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Molecular and pathological diagnosis of seedborne barley scald disease

by

Hyun-Kyung Lee

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Plant Science

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta

Spring 2002

University of Alberta

Library Release Form

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Title of Thesis: Molecular and pathological diagnosis of seedborne barley scald disease

Degree: Doctor of Philosophy

Year this Degree Granted: 2002

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For my parents

Thank you for your love and support

Abstract

Scald of barley caused by *Rhynchosporium secalis* (Oud.) J. J. Davis is a major foliage disease resulting in yield and quality losses of barley worldwide. The fungus is stubble and seedborne. Although the seedborne phase of the disease has received less attention than stubble-borne phase of the disease, this source of inoculum is of particular importance in long-range dispersal of the pathogen and dissemination of new races. Management of the pathogen carried with seeds can be facilitated by proper seed health testing in combination with other disease management strategies. The present study focused on developing pathological and molecular diagnostic methods for the seedborne scald and evaluating their potential in studies of the barley scald disease.

Histopathological study of head-infection in barley by *R. secalis* using light and scanning electron microscopy showed that the scald symptoms were distributed over the lemmas, paleas, awns, glumes and rachises, but were more common on glumes and awns. Fungal growth consisting of hyphae and conidia was restricted to the inner surfaces of lemmas, paleas and glumes in masses surrounded by what appeared to be mucilaginous material. Calcium-containing crystals were observed on the inner surfaces of scald lesions on glumes. This suggests and supports an earlier report that pathogenesis by *R. secalis* partly involves sequestration of calcium ions from host plants as found in some other pathosystems.

Rhynchosporium secalis was successfully isolated from infected seeds using an agar plating method developed in this study. This appears to be the first report of successful isolation of the scald fungus from infected barley seed.

PCR-based diagnostic assay was developed to detect *R. secalis* in barley seed using species-specific primers designed from the internal transcribed spacer (ITS) regions of ribosomal RNA genes of this pathogen. These primers specifically amplified a single diagnostic band from the DNA of all *R. secalis* isolates tested, but not from the DNA from other species and allowed for the detection of the scald pathogen in barley seed.

Symptomless infection of barley seed was detected in the susceptible cv. Harrington using cultural and PCR-based assays.

The potential of the competitive PCR assay for quantification of seedborne infection by *R. secalis* in barley was examined. A standard calibration curve for quantification of the levels of seed infection was constructed and used for detecting scald infection in naturally infected barley seeds collected during the 1995 to 1999 growing seasons. The assay showed the potential as an alternative seed health testing method allowing for qualitative as well as quantitative detection of *R. secalis* in barley seeds.

The differential effects of fungicide-timing and rotation sequences on the severity of head infection of scald were investigated using a competitive PCR assay in comparison with conventional visual disease assessment (VDA). Overall, competitive PCR assay allowed discrimination of the influence of Tilt®-timing and rotation on head-infection of barley and correlated better with other disease variables and yield losses than VDA.

Acknowledgements

I would like to express my deep appreciation to my supervisor, Dr. J. P. Tewari for his encouragement and support throughout the program. I also want to thank my supervisory committee members, Dr. T. K. Turkington, Dr. K. G. Briggs, and Dr. F. C. Yeh for their time and support. I wish to thank Lynne Sigler, Dr. P. V. Blenis and Dr. A. Tekauz for valuable suggestions and corrections in this manuscript.

There are many people who have supported me and given me valuable advice about my research. I would like to express my gratitude to Dr. K. Roy for helping with sequence analysis, to Dr. J. Robb for sharing useful information regarding molecular seed health testing and to Dr. P. Blenis for consultation with statistical analysis. Mr. George D. Braybrook I thank for assisting with scanning electron microscopy and X-ray microanalysis. I also want to thank Shirley Brezden, Renate Meuser, and Eleanor Degenhardt for their technical help and support.

I would like to thank the Government of Alberta, Dr. J. P. Tewari, Dr. T. K. Turkington, and the Department of Agricultural, Food, and Nutritional Science for providing me with financial support during my program.

I am also grateful for the support, my nice colleagues have provided throughout my program. I would like to give my thanks to Tracy Shinners-Carnelley, Amit Mukerji, Ali Navabi, Bezhad Sorkhilalehloo, and Bruce Moltzan.

It has been a great journey both in exploring a new world of research and in my own personal growth. I would like to express my sincere gratitude to Dr. Sung Jae-mo who guided me to the world of science and let me pave my own way.

Last but not least I want to thank my parents and two brothers, Cheong and Kil-sang, who have given me endless love, support and encouragement in every step of my life.

Thanks again to all of you.

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Chapter 1

Literature Review

1. 1 Introduction

Barley (*Hordeum vulgare* L. emend. Bowden) is an important feeding and malting crop, which is grown in most temperate regions of the world (Mathre, 1997). It belongs to the grass family Poaceae, tribe Triticeae. Although its major use is animal feed, with a recent increase in beer consumption worldwide, demands for high quality malting barley have increased. Canada is now the world's largest barley producer on a country comparison basis with an annual production of 13.2 million tons. Most of the barley is produced on the prairies with about one-half coming from Alberta (McLelland, 1989).

Scald caused by *Rhynchosporium secalis* (Oud.) J. J. Davis is a common foliage disease of barley, rye, and other grasses (Caldwell, 1937). Since Oudemans of the Netherlands first recorded scald on rye in 1897 under the name, *Marssonia secalis* Oud., it has been reported in cooler and semi-humid barley growing areas of up to nearly 50 countries including Canada, the U. S. A., U. K., the Netherlands, and Australia (Shipton *et al.*, 1974). Scald disease causes serious yield losses in western Canada as well as other major barley–growing areas. Yield losses due to the disease have been estimated to be as high as 35-40 % for severe epidemics, but more commonly, losses are in the 1-10 % range (Khan and Crosbie, 1988; Schaller, 1951; Shipton *et al.*, 1974). Yield loss is due primarily to a reduction in kernel weight, and is proportional to the percentage of leaf area damaged on the top two leaves of the plant; these leaves supply most of the energy required to produce well-filled grain. In addition, severe scald disease lowers quality characteristics such as kernel plumpness, and causes kernel discoloration. This reduction of quality is especially detrimental for malting barleys (Khan and Crosbie, 1988).

In western Canada, especially in cool and humid areas of the prairies, the incidence of scald disease has increased since the early 1950s as a consequence of the

use of improper cultural practices, the widespread use of conservation tillage and expansion of susceptible barley variety cultivation (Buchannon and Wallace, 1962; Skoropad, 1960; Xue *et al.*, 1994). In Alberta, yield losses of up to 30% have been reported and the average yield loss is estimated to be 2.4 % (Skoropad, 1960; Xue *et al.*, 1994). None of the registered malting barley cultivars currently grown in western Canada are resistant to scald. In Ontario, the disease has become increasingly widespread and severe in winter barley fields (Xue and Hall, 1991b).

1. 2 Barley scald disease

Causal organism and symptoms

Rhynchosporium secalis is a haploid (mitosporic) fungus placed within the Deuteromycota. Oudemans (1897) in the Netherlands described lesions on rye and named the causal organism as *M. secalis.* Four months later, in Germany, Frank (1897) isolated the fungus from barley and rye and identified it as *R. graminicola* Heinsen. Davis (1919) proposed the new combination *R. secalis* (Oud.) J. J. Davis as the correct name of the pathogen.

On the host, *R. secalis* produces hyaline to light gray subcuticular mycelium. Conidia (12-20 x 2-4 μm) are borne sessile on conidiogenous cells and are hyaline, one-septate, and cylindrical with a short apical beak (Fig. 1-1) (Ayesu-offei and Clare, 1970; Caldwell, 1937). Skoropad and Grinchenko (1957) reported globose microconidia produced from one-celled flask-like phialides, but their function is not known.

The pathogen, *R. secalis* commonly attacks barley, rye, and many other grass species but rarely wheat and oat (Caldwell, 1937). Barley scald is primarily a foliage disease, attacking the leaves. To a lesser extent, leaf sheaths and auricles are also infected, and barley heads are also susceptible to pathogen attack.

Typical scald symptoms are characterized by oval to elliptical water-soaked lesions with bluish-gray pale centers surrounded by dark brown margins (Fig. 1-2a). Infection is first evident as dark or pale gray, bluish gray lesions and as the disease

progresses, the centers of the lesions dry out and turn a light gray or grayish white. Margins of the lesions usually become dark brown (Caldwell, 1937; Shipton *et al.*, 1974). When infection is severe, adjacent lesions coalesce often leading to complete leaf death. However, atypical symptoms characterized by mild chlorotic lesions producing a much smaller number of conidia were also observed by Fowler and Owen (1971) and Davis *et al.* (1994). A controlled environment experiment suggested that atypical lesions could occur in barley crops when dry weather conditions coincided with the end of the latent period (Davis *et al.*, 1994). Symptomless infection of barley leaves also was reported by Davis and Fitt (1990).

In the head regions, most infection by *R. secalis* is present on the floral bracts, awns and pericarps (Fig. 1-2b). Scald lesions on barley seeds are usually found towards the distal end of lemmas. (Kay and Owen, 1973b; Skoropad, 1959).

Epidemiology of the disease

The disease cycle of barley scald is shown in Fig. 1-3. The pathogen is residue- and seedborne. *Rhynchosporium secalis* overwinters primarily in barley residues left in the field and these are the major source of primary inoculum (Caldwell, 1937; Shipton *et al.*, 1974; Skoropad, 1960). *R. secalis* survives longer in infected plant debris above the soil surface (up to 37 weeks), than on the soil surface (30 weeks) or underneath the soil surface (20 weeks) (Mayfield and Clare, 1984). Skoropad (1965) also indicated that the fungus could sporulate for up to 340 days in the field. Primary infections in early spring start mainly from splash-dispersed conidia newly produced on infected barley residues (Ayesu-offei and Carter, 1971). The greatest increases in production of primary inoculum occur in early spring after rain and subsequent drying and wetting periods (Davis and Fitt, 1992). Disease symptoms appear, if the seedling emerges close to infected debris when conditions are favorable. Conidia produced on the basal leaves serve as secondary inoculum and are dispersed in rain-splashed dropletes causing subsequent infections on adjacent plants (Ayesu-offei and Carter, 1971; Davis and Fitt, 1992; Skoropad, 1960). In the absence of infected host residues, infected seed can be an important source of primary inoculum. Seedborne inoculum is

particularly important in the long-range dissemination of the pathogen and dispersal of new races (Habgood, 1971; Jackson and Webster, 1976a; Kay and Owen, 1973b; Reed, 1957; Skoropad, 1959). Seed transmission of disease occurs through contact of the emerging coleoptile with pathogen inoculum present mainly on the inner surface of the lemma lesion (Skoropad, 1959). One disease cycle takes approximately 14 days during optimum environmental conditions. Head infection may occur if rain-splashed spores reach adjacent spikes (Skoropad, 1959). At the end of the season, the pathogen mainly persists on residues.

Many workers have investigated factors affecting sporulation of the pathogen, such as light, temperature, duration of leaf wetness and inoculum concentration (Caldwell, 1937; Davis and Evans, 1990; Rotem *et al.*, 1976; Ryan and Clare, 1975; Skoropad, 1965; Xue and Hall, 1992). Abundant sporulation occurred from stroma in scald lesions within 48 hrs at 10-18 °C under wet conditions (Caldwell, 1937). The optimum temperature for disease development ranged from 15 to 25°C when plants were kept wet for 14 hrs or more after inoculation (Ryan and Clare, 1975). They also found that plants kept in the dark after inoculation developed more lesions. The optimum length of wetting periods for sporulation of *R. secalis* in barley leaf lesions decreased as the temperature increased (Rotem *et al.*, 1976). Lysis of spores occurred when wetting periods increased (Skoropad, 1965; Rotem *et al.*, 1976). The fungus sporulated poorly below 5 °C or above 30 °C (Caldwell, 1937).

Pathogenic variability

Even though *Rhynchosporium secalis* has no known perfect stage, the fungus is notoriously variable for pathogenic ability. As much variation in pathogenicity has been reported among single spores isolated from the same lesion as between isolates from different lesions collected from the same or different locations (Brown, 1990). Knowledge of pathogenic variability of the fungus is critical to the long-term success of a breeding program aiming to incorporate resistance to *R. secalis* into barley cultivars. Many efforts have been made to identify pathogenic races by inoculating pathogen isolates onto host differentials, and high variability has been demonstrated

repeatedly in many parts of the world. In Canada, Tekauz (1991) identified 45 pathotypes of *R. secalis* using a set of 10 differentials, and 20 pathotypes were identified from 352 isolates obtained from Ontario by Xue and Hall (1991c). In Norway, Salamati and Tronsmo (1997) reported 32 pathotypes, while Jorgensen and Smedegaard-Petersen (1995) identified 28 races among Danish isolates. In Australia, Ali *et al.* (1976) and Brown (1990) recognized 35 and 20 different pathotypes, respectively. In the U.S.A., Jackson and Webster (1975) found that the race composition of *R. secalis* was complex, and at least 75 different pathogenic races existed in California, based on the reactions of 175 *R. secalis* isolates on 14 differential cultivars.

Genetic variability within the scald population has been studied using markers such as isozymes, morphological traits, cultural characteristics, ribosomal DNA, and genomic restriction fragment length polymorphism (RFLP) DNA markers (Burdon *et al.*, 1994; Goodwin *et al.*, 1993; McDermott *et al.*, 1989; McDonald *et al.*, 1999). These studies confirmed that most populations of *R. secalis* were highly variable for many genetic markers and that this genetic diversity can be distributed over a small scale; as well, there can be a high degree of genetic similarity among populations from widely separated geographic regions (Goodwin and Allard, 1992; Goodwin *et al.*, 1993; McDonald *et al.*, 1999; Salamati *et al.*, 2000). Goodwin *et al.* (1992, 1993) used isozyme markers to study the genetic structure of *R. secalis* populations obtained from different geographic regions and found that a large portion of the total genetic diversity was distributed within local populations. McDonald *et al.* (1999) also showed that the majority (76 %) of genetic diversity of the Australian *R. secalis* population was distributed within restricted sampling sites based on the results of RFLP markers. These results suggested that the diverse *R. secalis* populations could rapidly adapt to changes in the environment including the introduction of new resistant genes. More recently, researchers in several countries have studied genetic variability in the pathogenicity of the fungus and the corresponding genetic variability for resistance in barley. Changes in resistance of host plants have resulted directly in response to evolutionary processes in the pathogen (McDonald *et al.*, 1989). In California, evolutionary changes in resistance to *R. secalis* in barley have been studied

using composite cross populations developed by intercrossing cultivars from diverse barley-growing regions of the world, thus representing a broad sample of genetic diversity for reaction to scald disease (Jackson *et al.*, 1982; Muona and Allard, 1982; Saghai *et al.*, 1983; Webster *et al.*, 1986). These studies indicated that the frequency of host barley that is resistant to groups of pathogen races increased from early to late generations in response to biological stresses caused by the variable and shifting pathogen population.

