore difficult. It takes several years to develop a new barley cultivar, during which the pathogen can change. By the time such a cultivar is ready the pathogen can have a completely new quality allowing it to overcome resistance. Molecular markers offer a new tool to overcome this difficulty. Applying biotechnology techniques permits completing of the breeding process rapidly while using fewer plants. Moreover, the breeding operation does not have to be limited to the area in which the pathogen is present and can be done anywhere in the world. Only the final stages of the development of the new resistant cultivar should be performed in the area that the disease is present.

Molecular markers also simplify the screening process. In traditional breeding, the necessary presence of uniform disease pressure is hard to maintain from year to year. In some years the weather does not create proper conditions for the pathogen to develop at a level needed for accurate disease evaluation. Therefore, multiple locations with multiple replications are necessary for proper selection of resistant plants. Some pathogens require additional artificial inoculum

applied in a disease nursery and an overhead watering system to assure proper relative humidity. Moreover, a pathogen can arrive late in the season making it impossible to conduct the disease selection, as is the case of stripe rust *Puccinia striiformis* Westend. f. sp. *hordei* (Mathre, 1997).

With the use of molecular markers, a smaller number of plants allows the tests to be performed in greenhouses or growth chambers where the necessary conditions for plants to grow could be easily replicated. Another advantage of using greenhouses or growth chambers for screening, is a possibility of obtaining more generations in a specified time period, whereas traditional methods allow for only two, or maximum three, generations per year.

Table 3 illustrates a simple scenario where incorporating only one gene of interest could be performed faster using the molecular markers method. If more than one gene is involved, the process becomes more complicated, especially in case of traditional breeding. Also, different breeding methods would require specific modifications resulting in altered numbers of necessary plants in each generation.

Table 3. Hypothetical screening schedule for disease resistance comparing traditional and molecular methods

	Traditional Methods	Marker Assisted Selection
Year 1	Plant parents	Plant parents
	Cross	Cross
	Seed increase F1: F2	Seed increase F1: F2
	Plant F2 in the nursery/greenhouse Screen with pathogen	Plant F2 in the nursery/greenhouse Screen with molecular marker and select resistant plants
	Make a selection for resistant plants	Plant F3, check random plants for presence of the gene (s)
Year 2	Plant F3 generation, select Resistant plants	Advance plants, selecting for other agronomical traits
	If possible use winter nursery For advancing and selection	
Year 3	Plant F5 (F4 in case of lack of winter nursery) plants	
	Repeat until F7 or F8 generation when homozygocity reaches 99%	

While the molecular marker technique is a viable alternative to traditional methods, its main disadvantage remains the availability of the marker. Another important barrier is availability of the equipment and cost of materials involved in the screening process. Also, the complexity of the entire PCR process, despite

advances in molecular marker science, requires well trained personnel. Often the results obtained in one laboratory cannot be immediately replicated in another lab leading to a situation where, in some cases, results could be obtained easier with traditional screening methods. Even though newer techniques are being designed with safety in mind, they still require extensive knowledge of safe handling of chemicals.

METHODS AND MATERIALS

Crossing Procedure

The initial crosses were planted in approximately three feet wide greenhouse beds filled with 50/50 mixture of top soil and Sunshine Mix #1 (Sun Gro Horticulture Canada, Ltd., 15831 NE 8th Street, Bellevue, WA 98008), a commercial soil mixture containing peat moss, wetting agent, as well as perlite and starter nutrients. Two rows of ten seeds each were planted with five inch space between the seeds. Two separate plantings were performed for Harrington, SM 89010 and CDC Silky to nick (coordinate the flowering dates of) the crosses. Peters 20-20-20 fertilizer was applied at the two leaf stage with approximately 200 ppm of nitrogen per gallon of water.

Barley is a self-fertilizing plant and emasculations or removal of anthers from florets of the female plants are necessary prior to crossing to prevent selfing. The emasculations were performed before the anthers reached maturity, usually at the time when awns emerged about an inch from the collar of the flag leaf. Small forceps and scissors were used to remove the anthers. The flag leaf was cut right above the end of the tip of the spike and the sheath was split into a few sections and unrolled to uncover the spike. Each of the florets was carefully cut to expose the anthers that were later removed with forceps by pulling them gently out of the

floret. Since the sections of the spike are very fragile at this stage the emasculation, as well as pollination, procedures must be done with care so that the spike is not broken or twisted. During the removal of the anthers the delicate stigma should not be disturbed. The entire procedure is performed quickly to prevent the florets from desiccation.

A glassine bag was placed on the plant head after the anthers were removed. The emasculated plant was labeled with the appropriate date. Pollinations were performed 2-3 days later when the lemma and palea were slightly open. All pollinations were usually done in the morning under additional high sodium light provided to enhance maximum extrusion of the anthers. Male spikes were cut from the plant, placed above the female plant upside down and twirled to shed the pollen. This technique is fittingly called the twirl method (Foster, 1987). After the completed pollination the flag leaf was secured around the spike to prevent further desiccation.

Populations

Barley populations for these studies were derived from crosses of the scald susceptible cultivar Harrington and two scald resistant parents, CDC Silky and SM89010 (Table 4). Harrington is a cultivar used in malting and brewing. It was released to growers in 1981 for the areas of the United States and Canada where two-row barley is traditionally grown (American Malting Barley Association,

Inc., 1984). Harrington, developed by the University of Saskatchewan, constitutes 60% of the two-row malting cultivars grown in the intermountain area.

Table 4. Pedigrees of the parents included in the crosses

Parent	Year of Release	Pedigree
Harrington	1981	Klages/3/Gazelle/Betzes//Centennial
SM89010	1994	Nairn/Manley
CDC Silky	1994	SB83620/Duke

Starting at the F2 stage, seeds were planted in separate plastic cells, 12 inch long and 1.5 inch diameter, arranged within Cone-tainer trays from Hummerts International (4500 Earth City Expwy., Earth City, MO 63045). Each plastic cell was filled with commercial soil mixture Metro Mix 200 from Scotts Sierra Horticultural Products Company (14111 Scottslawn Road, Marysville, OH 43041) in all experiments. Six cone trays, each holding 98 cones, were placed in metal trays resting on greenhouse benches. The temperature in the greenhouse was controlled by the ARGUS system (Argus Control System Ltd., 1281 Johnson Road, White Rock, BC V4B 3Y9, Canada). Day length was increased up to 16 hours by adjusting temperature and providing artificial light with high pressure sodium lights maintained at minimum 300 candle foot. The temperature settings were: 24° C for the day and 20° C for the night time. Plants were fertilized at the 3 leaf stage, Zadoks stage 13 (Nelson et al., 1988), with the Peters 20-20-20

nitrogen fertilizer at the rate of 3/4 cup per gallon of water . Fertilizer was applied with a siphon mixer (Scotts Brass, Marysville, OH 43041) attachment that provided additional dilution factor of 16:1 ratio. Before planting, all the seeds were treated with the systemic insecticide Imidacloprid (Gaucho 480 F) (Gustafson, Inc. 1400 Preston Road, Suite 400, Plano, TX 75093) at the rate of 2 ounces per 100 seeds. Gaucho's active ingredients are 1-((6-Chloro-3-pyrimidinyl)methyl)-N-niro-2-imidazolidinimine and Ethylene glycol. This insecticide prevents seedlings from aphid infestation for up to 90 days. Although this eliminated or lowered the subsequent use of insecticides, the process was time consuming due to the disparate amount of the seeds used in different parts of the experiment. Screening was restricted to fall, winter and spring, since *R. secalis* isolates require relatively low temperature to infect barley effectively. Even with computerized greenhouse controls it was impossible to maintain such low temperature in the greenhouse during summer. This required advanced planning and a rigid schedule. Very low relative humidity in Colorado necessitated additional plant misting with water. Plants were misted twice a day with the Fogg-it "Waterlog" misting nozzle (Fogg-it Nozzel, Co., P.O. Box 16053, San Francisco, CA 94116) following the inoculation period and before obtaining the disease notes.

The initial cross for Harrington was made in 1972 followed by its release in 1981 (American Malting Barley Association, Inc., 1984). CDC Silky is a hulless, semi-dwarf, feed type, six row cultivar, released in 1994 (Rossnagel et al., 1994).

CDC Silky's name is derived from its very smooth awn. This cultivar is a candidate for becoming an important forage barley in areas producing barley for silage (Rossnagel et al., 1994). SM89010 is an experimental line with good malting qualities (Penner et al., 1996).

An additional cross between the two donor parents, SM89010 and CDC Silky, was also done for mapping purposes. Both donor parents are resistant to *R. secalis* isolate 1493, while CDC Silky is also resistant to *R. secalis* isolate 1824 (Figure 2). The other parent, SM89010, is moderately susceptible to isolate 1824 (Table 5). CDC Silky and SM89010 both possess the Rh gene and a RAPD marker for this gene is available (Penner et al., 1996). Mapping was done to develop the marker associated with the resistant gene in CDC Silky.

Table 5. Parent reactions to *R. secalis* isolates 1824, 1493 and the SM1000 marker

Cultivar	1824	1493	SM1000 Marker
Harrington	S[1]	S	-[2]
SM89010	MS	MR	+[3]
CDC Silky	R	R	+

[1] R = resistant, MR = moderately resistant, MS = moderately susceptible, and S = susceptible
[2] - = band absent
[3] + = band present

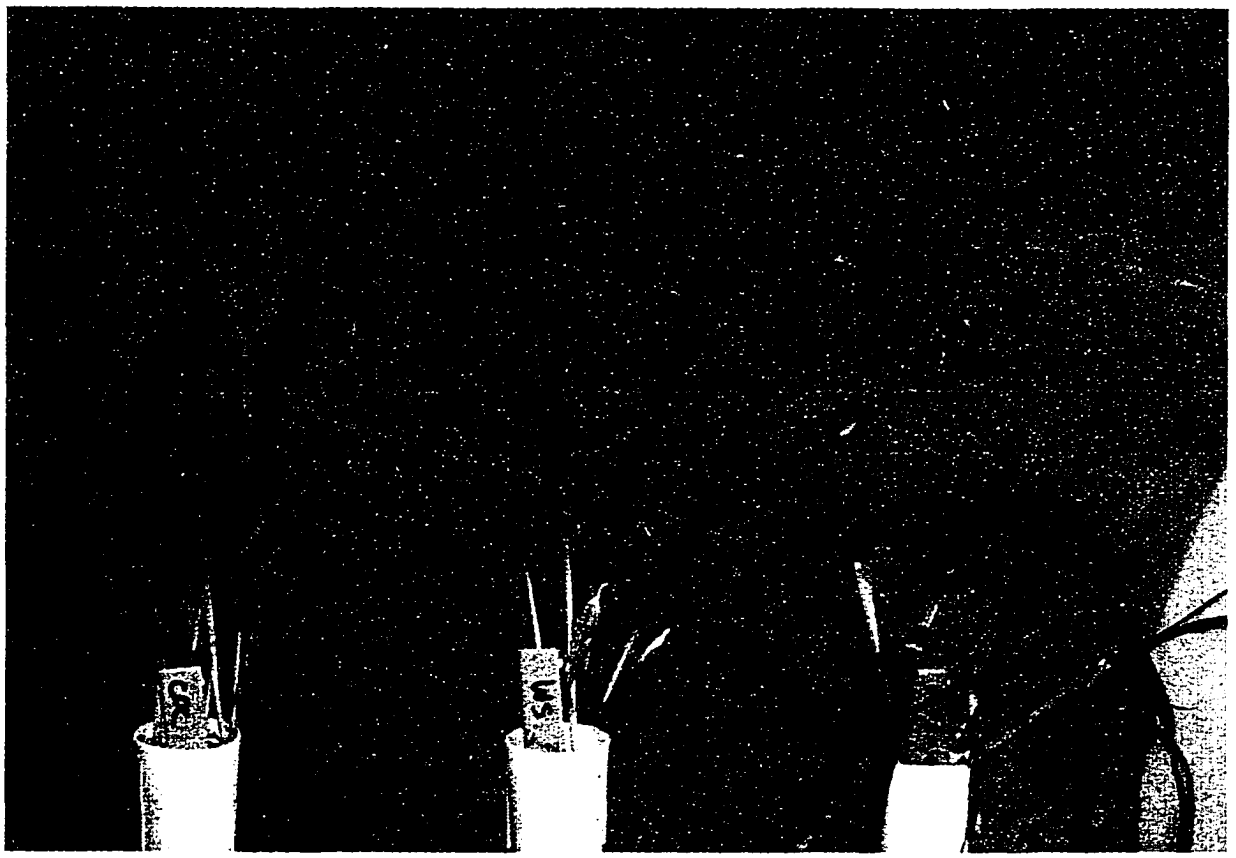

Figure 2. Typical parent reaction to *Rhynchosporium secalis* isolate 1824. CDC Silky = resistant, SM89010 = moderately susceptible and Harrington = susceptible.