In fungal pathogens, which have no known sexual stage, at least four mechanisms may result in increased genetic diversity in populations: spontaneous mutation, migration, unobserved sexual stage, or parasexual recombination. Of those mechanisms, parasexuality has generally been thought to be of great importance in the generation of variation among deuteromycetous fungi. Jackson and Webster (1975) co-inoculated five pathotypes on susceptible barley cultivars in the greenhouse and after two asexual fungal generations, found that only 17 among 100 re-isolates had a pathotype of the original five pathotypes. By this experiment, they concluded that there must have been some mechanism of recombination and segregation that caused changes in the pathotypes. Newman and Owen (1985) reported asexual recombinants on one of 20 lesions resulting from co-inoculation with isolates of *R. secalis* that differed for isozymes of esterase and ß-glucosidase. In contrast, Goodwin *et al.* (1994) suggested that mutation and/or migration could be very important sources of genetic variation within *R. secalis* populations, and that parasexual recombination plays only a minor role in generating pathogenic variability. McDermott *et al.* (1989) and Goodwin *et al.* (1993) proposed a frequency dependent selection as an explanation for the maintenance of variation in pathogenicity in the fungus. Although the sexual stage of *R. secalis* has not been reported, a study using diverse Australian field isolates showed that most of the alleles at isozyme loci were in gametic equilibrium, which is an indication of genetic exchange and recombination (McDonald *et al.*, 1999). Most recently, it has been suggested that *R. secalis* populations in Norway, Finland, and Australia undergo regular recombination, based on the analysis of the genetic structure of field populations of *R. secalis* from three continents using RFLP markers (Salamati *et al.*, 2000). They also postulated that the

telemorph of *R. secalis*, if present, would be closely related to the Helotiales that form an apothecium, based on results of sequence comparison of the internal transcribed spacers (ITS) regions of *R. secalis* rDNA.

The possibility that host plants other than cultivated barley contribute a degree of pathogenic variability to the *R. secalis* population has also been investigated. In a few limited studies, strict host specialization was observed (Caldwell, 1931; Reed, 1957). However, the majority of studies indicated that there was less host specialization (Ali, 1981; Jorgensen and Smedegaard-Petersen, 1995; Kay and Owen, 1973). Volunteer barley grasses (*H. leporinum* Link and *H. murinum* L.) are other sources of inocula (Ali, 1981; Brown, 1990) and Brown (1990) identified 19 different pathogenicity groups from 182 single spore isolates of *R. secalis* obtained from *H. leporinum*, on the basis of the reactions of 15 barley varieties.

Host-pathogen interaction

Infection processes of the pathogen have been investigated microscopically in barley leaves naturally infected in the field or artificially inoculated in the greenhouse. (Ayesu-offei and Clare, 1971; Howlett and Cooke, 1987; Lehnacker and Knogge, 1989; Jorgensen *et al.*, 1993; Ryan and Grivell, 1974). Fungal development has been characterized by the growth of subcuticular mycelium at an earlier stage, followed by the penetration of epidermal cells and invasion of the mesophyll at later stages of infection (Ayesu-offei and Clare, 1970; Caldwell, 1937). Based on the observations under Transmission electron microscope (TEM), Ryan and Grivell (1974) showed that the pectic and cuticular layers remained largely intact in leaf lesions until conidia were produced, whereas the cell wall was degraded and replaced by hyphae.

Once the pathogen penetrates, nutrient availability is critical for further development of the pathogen. *Rhynchosporium secalis*, unlike other pathogens, does not produce haustoria that allow the pathogen-intake of nutrients from host cells. Jones and Ayres (1972) showed that the early stages of pathogenesis were characterized by an increased permeability of host cell membranes, epidermal cell collapse and intact plant cell walls. Furthermore, early symptoms often developed at a

distance from fungal hyphae, and there was no direct contact between fungal hyphae and plant plasmalemmas during the recognition phase (Ayesu-offei and Clare, 1970; Jones and Ayres, 1974; Lehnackers and Knogge, 1990). Based on these observations, the involvement of toxic fungal metabolites in disease development has been suggested. Mazars *et al.* (1989, 1990) isolated a low molecular weight toxin, rhynchosporoside and a high molecular phytotoxic glycoprotein from culture filtrates. In a bioassay, rhynchosporoside and the phytotoxic glycoprotein were proved to be responsible for necrosis of leaf tips and margins, and subsequent chlorosis, in addition to similar symptoms on leaf blades, possibly by plugging xylem vessels (Auriol *et al.*, 1978). Wevelsiep *et al.* (1991) purified a new class of toxic compounds, three necrosis-inducing peptides (NIPs) with molecular masses of <10kda, from *R. secalis* culture filtrates. In bioassay, NIPs were found to be host non-specific.

Histological studies have been carried out to address potential mechanisms of resistance. Reduced sporulation and increased latent period have been found to be associated with race-nonspecific resistance (Fowler and Owen, 1971). Based on a comparative analysis of fungal development on susceptible and resistant cultivars, Lehnackers and Knogge (1990) suggested that inhibition of spore germination on the leaf surface and the prevention of establishment of subcuticular mycelium development were critical for plant defense. Jorgensen *et al.* (1993) observed the formation of papillae and halos in the cell walls around the pathogen's penetration pegs associated with the penetration process and concluded that the formation of papillae was a primary resistance mechanism against fungal penetration and subsequent tissue colonization. They also suggested that, to a lesser extent, halo size was correlated with plant resistance. Recently, such halos have been shown to be rich in silicon (Tewari J. P., unpublished data). More recently, Xi *et al.* (2000) proposed that host cell wall alteration might be one of the mechanisms responsible for resistance that may produce chemical barriers that help to prevent penetration of the pathogen.

Disease control

Cultural control

Rhynchosporium secalis overwinters primarily in infected barley residues left in farm fields. However, infected seeds also carry the fungus and serve as a primary source of inoculum. Studies have shown that the fungus may survive on residue for as long as the residue persists, which may be for one or two years (Mayfield and Clare, 1984; Skoropad, 1965). Therefore cultural practices involving residue management and crop rotation should be effective in reducing the severity of disease.

Because the fungus is a weak saprophyte, proper tillage practices that incorporate the residue and aid in the breakdown of the residue will reduce the abundance of the inoculum. However, this disease reduction achieved with tillage must be balanced against increased soil erosion potential. Crop rotation is an another effective management option to reduce inoculum levels. Rotating out of barley for one year will significantly reduce the potential for serious disease problems, although a two year period would be more effective in controlling the disease.

The reduction of scald disease was observed when cultivar mixtures containing lines differing in resistance and susceptibility to four diagnostic pathotypes of *R. secalis* were grown (McDonald *et al.*, 1988).

Chemical control

Application of fungicide provides an effective control means to reduce the severity of disease. In Canada, the systemic fungicide Tilt® (propiconazole) is the only registered product for the control of foliar diseases of barley (Shaffeek, 2000). It has been effectively used to control barley scald disease as well as net blotch (Jordan *et al.*, 1982; Martin and Sanderson, 1988; Scott *et al*, 1992; Sutton and Steele, 1983). Significant yield increases (kernel weight and kernel size) occurred when Tilt® was applied (Jordan *et al.*, 1982; Scott *et al*, 1992). Scott *et al.* (1992) reported that two timely applications of Tilt®, one at formation of the first node on the main tiller and

the second at flag-leaf emergence, resulted in almost complete control of scald disease and a yield increase of up to 37%. However, laboratory tests involving 2000 isolates of *Rhynchosporium secalis* and three demethylation inhibitor (DMI) fungicides, triadimenol, propiconazole, and prochloraz, revealed a decline in sensitivity to triadimenol and propiconazole, but not to prochloraz (Kendall *et al.*, 1993). The occurrence of pathotypes of *R. secalis* that are resistant to commonly used fungicides may reduce the fungicide effectiveness in the future. Therefore it has been suggested that mixtures of these fungicides with carbendazim would improve the fungicidal performance (Kendall *et al.*, 1993).

Fungicides applied as seed treatments can help reduce seedborne inoculum and protect disease-free areas. Currently, Baytan 30 (Triadimenol) is available for seed treatment to control scald disease on barley in Canada (Shaffeek, 2000). Although significant variation in the sensitivity of *R. secalis* to the fungicide triadimenol appeared to be present, based on the *in vitro* assay, Baytan seed treatment resulted in control of disease development of seedborne *R. secalis* (Hollomon, 1984).

Overall, fungicide applications are one of the most effective means of controlling scald disease. However, concerns regarding the development of fungicide resistant strains, their cost, and environmental protection may limit use of fungicides for control of the scald disease.

Breeding for resistant cultivars

Growing resistant cultivars is probably the most appealing, economical, and environmentally-friendly management option available. An understanding of the inheritance of resistance to scald in barley is essential to develop resistant cultivars. Previous studies on sources of resistance to *Rhynchosporium secalis* indicated the presence of several types of resistance in barley. Since Mackie (1929) first reported that scald resistance was controlled by a single recessive gene in an unnamed cultivar, various major genes conferring race-specific resistance to *R. secalis* have been identified. In many of the studies on the inheritance of resistance to *R. secalis*, resistance proved to be governed by alleles at single loci. Alleles for resistance were

usually dominant to alleles for susceptibility, but in a few cases resistant alleles have been only partly dominant, or recessive. Bryner (1957) designated a scald resistant gene present in the cv. Brier as R*h*. Dyck and Schaller (1961a, 1961b) identified five additional genes, designated as R*h*2, R*h*3, R*h*4, R*h*4^2 (an allele of R*h*4) and R*h*5. Genes R*h*2 and R*h*5 were independent, but R*h*3 and R*h*4 were closely linked on chromosome 3 and allelic to the R*h* locus described by Bryner (1957). Habgood and Hayes (1971) suggested that R*h*, R*h*3 and R*h*4 all were alleles and should be designated as R*h* with allelism being denoted by superscript number. In Canada, Penner *et al.* (1996) identified two resistant genes (R*h* and R*h*2) by evaluating reactions of western Canadian barley cultivars to *R. secalis* isolates. According to this study, the gene in Falcon was the R*h*2 gene derived from Atlas, and the gene(s) in CDC Silky was located within the R*h*/R*h*3/R*h*4 cluster and similar to the R*h* gene in Hudson.

Attempts to control scald disease have focused on identification and incorporation of individual vertical resistant genes into cultivars. However, due to the presence of great pathogenic variability in pathogen populations, resistance based on a single gene has proved to be unsuccessful. Therefore, much of the ongoing research has been focused on finding sources of long lasting durable resistance and new resistant genes (Garvin *et al.*, 1997). Genotypes with moderate resistance or adult-plant resistance may be more durable as sources of resistance. Several cultivars that were shown to be susceptible at the seedling stage in the greenhouse appeared resistant or race-nonspecific as adult-plants under field conditions (Cselenyi *et al.*, 1998; Xue *et al.*, 1995). Van Ginkel and Vivar (1986) detected slow scalding as a stable trait that contributes to durable resistance. More recently, Sorkhilalehloo *et al.* (2000, 2001) conducted field studies using western Canadian barley cultivars and detected slow-scalding type of resistance, which exhibited low to intermediate levels of disease incidence and severity. It has been suggested that components of quantitative resistance to scald are related with a longer incubation period, smaller lesions, and reduced sporulation capacity (Kari and Griffiths, 1993, 1997; Robinson *et al.*, 1997; Xi et al., 2000; Xue and Hall, 1991a).

Wild species related to cultivated crops are a potential source of genes for resistance to a range of diseases afflicting those crops. Abbott *et al.* (1992) screened accessions of *Hordeum vulgare* ssp. *spontaneum*, the wild progenitor of barley, for seedling response to four isolates of *R. secalis* and found that five genetically independent resistances were available for combining together for deployment in barley.

The selection of resistant lines in breeding programs can be difficult due to effects of environmental factors on the development of symptoms of host plants. Therefore, identification of molecular markers linked to genes for resistance, which can be used in marker-assisted selection would be useful to barley breeders. Barua *et al.* (1993) identified RAPD markers linked to the *R. secalis* resistance locus (R*h*) on chromosome 3L and RFLP markers linked to scald resistance gene R*h*2 were also identified by Schweizer *et al.* (1995).

Biological control

Biological control of scald disease has received less attention compared to other control measures. Jorgensen *et al.* (1996) showed that preinoculation of barley leaves with either of the two non-barley pathogens, *Bipolaris maydis* from maize and *Septoria nodorum* from wheat induced protection against important barley pathogens including *Drechslera teres, B. sorokiniana, Rhynchosporium secalis,* and *Erwinia graminis* f. sp. *hordei.*

Regulatory control

Seeds are not only the propagative materials for many crop plants, but also effective carriers for a number of plant pathogens. Many plant pathogens are perpetuate by infecting seeds of their host plants and are therefore termed seedborne (Neergaard, 1977). Almost 1500 microorganisms on about 600 genera of agricultural, horticultural, and tree crops have been recorded as seedborne (McGee, 1981). Seed transmission of pathogens is of particular significance in distributing random infection

foci in a field, long-distance dissemination, selection of host-specific races of pathogens, and providing prolonged transmission capacity for pathogens (Baker, 1980). Seed transmission of the pathogen is important not only when transmission rates are high, but also when small percentages of seeds are infected, as these equally represent sufficient sources of inoculum by which the pathogen can be introduced into uncontaminated areas (Gamboi, 1983). With the exception of cereal smuts, monocyclic pathogens, even very low levels of seed infection can lead to severe epidemics.

The spread of seedborne diseases can be restricted in a number of ways. One of them, chemical seed treatment, has been effectively used to control many seedborne pathogens, but it can be expensive and increasing environmental concerns limit its use. As a part of an integrated crop management program, a seed health inspection program is worthwhile (McGee, 1981). Seed health tests can be used to establish the commercial value of seed, to help to make plant disease management decisions, to investigate reasons for poor germination caused by seedborne pathogens, and for research (Reeves, 1995). Currently used seed health tests include field inspection of seed crops for the presence of disease, direct visual examination of dry seed, incubation tests involving growing the pathogen on selective media, grow-out tests of seeds, serological assays, and nucleic acid-based assays (McGee, 1995).

Barley is attacked by a number of pathogens. Of these, scald of barley caused by *Rhynchosporium secalis* is a major foliage disease throughout the cooler, semi-humid barley growing areas. *Rhynchosporium secalis* overwinters predominantly as dormant mycelium on barley residues (Cladwell, 1937; Reed, 1957; Skoropad, 1959). However, in the absence of residue-borne inoculum, seedborne inoculum serves as a primary inoculum (Habgood, 1971; Jackson and Webster, 1976a; Kay and Owen, 1973b; Reed, 1957; Skoropad, 1959). Habgood (1971), Kay and Owen (1973b), and Skoropad (1959) have reported seed infection of up to 14.5% and subsequent seedling infection of 2%. In the U.S.A., Jackson and Webster (1976a) reported up to 36.5% incidence of seedborne *R. secalis* and a transmission rate of 26.5% from seed to seedling. Although the importance of the seedborne inoculum has been recognized,

assessment of scald infection of barley seed has been based on visual examination of the external symptoms, which is considered to be less sensitive.

1. 3 Objectives

Seedborne inoculum may be responsible for the early start of epidemics, for the introduction of *Rhynchosporium secalis* into new areas, and for the dispersal of new races. Since no resistant malting barley varieties have been registered in Canada and there is high variability of the pathogen, protection of the crop from seedborne source of inoculum is important for developing an integrated crop management program. The objectives of this study were:

1. To examine infection of barley head by *R. secalis* using scanning electron microscopy and energy dispersive x-ray microanalysis.

2. To develop an agar plate technique to isolate the pathogen from *R. secalis*-infected seed

3. To develop a species-specific PCR-based diagnostic assay to detect *R. secalis* in barley seed.

4. To detect the presence of the symptomless *R. secalis* infection.

5. To adapt a qualitative PCR assay for quantitative analysis and examine the potential of this assay to determine the levels of *R. secalis* infection in barley seed.

6. To study the effects of the timing of Tilt® fungicide application and rotation trials on control of the barley scald disease and compare quantitative PCR assay and visual disease assessment (VDA) diagnostic methods.

1. 4 Figures

Figure 1-1. Scanning electron micrograph of *Rhynchosporium secalis* conidia on barley leaf surface.

Figure 1-2. Typical symptoms of barley scald disease on (a) leaf (Photo courtesy of Dr. J. P. Tewari) and (b) head.

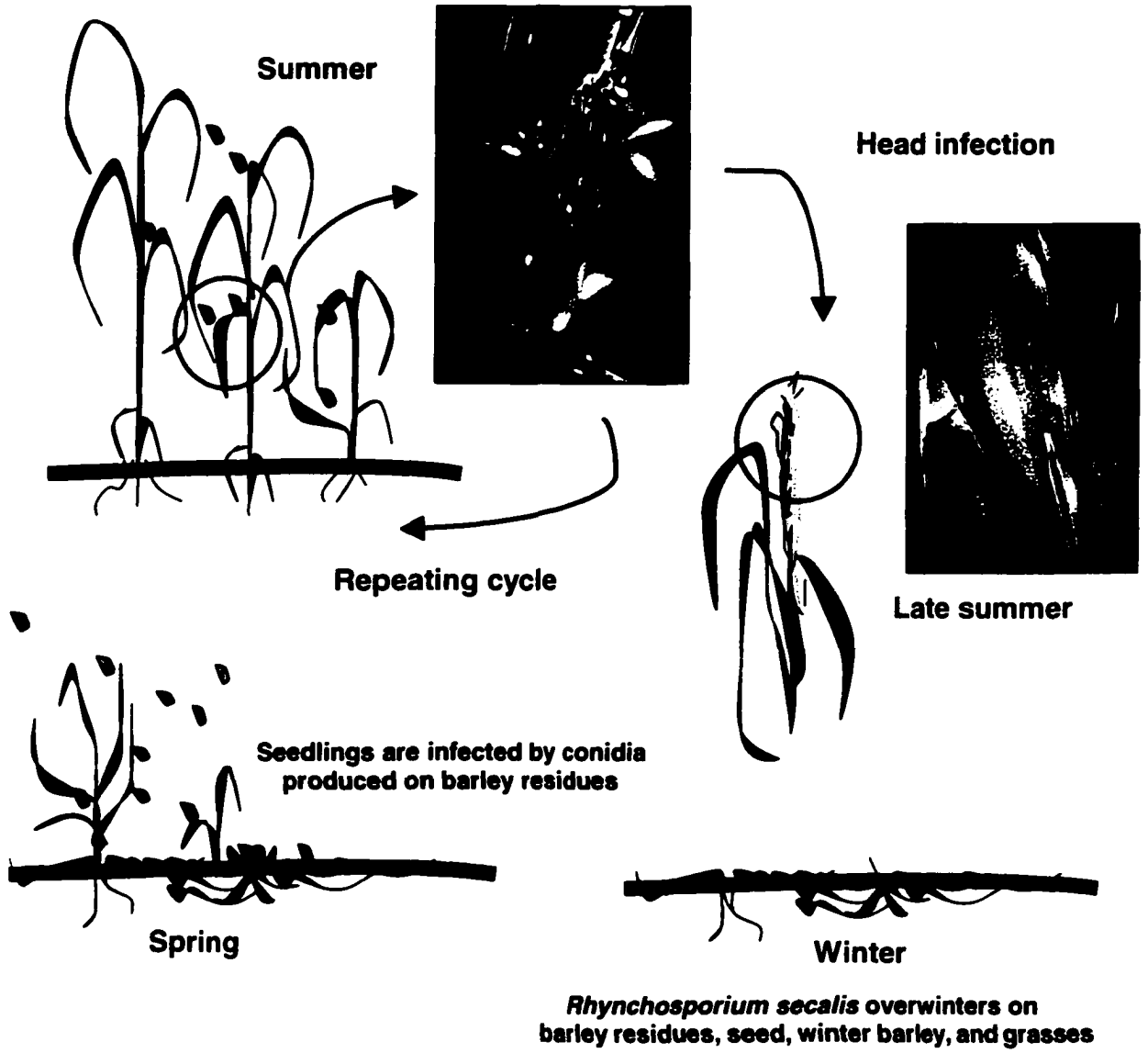

Figure 1-3. Disease cycle of barley scald

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Chapter 2

Symptomatology and histopathology of barley heads infected by *Rhynchosporium secalis* [1]

2. 1 Introduction

Since Smith (1937) first described scald symptoms on barley seeds, the seedborne nature of the disease has been studied on several occasions, mostly in relation to seed transmission of the disease either under greenhouse or field conditions. In earlier studies, Habgood (1971), Kay and Owen (1973), and Skoropad (1959) have reported *Rhynchosporium secalis* infection of barley seed of up to 14.5 % and subsequent seedling infection of 2 %. Jackson and Webster (1976) also reported a seed infection rate of up to 36.5 % and a transmission rate of 26.5 % from seed to seedling. Although seed transmission experiments conducted in different locations and conditions resulted in inconsistent seed transmission rates of the disease due to the environmental conditions, in general the results suggested that infected seed may serve as a source of inoculum for barley scald epidemics and for dispersal of new races of the pathogen. The infection route and location of pathogens on and in seeds affect the capacity for survival in the seedborne phase, and ultimately, their transmission from seeds to seedlings (Maude, 1996). In Canada, Skoropad (1959) reported on scald symptoms on seeds and also on the way by which barley seeds are infected and the disease eventually transmitted. According to his study, seed transmission of disease occurs through the contact of the emerging coleoptiles with inoculum present mainly on the inner surfaces of the lemma lesions. However, 20 % of the seedlings arising from diseased seeds escaped infection due to the manner of germination of seeds (Skoropad, 1959).

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[1] *A part of this chapter has been published*: Lee, H. K., Tewari, J. P., and Turkington, T. K. 1999. Seed Sci. & Technol. 27: 477-482.

Kay and Owen (1973) also described the symptoms of the seed infection by *R. secalis* based on the results of microscopic observations. Both studies indicated that the fungus infected barley seeds mainly by colonizing the inner surfaces of seed coats. Since then, although the importance and contribution of the seedborne inoculum to the epidemics of the scald disease have been noted, seedborne barley scald has received less attention than stubble-borne scald.

The present study was undertaken to examine scald symptoms on barley heads in different barley cultivars, using light microscopy as well as scanning electron microscopy (SEM).

2. 2 Materials and Methods

Plant materials

Rhynchosporium secalis-infected barley seeds were examined using seeds stored for two years and others collected in the same year from the barley fields. Seed lots of the cvs Harrington and Manley stored at room temperature were obtained from the Gateway Research Organization, an applied research organization at Westlock, AB in 1996 and individual seeds were examined for the presence of scald lesions. In order to examine various parts of barley heads infected under natural conditions, heads of different cultivars with scald lesions were collected during the 1997 to 2000 growing seasons from fields at Calmar and the Edmonton Research Station of the University of Alberta.

Light microscopy

Selected seed samples with putative scald lesions were soaked in distilled water for 15 min. before separating the lemmas and paleas. The lemmas and paleas were stained with Lactophenol dye and examined under a light microscope.

Scanning electron microscopy and energy dispersive X-ray microanalysis

In the case of stored seed samples, lemmas and paleas exhibiting scald symptoms were separated after soaking seeds in distilled water for 15 min. Barley

heads collected from fields were dissected into lemmas and paleas, awns, glumes and rachises. For scanning electron microscopy, these barley head parts were placed onto filter paper and vapor-fixed with 2% (v/v) osmium tetroxide in water overnight. Samples were then air-dried overnight at room temperature and mounted onto stubs and secured with Marivac colloidal carbon paint. The samples were coated with gold and subsequently examined in a Scanning Electron Microscope (JSM 6301-F). Energy-dispersive X-ray microanalyses were conducted using a Link eXL energy-dispersive X-ray system with a light element detector.

2. 3 Results

The cvs Harrington and Manley seeds stored for two years were examined for the presence of scald symptoms. Scald symptoms were characterized by oval to elliptical lesions with pale centers surrounded by dark brown margins, which were mainly situated towards the upper ends of the lemmas (Fig. 2-1a). However, some symptoms were ambiguous if typical light center lesions were not present. Whole mount preparations of the lemmas and paleas had abundant thick and short-celled dormant mycelia mainly restricted to the lesions as viewed under a light microscope (Fig. 2-2). Conidia of *R. secalis* were rarely observed along the lesions. Scanning electron micrographs showed the same hyphal colonization and a few detached conidia of *R. secalis* confined to the inner surface of lesions. For seeds with ambiguous symptoms, typical *R. secalis* dormant mycelia or conidia were not always observed. Although the infection of lemmas and paleas with *R. secalis* was confirmed by the presence of symptoms using visual identification, some samples appeared to be heavily colonized by microorganisms other than *R. secalis* such as *Drechslera teres*, *Cladosporium* sp., and mycelia of unknown fungi.

In two-row (cvs. Harrington, Manley, Seebe, TR129, TR150), six-row (cvs. AC Rosse, BT433, BT922, BT951, Kasota), and hulless (cvs. HB329, HB805) barley cultivars, scald symptoms were distributed over lemmas, paleas, awns, glumes and rachises, but were most common on glumes and awns. Cultivars with severe leaf infections tended to have more head infection reflecting their susceptibility. In most

cultivars examined, awns showed lots of lesions. The scald lesions on awns were in the form of tiny dark brown to black dots on the awn surface. Oval to elliptical scald lesions were rarely observed on the awns. Scald symptoms on seeds were mostly on the lemmas and rarely on paleas as those described by Skoropad (1959) and Kay and Owen (1973). Scald lesions on lemmas tended to appear towards their upper regions and were distributed around the margins of lemmas, where the lemmas seemed to trap rain-splashed conidia (Fig. 2-1b). Occasionally, typical scald symptoms similar to leaf lesions were also observed on the center of the lemma. These symptoms were characterized by somewhat elliptical shapes with pale yellowish centers surrounded by dark brown margins. Even when distinct lesions were present on the surface of lemmas and paleas, conidia and mycelia were only observed on their inner surfaces and on the outer surface of the pericarp, as described by Skoropad (1959) and Kay and Owen (1973). Conidia and mycelia were not present on the outer surfaces of lemmas and paleas. Scanning electron micrographs indicated the conidia were present in masses surrounded by what appeared to be mucilaginous material (Fig. 2-3b). The lesions on glumes and lemmas were generally similar. Scald lesions on glumes also tended to be located towards their upper regions and were in the form of elongated or elliptical lesions with pale yellowish centers surrounded by dark brown margins. Scanning electron micrographs showed that conidia and mycelia were distributed along the awn surface and on the inner surfaces of lesions on glumes and (Fig. 2-3a & 2-3c). Symptoms on rachises, in the form of small irregular brownish dots were rare.

In the case of glumes of cv. Harrington, numerous rod-like crystals were observed and these crystals were associated with conidia of the pathogen colonizing the inner surface (Fig. 2-3c). Crystals were only rarely observed in other parts of barley head. The X-ray energy-dispersive emission spectra revealed that the major element present in crystals from such lesions was calcium (Fig. 2-4 & 2-5).

2. 4 Discussion

Scald symptoms on seeds observed in the present study were generally similar to those described by Skoropad (1959) and Kay and Owen (1973). However, unlike

seeds of barley heads collected from the fields, there were difficulties in assessing seed infection using 2-yr old seed samples due to the presence of ambiguous symptoms that could be confused with those caused by other pathogens such as *Drechslera teres, Fusarium poae, Pseudomonas syringae* and kernel discoloration caused by saprophytes. Net blotch, caused by *D. teres* is one of the most common diseases of barley found in association with barley seeds. Seed infection by *R. secalis* occasionally results in a diffuse, dark or pale coloration, which makes it difficult to distinguish from net blotch infection. Unlike symptoms of net blotch, typical symptoms on seeds caused by *Fusarium poae, Pseudomanas syringae* pv. *syringae* are similar to those caused by *R. secalis* (Mathre, 1997). In the present study, some of the seeds with ambiguous lesions examined under light or scanning electron microscopes appeared to be colonized by other microorganisms, such as *D. teres, Cladosporium sp.*, and some unknown fungi, rather than *R. secalis*.

The microscopic studies showed that in seeds (both stored and collected from the barley fields), the mycelia and conidia of *R. secalis* were primarily distributed between the pericarp and lemma or palea, and colonization was restricted to the inner surfaces of lesions. No lesions were observed near the embryo end. Most sampled heads had heavy awn infections and these structures appeared to trap numerous spores dispersed by rain splash. As suggested by Skoropad (1959), it can be assumed that head infection occurs primarily when kernels, which are enclosed by floral bracts are half filled. Rain-splashed water droplets containing spores enter through the opening near the upper lemma end where the infection is initiated. The edges of the lemmas and glumes that form the groove appeared to be the most susceptible areas for scald infection since they act as ideal incubators for infection and sporulation of the pathogen due to the accumulation of water. Many necrotrophic pathogens are also known to locate internally, mainly between the seed coat and pericarp, as in *R. secalis*. Although this location allows the pathogen to survive protected for many years, the growing coleoptile needs to contact the inoculum confined in the lesions in order to transmit the disease (Jorgensen, 1977; Skoropad, 1959). This location of the lesions may partly contribute to the discrepancies in seed transmission rates of the pathogen reported elsewhere (Jackson and Webster, 1976; Kay and Owen, 1973).

Rain-splashed conidia are known to be responsible for the dispersal of *R.secalis* (Reed, 1957; Shipton *et al.*, 1974). Mucilage associated with conidia and mycelia of many of the fungal pathogens belonging to the group Deuteromycetes protects and controls the release of conidia by rain splash (Maude, 1996). Scanning electron micrographs revealed that conidia of *R. secalis* present on the inner surface of lemma were surrounded by what could be mucilaginous material.

Barley heads of most cultivars examined in this study appeared susceptible to scald infection. However, some cultivar-related differences in overall seed infection by *R. secalis* were observed which may be a reflection of differential host susceptibility.

In many host plant-pathogen interactions, calcium sequestration from the host cell walls by oxalic acid produced by the pathogen has been observed during pathogenesis (Kaile *et al.*, 1991; Rao and Tewari, 1987; Yang *et al.*, 1993). The role of oxalic acid in pathogenesis is to acidify host tissues and sequester calcium from host cell walls, thus weakening the host cell walls by the resulting production of calcium oxalate crystals. Weakening of the host cell walls allows polygalacturonase to cause degradation more rapidly in a synergistic response (Martin and Evans, 1996). In the present study, rod-shaped calcium-containing crystals associated with conidia *of R. secalis* were often abundant on the inner surface of glume-lesions in the susceptible cv. Harrington, but were rarely found on the other parts of barley heads, or in other cultivars. Tewari *et al.* (1995) first observed cation sequestration by *R. secalis* in scald-infected leaf lesions of the susceptible cv. Manley. Mukerji *et al.* (1998) demonstrated that the levels of calcium were related to the levels of scald resistance of host plants. Calcium sequestration found in the present study suggests and supports an earlier report (Tewari *et al.*, 1995) that pathogenesis by *R. secalis* partly involves sequestration of calcium ions from host plants, as is found in some other pathosystems.