The initial crosses (Cross1, Cross 2 and Cross 3) were made in the fall of 1996 and plants harvested in 1997. The F1 increase was performed and F2 progenies were screened for resistance with *R. secalis* isolate 1493 (Cross 1 and 2) and 1824 (Cross 3). Resistant plants were selected and advanced into F3 generation. At the F3 stage, six seeds were planted from Cross 1 and four seeds from Cross 2 to allow the expression of possible genotypes. For Cross 1 (Harrington/CDC Silky), only two-row resistant plants were selected. Crosses segregated in a 3:1 ratio for resistance suggesting presence of one dominant gene. Plants were screened at the two leaf stage, Zadoks 12 (Nelson et al., 1988), and evaluated on the 0 to 3 scale developed from Schein (1958); 0 = resistant and 3 = susceptible (Bockelman, 1977). Each screening population included a set of controls that consisted of all the parents and the additional scald susceptible two-row cultivar Klages. Both Harrington and Klages have good malting qualities, but are susceptible to scald.

At the F4 generation, resistant progenies of crosses 1 and 2 were also screened with SM1000 marker for the presence of the Rh gene (Penner et al., 1996). Resistant F4 plants were crossed and the obtained F1 seeds increased for Cross 4 (Table 6). F2 plants from Cross 4 were screened with the mixture of *R. secalis* isolates 1493 and 1824 to select plants resistant to both isolates. Randomly selected resistant plants were tested with SM1000 marker.

Table 6. Crosses used in the studies of developing *R. secalis* resistant line

Cross	Cross Name	Resistant Parent	Susceptible Parent
1	C97-X374	CDC Silky	Harrington
2	C97-5162	SM89010	Harrington
3	C97-7000	CDC Silky	SM89010
4	C98-2984	C97-X374	C97-5162

Inoculation Techniques

R. secalis isolates 1824 and 1943 used in screening of the barley populations were obtained from Dr. A. Tekauz of the Agriculture and Agri-Food Canada, Winnipeg Research Centre, under USDA permit number 32382. Seeds for the test plants were obtained from Agricore (formerly Alberta Wheat Pool). Plants of the parents used in crosses were inoculated with either isolate 1493 or 1824 separately. Diseased leaf tissue was collected and stored in paper envelopes in the refrigerator at 4° C as the source of single spore cultures for inoculation purposes. Seeds from the diseased plants were also collected for future use in the studies.

For each inoculation, the fungus was re-isolated from the originally collected diseased tissue. Infected leaves were surface disinfested in a mixture of 5% ethyl alcohol, 15% NaOCl and 80 % sterile distilled water for 3 minutes and

plated in petri dishes on Water Agar media from DIFCO Laboratories (Detroit, MI 48232-7058). Since *R. secalis* is a slow growing fungus the cultures were placed under cool temperatures to slow bacterial growth. Growth chamber settings were: 12 hours light alternating with 12 hours of darkness, 16° C for the day and 12° C for the night. Fungal growth in the media was concentrated under the leaf tissue. The fungus appeared as shiny, slightly pinkish hyphae with profound branching. Conidia were abundant in the culture.

Small pieces of agar with conidia were plated with a transfer needle into petri dish containing Lima Bean Agar (DIFCO) or Malt Agar media (20 grams of finely ground barley malt, 15 grams of BactoAgar (DIFCO) dissolved in 1000 ml of distilled water, then autoclaved for 20 minutes at 121° C). Subsequent transfers were made by applying small amounts of distilled water (DI) on the culture and scraping with a sterile cover glass and transferred by pipette into a petri dish containing Malt Agar media. Eight days later conidial suspension inoculum was prepared by scraping the culture with cover glass, diluting with sterile distilled water and filtering through four layers of cheese cloth to remove pieces that would cause clogging of the inoculating device.

Conidia were further diluted with distilled water to obtain desired concentration. Concentration of the inoculum was calculated using a Spencer Brightline Hemacytometer. The concentration used for all inoculations was 2.5 x 10^5 spores per milliliter of autoclaved distilled water. Approximately 0.5 ml of

the conidial suspension per plant was applied using DeVilbiss atomizer No. 152 (DeVilbiss Division, Sunrise Medical, Somerset, PA 15501-0635).

Plants were inoculated at the two leaf stage, Zadoks stage 12 (Nelson et al., 1988), when they were approximately ten days old (Figure 3). The inoculum contained approximately 10 drops of Tween 20 (Fisher Scientific, 9999 Veterans Rd., Houston, TX) solution (poloyoxyethylene-20-sorbitan monolaureate) per 100 ml of conidial suspension. Plants inoculated with *R. secalis* isolate 1493 were placed for 24 hours in the chamber with 100% relative humidity while plants inoculated with isolate 1824 were kept in the chamber for 48 hours (Figure 4). The inoculation method for *R. secalis* isolate 1824 was based on the procedure described by Tekauz (1991). *R. secalis* isolate 1943 did not require 48 hours of 100% relative humidity, since sufficient scald symptoms developed after 24 hours. Humidity was controlled by Model 500 Humidifier (Herrmidifier Co. Inc., Lancaster, PA) with the approximate output of 2 pints per hour. Disease was evaluated fourteen days after the inoculation. Selected resistant plants were saved and grown for seeds. Seeds from susceptible plants were collected , but not used in later studies. Each generation of screened plants included a set of the parents used for the crosses as well as the additional susceptible check, Klages.

Figure 3. Inoculated barley plants at the two leaf stage (Zadoks stage 12).

Figure 4. Barley plants in the inoculation chamber. Each tray contains 92 plants and 4 checks: Harrington, SM89010, CDC Silky and Klages.

Disease Evaluation

Three seeds per cone were planted for each parent and one seed per cone for each tested plant. Seeds were planted in the trays holding ninety-eight cells. Each tray of 94 tested plants included four differentials consisting of Harrington, SM89010, CDC Silky, and an additional susceptible Klages cultivar. Each group inoculated with conidial suspension also included a control set of the same four differentials inoculated with sterile distilled water. Fourteen days after the inoculation, disease notes were taken from the inoculated plants. The scale used was based on four steps varying from zero to three (Table 7) (Bockelman, 1977), with zero being the resistant response and three the susceptible (Figure 5). Only resistant plants were used in subsequent studies.

Table 7. Scald reaction based on a scale by Schein (1958)

Scale Point	Response	Reaction
0	No visible symptoms	R[1]
1	Very small lesions usually along leaf margin	MR
2	Larger lesions at leaf margin or in central portions of leaf blade	MS
3	Large coalescing lesions or total collapse of entire leaf	S

[1]R = resistant, MR = moderately resistant, MS = moderately susceptible, and S = susceptible

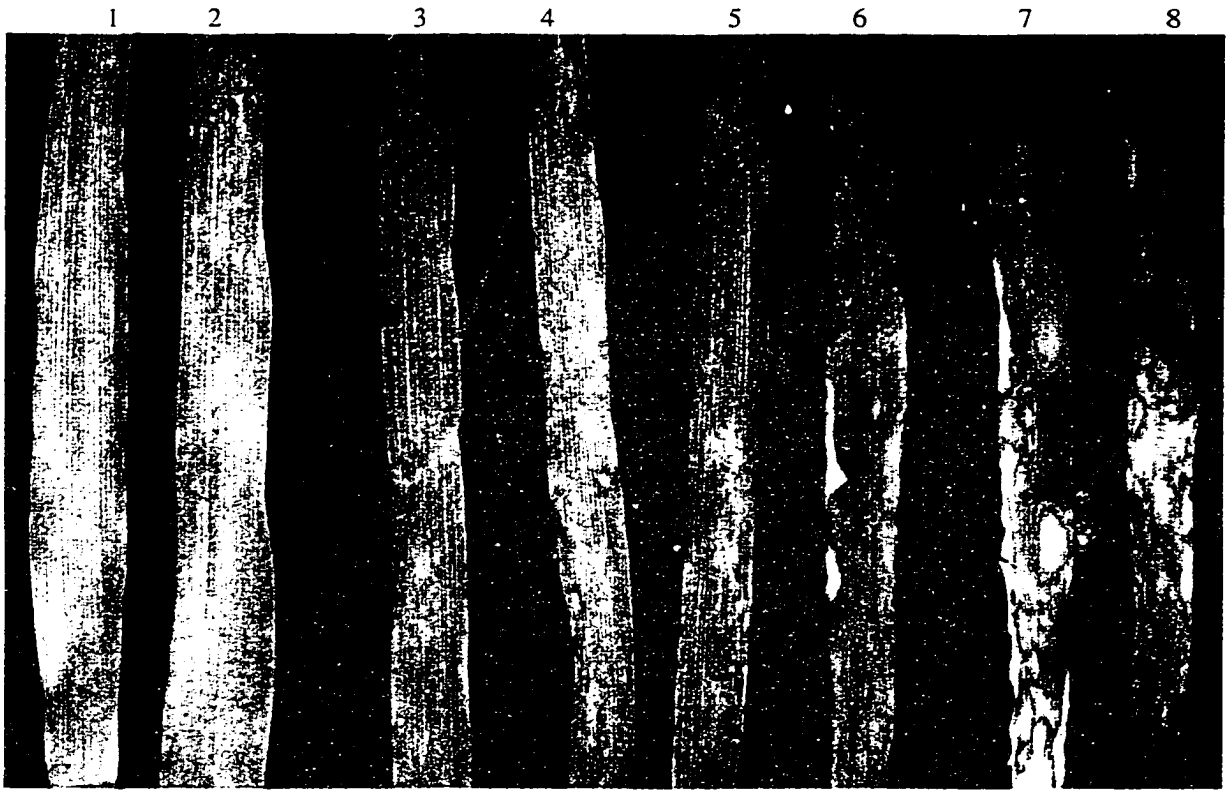

Figure 5. Scald symptoms. Leaf 1 = resistant reaction, leaf 2 = moderately resistant reaction, leaves 3-5 = moderately susceptible reaction, leaves 6-8 = susceptible reaction.

Cross 1: C97-X374

Harrington, a commercial two-row malting cultivar, was crossed with the six-row feed cultivar CDC Silky used as the male parent. The cross, which consisted of 10 plants of each parent, was made in the fall of 1996 and seeds were harvested in January of 1997. F1 plants were grown in the greenhouse and screened at the two leaf stage using *R. secalis* isolate 1493. The F2 population consisted of 300 plants. The segregation ratio fit the 3:1 model, with three fourths of the plants showing resistance and one fourth showing susceptibility, indicating a single dominant resistance gene present. The F2 plants also segregated for type of the spike, since Harrington is a two-row cultivar and CDC Silky a six-row. Only two-row plants were harvested and advanced into the next generation.

Resistant plants were selected and screened again with *R. secalis* isolate 1493 in the F3 generation. As with the F2 generation, six-row plants were not included in subsequent studies. At the F4 stage, 20 plants were selected and again screened with *R. secalis* isolate 1493 and with the SM1000 marker for the presence of the Rh gene (Penner et al., 1996). Resistant plants from Cross 1 (Harrington/CDC Silky) were crossed with resistant F4 plants from the Cross 2 (Harrington/SM89010). Cross 1 was chosen to be a pollen donor.

Cross 2: C97-5162

Harrington, male parent, was crossed with SM89010, female parent, a two-row malting quality line resistant to isolate 1493 (Penner et al., 1996). This cross

was made in the fall of 1996 and plants were harvested in January, 1997. F1 plants were increased and screening *R. secalis* isolate 1493 was performed on the F2 seedlings. Three hundred F2 plants were tested. The segregation ratio fit the 3:1 model, with three fourths of the plants showing resistance and one fourth of plants being susceptible.

Seeds from resistant plants were planted and the screening with *R. secalis* isolate 1493 performed on F3 generation. Four F3 seeds were planted from each resistant or moderately resistant F2 plant. At the F4 stage twenty plants were selected and crossed with F4 plants from Cross 1 (Harrington/CDC Silky). Since Cross 2 was a two by two row spike type, its plants were chosen as a female parent for the final Cross 4.

Cross 3: C97-7000

Cross 3 was made between SM89010 and CDC Silky, both of which possess the Rh gene detectable with the SM1000 amplicon. The SM89010 malting line and CDC Silky cultivar are both resistant to *R. secalis* isolate 1493, but CDC Silky is also resistant to *R. secalis* isolate 1824. F2 plants from this cross were inoculated with *R. secalis* isolate 1824. Random primers were used to determine any polymorphism between the donor parents. The results were combined with phenotypic disease symptoms in an effort to detect the existence of a marker associated with the gene for resistance. Two primers from Genosys

Biotechnologies, Inc. (1442 Lake Front Circle, Suite 185, The Woodlands, Texas 777380-3600) OPH-G-1 and OPH-G-3, showed a polymorphism.

R. secalis isolate 1824, used for the screening of F2 plants, was also used to screen the F3 population. At the F3 stage seeds were planted to observe four possible genotypes. The number of plants varied for each entry from 4 to 11 plants. Phenotypic data from both screened generations along with the primer data were analyzed using MAPMAKER computer software (Lander, 1987).

Cross 4: C98-2894

Cross 4 involved F4 plants from Cross 2 used as a female parent and plants from Cross 1 that were the pollen donors. F1 seeds were increased during the summer of 1998. F2 plants were screened in the spring of 1999 using a mixture of *R. secalis* isolates 1943 and 1824. *R. secalis* isolate 1943 was used to select progenies with the Rh gene which was present in both parents. *R. secalis* isolate 1824 was used to select progenies resistant to this isolate. Fourteen randomly selected resistant plants and all fourteen susceptible plants were screened with the SM1000 marker for the presence of the Rh gene (Penner et al., 1996).

DNA Extraction Techniques

Most of the techniques available for DNA extraction were not applicable to this study. The objective of this research was to use techniques that are safe, quick and require a minimum amount of plant material. These requirements were also

affected by the need to screen large numbers of plants quickly and at minimum expense. In spite of the multitude of existing methods, it was difficult to find one that would fit the required regime. Moreover, most of the presently available methods use highly toxic chemicals and call for special handling and disposal of residue which also had to be taken under consideration in this project.

After testing several available methods, the NucleoSpin Plant Kit (K3060-1) from Clontech Laboratories, Inc. (1020 East Meadow Circle, Palo Alto, CA 94303-4230) was chosen. This kit seems to be the most safe and efficient method of harvesting DNA for this study. It requires only small pieces of the plant material (approximately 50 mg) for the extraction and the entire procedure is safe and easy to follow with the adequate quality of extracted DNA for the PCR method.

The DNA concentration was determined by taking absorbance readings at 260 nm and 280 nm using Beckman spectrophotometer model Du-65 (Beckman Instruments, Inc., Fullerton, CA 92634) and calculating the applicable ratio. The reading at 260 nm allows calculation of the nucleic acid concentration in the sample. An OD (optical density) of 1 corresponds to approximately 50 ug/ml for double stranded DNA and 40 ug/ml for single stranded DNA. The ratio of DNA with the sufficient purity for PCR use should be 1.8 (Sambrook et al., 1989). Any contamination with protein or chemicals used in extraction would significantly lower this ratio making it impossible to accurately quantify the amount of DNA.

DNA Amplifications

The PCR technique has many advantages, one of them being the small amount of the plant DNA required. However, this technique is very sensitive to any changes in the testing environment and is not always sufficiently reliable. Researchers often have difficulty repeating PCR protocols.

The first reactions in this study were performed at volume of 50 μl. This relatively high volume was chosen to minimize the pippeting error and maximize the number of successful reactions. However, those higher volumes raised the cost of the reagents and Taq Polymerase used. Subsequently one of the research objectives was to minimize the reaction volume.

SM1000 amplicon was used to screen for the Rh gene (Penner et al., 1996). SM1000 are RAPD 10mers were used with the following sequences:

SM1000 F "TGTGTACGTACGCAGCATCTC"

SM1000 R "TTGACCGGGGATAAAGACATG".

The reactions were initially performed on the DNA extracted from the parents for planned crosses: Harrington, SM89010 and CDC Silky. Harrington, as a susceptible parent, lacks the Rh gene and reveals absence of the band at 1000 kb while the resistant parents showed clear presence of that band.

Two other primers used in the study were obtained as a part of a random primers package from Genosys Biotechnologies, Inc. Sequences (5'-3') for these two primers showing polymorphism between the parents are:

OPH-G-1: "GGTCGGAGAA"

OPH-G-03: "AGACGTCCAC "

In addition to the primers from Genosys Biotechnologies, Inc. over fifty random primers (Set # 6, UBC Lot # 2) from the Nucleic Acid–protein Service Unit, (University of British Columbia, Room 237-Wesbrook Building, 6174 University Boulevard, Vancouver, B.C. V6T 1Z3, Canada) were tested to detect presence of polymorphism between the parents: Harrington, SM89010 and CDC Silky.

PCR Reactions

To calculate the correct amounts of reagents required for PCR reactions a template created with Microsoft Excel (Microsoft Corporation, One Microsoft Way, Redmont, WA 98052-6399) was used. The calculations included such variables as pipetting loss, number of reactions per master mix and concentration of reagents per sample (Table 8). Final volume (µl) column was calculated based on the number of samples.

Table 8. A sample of master mix for PCR reactions

Components	Volume per Sample (µl)	Number of Samples	Final Volume (µl)	Concentration per Sample
25 mM MgCl	6	5	30	3 mM
10 X Buffer	5	5	25	1 X
10 mM dNTP	4	5	20	0.8 mM
SM1000F	0.26	5	1.3	20 pMOL
SM1000R	0.3	5	1.5	20 pMOL
TAQ	0.5	5	2.5	2.5 U
Water	32.94	5	164.7	
DNA	1	5	5	
Total Volume	50		250	

Reactions for the SM1000 amplicon were performed in volumes of 50 µl, followed by volumes of 25 µl and 15 µl respectively, each containing 1X Tris-Cl Buffer, pH 8.3, 3.0 mM $MgCl_2$, 0.8 mM dNTP's, 20 pM of each primer and 1 unit of Taq Polymerase per reaction. In the random primer reactions the amount of primer was adjusted to 40 pM per reaction. Concentration of magnesium and 10X buffer was kept at the same level as in the SM1000 amplicon reaction. Amplifications were performed in Thermocycler Series 669 (Barnstead Thermolyne Corporation, 2555 Kerper Blvd., Dubuque, IA 52004-0797).

When heated, DNA molecules start to vibrate, then hydrogen bonds begin to come apart and DNA separates into two strands at the specific temperature for

each DNA molecule known as the melting temperature (Tm). The Tm for each primer vary due to the different ratios of base pairs. There are four bases in DNA: adenine (A), thymine (T), guanine (G) and cytosine (C). GC base pairs have three hydrogen bonds compared to two holding the AT base pairs which results in CG base parts being stronger than AT base pairs. When the temperature rises, the regions of the DNA with more AT base pairs come apart first, before any GC base pairs denature. Thus the Tm is higher for the primers with more GC base pairs than for primers with more AT base pairs (Clark and Russell, 1997).

Therefore, different melting temperature (Tm) for each primer required the application of separate programs for primers from UBC, Genosys Biotechnologies, Inc. and SM1000 amplicon. The program used for SM1000 amplicon had 35 cycles with 6 minutes of hot start at 94° C, followed by 1 minute at 94° C to denature the DNA, 1 minute at 60° C for an annealing and 1 minute and 30 seconds at 72° C degree for extension. Another program with similar timetable but a lower annealing temperature of 36° C was used for the random primers. For primers OPH-G-1 and OPH-G-3 the annealing temperature was 32° C. Additionally, seven minutes of extension at 72° C at the very end of the program was followed by a cooling period at 4° C. The results were analyzed by electrophoresis on 1.5% low melting agarose gel and detected by staining with ethidium bromide using Tris-acetate buffer.

The 1 kb DNA ladder (Promega Corporation, 2800 Woods Hollow Road, Madison, WI) was used to determine the sizes of double stranded DNA from 250 –10,000 base pairs (bp). The 1,000 and 3,000 bp fragments in the ladder have an increased intensity relative to the other bands and serve as reference indicators. For SM1000 amplicon 1 kb DNA ladder is also used since the expected band is exactly at 1,000 bp.

STATISTICAL PROCEDURES

F2 Populations

The data were analyzed by using the statistical package included with the Microsoft Excel (Microsoft Corporation, One Microsoft Way, Redmont, WA 98052-6399) software. At the F2 stage all crosses were tested with the goodness-of-fit method to determine the segregation ratio. Along with the hypothesis stated below, the level of significance was chosen at the 0.05. Chi-square test and p-value were also calculated.

The null and the alternative hypothesis are:

Ho: The F2 plants are segregating in 3:1 ratio, with three fourths parts of the plants showing resistance and one fourth showing susceptibility.

Ha: The F2 plants don't fit the 3:1 ratio.

The next step was to record the observed data for resistant and susceptible plants. The expected ratio that would fit the Ho hypothesis was calculated and the goodness-of-fit test performed. Values of χ^2 for Crosses 1, 2 and 3 are presented in Table 8. Based on the comparison of the computed value and table value a statistical decision was made allowing for one degree of freedom. At the $\alpha = 0.05$ significance level and $k - 1 = 2 - 1 = 1$ degrees of freedom, the critical value of χ^2

is 3.841. With the computed values of χ^2 lower than the critical value, the Ho hypothesis was accepted to fit the simple model 3:1. Frequency distribution of the F2 populations in Crosses 1 and 2 consisted of two discrete classes, representing a 0 to 3 scale (Schein, 1958) used in rating scald symptoms (Table 9). Resistant and moderately resistant plants were grouped in one resistant class. Also, moderately susceptible and susceptible plants were grouped as one susceptible class. The data from Crosses 1 and 2 fit the hypothesized one locus 3:1 model with the probability of 0.11 and 0.594 respectively. The data from Cross 3 or Cross 4 did not fit the single locus model.

Table 9. Chi-square test of hypothesized ratios of *R. secalis* resistant and susceptible barley plants in F2 populations of Crosses 1, 2, 3 and 4

			Distribution			
Cross	Test ratio	Type	Resistant	Susceptible	χ^2	Probability
Cross 1	3:1	Observed	238	62	2.56	0.110
		Expected	225	75		
Cross 2	3:1	Observed	229	71	0.28	0.594
		Expected	225	75		
Cross 3	3:1	Observed	93	17	5.3	0.021
		Expected	82.5	27.5		
Cross 4	3:1	Observed	237	14	50.5	<0.001
		Expected	188.25	62.75		

In Cross 1, where two row Harrington was crossed with a six-row hulless cultivar, the test of goodness-of-fit also was applied for plants segregating for the presence or absence of the hull, and segregating for two-row or six-row type of spike. The χ^2 value for the segregation for the presence of the hull was 2.56 with the probability of 0.11 and the χ^2 value for spike type segregation was 1.44 with the probability of 0.23. In both cases the null hypothesis was accepted at the $\alpha = 0.05$ significance level to fit the segregating ratio 3:1.

Since the results for Cross 3 and Cross 4 did not fit the simple 3:1 model, a two locus model 9:3:3:1 was tested. The χ^2 value of 12.73 for Cross 3 was obtained with the probability of 0.00523 while the χ^2 value of 52.94 was obtained with the probability of <0.0001 (Table 10).

Table 10. Chi-square test of hypothesized ratios of *R. secalis* resistant and susceptible barley plants in F2 populations of Crosses 3 and 4

			Distribution[1]					
Cross	Test ratio	Type	R	MR	MS	S	χ^2	P[2]
Cross 3	9:3:3:1	Observed	62	31	17	0	12.73	0.0053
		Expected	61.9	20.625	20.625	6.875		
Cross 4	9:3:3:1	Observed	187	50	10	4	52.94	<0.0001
		Expected	141.2	47.1	47.1	15.69		

[1] R = resistant; MR = moderately resistant; MS = moderately susceptible; and S = susceptible
[2] P = Probability

Neither Cross 3 or Cross 4 fit the two locus model 9:3:3:1. A model for three locus was also tested but again the crosses did not fit that model either.

However, Cross 3 fit the 13:3 model with the χ^2 value of 0.784 and probability of 0.376 and Cross 4 fit hypothesized two locus model 15:1 with χ^2 value of 0.234 and probability of 0.629 (Table 11).

Table 11. Chi-square test of hypothesized ratios of *R. secalis* resistant and susceptible barley plants in F2 populations of Crosses 3 and 4

			Distribution			
Cross	Test ratio	Type	Resistant	Susceptible	χ^2	Probability
Cross 3	13:3	Observed	93	17	0.784	0.376
		Expected	89.375	20.625		
Cross 4	15:1	Observed	237	14	0.234	0.629
		Expected	247.31	15.685		

Mean values were computed for populations from Crosses 1, 2, 3 and 4 at the F2 stage. Mean for Cross 4 has a lower value than its parents Crosses 1 and 2. Out of 251 plants screened only ten plants were moderately susceptible and four plants were susceptible (Table 12).

Table 12. Mean values for F2 generation barley plants for Crosses 1, 2, 3 and 4

Cross number	Plants screened	Mean
Cross 1	300	0.587
Cross 2	300	0.647
Cross 3	110	0.564
Cross 4	251	0.327

F3 Populations

At the F3 generation populations of Crosses 1, 2 and 3 were analyzed with the goodness-of-fit test. The single locus model (3:1) was used. Inoculated plants were classified based on the phenotypic response to *R. secalis* isolates. Crosses 1 and 2 were screened with isolate 1493 while Cross 3 was screened with the 50:50 ratio mixture of isolates 1493 and 1824. Plants with moderate and resistant reactions were combined as one resistant category and moderately susceptible along with the susceptible plants as one susceptible category. At the F3 generation Chi-square value for all three crosses fit the single locus model, with χ^2 values smaller than the tabulated critical value and the 0.05 significance level (Table 13).

Table 13. Chi-square test and probabilities for the F3 populations from Cross 1, Cross 2 and 3

			Distribution			
Cross	Test ratio	Type	Resistant	Susceptible	χ^2	Probability
Cross 1	3:1	Observed	98	34	0.04	0.84
		Expected	99	33		
Cross 2	3:1	Observed	134	57	2.497	0.114
		Expected	143.25	47.5		
Cross 3	3:1	Observed	502	144	2.53	0.112
		Expected	484.5	161.5		

It was not possible to make an accurate assessment of the number of loci segregating for *R. secalis* resistance in the Crosses 3 and 4 based on the results

obtained with the above evaluation methods and the population sizes in the F2 and F3 generations. However, Crosses 1 and 2 fit the single loci model in both generations. Therefore an assumption is made that these crosses segregated for one dominant resistance gene.