2. 5 Tables and Figures

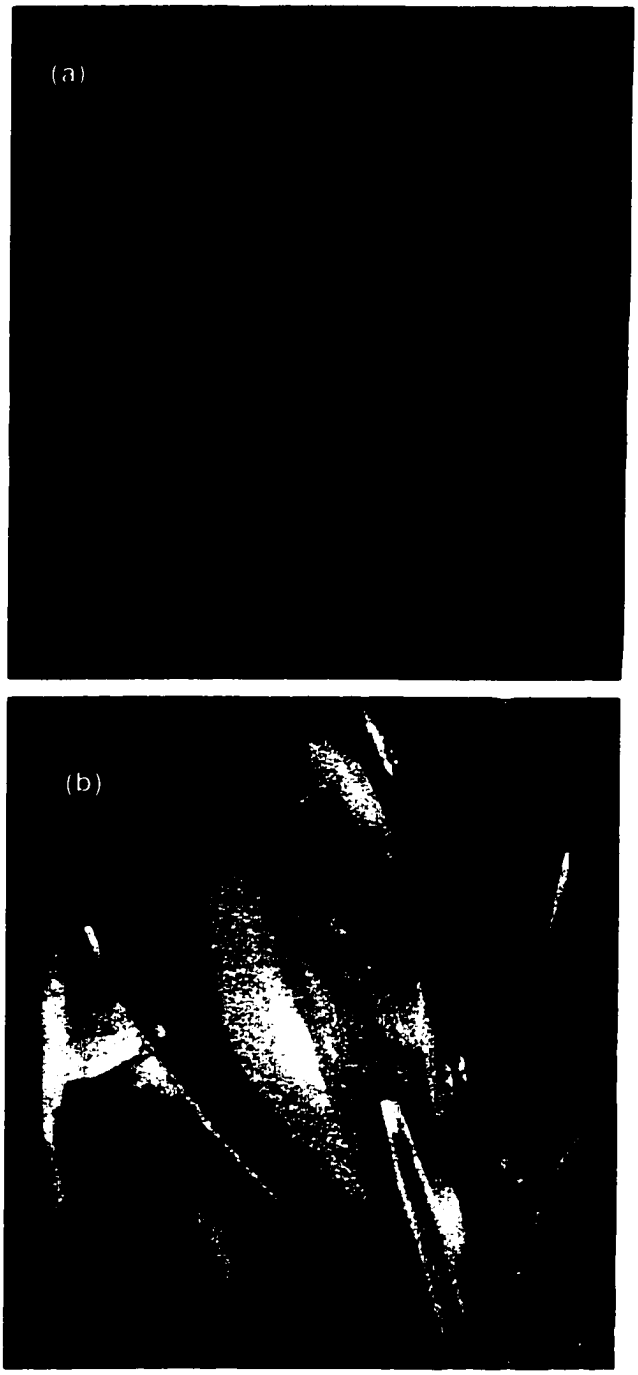


Figure 2-1. Typical scald symptoms found on (a) seeds stored for two years and (b) collected from the barley fields.

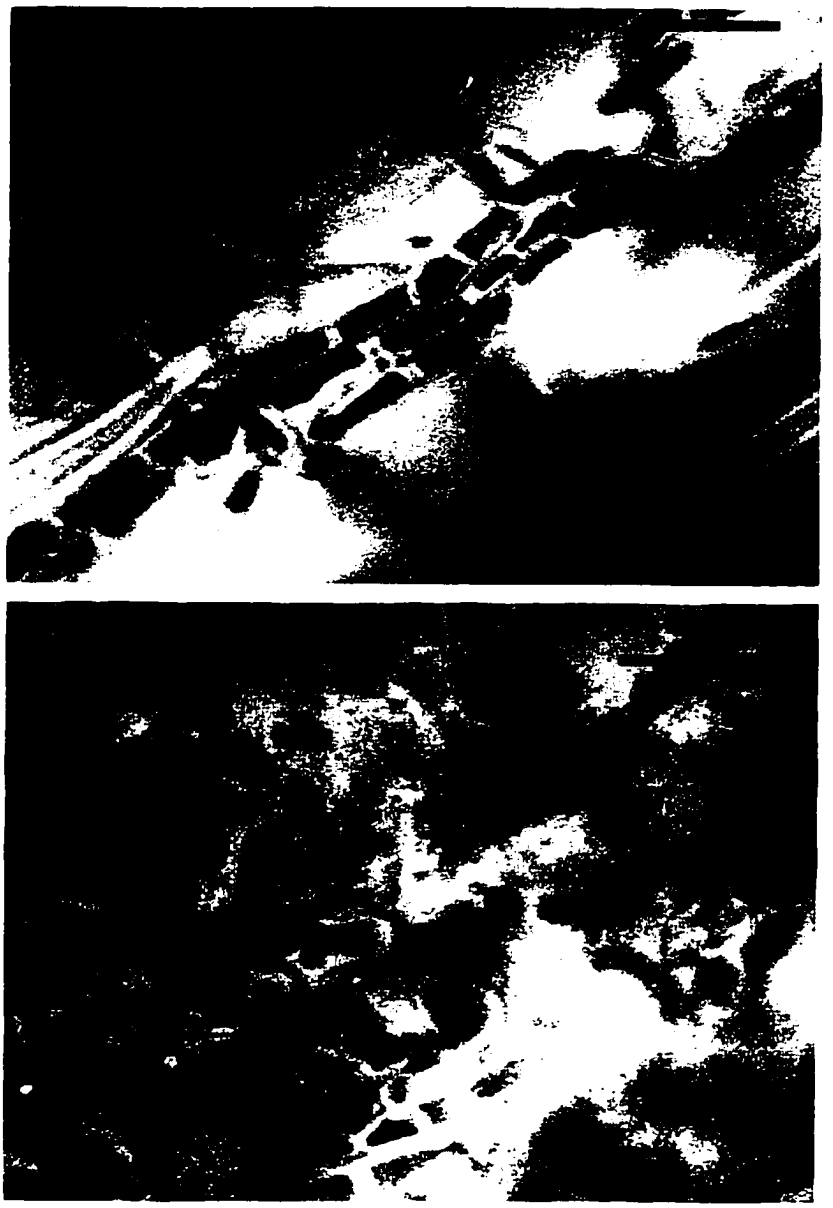
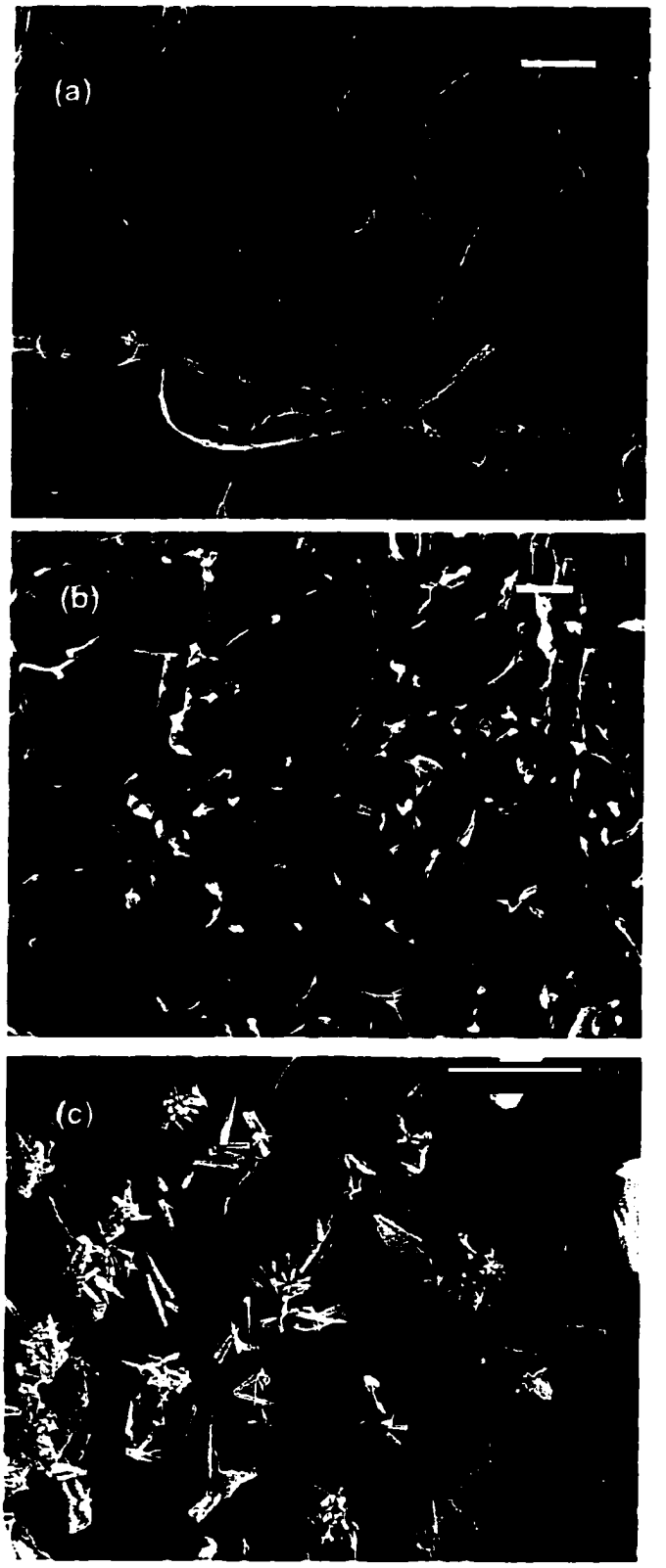


Figure 2-2. Light micrographs of the inner surface of the lemma lesions showing compact mycelia and conidia of *Rhynchosporium secalis*.
Bar =10 μm.

Figure 2-3. Scanning electron micrographs of scald lesions (a) on surface of the awn and (b) on the inner surface of the lemma of cv. Harrington showing hyphae and conidia of *Rhynchosporium secalis* surrounded by mucilage-like material. (c) Inner surface of glumes showing *R. secalis* conidia and calcium-containing crystals. Bar= 10 μm.



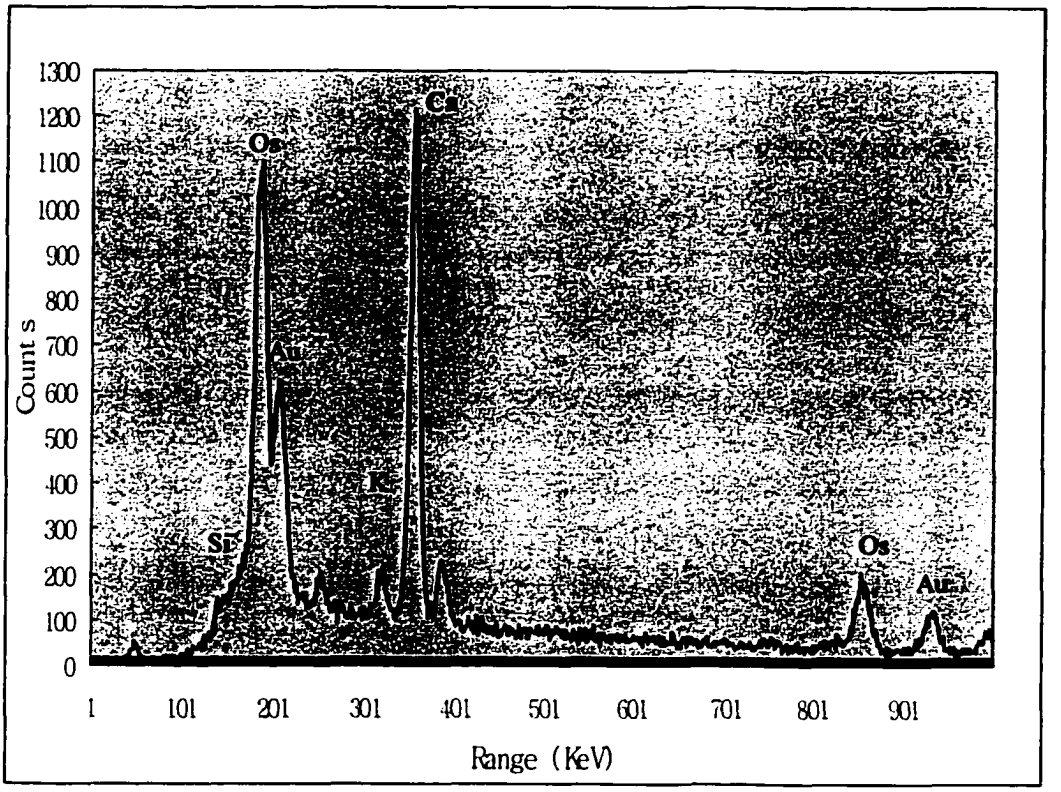


Figure 2-4. Energy-dispersive X-ray spectrum showing a high peak of calcium from a crystal formed on inner surface of a glume infected with *Rhynchosporium secalis.*

Note the peaks for silicon and potassium arising from elements present in the glume. Osmium is from chemical fixation of the material with osmium tetroxide and gold was used to coat the material before examination in the SEM

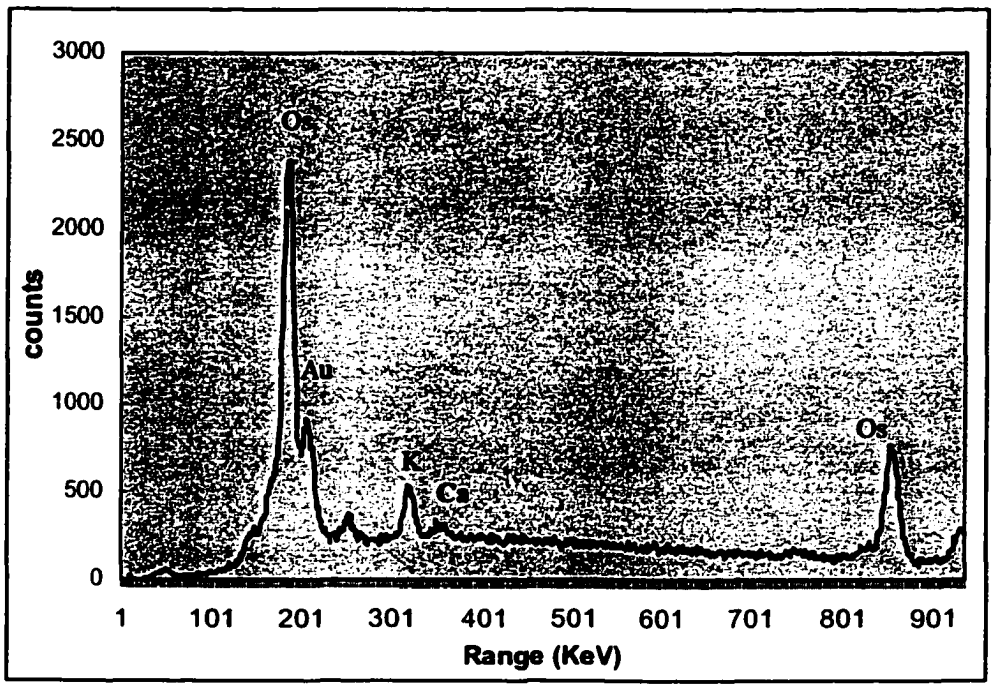


Figure 2-5. Energy-dispersive X-ray spectrum of an uninfected area on the inner surface of a glume.