RESULTS

Control Cultivars

Each set of the screened plants was accompanied by Harrington, SM89010, CDC Silky and an additional susceptible cultivar Klages (Betzes/Domen). The set of parents was included in each screened group of 94 plants. The control group inoculated with the distilled water was also included. Disease data for control cultivars were recorded for each group of the screened plants to assure that none of the group expressed ratings different than expected. For the susceptible cultivars Harrington and Klages, a score of 3 was the expected value on the 0 to 3 scale (Table 6), although a score was 2 instead of 3 was given in some cases, as more appropriate reading. Some difficulties occurred during screening of Cross 3 at the F2 and F3 stage with *R. secalis* isolate 1824 where a score of 2 was expected for SM89010, corresponding to the moderately susceptible reaction. However it was given a score 1 in some cases, a moderately resistant reaction (Table 14).

Table 14. Disease reaction of the parents

Cultivar	Disease reaction isolate 1493	Disease reaction isolate 1824
Harrington	3/S	2, 3/S
SM89010	0/R	1, 2/MR, MS
CDC Silky	0/R	0/R
Mean	1.0	1.6

The variable ratings could be a result of the plants location in the inoculation chamber or their position on the greenhouse bench between the inoculation and obtaining disease notes. This factor, however, might have played a detrimental role in the mapping population of Cross 3 at the later stages of the study.

Analysis of Progeny Distributions

F2 Plants Data – Crosses 1 and 2

The results obtained for F2 plants were not distributed uniformly and were skewed toward resistance. In Cross 1, out of 300 screened plants, 237 plants obtained score 0, on the 0 to 3 scale (see Table 7), corresponding to resistant plants (R). One plant obtained score 1, which corresponds to a moderately resistant score, thirteen plants scored 2, and fifty plants 3, moderately susceptible

(MS) and susceptible (S), respectively. Also, a significant lack of germination (approximately 23%) and, later, sterility was observed. Although the seeds for both crosses were handled the same way, possibly CDC Silky requires a longer dormancy period between plantings.

Resistant plants were advanced into the F3 generation. Hulless plants from Cross 1 were also included in future studies. Six-row or segregating plants from that cross were not included in the advancement since the objective of this study was to develop a two row barley line targeted for the intermountain region and Canada.

F3 Plants Data – Crosses 1 and 2

At the F3 stage, 132 families were planted again, and screening was repeated with the 1943 isolate. At this generation the results were also skewed towards resistance. There were 98 homozygous resistant families and 34 segregating families in Cross 1. In Cross 2, of the 191 families 134 were homozygous resistant, 4 homozygous recessive and 53 were still segregating (Table 15).

Table 15. Results for populations from Cross 1 and 2 at the F3 generation inoculated with *R. secalis* isolate 1943

Cross	Homozygous resistant	Homozygous susceptible	Segregating	Total
1	98	0	34	132
2	134	4	53	191

F4 Plants Data

The seeds of the homozygous families showing early maturity and the highest seed yield were chosen for planting as F4 generation. Plants were screened with *R. secalis* isolate 1493 and the SM1000 amplicon. Resistant plants were crossed with each other to obtain the F1 seeds. This cross attempted to combine malting qualities of Harrington and to incorporate genes for resistance from SM89010 and CDC Silky. This is a slight modification of the recurrent selection method. The F1 seeds from Cross 4 (C98-2084) were increased and advanced into the F2 stage, where plants were screened with the mixture of *R. secalis* isolates 1493 and 1824. The isolate 1943 was used to confirm the presence of the Rh gene whereas the isolate 1824 to eliminate plants susceptible derived form the SM89010.

Figure 6 displays agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products of the DNA extracted from F4 plants. Specific primer SM1000 was used to determine presence of the band at 1,000 base-pair

which confirms presence of the Rh resistance gene . The lines B-O show reaction for F4 plants from Cross 1. The second part of the figure, lines W-10 consist of 14 F4 plants from Cross 2. Lines Q-T consist of the reaction for the checks and a negative (no DNA added). Harrington (line Q) lacks the band at 1,000 kb since it does not possess the Rh gene. SM89010 (line R) and CDC Silky (line S), both have the band at 1,000 kb. The negative was added as a check for detecting any contamination that might have occurred during the preparation of the reactions.

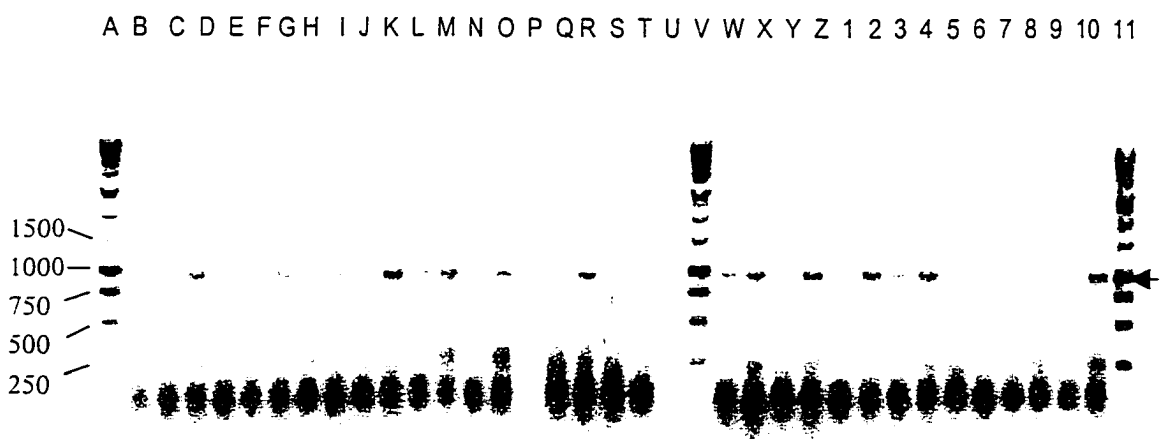

Figure 6. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with SM1000 amplicon from F4 plants (Cross 1 and Cross 2). Line A, V and 11 = 1 kb DNA ladder. Lines B-O = F4 (1-14) plants from Cross 1. Lines W-10 = F4 (1-14) plants from Cross 2. Line Q = Harrington, line R = SM89010, line S = CDC Silky, line T = negative. The approximately 1000-base pair (bp) band was present in SM89010, CDC Silky and F4 plants (marked with an arrow).

Reactions for F4 plants were repeated to confirm the presence of the 1,000 bp band. Consequently, only plants with the 1,000 bp band present, i.e. those that possess Rh resistance gene, were used in the subsequent development of Cross 4.

F2 and F3 Plants Data – Cross 3

This population was developed primarily for the mapping purposes. SM89010 is moderately susceptible to *R. secalis* isolate 1824, while CDC Silky is resistant, therefore there was a possibility of presence of additional gene(s) for resistance. One hundred and ten F2 plants were screened with *R. secalis* isolate 1824. Phenotypic data was recorded for future use in the MAPMAKER program. Chi-square test was used to test hypothesized models 3:1 and 15:1. Cross 3 did not fit hypothesized single gene or two genes model, but fit the model 13:3 for two genes, one dominant and one recessive.

Random primers were tested to detect polymorphism between SM89010 line and CDC Silky cultivar. Two primers were later tested on the F2 population of 110 plants from Cross 3. The 900 base-pair band was present in CDC Silky and some of the F2 plants (Figures 7-11) and absent in SM89010. To reconfirm the presence of the 900 (bp) band, reactions were repeated for plants where its presence was not obvious (Figure 10, lines C-O and Figure 11, lines C-I).

Figure 7. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-1 from F2 plants (Cross 3). Lines C-7 = F2 plants (1-31). Line 10 and 12 = SM89010, line 11 and 13 = CDC Silky. Lines A and 14 = 1 kb DNA ladder. The approximately 900-base pair (bp) band was present in CDC Silky (marked with an arrow).

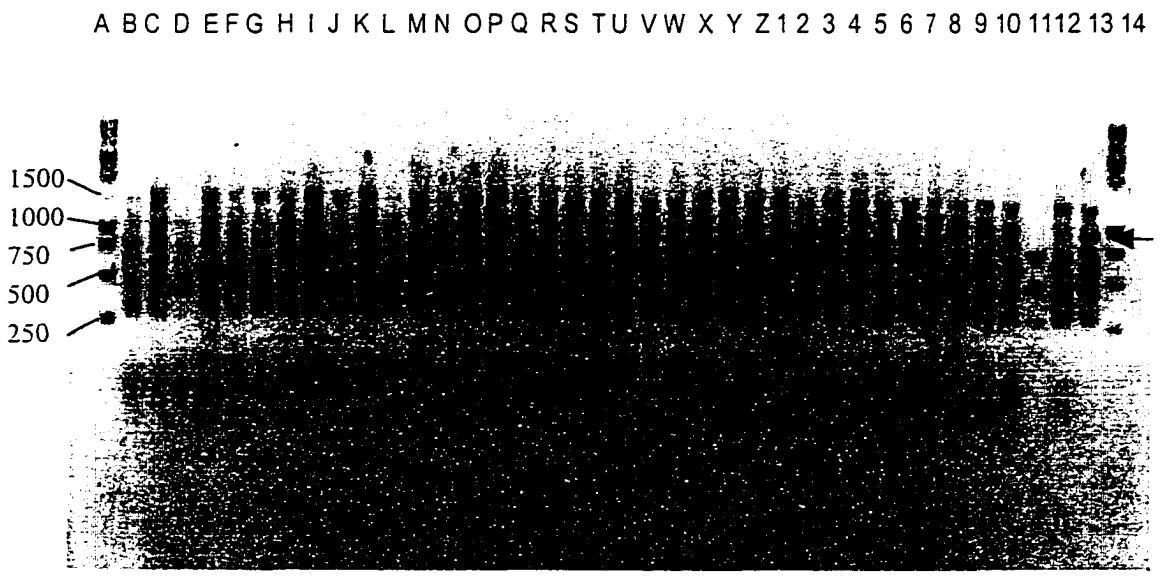

Figure 8. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-1 from F2 plants (Cross 3). Lines B-10 = F2 plants (32-66). Lines 11 and 13 = CDC Silky, line 12 = SM89010. Lines A and 14 = 1 kb DNA ladder. The approximately 900-base pair (bp) bend was present in CDC Silky (marked with an arrow).

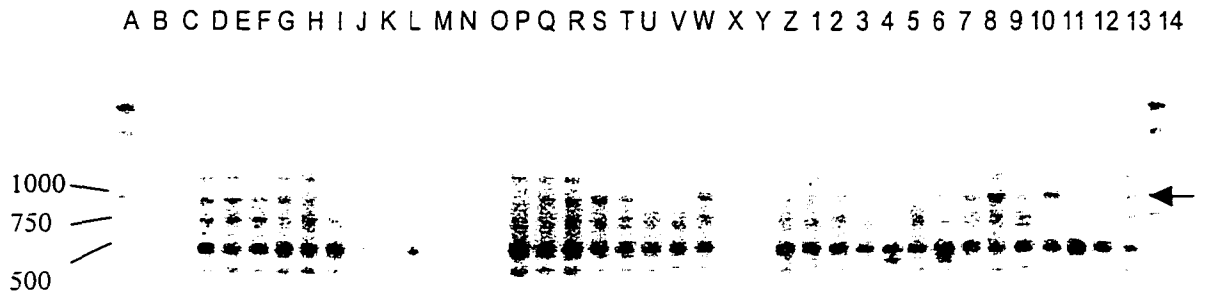

Figure 9. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-1 from F2 plants (Cross 3). Lines B-10 = F2 plants (67-100). Line 11 and 13 = CDC Silky, line 12 = SM89010. Lines A and 14 = 1 kb DNA ladder. The approximately 900-base pair (bp) bend was present in CDC Silky (marked with an arrow).

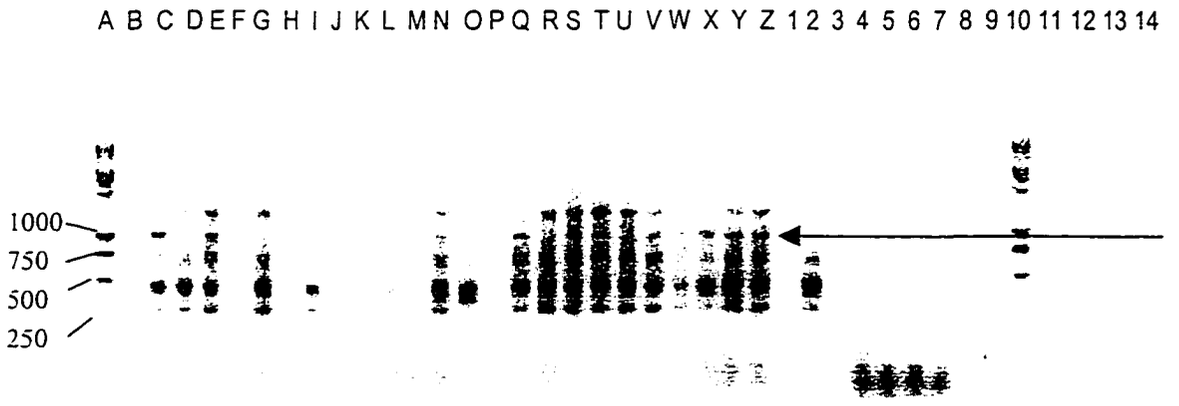

Figure 10. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-1 from F2 plants (Cross 3). Line G = F2 plant 67, lines H-L = F2 plants 76-80, lines M and N = F2 plants 90 and 91, line O = F2 plant 95, line Q-Z = F2 plants 101-110. Line C and E = CDC Silky, line D = SM89010. Lines A and 10 = 1 kb DNA ladder. The approximately 900-base pair (bp) bend was present in CDC Silky (marked with an arrow).

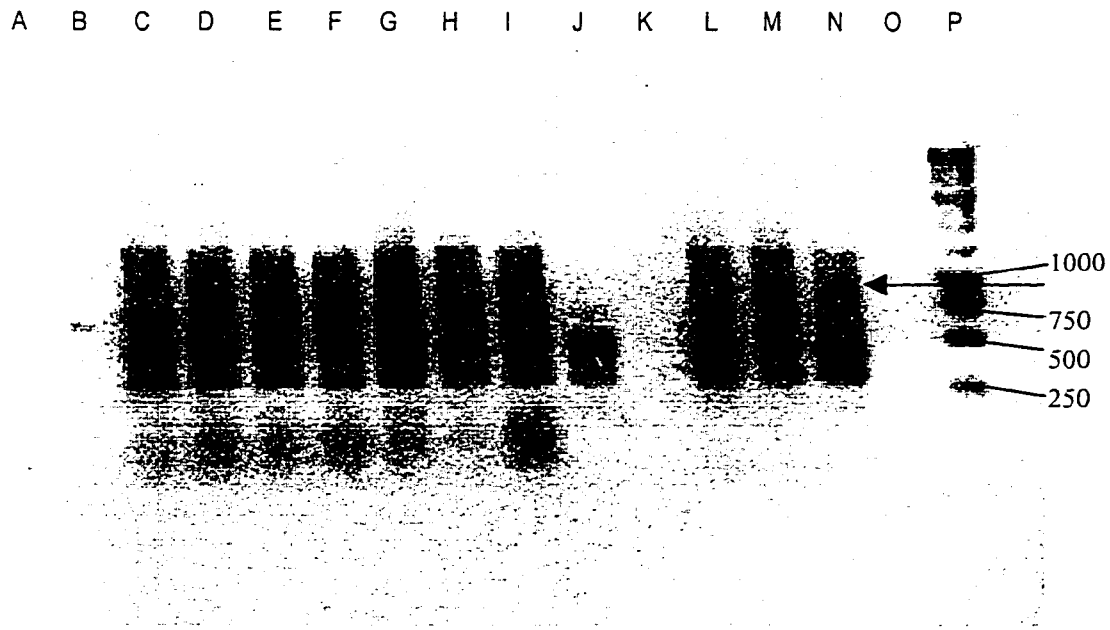

Figure 11. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-1 from F2 plants (Cross 3). Lines C-F = F2 plants (77-80), lines G and H = F2 plants 89 and 90, line I = F2 plant 100. Line L and N = SM89010, line M = CDC Silky. Lines P = 1 kb DNA ladder. The approximately 900-base pair (bp) bend was present in CDC Silky (marked with an arrow).

Another RAPD primer, OPH-G-3, also showed polymorphism between both SM89010 and CDC Silky cultivar. Here a band was present in SM89010 and some of the F2 plants and absent in CDC Silky. The size of the band was approximated at the 1,100 kb (Figures12-15). Again some of the F2 plants lack this band.

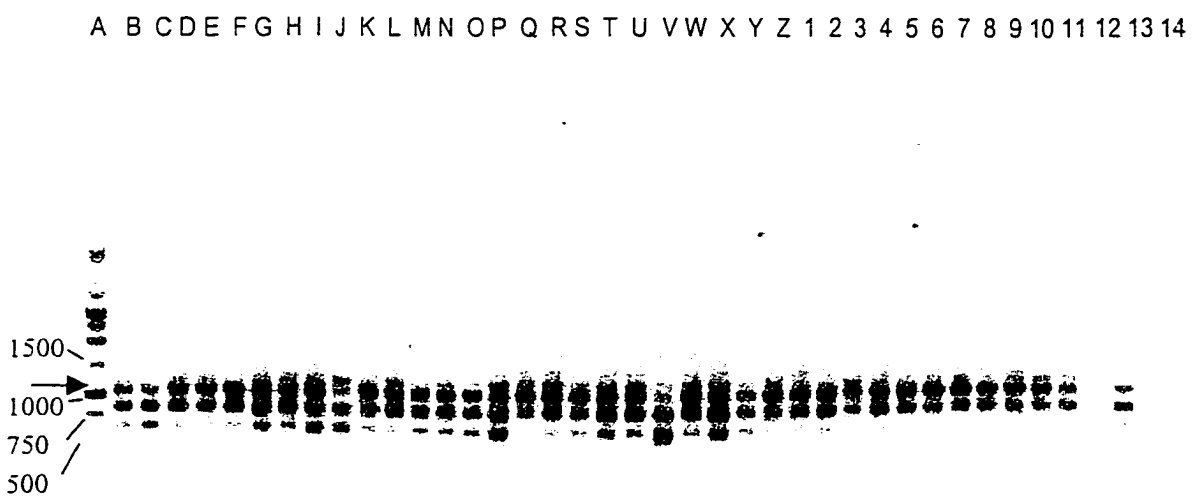

Figure 12. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-3 from F2 plants (Cross 3). Lines B-10 = F2 plants (1-35). Line 11 = SM89010, line 13 = CDC Silky. Lines A and 14 = 1 kb DNA ladder. The approximately 1100-base pair (bp) bend was present in SM89010 (marked with an arrow).

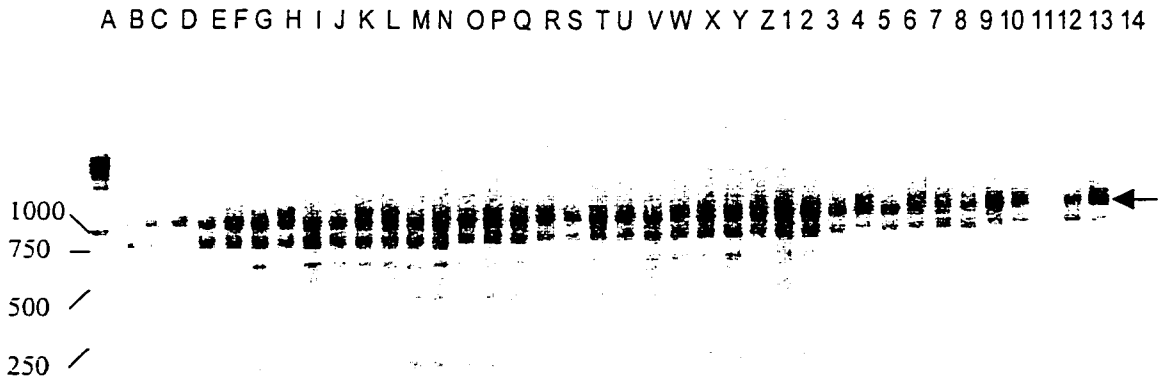

Figure 13. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-3 from F2 plants (Cross 3). Lines B-10 = F2 plants (36-70), line 12 and 13 = F2 plants 71 and 72. Line A = 1 kb DNA ladder. The approximately 1100-base pair (bp) bend was present (marked with an arrow).

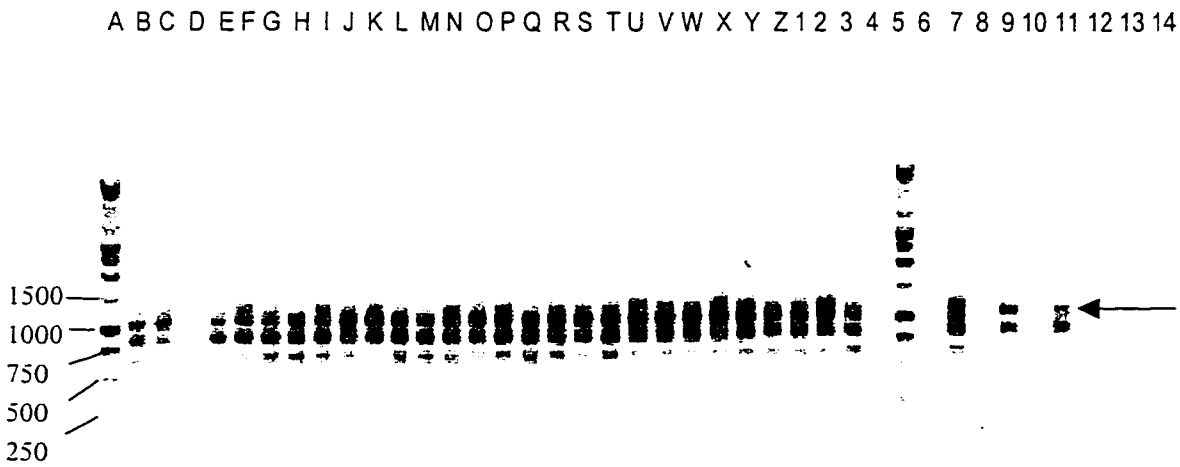

Figure 14. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-3 from F2 plants (Cross 3). Lines B-3 = F2 plants (73-100). Line 7 = Harrington, line 9 = SM89010, line 11 = CDC Silky. Lines A and 5 = 1 kb DNA ladder. The approximately 1100-base pair (bp) bend was present in SM89010 (marked with an arrow).

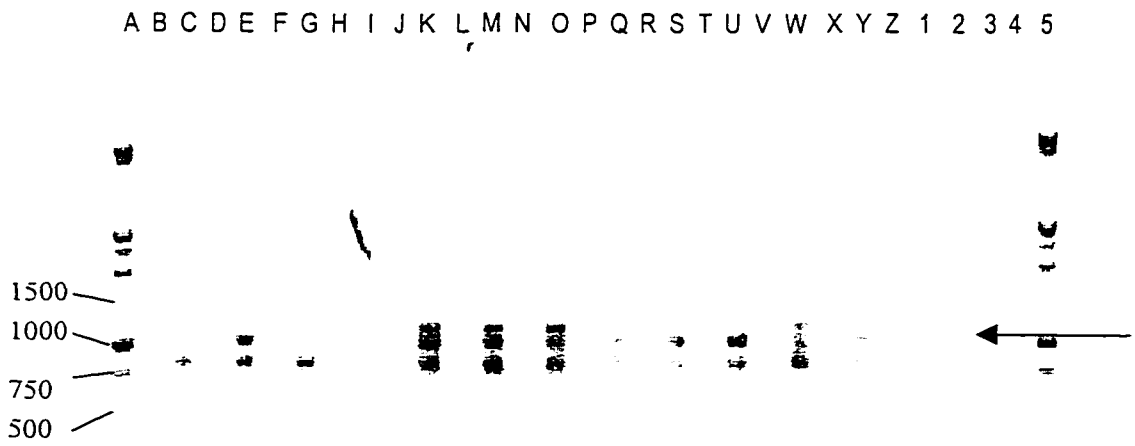

Figure 15. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with random primer OPH-G-3 from F2 plants (Cross 3). Lines C, E, G, I, K, M, O, Q, S, U = F2 plants (101-110). Line W = Harrington, line Y = SM89010, line 1 = CDC Silky. Lines A and 5 = 1 kb DNA ladder. The approximately 1100-base pair (bp) bend was present in SM89010 (marked with an arrow).

Segregation for resistance was checked at the F3 generation. Screening with *R. secalis* isolate 1824 was repeated to establish the homozygocity of the families. The number of plants per each F3 family varied from 4 to 11 plants, with probability from 0.1 to 0.001 respectively and 0.5 probability of failure (Hanson, 1959). Phenotypic data was obtained for each individual plant within the F3 family. A final score was obtained for each of the families which was subsequently used in the MAPMAKER program along with the polymorphism data detected with the RAPD primers. The scores were based on either the segregation or lack of it in individual plant families. Score one was given to the families which expressed only resistant phenotypic reaction, corresponding to 0 or 1 on the 0 - 3 scale (see Table 7). Segregating families obtained score 2 and families with all plants susceptible obtained score 3. Phenotypic data and the results from OPH-G-1 and OPH-G-3 primers were combined and analyzed using MAPMAKER to calculate the distance of markers from the resistance gene. The results were not satisfactory and marker associated with the gene for resistance was not mapped. The distance of the marker from possible resistance gene location was beyond the 25 cM, which is the maximum acceptable distance.