Note a small peak for calcium. See the legend for Figure 2-4 for explanation of other peaks.

2. 6 References

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Chapter 3

Development of an isolation technique for *Rhynchosporium secalis* from infected barley seed[1]

3. 1 Introduction

Although the telemorph of *Rhynchosporium secalis* has not yet been identified, genetic variation in *R. secalis* populations is extremely high (McDermott *et al.*, 1989; McDonald *et al.*, 1999; Tekauz, 1991). However, studies have shown a high degree of genetic similarity among populations collected from widely separated geographic regions (McDonald *et al.*, 1999; Salamati *et al.*, 2000). Significant long-distance dispersal of races poses a threat to deployment of resistance genes tailored to local pathogen populations.

Seedborne inoculum may be responsible for the genetic similarity found among geographically separated regions since it plays an important role in dispersal of new races. Therefore, it is important to monitor the levels of seed infection to manage the disease and restrict introduction of new races.

Direct examination of seed samples and seed incubation tests are important methods for detecting pathogens in general seed pathological research and in seed health testing (Agarwal and Sinclair, 1997). Visual inspection of external symptoms on seeds has been the method employed for diagnosing seedborne infection of *R. secalis*. However, scald symptoms can be confused with other diseases, and the number of symptomless seeds can be underestimated (Kay and Owen, 1973; also see Chapter 2).

Cultural methods of assessment based on the growth of the pathogen on agar plates or filter paper give an indication of the presence of viable inoculum in an infected seed sample.

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[1] *A part of this chapter has been published*: Lee, H. K., Tewari, J. P., and Turkington, T. K. 1999. Seed Sci. & Technol. 27: 477-482.

These methods are most useful to detect high-incidence pathogens (Jorgensen, 1977). Although there have been reports of the presence of numerous *R. secalis* conidia on scald lesions on seed, it has not been possible to isolate the pathogen from infected seeds due to the slow-growing nature of the pathogen and the presence of other contaminants (Kay and Owen, 1973; Professor C. Cappelli, personal communication).

Therefore, the present study was conducted to develop a cultural method that could be used for isolating the pathogen from seeds and for seed health testing.

3. 2 Materials and Methods

For development of an isolation method, *R. secalis*-infected seeds of the cvs. Harrington and Manley were used. These seeds were derived from samples that were heavily infected and were kept in storage at room temperature. The lemmas and paleas with lesions from each seed were peeled-off and washed 3-4 times with sterile distilled water in microfuge tubes by vortexing to remove as many contaminants as possible. After washing, the lemmas and paleas were blotted with sterilized filter paper to remove excess moisture and placed into microfuge tubes with 50 µl sterilized distilled water containing 100 mg L^{-1} streptomycin. The lemmas and paleas were incubated for various periods at 16 C to enrich the scald fungus in relation to the contaminants present. The incubation times tested were 0, 12, 24, 36, and 48 hours. For each incubation time, lemmas and paleas from 3 seeds were examined. After incubation, the materials were teased with a glass rod to dislodge the scald fungus and streaked on lima bean agar plates supplemented with 200 mg L^{-1} streptomycin and 50 mg L^{-1} rose bengal using a sterile loop. Three replicate plates for each seed were used for scald colony development and examined after 7 to 10 days incubation at 16 C. While the experiment was repeated eleven times, results from only five repeats of this experiment are given in this chapter.

3. 3 Results and Discussion

The number of seeds which gave rise to colonies of *R. secalis* did not vary at different incubation times in each set (Table 3-1). However, as the incubation time

was increased, contaminants often overwhelmed the developing *R. secalis* colonies. Seed contaminants were mainly bacteria and different kinds of fungi such as *Alternaria alternata, Fusarium* sp., *Cladosporium* sp., *Sporobolomyces* sp., *Penicillium* sp., *Ulocladium* sp., and sterile mycelium of an unidentified fungus. Therefore, either no incubation or an incubation period of 12 hour would be suitable for the described protocol for isolating *R. secalis* from seed. These incubation times promoted less growth of contaminants from lemmas and paleas that were used to isolate *R. secalis*. The results showed that enrichment of the scald fungus is not required for isolation of the pathogen from barley seed.

It is not quite clear why isolation of *R. secalis* from barley seed has so far not been possible. Kay and Owen (1973) attributed the lack of successful isolation of *R. secalis* to the slow growing nature of the fungus and inhibition by contaminants. In terms of scald colony formation, there was a greater difference between individual seed samples than incubation time. Therefore, this method could be useful for examining the distribution and viability of the seedborne inoculum of *R. secalis*. In our investigation, presence of *R. secalis* could be consistently confirmed after 7 to 10 days incubation on lima bean agar plates (Fig. 3-1). This appears to be the first report on successful isolation of *R. secalis* from infected barley seed.

3. 4 Tables and Figures

Table 3-1. Number of seeds of cvs. Harrington and Manley that gave rise to scald colonies after different incubation periods.

Experimental set[†]	Incubation time (hr)[‡] Number of seeds which gave rise to *Rhynchosporium secalis* colonies[*]				
	0	12	24	36	48
Set 1	3	3	2	2	3
Set 2	1	2	0	0	2
Set 3	2	2	0	2	1
Set 4	1	1	0	1	1
Set 5	2	0	1	0	1
Set 6	2	2	2	1	1

[†]Sets 1 - 3, cv. Harrington; Sets 4 - 6, cv. Manley.

[‡]Incubation time (hr) in sterile distilled water amended with streptomycin before streaking on lima bean agar plate.

[*]Lemmas and paleas derived from each of three seeds per treatment were used to assess seed infection with *R. secalis*.

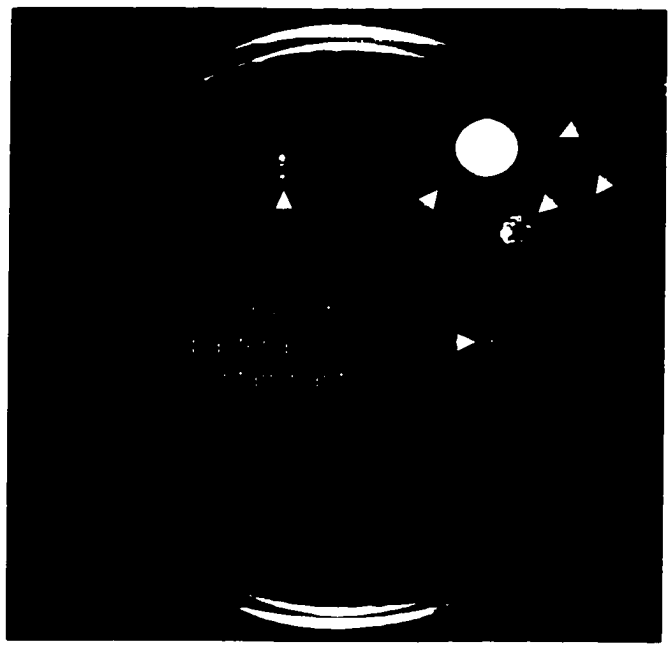

Figure 3-1. Lima bean agar plate incubated for 24 hrs at 16 °C showing colonies of *Rhynchosporium secalis* isolated from barley seed.

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Chapter 4

Development of a rapid and sensitive PCR-based diagnostic method for *Rhynchosporium secalis* in infected barley seed[1]

4. 1 Introduction

Visual inspection of external symptoms on seeds has been the traditional method employed for diagnosing seedborne infection of *Rhynchosporium secalis*. However, this method of assessing seedborne inoculum has not been sensitive enough to be of practical value. A recently developed cultural method based on the actual isolation of the scald pathogen from seed offers another diagnostic choice (Lee *et al.*, 1999). The cultural method is superior to conventional visual disease assessment as it also gives information about the viability of inoculum of the pathogen. However, this method is not practical for routine seed testing because it is laborious and time consuming. The introduction of disease into areas previously unsown with barley, or which have been substantially free from the disease, may be due to the pathogen carried with seed (Kay and Owen, 1973). Therefore, it is desirable to develop a reliable, rapid and sensitive diagnostic method for detecting seedborne infection by this pathogen.

In the last decade, molecular approaches have revolutionized the detection, identification, and quantification of phytopathogenic microbes. The polymerase chain reaction (PCR) assay is one approach that allows detection of extremely small quantities of specific DNA in complex environments. The utility of PCR as a specific and sensitive assay for identification of plant pathogens is well documented (Henson and French, 1993). The PCR is highly sensitive and reproducible for amplification of diagnostic molecular markers, and could easily be used for identification and detection if species-specific primers are available.

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[1] *A part of this chapter has been published*: Lee, H. K., Tewari, J. P., and Turkington, T. K. 2001. Plant Dis. 85: 220-225.

Although molecular methods have been widely used for detection, identification, and phylogenetic study of various phytopathogenic fungi, attempts to use molecular approaches for detecting seed infection have gained attention only recently (Doohan *et al.*, 1998; Huff *et al.*, 1994; Parry and Nicholson, 1996; Reeves, 1995; Smith *et al.*, 1996; Taylor, 1993; Yao *et al.*, 1990).

Ribosomal genes and the spacers between them provide attractive targets for detection and phylogenetic studies, because more than 50 copies may be present per genome. This suggests a high sensitivity of detection. The ribosomal genes and spacers between them possess conserved sequences as well as variable sequences, so they can be easily amplified and sequenced with universal primers based on their conserved sequences (White *et al.*, 1990). Nuclear rDNA consists of the small and large subunits, 5.8S, and the internal transcribed spacer (ITS) region in fungi and each sequence is variable at the family, genus, or species level (Fig. 4-1a). The ITS regions between the 18S and 28S rDNA subunits, which are non-transcribed regions, have considerably greater sequence variation between species. For these reasons, ITS regions have been widely used for developing species-specific probes or for designing primers for detection of pathogens of interest (Beck and Ligon, 1995; Goodwin *et al.*, 1995; Johanson and Jeger, 1993; Kageyama *et al.*, 1997; Lovic *et al.*, 1995a; Lovic *et al.*, 1995b; McLelland, 1989).

The objective of the present study was to develop a PCR-based detection method for *R. secalis* using pathogen-specific primers and evaluate its use for diagnosis of scald infection in barley seed.

4. 2 Materials and Methods

Sources of isolates and maintenance of cultures

Rhynchosporium secalis isolates from different locations in Canada and originating from different barley cultivars were chosen for this study (Table 4-1). Cultures of *R. secalis* were revived from stock cultures stored in 10% aqueous glycerol in liquid nitrogen at −196°C and maintained on lima bean agar (LBA) plates

at 16°C. All other cultures used in this study (Table 4-1) were maintained on potato dextrose agar (PDA) plates at 20°C.

DNA extraction

Total fungal genomic DNA was extracted from cultures growing on sterile cellophane discs (Biorad# 165-0963) overlaid on LBA or PDA. Fungal mycelium was scraped off the cellophane disc and transferred to 1.5-ml microfuge tubes for DNA extraction according to the method of Lecellier and Silar (1994) with some modifications. The DNA pellet was dissolved in 100 µl Tris-EDTA buffer, treated with RNase A (100 µg ml^{-1}) at 37°C for 30 min, extracted once with chloroform/isoamylalcohol (24:1) and precipitated with isopropanol. The resulting pellet was dissolved in Tris-EDTA buffer. Total bacterial genomic DNA was extracted from cultures growing on PDA plates according to the method of Li and DeBoer (1995). Total DNA from barley seed was extracted using the hexadecyl trimethylammonium bromide (CTAB)-method described by Weising *et al.* (1995). Barley seeds were surface sterilized with 70% ethanol for 30 sec and washed several times with sterilized distilled water. Seeds were ground for 15 min to a fine powder using a mixer mill grinder (Brinkmann, Rexdale, Ontario, Canada). Ground seed powder (0.1 g) was suspended in the extraction buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, containing 2% CTAB and 0.2% ß-mercaptoethanol). The suspension was incubated at 65°C for 30 min and extracted twice with chloroform/isoamylalcohol (24:1, v/v). The aqueous phase was incubated at 37°C for 1 h with RNase (final concentration of 100 µg ml^{-1}). The DNA was recovered by precipitation with an equal volume of isopropanol, washed with 70% ethanol containing 10 mM ammonium acetate, and dissolved in 100 µl Tris-EDTA buffer. DNA stocks were further diluted in sterilized distilled water and the equivalent of 0.1 mg dry weight in 3 µl was used for PCR reactions. The concentration of DNA stocks was estimated using Gene Quant, RNA/DNA calculator (Pharmacia LKB Biochrom Ltd., Cambridge, England) at 260 nm.

PCR amplification

The universal primers ITS1 and ITS4, derived from conserved sequences of 18S and 28S rDNA, were used to amplify the ITS region of *R. secalis* rDNA as described by White *et al.* (1990). PCR amplification was carried out in 25 µl of the reaction mixture containing 1X PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 250 nM of each forward and reverse primer, 0.6 units *Taq* DNA polymerase (GIBCO-BRL), and 30 ng genomic DNA. PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) with the following amplification conditions: initial denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min with the final extension at 72°C for 10 min. A 10 µl aliquot of the PCR amplified products was separated on a 1.5% agarose gel in Tris-borate-EDTA buffer at 100 V for 1 h. The gel was stained with ethidium bromide for 15 min and the PCR products were viewed using a UV transilluminator.

DNA sequencing and primer design

Single DNA fragments amplified by the PCR with the primers ITS1 and ITS4 from two isolates of *R. secalis* from cvs. Harrington (RS020) and Manley (RS006) and one isolate of *R. orthosporum* were excised from the agarose gel and purified with the Agarose Gel DNA Extraction Kit (Boehringer, Mannheim, Germany). The purified fragments were sequenced at the DNA Services Laboratory, Department of Biological Science, University of Alberta. Sequence data were compared with the aid of the computer program GenejockyII (P.L. Taylor, Cambridge, UK) and potential primers were designed using the program Amplify 1.2 (Bill Engels, Madison, WI). Nine primer sequences within the ITS regions 1 and 2 were selected based on the sequence differences found between *R. secalis* and *R. orthosporum* and an average G-C content ranging from 40% to 55% (Table 4-2). These are referred to as primers RS1, RS2, RS3, RS4, RS5, RS6, RS7, RS8, and RS9. The primers were synthesized at the DNA Services Laboratory, Department of Biological Science, University of Alberta.

Test of primer specificity

Five primer sets were tested for the amplification of the predicted-size fragments and for specificity to *R. secalis* at different annealing temperatures (55°C, 60°C, 65°C, and 70°C) using the *R. secalis* isolate RS020 and *R. orthosporum*. The same five primer sets also were used to amplify DNA from the isolates of *R. secalis* from a range of geographic origins and from different barley cultivars. DNA from isolates of contaminants isolated from barley seeds, taxonomically related species, and microbes from the same ecological niche as *R. secalis* also were used to validate primer specificity (Table 4-1).

Detection of *R. secalis* in barley seed

Primers also were tested for the detection of *R. secalis* from barley seed. One hundred seeds of the cv. Harrington with typical scald symptoms, and 100 healthy-looking seeds were sorted out and ground separately into powder as described previously. Various amounts of putatively *R. secalis*-infected seed powder were serially mixed with putatively uninfected seed powder to determine the minimum level of detection using species-specific primers. A total of 0.1 g of ground powder from each sample mixture was used for subsequent tests. The artificially infested ground powder had a 100%, 80%, 60%, 40%, 20%, 10%, 8%, 6%, 4%, 2%, 1% and 0% level of infestation with the *R. secalis*-infected seed powder. Control amplifications of DNA from seeds of the *R. secalis*-resistant cultivar Seebe, and PCR reaction mixture lacking DNA were included as negative controls. Five replicates were used for each level of artificial *R. secalis* infestation in the ground seed powder and PCR amplification. Forty PCR cycles were used to amplify DNA from seed samples.

4. 3 Results

Sequence of the ITS region of *R. secalis*

Amplification of *R. secalis* and *R. orthosporum* DNA with ITS1 and ITS4 universal primers resulted in 627-bp and 625-bp single fragments, respectively. The sequences of ITS regions containing 5.8S rDNA, ITS1, and ITS2 in the two isolates of *R. secalis* from different cultivars were identical. Sequence alignment of *R. secalis* with *R. orthosporum* revealed overall 93% homology, 86% homology in the ITS1, and 95% homology in the ITS2 regions. However, the sequences of the 3' end of the 18S and 5.8S genes, and the 5' end of the 28S gene were identical between *R. secalis* and *R. orthosporum* (Fig. 4-1b).

Primer selection and specificity to *R. secalis*

Based on sequence differences found within the ITS regions of two species, five sets of primers (A, B, C, D and E) were selected and tested for their specificity in detecting *R. secalis* (Table 4-2). All primer sets amplified the expected size of single fragments from all isolates of *R. secalis* tested when the annealing temperature was 55°C. However, except for primer set E (RS8 and RS9), sets A, B, C, and D amplified a similarly sized single fragment from the DNA extracts of *R. orthosporum,* showing different intensities. These fragments could be eliminated by increasing the annealing temperature to 65°C and 70°C for the primer sets A and B, and the primer sets C and D, respectively. The primer set E amplified a 264-bp fragment from all DNA extracts of *R. secalis* isolates, but not from those of other species considered taxonomically and ecologically most closely related to *R. secalis*. Therefore primer set E was used for further experiments to detect *R. secalis* in barley seeds.

Sensitivity of PCR detection assay

Amplification reactions were conducted with decreasing amounts of the genomic DNA of *R. secalis* to determine the minimum amount of input DNA required to produce a detectable product using the primer sets A and E. These primer pairs

were able to direct the amplification of the target DNA sequence from as little as 1 pg DNA for set A and 10 pg DNA for set E (Fig. 4-2).

PCR detection of *R. secalis*-DNA in extracts from barley seeds

The primer set E was used to detect *R. secalis*-DNA in extracts of seed of cv. Harrington (Fig. 4-3). PCR products from initial DNA extracts were not detected by electrophoresis. However, when the DNA extracts were diluted from 10- to 100-fold with sterilized distilled water, distinct bands for the pathogen-DNA amplification products were observed. DNA extracts were diluted 30-fold with sterilized distilled water in subsequent experiments. The results of five replicates using samples of ground *R. secalis*-infected seed powder that were serially mixed with ground uninfected seed powder are summarized in Table 4-3. In the five independent experiments, the detection levels at 0, 1, and 2% of infestation with the infected seed powder were variable. The negative controls (DNA extract from seeds of the resistant cultivar, Seebe, and the reaction mixture without DNA) did not reveal any amplification while the positive control (*R. secalis* DNA) showed strong positive PCR product amplification (Fig. 4-3).

4. 4 Discussion

In this study, a PCR-based diagnostic assay was developed to detect *R. secalis* in barley seed using pathogen-specific primers derived from the ITS region of rDNA of *R. secalis*. Nuclear ribosomal DNA sequences have been an attractive source for designing species-specific primers for detection or differentiation of the pathogen of interest, since they are generally highly conserved within species, yet variable among species (Beck and Ligon, 1995; Goodwin *et al.*, 1995; Johason and Jeger, 1993; Kageyama *et al.*, 1997; Lovic *et al.*, 1995a; Lovic *et al.*, 1995b; McLelland, 1989; Smith *et al.*, 1996; Willitis and Sherwood, 1999). This study revealed that DNA sequences of the two isolates of *R. secalis* from cvs. Harrington and Manley were identical over a 627-bp stretch from the 3' end of the 18S rRNA gene to the 5' end of the 28S rRNA gene. The complete homology of the ITS regions of the two isolates is

consistent with the view that the ITS region is conserved within the species (White *et al.*, 1990). However, there was 93% homology in the ITS regions between *R. secalis* and *R. orthosporum*. Besides *R. secalis*, *R. orthosporum* and *R. alismatis* are the only two other known species belonging to the genus *Rhynchosporium* (Caldwell, 1937; Cother and Gilbert, 1994; Ferrandez and Welty, 1991). Whereas, *R. alismatis* is known to attack some weed species of the family Alismataceae, *R. orthosporum*, the causal agent of the leaf scald disease of orchardgrass and some other grasses, has a broad host range including some common grass species that are also known to be attacked by *R. secalis* (Caldwell, 1937; Cother and Gilbert, 1991; Ferrandez and Welty, 1994). The pathogen, *R. orthosporum*, produces symptoms identical to those of *R. secalis* and has similar cultural characteristics, except for the production of uniformly cylindrical conidia (Caldwell, 1937; Ferrandez and Welty, 1994). Therefore, in this study, *R. orthosporum* was used to compare its ITS sequence with that of *R. secalis*. Sequence divergence found in the ITS1 (95% identity) and ITS2 (86% identity) regions between the two species suggested the potential value of the ITS sequence as a molecular marker for *R. secalis*. Five sets of primers, constructed from the divergent sequences within the ITS1 and ITS2 regions of *R. secalis*, produced fragments of expected size from the DNA extract of *R. secalis*. However, the primer sets A, B, C, and D, but not E, failed to differentiate *R. secalis* from *R. orthosporum* at 55°C, the initial annealing temperature. The reduced sensitivity of these primers could be explained by the fact that the primer sets A, B, C, and D were derived from the less divergent sequences of the ITS region, that included one or several sequence differences between the two species. Increasing the annealing temperature for those primer sets allowed differentiation between *R. secalis* and *R. orthosporum*. However, since *R. orthosporum* is not known to attack barley, those primer sets showed also be useful to detect *R. secalis* in barley seed without concerns of cross reactivity with *R. orthosporum*. Although all primer sets appeared to be species-specific after optimization of the PCR cycle, primer set E was selected for this study due to its high specificity. All primer sets produced detectable fragments from as little as 1 to 10 pg *R. secalis* DNA.

Scald symptoms are distributed over the lemmas, paleas, awns, glumes, and rachises of barley heads (Habgood, 1971; Kay and Owen, 1973; Skoropad, 1959). Lemma and palea lesions have been the basis for visual scald disease assessment (Kay and Owen, 1973). Therefore, in sampling for PCR-based detection, seeds bearing no symptoms on the lemma and palea and seeds with visible symptoms were separately sorted and tested. PCR amplification of fungal DNA in a crude DNA extract from final 0.1 g of seed samples allowed for the detection of presumably a small biomass of *R. secalis*. Effects of inhibitors of the PCR reaction commonly found in the DNA extracts from plant materials were overcome by diluting the *R. secalis* DNA from 10- to 100-fold with sterilized distilled water. The PCR amplification revealed the presence of *R. secalis* in the series of *R. secalis*-infested powdered seed samples. Even though a difference in the band intensity for each level of *R. secalis*-infested samples was found, sample to sample variation was observed in different replications of samples, particularly those prepared for the lower levels of *R. secalis* infestation (<2%). The variability in PCR results for the five independent replications may have been due to variations in inoculum loads in the samples. In replications 1 and 4, the DNA extracts from seed powder that had 0% infestation with *R. secalis* produced a faint band, while the negative control samples without any DNA did not produce any bands. This indicated that the results were not a false positive by PCR contamination and the fact that *R. secalis* was detected in the asymptomatic sample may be due to the high sensitivity of the PCR assay. Symptomless infection of barley seeds by *R. secalis* was reported by Kay and Owen (1973) and Lee *et al.* (2001b).

Although, there has been much interest in the use of PCR assays for detection and characterization of phytopathogenic microbes in general, PCR for the detection of seedborne pathogens has received little attention (Doohan *et al*, 1998; Smith *et al.*, 1996; Taylor, 1993; Yao *et al.*, 1990). In this study, attempt to construct *R. secalis*-specific primers to detect the pathogen from barley seed was successful. In comparison to those PCR-based assays used for detection of other seedborne pathogens, which require long incubation periods for completion of the assay (Smith *et al.*, 1996; Taylor, 1993), the methodology described here only takes about one working day from DNA extraction to PCR detection and does not require a pathogen

enrichment period. In addition, the method described is more sensitive and less laborious compared to the cultural method, which requires up to 10 days of incubation (Lee *et al.*, 1999). The PCR-based diagnostic assay described here for testing barley seed health provides several benefits over conventional methods for *R. secalis*. Furthermore, since the assay is based on the presence or absence of a diagnostic molecular marker, it should be useful in epidemiological studies of the pathogen.

4. 5 Tables and Figures

Table 4-1. Isolates and results of the PCR assays using RS8 and RS9 primers.

Species	Isolates	Host	Origin	PCR product
Rhynchosporium secalis	RL 001	Falcon [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 006	CDC Guardian[a]	Edmonton, AB	+
Rhynchosporium secalis	RL 009	Abee [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 013	Harrington [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 018	Tukwa [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 024	Duke [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 027	AC Stacey [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 030	Leduc [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 038	AC Lacombe [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 042	Jackson [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 044	Manley [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 048	Galt [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 053	Klages [a]	Edmonton, AB	+
Rhynchosporium secalis	RL 057	AWP1602[a]	Edmonton, AB	+
Rhynchosporium secalis	RL 059	B1215[a]	Edmonton, AB	+
Rhynchosporium secalis	RL 083	Tukwa [a]	Bowden, AB	+
Rhynchosporium secalis	RL 087	Harrington [a]	Bowden, AB	+
Rhynchosporium secalis	RL 092	Unknown [a]	Red Deer, AB	+
Rhynchosporium secalis	RL 099 (1824)[b]	Unknown [a]	Pilot Mound, MN	+
Rhynchosporium secalis	RL 100 (1395) [b]	Winter barley [a]	Guelph, ON	+
Rhynchosporium secalis	RL 101 (1826) [b]	Leduc [a]	Sylvan Lake, AB	+
Rhynchosporium secalis	RL 102 (1493) [b]	Unknown [a]	Tisdale, SK	+
Rhynchosporium secalis	RL 105 (1389) [b]	Winter barley [a]	Arnell Station, ON	+
Rhynchosporium secalis	RL 106 (1874) [b]	Unknown [a]	Medstead, SK	+
Rhynchosporium secalis	RL 107	Brome grass	Michener Park, AB	+
Rhynchosporium secalis	RS 006	Manley [a]	Westlock Gro site, AB	+
Rhynchosporium secalis	RS 014	Stander [a]	Westlock Gro site, AB	+

Rhynchosporium secalis	RS 015	CDC Earl [a]	Westlock Gro site, AB	+
Rhynchosporium secalis	RS 020	Harrington [a]	Westlock Gro site, AB	+
Acremonium sp.	Co#1	Harrington [a]	Westlock Gro site, AB	-
Microdochium sp.	Co#2	Harrington [a]	Westlock Gro site, AB	-
Penicillium chrysogenum	Co#3	Harrington [a]	Westlock Gro site, AB	-
Cladosporium sp.	Co#4	Harrington [a]	Westlock Gro site, AB	-
Alternaria alternata	Co#5	Harrington [a]	Westlock Gro site, AB	-
Mycelia sterilia	Co#6	Harrington [a]	Westlock Gro site, AB	-
Ulocladium sp.	Co#7	Harrington [a]	Westlock Gro site, AB	-
Pyrenophora graminea	720(on) [b]	Unknown [a]	Unknown	-
Cochliobolus sativus	718 (on) [b]	Unknown [a]	Unknown	-
Pyrenophora teres	858 WRS [b]	Unknown [a]	Unknown	-
Stagonospora nodorum	1899 WRS [b]	Unknown [a]	Unknown	-
Fusarium graminearum (Gibberella zeae)	G-1 [b]	Unknown [a]	Unknown	-
Colletotrichum graminicola	1725 WRS [b]	Unknown [a]	Unknown	-
Sporobolomyces sp.	Co#8	Harrington [a]	Westlock Gro site, AB	-
Gerlachia oryzae	ATCC 32154	Rice	Allen Parish farm, Louisiana	-
Rhynchosporium orthosporum	CBS 698.79	Orchardgrass	Unknown	-
Ustilago nuda		Harrington [a]	Westlock Gro site, AB	-
Pseudomonas syringae pv. *atrofaciens*	#3894 [b]	Unknown [a]	Unknown	-
Xanthomonas campestris pv. translucens	#4790 [b]	Unknown [a]	Unknown	-
Unidentified bacterial isolate	Co#9	Harrington [a]	Westlock Gro site, AB	-
Unidentified bacterial isolate	Co#10	Harrington [a]	Westlock Gro site, AB	-
Unidentified bacterial isolate	Co#11	Harrington [a]	Westlock Gro site, AB	-

[a] Host barley cultivars.

[b] Isolates provided by Dr. Andy Tekauz, Agriculture and Agri-Food Canada, Winnipeg, Manitoba.

ATCC: American Type Culture Collection, Rockville, MD, U.S.A.

CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Co: Contaminants isolated from barley seeds. Some of these were identified with the help of Identification Service, CABI Bioscience, Surray, U. K.

+ Presence of 264-bp DNA fragment after PCR amplification.

- Absence of 264-bp DNA fragment after PCR amplification.

RL: Isolated from leaf lesions.

RS: Isolated from infected seed.

Table 4-2. Sequences of primer sets derived from the ITS region of *Rhynchosporium secalis*.

Set	Primer designation	Sequence (Match at)	Expected fragment size (bp)
A	RS1	AAGAAGCCTGGTTCAGACCTCC (115)	371
	RS3	ACCGCCACTGATTTTAGGGG (486)	
B	RS2	AAACTACCTCTGTTGCTTTGGCAGG(147)	339
	RS3	ACCGCCACTGATTTTAGGGG (486)	
C	RS4	ATAGAGCAATGAACAGTCGG (31)	456
	RS5	CACCGCCACTGATTTTAGGG (487)	
D	RS6	CTCCACCCTTGAATAAACTACC(133)	419
	RS7	GTTGTTGGCAAGTAGACCAGCC (552)	
E	RS8	TTGTTTTTAGTGATGTCTGAG (226)	264
	RS9	AGGCACCGCCACTGATTTTAGGG (490)	

Table 4-3. Results of detection of *Rhynchosporium secalis* in preparations of infected and uninfected barley seed (cv. Harrington) using PCR.