Future studies would be required to obtain a useable marker. Since the phenotypic reaction obtained with *R. secalis* isolate 1824 on F2 and F3 generation was not satisfactory, with SM89010 obtaining score of 1 instead of 2 on the 0 - 3 scale, this population should be screened with another isolate to which one of the

parents would be truly susceptible. Since SM89010 is only moderately susceptible to *R. secalis* isolate 1824 it produced poor segregation in F2 and F3 generations. Additional random primers tested for polymorphism between SM89010 and CDC Silky were not tested on the F2 plants from this cross. Primers 13, 16, 18, 19, 21, 27, 35, 37, 46 and 47 from the (Set # 6, UBC Lot #2) show the polymorphism between SM89010 and CDC Silky (Figures 16 -19).

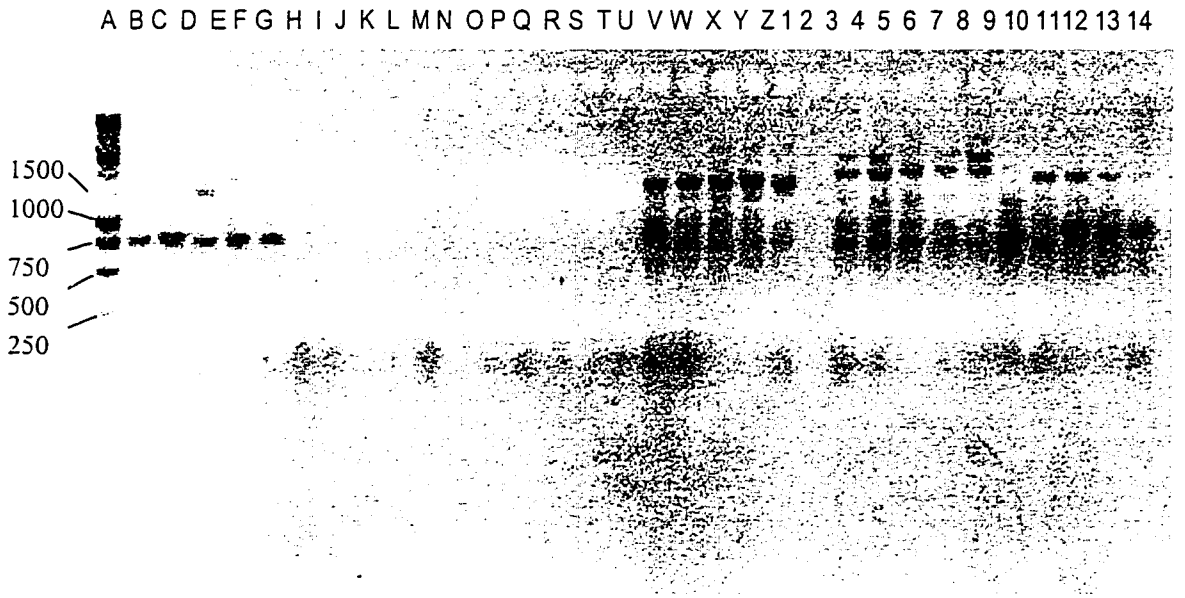

Figure 16. Agarose gel electrophoresis (1.5 %) of polymerase chain reaction products obtained with RAPD primers (Set #6, UBC Lot #2), UBC Biotechnology Laboratory, showing polymorphism between parents, resistant bulk from Cross 1 and susceptible bulk from Cross 1. Each primer set includes five reactions: Harrington, SM89010, CDC Silky, resistant bulk and susceptible bulk, respectively. Lines B-F = primer 13, lines S-W = primer 16, lines 1-5 = primer 17, lines 6-10 = primer 17. Line A = 1 kb DNA ladder.

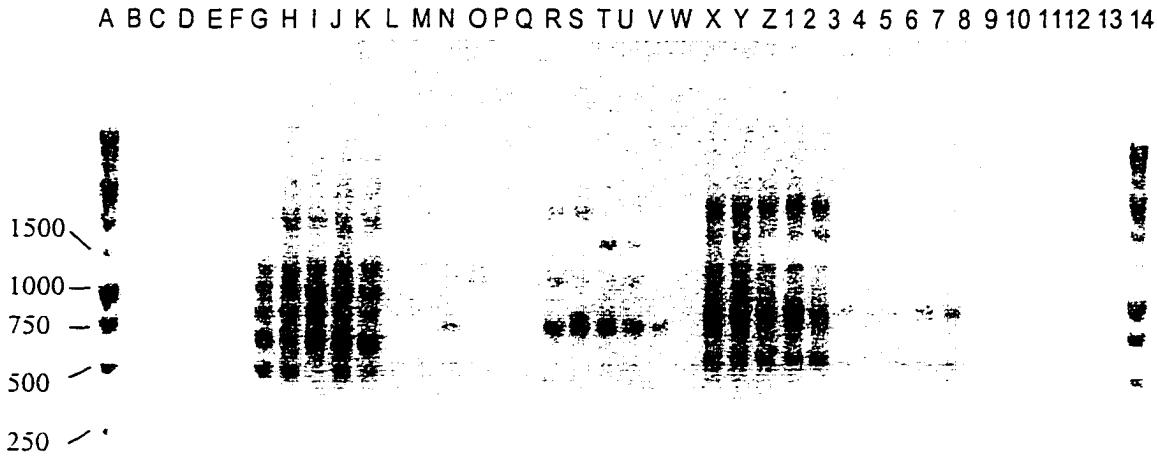

Figure 17. Agarose gel electrophoresis (1.5 %) of polymerase chain reaction products obtained with RAPD primers (Set #6, UBC Lot #2), UBC Biotechnology Laboratory, showing polymorphism between parents, resistant bulk from Cross 1 and susceptible bulk from Cross 1. Each primer set includes five reactions: Harrington, SM89010, CDC Silky, resistant bulk and susceptible bulk, respectively. Lines G-K = primer 19, lines S-W = primer 21, lines Y-3 = primer 22. Lines A and 14 = 1 kb DNA ladder.

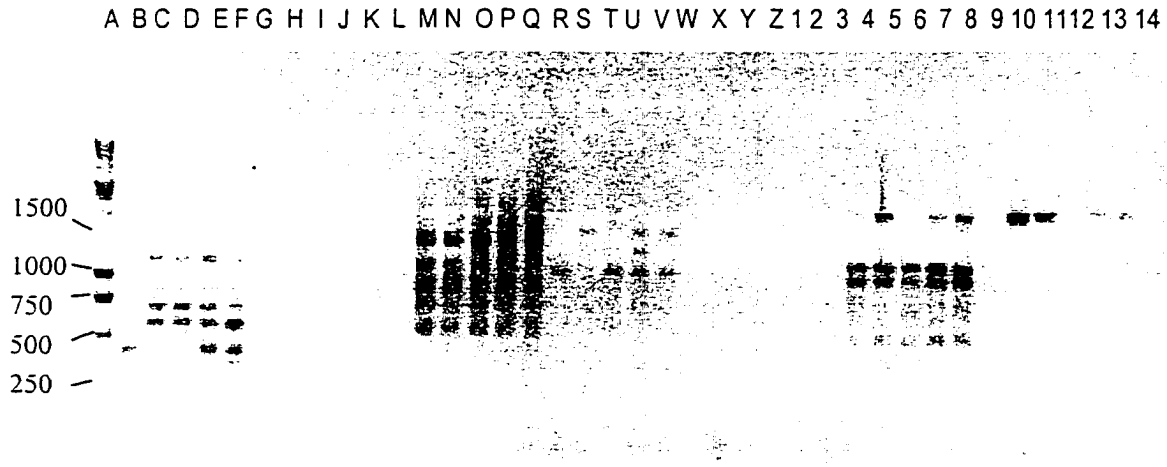

Figure 18. Agarose gel electrophoresis (1.5 %) of polymerase chain reaction products obtained with RAPD primers (Set #6, UBC Lot #2), UBC Biotechnology Laboratory, showing polymorphism between parents, resistant bulk from Cross 1 and susceptible bulk from Cross 1. Each primer set includes five reactions: Harrington, SM89010, CDC Silky, resistant bulk and susceptible bulk, respectively. Lines B-F = primer 32, lines M-Q = primer 34, lines R-W = primer 35, lines 4-7 = primer 37. Line A = 1 kb DNA ladder.

Figure 19. Agarose gel electrophoresis (1.5 %) of polymerase chain reaction products obtained with RAPD primers (Set #6, UBC Lot #2), UBC Biotechnology Laboratory, showing polymorphism between parents. Each primer set includes three reactions: Harrington, SM89010, CDC Silky, respectively. Lines E-F = primer 19, lines H-J = primer 21, lines K-M = primer 27, lines N-P = primer 31, lines Q-S = primer 35, lines W-Y = primer 47, lines Z-2 = primer 56, lines 3-5 = primer 57, lines 9-11 = primer 59. Line A = 1 kb DNA ladder.

F2 Plant Data – Cross 4

Cross 4 was developed by crossing progenies from Crosses 1 and 2. At the F2 stage plants were screened with the mixture of the *R. secalis* isolates 1824 and 1493. The 50:50 ration mixture of the isolates was used to detect any plants that do not possess the Rh gene. Out of 251 plants assessed, using the scale from 0 to 3, 186 plants were resistant, 51 plants moderately resistant , 10 moderately susceptible and 4 susceptible. Segregation ratio was tested using χ^2 test. Although SM89010 is moderately susceptible to *R. secalis* isolate 1824 and CDC Silky is resistant to it, the segregating ratio fit two dominant genes hypothesized model 15:1. Since the marker for an additional gene was not successfully mapped, all the plants expressing moderately susceptible and susceptible symptoms were screened with SM1000 amplicon for the presence of the 1,000 base-pair band. Also, random resistant and moderately resistant plants were tested with SM1000 amplicon. With exception of one plant from the susceptible group all showed the presence of the band at 1,000 bp, which confirmed presence of the Rh gene. However, the phenotypic reaction was still 3 on the 0-3 scale. Each of the ten moderately susceptible plants also possessed this band. Within a moderately resistant group of six plants only one did not have the 1,000 bp band. Each of the eight resistant plants indicated the 1,000 bp band present. Reactions were repeated several times to confirm the presence of the 1,000 bp band. Figures 20-23 show

groups of the F2 plants along with the parents. Three figures are included since some of the bands are either not present or weak in reactions from different days.

SM1000 is a specific primer and the reactions were repeated due to the absence of the bands. Figure 20 shows susceptible plants in lines F, H, L, and M. Moderately susceptible plants are located in the lines: E, G, M-O, U-Z. Resistant plants are in the lines 1-6, each with the band present at the 1,000 bp. Figure 21 is an example of how sensitive the SM1000 amplicon is. It shows reactions performed the same day with two different batches of the dNTPs. One of the master mixes was done from the freshly defrosted batch from -20° C freezer, while the other was used previously, but stored according to manufacturer recommendations at -20° C. The first batch produced successful results (lines A-I) and the second one did not work (lines J-R). Reaction from moderately susceptible plants was repeated to confirm the presence of the band.

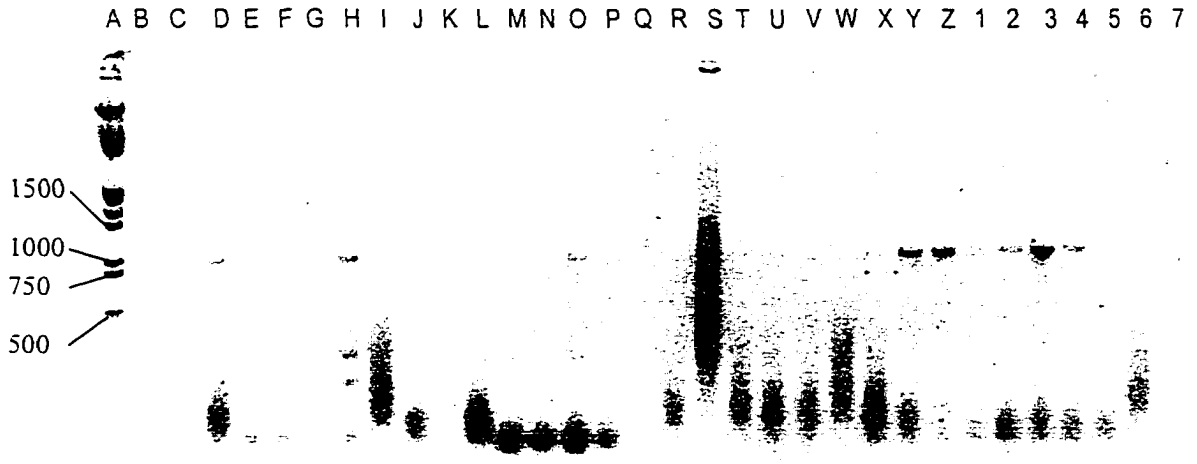

Figure 20. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with SM1000 primer. Lines E-H, L-O, U-Z = susceptible F2 plants from Cross 4. Lines 1-5 = resistant F2 plants from Cross 4. Line D = CDC Silky. Line A = 1 kb DNA ladder.

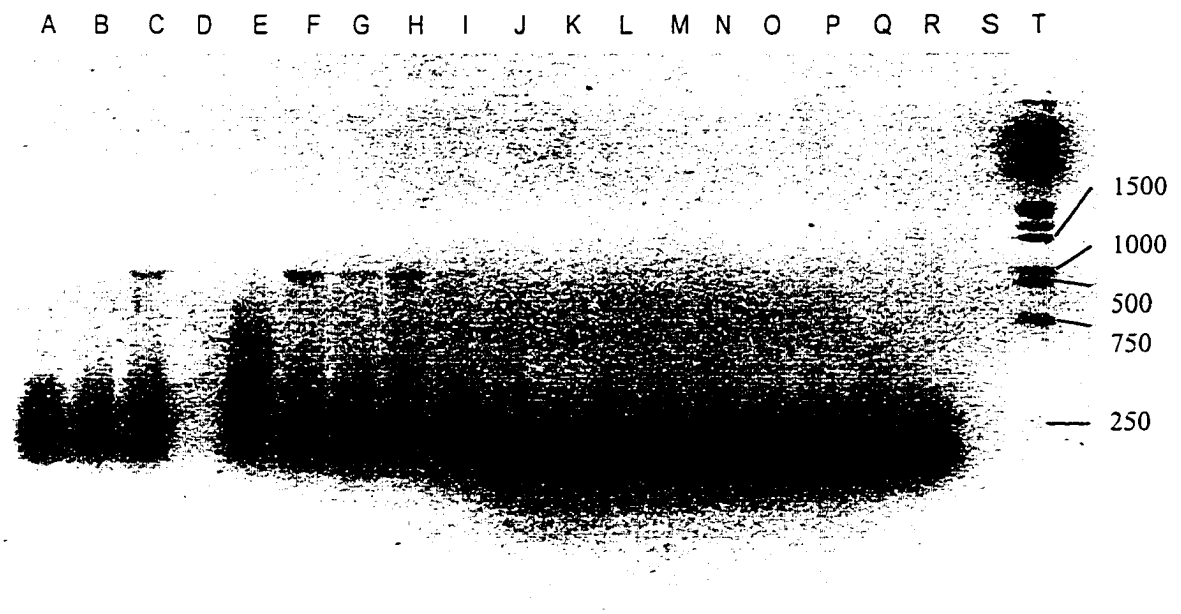

Figure 21. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with SM1000 primer. Lines E-I = susceptible F2 plants (177B, 178, 179, 182, 184) from Cross 4. Lines A-C = Harrington, SM89010, CDC Silky. Line T = 1 kb DNA ladder.

In Figure 22 line D contains the reaction for CDC Silky parent and the band at 1,000 base pair. Reaction for susceptible plants is shown in lines G, H and I. Moderately resistant plants are shown in lines F, and J-M. Lines N-Q contain the reaction product for resistant plants. Figure 23 includes all tested plants organized into groups. Lines B-O contain all the susceptible plants. The first two plants (lines B-C) do not show the 1,000 bp band, although this band was observed in all previous reactions. Line J contains the reaction for susceptible band that uniformly in all the reactions lacks the presence of the 1,000 bp band. All resistant plants that obtained a score of 0 on the 0-3 scale (lines Q-X) show a band present at 1,000 bp. One plant from a moderately susceptible group (line 3) lacks this band.

Results from screening with SM1000 amplicon point to an assumption that the Rh gene was lost during the crossing procedure, since all the F4 plants used in the cross were previously screened with the marker and the presence of the 1,000 bp band was demonstrated.

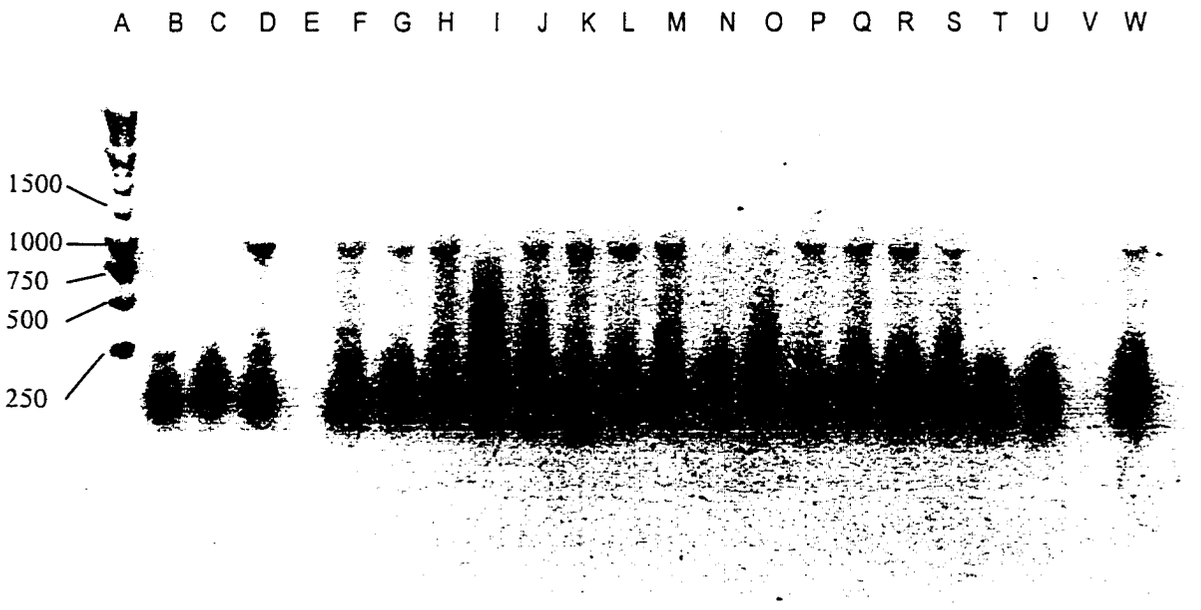

Figure 22. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with SM1000 primer. Lines F-M = susceptible F2 plants (22, 27, 142, 147, 177, 184, 188, 189) from Cross 4. Lines N-Q = resistant F2 plants (110, 86, 180, 140) from Cross 4. Line R-U = moderately resistant F2 plants (60, 116, 84, 136) form Cross 4. Line D and W = CDC Silky. Line A = 1 kb DNA ladder.

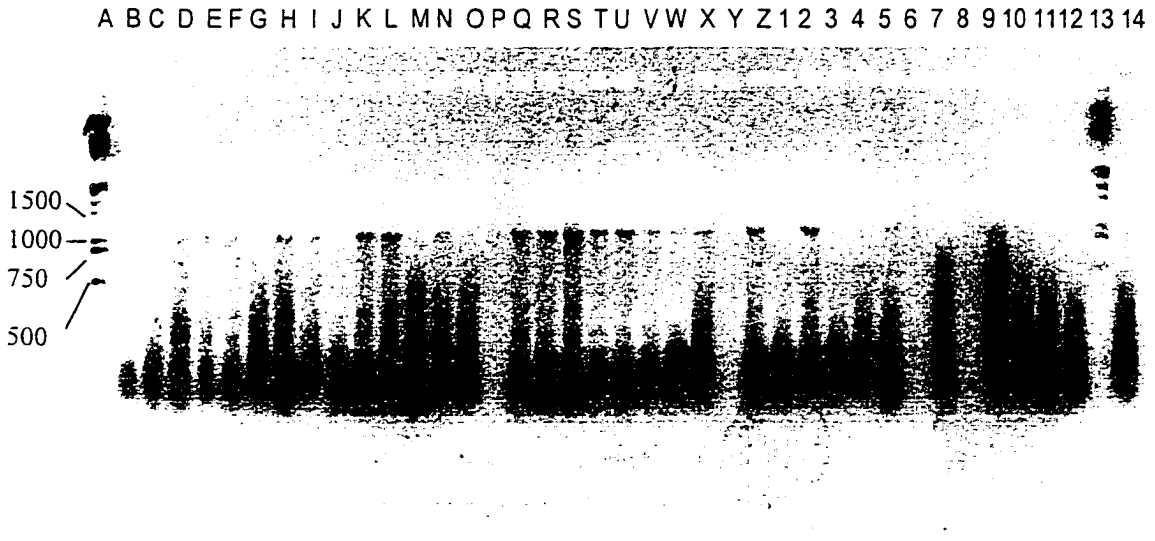

Figure 23. Agarose gel electrophoresis (1.5%) of polymerase chain reaction amplification products obtained with SM1000 primer. Lines B-O = susceptible F2 plants (3, 21, 22, 27, 142, 147, 177, 177 B, 182, 184, 188, 189) from Cross 4. Lines Q-X = resistant F2 plants (1, 8, 37, 61, 110, 86, 180, 140) from Cross 4. Lines Z-6 = moderately resistant F2 plants (40,60, 116,84,185,136) form Cross 4. Line 8 = Harrington. Line 14 = CDC Silky. Line A and 13 = 1 kb DNA ladder.

Random primers

Over sixty random primers were tested for polymorphism between SM89010 and CDC Silky. Harrington was also included in the tests (Figures 10-16). Since there were some difficulties with the phenotypic reaction of *R. secalis* isolate 1824 on SM89010, a bulked DNA from ten susceptible and ten resistant plants from Cross 1 was also included in random primer tests. Figure 17 summarizes those primers that could possibly be used in future mapping of the population from Cross 4. Especially primers 19, 47 and 59 can be useful in the future studies. Primer 19 shows opposite reactions in CDC Silky and SM98010. At 500 bp a band is present in SM89010 and absent in CDC Silky, while at 1,200 bp SM89010 lacks a band and CDC Silky shows the band present. Primers 47 and 59 show polymorphism between SM89010, CDC Silky and Harrington. Figures 16-18 include, along with Harrington, SM89010 and CDC Silky, the resistant and susceptible bulk from Cross 1.

DISCUSSION

Combining traditional breeding methods with RAPD type molecular markers to develop *R. secalis* resistant barley germplasm was demonstrated to be possible in a commercial environment. This could become a standard way of breeding for resistance to pathogens with a high degree of heterozygocity in future. Both the traditional method and the application of markers were not always reliable due to environmental conditions during inoculation and problems with repeatability of PCR reactions, as demonstrated in this study. Problems with obtaining expected results with *R. secalis* isolate 1824 occurred mainly during the screening of F2 and F3 generations of Cross 3. Even though the environmental conditions in the greenhouse were recreated as close as possible to the ones described in previous studies with *R. secalis* isolate 1824, the expected results were not uniform. Climate specific to Colorado with its extremely low humidity could be one of the reasons for such variability in assessment of scald symptoms while screening with *R. secalis* isolate 1824.

The results of this study suggest using other isolates of *R. secalis* in future research on crosses between SM89010 and CDC Silky. Moreover, using at least three additional *R. secalis* isolates with different virulence would allow more

accurate selection of parents and their progenies. Ideally, both parents should be susceptible to one isolate. Also, the first parent should be resistant to the second isolate and susceptible to the third one while the second parent should be susceptible to the second isolate and resistant to the third one. Selected isolates should be tested several times prior to the beginning of each research to assure uniformity of obtained results.

Due to large amounts of tissue required for the extraction methods and chemicals that demanded special handling, several extraction methods used by other laboratories for barley DNA extraction were rejected as not applicable in this project. The NucleoSpin Plant extraction kit from Clontech was selected as most appropriate and reliable among other tested protocols. However, even with this method, there were inconsistencies in the final amplification products resulting in the need to repeat reactions to confirm the presence or absence of the desired bands. This issue is not an isolated case. Studies on the reproducibility of RAPD technique prove this method is often not repeatable in different laboratories (Jones et al., 1997).

For this study, it was determined that DNA could be extracted from approximately 100 plants within one working day. It was possible to obtain final results in additional 48 hours. Once the DNA was extracted the reactions were repeated with several other primers to confirm the presence or absence of additional resistance genes.

Progenies obtained from the four crosses in this study expressed resistance similar to that of the SM89010 and CDC Silky parents. The mean of 0.587 for Cross 1, 0.647 for Cross 2, 0.564 for Cross 3 and 0.327 for Cross 4 were observed for resistant F2 plants. On the scale of 0 (R) to 3 (S), these reactions fall between a resistant and moderately resistant rating. Means for parents were 1.0 for *R. secalis* isolate 1493 and 1.5 for isolate 1824. Resistance of the progenies in Crosses 1 to 4 was greatly improved in comparison to susceptible parent Harrington. Harrington consistently scored 3 (S) on the rating scale.

The frequency distributions for the F2 plants from Cross 1 and Cross 2 did not form discrete classes for each of the groups. Both of the crosses had only one plant rated as moderately resistant. Therefore, resistant and moderately resistant plants were grouped as a resistant class while moderately susceptible and susceptible ones were grouped as a susceptible class. Frequency distributions were tested with the goodness of fit test for the hypothesized segregation ratio. Cross 1 and 2, inoculated with *R. secalis* isolate 1493, fit the hypothesized single gene model 3:1 for dominant resistance gene.

Cross 3 was screened with *R. secalis* isolate 1824 and Cross 4 with the mixture of isolates 1493 and 1824. The data from Crosses 3 and 4 did not fit the hypothesized one locus model, although Cross 3 fits the hypothesized 13:3 model for two genes, one dominant and one recessive, while Cross 4 fits the hypothesized 15:1 two locus model for two dominant genes.