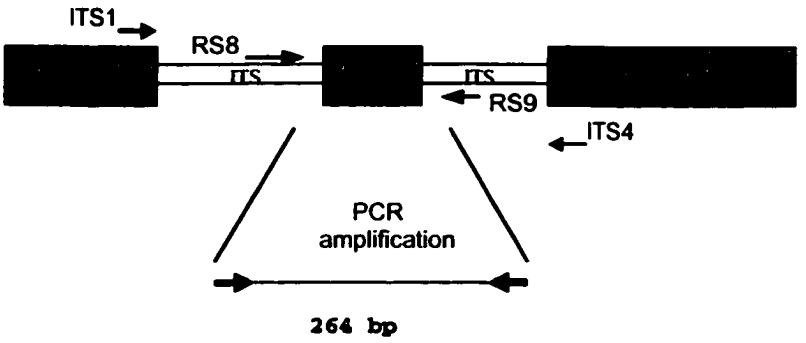
% infected seed powder	Detection of *R. secalis* by PCR amplification					% detection level by PCR
	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	
100%	+	+	+	+	+	100%
80%	+	+	+	+	+	100%
60%	+	+	+	+	+	100%
40%	+	+	+	+	+	100%
20%	+	+	+	+	+	100%
10%	+	+	+	+	+	100%
8%	+	+	+	+	+	100%
6%	+	+	+	+	+	100%
4%	+	+	+	+	+	100%
2%	+	-	+	+	+	80%
1%	+	-	-	+	-	40%
0%	+	-	-	+	-	40%

+ : Presence of *R. secalis*-specific band after PCR amplification.

- : Absence of *R. secalis*-specific band after PCR amplification.

Figure 4-1. Development of a species-specific primer set from the internal transcribed spacers (ITS) region of *Rhynchosporium secalis*. (a) Schematic diagram of the ITS region. (b) Sequence comparison of the ITS region and 5.8s rRNA gene from *R. secalis* (RS20) and *R. orthosporum*. The sequences typed in bold letters indicate the different nucleotide sequences found in the ITS regions of the two species.

a.



b.

18s rRNA

```
1 TCCGTAGGTGAACCTGCGGAAGGATCATTA ATAGAGCAAT GAACAGTCGG R.secalis
1 TCCGTAGGTGAACCTGCGGAAGGATCATTA ATAGAGCAAT GAACAGTCAG R.orthosporum
```

ITS 1 →

```
51 CGCCCCAGGA GAAATCCTGG GGGCTACCCT ACTTCGGTGG GGTTTAGAGA R.secalis
51 CGCCCCAGGA GCGATCCTGG GGGCTACCCT -CTTCGG-AG GGTTTAGAGA R.orthosporum

101 CGTCAGGCCG CTCGAAGAAG CCTGGTTCAG ACCTCCACCC TTGAATAAAC R.secalis
 99 CGTCGGGCCC TTCGGGGATA CTCGGTTCAG ACCTCCACCC TTGAATACAT R.orthosporum

151 TACCTCTGTT GCTTTGGCAG GCCGCCTAGC GCCAGCGGCT TCGGCTGCTG R.secalis
149 TACCTTTGTT GCTTTGGCAG GACGCCTCGT GCCAGCGGCT TCGGCTGTTG R.orthosporum
```

RS 8 →

```
201 AGTGCCTGCC AGAGGACCAC AACTCTTGTT TTTAGTGATG TCTGAGTACT R.secalis
199 AGTGCCTGCC AGAGGACCAC AACTCGTGAA ACATATGAAG TCTGAGTACT R.orthosporum

251 ATATAATAGT TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT R.secalis
249 ATATAATAGT TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT R.orthosporum

301 GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA R.secalis
299 GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA R.orthosporum
```

5.8s rRNA

```
351 ATCATCGAAT CTTTGAACGC ACATTGCGCC CTCTGGTATT CCGGGGGGCA R.secalis
349 ATCATCGAAT CTTTGAACGC ACATTGCGCC CTCTGGTATT CCGGGGGGCA R.orthosporum

401 TGCCTGTTCG AGCGTCATTA TAACCACTCA AGCTCTCGCT TGGTATTGGG R.secalis
399 TGCCTGTTCG AGCGTCATTA TAACCACTCA AGCTCTCGCT TGGTCTTGGG R.orthosporum
```

← RS 9

```
451 GTTCGCGTCC TCGCGGCCCC TAAAATCAGT GGCGGTGCCT GTCGGCTCTA R.secalis
449 GTTCGCGTCC TCGCGGCTCC TAAAATCAGT GGCGGTGCCG GTCGGCTCTA R.orthosporum

501 CGCGTAGTAA TACTCCTCGC GATTGAGTCC GGCTGGTCTA CTTGCCAACA R.secalis
499 CGCGTAGTAA TACTCCTCGC GATTGAGTCC GGTCGGTCTA CTTGCCAACA R.orthosporum
```

28s rRNA

```
551 ACCCCCAAAT TTTTACAGGT TGACCTCGGA TCAGGTAGGG ATACCCGCTG R.secalis
549 ACCCCCCATT TTTTACAGGT TGACCTCGGA TCAGGTAGGG ATACCCGCTG R.orthosporum

601 AACTTAAGCA TATCAATAAG CGGAGGA R.secalis
599 AACTTAAGCA TATCAATAAG CGGAGGA R.orthosporum
```

← ITS 4

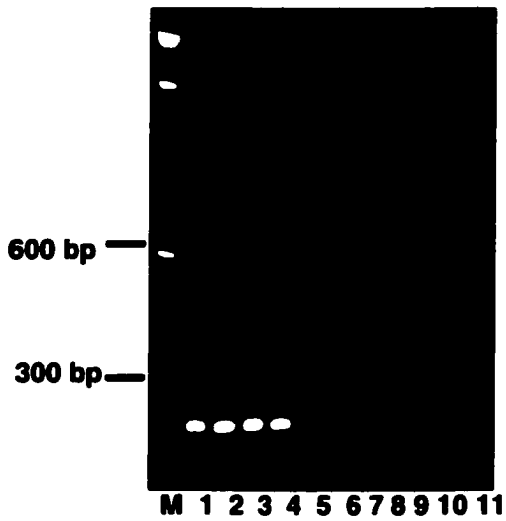


Figure 4-2. Determination of the minimum amount of input DNA of *Rhynchosporium secalis* using primer set E. M; 100-bp DNA marker, 1, 30 ng/μ; 2, 10 ng/μ; 3, 1 ng/μ; 4, 100 pg/μ; 5, 10 pg/μ; 6, 1 pg/μ; 7, 100 fg/μ; 8, 10 fg/μ; 9, 1 fg/μ; 10, 0; 11, *R. orthosporum* (30 ng/μ).

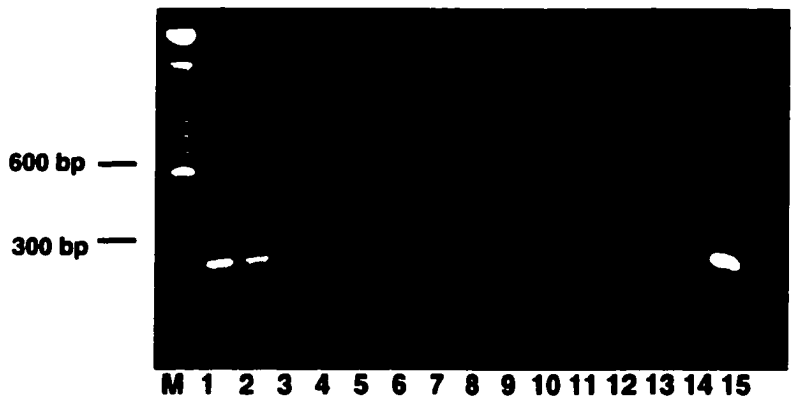


Figure 4-3. Amplification of *Rhynchsporium secalis* DNA at different levels of scald infection of barley seeds. M; 100-bp DNA marker, 1; 100% , 2; 80%, 3; 60%, 4; 40%, 5; 20%, 6; 10%, 7; 8%, 8; 6%, 9; 4%, 10; 2%, 11; 1%, 12; 0%, 13; DNA extract from resistant barley cultivar Seebe, 14; negative control (PCR reaction mixture lacking in DNA), 15; positive control (*R. secalis* DNA)

4. 6 References

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Chapter 5

Detection of symptomless infection on seed

5. 1 Introduction

The incidence of seedling infection of *Rhynchosporium secalis* is dependent upon spread of inoculum from residues, as well as from visible and latent (symptomless) seedborne inoculum (Jackson and Webster, 1976). In Canada, Skoropad (1959) surveyed infected crops and found an average of 2% seed infection and a subsequent seedling transmission rate of up to 85% from infected seed. He also found that infection of seed occurring during the late-dough stage of barley did not always progress sufficiently to express visible scald symptoms on the outer surfaces of the lemma and palea. These observations were based on artificial inoculation of the emerged spikes with *R. secalis*. In England, Habgood (1971) observed that 2% of the seeds from a heavily infected crop produced infected seedlings and that some seeds without scald lesions also gave rise to infected seedlings. Kay and Owen (1973) noticed some paleas from symptomless seeds to be colonized with *R. secalis*. They also used fragments of lemmas from seeds without symptoms to inoculate on barley leaves and observed the development of lesions. These observations indicated the presence of symptomless infection by *R. secalis* in barley seed.

Until recently, seed infection by *R. secalis* and its level have been evaluated based on visual symptoms on lemmas and paleas, characterized by irregular or elliptical lesions with dark brown margins and pale centers, although a PCR-based diagnostic method was described recently (Lee *et al.*, 2001a). However, visible seed symptoms may not necessarily account for total seed-infection by *R. secalis* (Skoropad, 1959; Kay and Owen, 1973).

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[1] *A part of this chapter has been published*: Lee, H. K., Tewari, J. P., and Turkington, T. K. 2001. Can. J. Plant Pathol. 23: 315-317.

The present study was conducted to investigate the presence of symptomless infection of barley seeds using cultural and PCR-based diagnostic methods.

5. 2 Materials and Methods

Seed samples of the susceptible two-row malting barley cv. Harrington, collected from an Alberta Agriculture, Food and Rural Development regional variety trial site at Calmar, Alberta in 1996, were used for this study. For the cultural assay, 6 sets of 12 seeds each were chosen based on visual inspection for the absence of scald infection. The seeds were washed several times with sterilized distilled water and plated according to the method of Lee *et al.* (1999). Lemmas and paleas from each seed without lesions were removed and incubated in a tube at 16°C for 12 h in 50 µl sterilized distilled water amended with 100 mg L^{-1} of streptomycin. The liquid from each tube was then streaked on three plates of lima bean agar supplemented with 200 mg L^{-1} streptomycin and 50 mg L^{-1} rose bengal. The resulting plates were examined for scald colony development after 10 days incubation at 16°C.

For the present study, a PCR-based assay (Lee *et al.*, 2001a; see Chapter 4) was used to monitor symptomless infection of barley seed. For DNA extraction, five sets of 100 seeds each of the *R. secalis*-susceptible cv. Harrington and two sets of 100 seeds each of the resistant cv. Seebe were used. All seeds appeared healthy-looking and lacked any visible symptoms of *R. secalis* infection. Seeds were surface sterilized with 70% ethanol for 30 s and washed several times with sterilized distilled water and ground to a fine powder using a mixer mill grinder (Brinkmann, Retsch, Ontario, Canada) for 15 min. Three subsamples of 0.1g of seed powder were prepared from each set and used for PCR amplification. Total DNA from barley seed was extracted using the hexadecyl trimethylammonium bromide (CTAB) method described by Weising *et al.* (1995). DNA extracts were diluted in sterilized distilled water and the equivalent of 0.1 mg dry weight in 3 µl was used for PCR reaction. The concentration of DNA stocks was estimated using the Gene Quant, RNA/DNA calculator (Pharmacia LKB Biochrom Ltd., Cambridge, England) at 260 nm. PCR amplification

was carried out in 25 µl of the reaction mixture containing 1X PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 250 nM of each forward and reverse primers, and 0.6 units of Taq DNA polymerase (GIBCO-BRL). A set of primers designed from the ITS regions of the pathogen, i.e. RS1 (5'-AAGAAGCCTGGTTCAGACCTCC-3') and RS3 (5'-ACCGCCACTGATTTTAGGGG-3'), were used to amplify the *R. secalis* DNA (Lee et al., 2001a). PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) with the following amplification conditions: initial denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. Control amplifications of DNA from seeds of the *R. secalis*-resistant cultivar Seebe, and PCR reaction mixture lacking DNA were included as negative controls. Total genomic-*R. secalis* DNA (Lee *et al.*, 2001a) was also used to monitor PCR efficiency as a positive control. A 8 µl aliquot of the PCR amplified product was separated on a 1.5% agarose gel in Tris-borate-EDTA buffer at 100 V for 1 hr. The gel was stained with ethidium bromide for 15 min and the PCR products were viewed using a UV transilluminator. The experiment was repeated once with similar results.

5. 3 Results and Discussion

In the cultural assay, in six independent sets of 12 seeds each, an average of 14% of the seeds without symptoms gave rise to scald colonies. In 6 sets, the percentage of seeds producing colonies of *R. secalis* were 25, 17, 0, 17, 8 and 17. This incidence of symptomless infection of seeds was somewhat higher than found in earlier report, which ranged from 6% to 10% (Kay and Owen, 1973). The use of the susceptible cv. Harrington, which had high seed infection rates may account for this result. When symptomless seed samples taken from the cv. Harrington were used for PCR amplification, a single *R. secalis*-diagnostic band was produced in all the subsamples tested. Results from the 15 subsamples are shown in Fig. 5-1. In the control experiment, no PCR products were amplified in DNA extracts from the cv. Seebe, whereas DNA extracts of subsamples taken from symptomless seeds of the cv.

Harrington all gave rise to scald-diagnostic bands (Fig. 5-2). The PCR-based assay confirmed the results of the cultural assay, namely that appreciable levels of symptomless infection were present in the seed lot. In earlier studies, transmission of the pathogen from infected seeds to seedlings appeared to vary depending on the conditions under which the seedlings emerged in either the greenhouse or the field (Skoropad, 1959; Habgood, 1971; Kay and Owen, 1973; Jackson and Webster, 1976). However, even when transmission rates are low, there can be sufficient infected seeds in commercial seed lots for an epidemic to begin under favorable weather conditions. The results of the present study suggest that conventional visual assessment of barley seeds underestimate the actual level of *R. secalis* infection of seeds. Improved diagnostic methods are warranted, since symptomless infection of seed may act as a source of the pathogen. Results reported here and elsewhere (Lee *et al.*, 1999, 2001a) indicate that both the cultural and PCR-based diagnostic assays would be useful for detecting barley scald infection in seeds with and without the presence of symptoms. However, unlike the cultural assay, the PCR assay reported here is only qualitative.