The frequency distributions for F3 generations for Crosses 1 and 2 fit the hypothesized single dominant gene model 3:1 segregating ratio. The single locus model in Cross 1 and Cross 2 at F2 and F3 generations confirms segregation for one dominant resistance gene. The number of resistant genes involved in Crosses 3 and 4 is not clear. Cross 3 fits the 13:3 ratio for two genes, one dominant and one recessive at the F2 generation, but the same cross in the F3 generation fits the single gene model. This could be due to the screening difficulties mentioned with *R. secalis* isolate 1824. There is a chance that the assessment of the disease was incorrect and plants which should be classified as moderately resistant were grouped into a susceptible category. Overall, the usual disease assessment with *R. secalis* isolate 1824 was unsatisfactory to identify the resistance gene.

Cross 4 fits the hypothesized two gene model at the F2 generation. Out of 251 screened plants only four from Cross 4 were rated as susceptible and ten as moderately susceptible. The results from screening with SM1000 marker for presence of the Rh gene show only one plant from the susceptible group lacking the Rh gene, with all moderately susceptible plants possessing it. Out of six moderately resistant plants, two lack the 1,000 bp band that confirms presence of the Rh resistance gene. Moreover, prior to obtaining Cross 4, F4 plants used as parents had been confirmed for the presence of the Rh resistance gene.

The RAPD type markers used in this study do not distinguish both alleles in the heterozygous individuals which offers a possible explanation for the results. The Rh locus along with Rh3 and Rh4 resistance genes form a complex locus

whose genetic structure remains unclear (Graner et al., 1996). An alternative hypothesis of multiple allelic series for the Rhs 1(Rh) locus was suggested by Hagbood and Hayes (1971). Another possible explanation for the presence of the resistance indicating bands in the susceptible and moderately susceptible plants could be the use of mixture of *R. secalis* isolates 1493 and 1824 on the F2 progenies from Cross 4. The selected susceptible and moderately susceptible plants could be progenies derived from SM89010, which is moderately susceptible to isolate 1824.

Disease assessment at all stages of this study was based on a scale described by Schein (Schein, 1958). The scale did not distinguish between marginal lesions and large coalescing lesion. Perhaps a scale described by Ali and Boyd (1973) that classified reactions into five categories could be more appropriate in future studies. A scale with five classes would provide more information and avoid the possible classification of moderately resistant plants into moderately susceptible category. A set of differentials chosen to accompany the tested populations should include cultivars with the reported resistance Rh3 and Rh4 genes to explain the segregation ratios since these two genes are within the same locus.

In future studies on developing *R. secalis* resistant germplasm, a choice of parents with available markers should also be considered. This study demonstrated that developing a new marker is a complex and laborious task. The laboratory available in this study had limited resources and was not equipped or

set up for marker development. The information obtained in this research, however, will influence the success of the future use of molecular markers in the barley breeding program, not only in breeding for *R. secalis* resistance, but for other pathogens as well. Germplasm obtained as a results of this study will be planted for further testing for agronomic qualities and disease resistance in the Intermountain area.

Conclusions

At the F2 generation Cross 4 fit the hypothesized ratio for the two dominant genes model. Selected progenies were resistant to both isolates used in traditional screening. Presence of the Rh gene was confirmed with the RAPD SM1000 marker. The mean for the disease assessment of Cross 4 was less than the parent crosses, indicating improved resistance to *R. secalis*.

Resistant progenies of final Cross 4 selected with the SM1000 marker and traditional techniques using *R. secalis* isolates 1493 and 1824 will be planted in headrows and evaluated for disease resistance and agronomics in the Intermountain area. At the F5 generation selected headrows would be tested for malting qualities. If markers for barley malting qualities become available they will be used in further evaluations of Cross 4 progenies.

Literature Cited

Abbott, D. C., A. H. D. Brown, and J. J. Burdon, 1992. Genes for scald resistance from wild barley (*Hordeum vulgare* ssp. *spontaneum*) and their linkage to isozome markers. Euphytica 61: 225-231.

Abbott, D. C., E. S. Lagudah, and A. H. D. Brown, 1995. Identification of RFLPs flanking a scald resistant gene on barley chromosome 6. J. Hered. 86: 152-154.

Ali, S. M., and W. J. R. Boyd, 1973. Host range and physiologic specialization in *Rhynchosporium secalis*. Aust. J. Agric. Res. 25: 21-31.

Ali, S. M., A. H. Mayfield, and B. G. Clare, 1976. Pathogenicity of 203 isolates of *Rhynchosporium secalis* on 21 barley cultivars. Physiological Plant Pathology 9: 135-143.

American Malting Barley Association, Inc., 1984. Barley Variety Dictionary. American Malting Barley Association. Milwaukee, Wisconsin, Rev. 1990.

Barua, U. M., K. J. Chalmers, W. T. B. Thomas, and C.A. Hackett, 1993. Molecular mapping of genes determining height, time to heading, and growth habit in barley (*Hordeum vulgare)*. Genome 36: 1080-87.

Baum, M. H. Sayed , S. Grando, S. Ceccarelli, G. Backes, W. Mohler, A. Jahoor and G. Fischbeck, 1996. QTL analysis of agronomic important characters for dryland conditions in barley by using molecular markers. V International Oat Conference & VII International Barley Genetics Symposium, Proceedings pp. 241-243.

Bockelman, H. E., E. L. Sharp, and R. F. Eslick, 1977. Trisomic analysis of genes for resistance to scald (*Rhynchosporium secalis*) and net blotch

(*Pyrenophore teres*) in several barley cultivars. Can J. Bot. Vol. 55(15): 2142-2148.

Borovkova, I. G., B. J. Steffenson, Y. Jin, J. B. Rasmussen, A. Kilian, A. Kleinhofs, B. G. Rossnagel, and K. N. Kao, 1995. Identification of molecular markers linked to the stem rust resistance gene *rpg4* in barley. American Phytopathological Society. Vol. 85, No. 2: 181-185.

Briggs, S. P., and G. S. Johal, 1994. Genetic patterns of plant host-parasite interactions. TIG Vol. 10, No. 1: 12-16.

Brown, J. S., 1985. Pathogenic variation among isolates of *Rhynchosporium secalis* from cultivated barley growing in Victoria, Australia. Euphytica 34: 129-133.

Brown, J. S., 1990. Pathogenic variation among isolates of *Rhynchosporium secalis* from barley grass growing in South Eastern Australia. Euphytica 50: 81-89.

Ceoloni, C., 1980. Race differentiation and research for sources of resistance to *Rhynchosporium secalis* in barley in Italy. Euphytica 29: 547-553.

Clark, D. P. and L. D. Russell, 1997. Molecular Biology Made Simple and Fun. Cache River Press. Vienna, IL.

Dyck, P. L. and C. W. Schaller, 1961. Inheritance of resistance in barley to several physiological races of the scald fungus. Canadian Journal of Genetics and Cytology 3, 153-164.

Ellingboe, A. H., 1981. Changing concepts in host-pathogen genetics. Ann. Rev. Phytopathol. 19: 125-43.

Flor, H. H., 1942. Inheritance of pathogenicity in melampsora lini. Phytopathol. Vol. 32, No. 8: 653-669.

Flor, H. H., 1946. Genetics of pathogenicity in melampsora lini. Journal of Agricultural Research Vol. 73, Nos. 11, 12: 335-357.

Flor, H. H., 1947. Inheritance of reaction to rust in flax. Journal of Agricultural Research Vol. 74, Nos. 9, 10: 241-262.

Foster, A. E., 1987. Barley. Walter R. Fehr, ed., Principles of Cultivar Development. MacMillan Publishing Company, pp. 83-125.

Fukuyama, T., and H. Takeda, 1992. Survey of resistance to scald in world collection of cultivated barley. Japan. J. Breed. 42: 761-768.

Goodwin, S. B., R. W. Allard, and R. K. Webster, 1990. A nomenclature for *Rhynchosporium secalis* pathotypes. Phytopathology Vol. 80, No. 12: 1330-1336.

Graner, A, A. Jahoor, J. Schondelmaier, H. Siedler, K. Pillen, G. Fischbeck, G. Wenzel, and R. G. Herrmann, 1991. Construction of an RFLP map of barley. Theor Appl Genet 83: 250-256.

Graner, A., and A. Tekauz, 1996. RFLP mapping in barley of a dominant gene conferring resistance to scald (*Rhynchosporium secalis*). Theor. Appl. Genet. 93: 421-425.

Habgood, R. M., and J. D. Hayes, 1971. The inheritance of resistance to *Rhynchosporium secalis* in barley. Heredity 27: 25-37.

Hahn, M., S. Jungling, and W. Knogge, 1993. Cultivar specific elicitation of barley defense reactions by the phytotoxic peptide *NIP1* from *Rhynchosporium secalis*. MPMI Vol. 6, No. 6:

Hansen, and H. A. Magnus, 1973. Virulence spectrum of *Rhynchosporium secalis* in Norway and sources of resistance in barley. Phytopathologishe Zeitshrift 76: 303-313.

Hanson, W. D., 1959. Minimum family sizes for the planning of genetic experiments. Agronomy Journal p. 711-715.

Harrabi, M., 1996. Breeding for resistance to the major fungal leaf pathogens of barley. Proceedings of V IOC and VII IBGS. Session 10: 314-326.

Hayes, P. M., F. Q. Chen, A. Kleinhofs, A. Kilian, and D. E. Mather, 1996. Barley genome mapping and its applications. P. P. Jauhar, ed. Methods of Genome Analysis in Plants. CRC Press, Inc., Boca Raton, FL.

Jones, C. J., K. J. Edwards, S. Castaglione, M. O. Winfield, F. Sala, C. van de Wiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daily, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp., 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding. 3: 381-390.

Khan, T. N., 1986. Effects of fungicide treatments on scald (*Rhynchosporium secalis* (Oud.) J. Davis) infection an yield of barley in Western Australia. Aust. J. Exp. Agric. 26: 231-5.

Kleinhofs, A., A. Kilian, D. Kudrna, M. A. Saghai Maroof, and B. J. Steffenson, 1994. The barley genome map based on the Steptoe x Morex cross. Plant Genome II January 1994.

Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, S. E. Lincoln, and L. Newburg, 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics. 1, 174-181.

Laurie, D.A., J. W. Snape, and M. D. Gale, 1992. DNA marker techniques for genetic analysis in barley. Peter R. Shewry, ed. Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. C. A. B. International, Wallingford, U.K., pp. 115-132.

Lyngs Jorgensen, H. J., and V. Smedegaard-Petersen, 1994. Pathogenic variation of *Rhynchosporium secalis* in Denmark and sources of resistance in barley. Plant Disease Mar. 1995: 297-301.

Mathre, D. E., 1997. Compendium of Barley Diseases. American Phytopathological Society, St. Paul, MN.

Metcalfe, D. R., 1978. Reaction of Ethiopian barleys to Canadian barley pathogens. Can. J. Plant Sci. 58: 885-890.

Nelson, J. E., K. D. Kephart, A. Bauer, and J. F. Connor, 1988. Growth staging of Wheat, barley and wild oat. NDSU Extension Service, North Dakota.

Nelson, R. R., 1977. Breeding Plants for Disease Resistance. Pennsylvania State University.

Penner, G. A., A. Tekauz, E. Reimer, G. J. Scoles, B. G. Rossnagel, P. E. Eckstein, W. G. Legge, P. A. Burnett, T. Ferguson, J. F. Helm, 1996. The genetic basis of scald resistance in western Canadian barley cultivars. Euphytica 92: 367-374.

Pollock, J. R. A., 1979. Brewing Science. Vol. 1. Academic Press. Reading, England.

Anonymous, 1997. Recommendations for the designation of the barley chromosomes and their arms. Barley Genetics Newsletter. Vol. 27:5-6.

Rossnagel, B. G., R. S. Bhatty, and B. L. Harvey, 1994. CDC Silky six-row semi-dwarf hulless barley. R. D. Horsley, ed. Barley Newsletter Vol. 38, p. 107.

Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989. Molecular Cloning – A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, New York.

Schein, R. D., 1958. Pathogenic specialization in *Rhynchosporium secalis*. Phytopathology. Vol. 48: 477-480.

Schweizer, G. F., M. Baumer, G. Daniel, H. Rugel, M. S. Röder , 1995. RFLP markers linked to scald (*Rhynchosporium secalis*) resistance gene RH2 in barley. Theor. Appl. Genet. 90: 920-924.

Shipton, W. A., 1974. Scald of barley. Phytopathology. Vol. 53, No. 11: 839-861.

Stoskopf, N. C., D. T. Tomes, B. R. Christine, 1993. "Plant breeding: theory and practice. Westview Press, Inc. Boulder, CO.

Tekauz, A., 1991. Pathogenic variation in *Rhynchosporium secalis* on barley in Canada. Canadian Journal of Plant Pathology 13: 298-304.

Webster, R. K., 1980. Sources of resistance in barley to *Rhynchosporium secalis*. Plant Disease. Vol. 64, No. 1: 88-90.

Welty, R. E., 1996. First report of scald of triticale caused by *Rhynchosporium secalis* in North America. Plant Dis. 80: 1220-1223.