5. 4 Tables and Figures

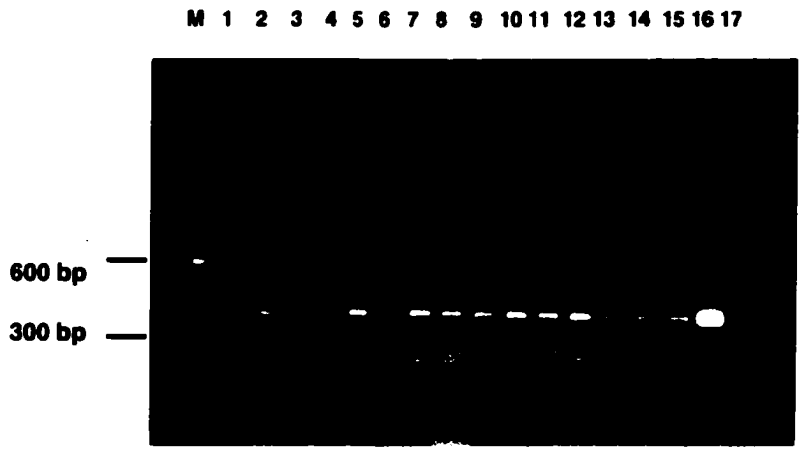

Figure 5-1. Amplification of *Rhynchosporium secalis* DNA from symptomless barley seed. M, 100 bp DNA marker; Lanes 1-15, DNA extracts of subsamples of 5 sets of the cv. Harrington; Lane 16, Positive control using *R. secalis*-DNA; Lane 17, Negative control lacking DNA.

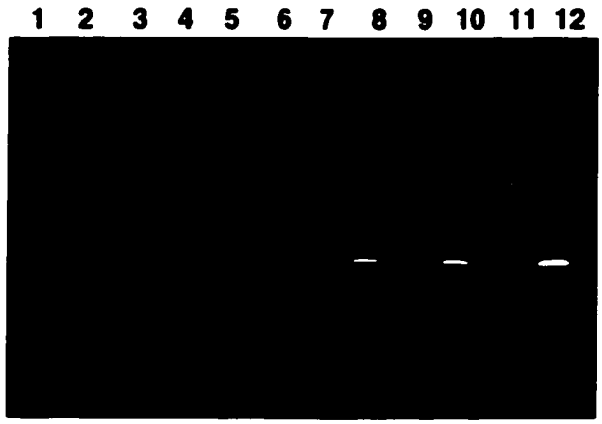

Figure 5-2. PCR amplification of seeds of the cvs. Harrington and Seebe using *Rhynchosporium secalis*-specific primers. Lanes 1-6, DNA extracts from subsamples of 2 sets of the cv. Seebe; Lanes 7-11, DNA extracts from subsamples of 5 sets of the cv. Harrington; 12, Positive control using *R. secalis*-DNA.

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Chapter 6

Development of a quantitative assay using a competitive PCR and its potential use in seed health testing

6. 1 Introduction

In the previous studies, a cultural assay based on isolation of the pathogen and a PCR-based assay using *Rhynchosporium secalis*-specific primers appeared to be useful to monitor seed infection by *R. secalis* (Lee *et al.*, 1999; Lee *et al.*, 2001a). The cultural method is superior to conventional visual disease assessment as it monitors symptomless infection and also gives information about the viability of inoculum of the pathogen (Lee *et al.*, 1999). However, due to the slow growing nature of the pathogen and laborious procedures involved, this method is not practical for routine seed health testing. Unlike the cultural method, the PCR-based detection method allowed for rapid and sensitive diagnosis of the pathogen present in barley seed (Lee *et al.*, 2001a).

In the last decade, molecular approaches, including PCR, have been widely used for the detection and identification of plant pathogens. However, attempts to use molecular approaches for detecting seed infection have received less attention (Doohan *et al.*, 1998; Doohan *et al.*, 1999; Nicholson *et al.* 1998; Parry and Nicholson, 1996; Smith *et al.*, 1996; Taylor, 1993; Reeves, 1995). In the majority of these cases, due to the exponential nature of amplification, PCR was used for specific detection of the pathogen in plant material, but not for its quantification. However, competitive PCR assays have been used for PCR-based quantification in some pathosystems (Doohan *et al.*, 1999; Hu *et al.*, 1993; Hu *et al.*, 1995; Nicholson *et al.*, 1998).

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[1] *A part of this chapter was accepted for publication* on November 2, 2001: Lee, H. K., Tewari, J. P., and Turkington, T. K. 2002. Plant Pathol.

The Competitive PCR assay uses an internal control that competes for the same primer set and subsequently amplifies at the same rate with target DNA during the reaction. Therefore, the ratio of PCR products between the target DNA and the internal control DNA allows for the quantification of the initial concentration of the pathogen DNA. Among the advantages of competitive PCR is that any variable that may affect amplification has the same effect on both target and internal control DNA, and that the final ratio of amplified products reflects exactly the initial ratio of targets, rendering the reaction independent of the number of amplification cycles (Celi *et al.*, 1993; Diviacco *et al.*, 1992). So far there have been only a few reports of the use of competitive PCR for quantification of seedborne inocula (Doohan *et al.*, 1999; Nicholson *et al.*, 1998). More recently, real-time quantitative PCR, obviating the need for post-PCR processing, has been also successfully applied to quantification of seedborne inoculum (Zhang *et al.*, 1999)

In an earlier study, a series of *R. secalis*-specific primer sets were designed from the internal transcribed spacer (ITS) regions of ribosomal RNA gene of the pathogen and used for detection of the pathogen in barley seed (Lee *et al.*, 2001a). Even though all primer sets appeared to detect the pathogen in barley seed with the conventional PCR assay, it was impossible to quantify the levels of seed infection as a result of the intrinsic unsuitability of this technique for quantification studies. The objectives of this work were to adapt the qualitative PCR assay for quantitative analysis by introducing an internal control, and examine the potential of the assay developed for detection and quantification of *R. secalis* infection in barley seed.

6. 2 Materials and Methods

Fungal isolates

Two single-spore isolates of *R. secalis* (RS006 and RS020 from Dr. J. P. Tewari's fungal culture collection at the University of Alberta) used in this study were originally obtained from seeds of the barley cvs. Manley and Harrington, collected during 1996 from Westlock, Alberta. Isolates were stored in liquid nitrogen at -196°C axenically as conidial suspensions in 10% aqueous glycerol. To revive the isolates,

thawed conidial suspension was pipetted directly onto sterile cellophane discs (Biorad) overlaid on lima bean agar (LBA) plates and the cultures were grown in a 16°C incubator for 2 weeks prior to being used for DNA extraction.

Plant materials and cultural assay

Seeds of the scald-susceptible cv. Harrington, grown in trials at Calmar, AB from 1995 to 1999 were used for this study. In order to compare conventional visual disease assessment and the cultural assay with competitive PCR, each sample used for the latter assay was first assessed for infection based on external visual symptoms. Since the cultural method developed for detecting *R. secalis* in barley seed involves the disruption of individual seeds (Lee *et al.*, 1999), seed taken from the same batch was used separately for the cultural assay. For the cultural assay, handful amounts of seeds were taken from the seed batch from each year and 50 seeds were separated and examined for symptoms of scald symptoms. Scald symptoms on seeds were recognized as round to elliptical lesions with pale centers surrounded by a darker zone on the lemmas and paleas, generally towards the distal end of the seed. Individual seeds were then plated on the LBA plates supplemented with 200 mg L^{-1} of streptomycin and 50 mg L^{-1} of rose bengal according to the method of Lee *et al.* (1999). Three plates were prepared for scald colony development from each seed and examined after 10 days of incubation at 16 °C.

DNA extraction

Total fungal genomic DNA was extracted from cultures (RS006 and RS020) growing on sterile cellophane discs overlaid on LBA plates as described in Chapter 4. Fungal mycelium was scraped off the cellophane disc and transferred to 1.5 mL microfuge tubes for DNA extraction according to the method of Lecellier & Silar (1994) with some modifications as described by Lee *et al.* (2001a).

For DNA preparation from seed, 100 barley seeds were surface sterilized with 70% ethanol for 30 s and washed several times with sterile distilled water. Seeds were subsequently dried at room temperature and ground to a fine powder using a mixer mill grinder (Brinkmann, Rexdale, Ontario, Canada) and a 0.1 g subsample of seed

powder was made for DNA extraction. Total DNA from barley seed was extracted using the hexadecyl trimethylammonium bromide (CTAB) method described by Weising *et al.* (1995). For the PCR reaction, the DNA equivalent of 0.1 mg dry seed weight in 1 µL of sterilized distilled water was used.

PCR amplification

PCR amplification was carried out in a 25 µL reaction mixture containing 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 250 nM of each forward and reverse primer, and 0.6 units of Taq DNA polymerase (GIBCO-BRL). In an earlier study, a series of primers were designed to detect the pathogen from *R. secalis*-infected barley seed (Lee *et al.*, 2001a). All primer sets previously designed from ITS regions of rDNA of the pathogen showed high specificity for detecting the pathogen in seed. In a previous study on the qualitative detection of the pathogen (Lee *et al.*, 2001a), primers RS8 and RS9 were selected since they did not show cross reactivity with *R. orthosporum*. However, for this study, RS1 (5'-AAGAAGCCTGGTTCAGACCTCC-3') and RS3 (5'-ACCGCCACTGA TTTTAGGGG-3') were used to amplify *R. secalis* DNA from barley seed. Although, primers RS1 and RS3 showed cross reactivity with *R. orthosporum* at low annealing temperature, i.e. 55°C, in preliminary experiments, they showed high sensitivity allowing for detection of pathogen DNA present in low abundance compared to other primer combinations. Since *R. orthosporum* is not known to attack barley, in the present study, the primers RS1 and RS3 were used to optimize sensitive detection of *R. secalis* DNA present in low abundance in total seed DNA. PCR amplification was performed in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) with the following amplification conditions: initial denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. An 8 µL aliquot of the PCR-amplified products was separated on a 1.5% agarose gel in Tris-borate-EDTA buffer at 100 V for 1 h. The gel was stained with ethidium bromide for 15 min, and the PCR products were visualized on a UV transilluminator.

Preparation of internal control template and competitive PCR

A heterologous internal control was prepared using a competitive DNA construction kit (Takara Shuzo Co. Ltd.; Catalogue number RR017). Competitor fragments had 5'- and 3'- termini identical to the fungal target primer sites (RS1 and RS3) but no internal sequence homology to the target sequence. The competitor fragment was generated according to the manufacturer's instructions and the amplified competitor fragment was 445 bp. Initial tests were carried out to determine the concentration of competitor DNA template for a primer pair that would result in approximately equal amplification of both fungal and competitor fragments when 100 pg of fungal DNA was used in the PCR reaction. Too high a proportion of competitor DNA template would saturate the reaction such that no fungal DNA could be detected whereas if the proportion was too low, all seed samples would appear equally heavily infected. Total fungal genomic DNA of *R. secalis*, in the range of 1 pg to 10 ng was then added to reagent mixtures containing the selected quantity of the competitor molecule prior to PCR. The reaction components and amplification conditions were the same as those for the conventional PCR assay described above. Following amplification, the PCR products of each reaction were separated by gel electrophoresis through 1.5 % agarose. Gels were stained with ethidium bromide, viewed under UV light on a Gel Doc 1000 system (Bio-Rad, U. K.) and analyzed using a Molecular Analyst Software (Bio-Rad, U. K.) to quantify densitometrically the relative degree of amplification of the fungal and competitor PCR products in each sample. The relationships between the PCR product ratios and the amount of fungal DNA added to the reaction for each dilution series were then determined. These data were used to generate a standard curve, by reference to which the quantification of the fungal DNA in barley seed could be estimated. Each PCR product ratio was based on the mean of five replicates of each PCR reaction.

Quantification of *R. secalis* in infected barley seed

To estimate the relative amounts of fungal DNA present in seed lots with various proportions of *R. secalis*-infected seeds, seed samples were prepared by

mixing healthy-looking seeds (seeds without distinct visible symptoms) of the resistant cultivar Seebe with *R. secalis*-infected seeds of the susceptible cultivar Harrington showing distinct scald symptoms. Since, in a previous study (Lee *et al.*, 2001b), some seed samples without external symptoms taken from the cv. Harrington were found to be infected with *R. secalis*, cv. Seebe, known to be resistant to scald, was used to prepare seed samples in the present study. Seed samples were prepared by mixing varying amounts of infected seeds of the cv. Harrington with seeds of cv. Seebe to obtain 1, 2, 5, 10, 20 percent infected seeds in a total of 100 seeds per sample. Three replicates were prepared for each level of infected seed mixtures. Two subsamples of 0.1g of the milled seed powder from 100 seeds were measured from each sample and used for DNA extraction. DNA extracts were used for competitive PCR analysis in the presence of constant amount of the internal control template DNA. The mean levels of the PCR product ratios obtained from three replicates of each level of infection were plotted against the percentage of seed infection. These data could be used to generate a standard curve, by reference to which the quantification of fungal DNA in field-infected barley seed could be estimated.

For quantitative PCR analysis of DNA from naturally-infected seed samples (cv. Harrington), handful amounts of seeds were taken from each yearly batch of field-infected seed from 1995-1999 and 100 seeds were counted and assessed for the presence of external scald symptoms. Five replicates, each consisting of 100 seeds, were made from each yearly batch. Two subsamples of 0.1g milled seed powder from 100 seeds were measured from each sample and used for DNA extraction. DNA extracts were then subjected to competitive PCR analysis. The mean levels of the PCR product ratios of the two subsamples were converted to ng fungal DNA mg^{-1} seed material using the *R. secalis* standard calibration curve. In order to estimate relative levels of seed infection in samples, the resulting DNA concentrations of each sample were compared with those in the calibration curve generated using the infected seed mixtures.

6. 3 Results

Quantification of fungal DNA by competitive PCR

The concentration of the competitor DNA template selected, as based on the results of preliminary quantification experiments, was 5 pg. *R. secalis* genomic DNA from the isolates RS006 and RS020 over the range of 1 pg to 10 ng was added to each reaction mixture containing the 5 pg of internal control template DNA. The amplification ratios for *R. secalis* indicated that quantification of fungal DNA over the range 5 pg to 10 ng could be achieved using 5 pg of the internal control template DNA (Fig. 6-1a). A standard curve was generated based on the results of five replicates of each concentration of *R. secalis* DNA (Fig. 1b). By this curve, quantification of *R. secalis* was possible over approximately a 2,000-fold range.

Quantification of inoculum load present in mixtures of healthy seed with *R. secalis*-infected seed

In order to construct a standard curve based on seed samples with different levels of infection, a series of seed samples with different numbers of infected seeds were used for the competitive PCR assay. The mean levels of PCR product ratios obtained from three replicates were plotted against increasing percentages of *R. secalis* seed infection. A linear relationship was found between the DNA concentration and the increasing percentage of *R. secalis* infected seeds (Fig. 6-2a and 6-2b). The equation of the curve was $y = 0.034x^{1.2675}$ ($R^2 = 0.94$). Extrapolation from a standard curve of *R. secalis* DNA and the PCR product ratio provided estimates of pathogen DNA of 0.0468, 0.0687, 0.1451, 0.9065, 1.6371 ng mg^{-1} at 1%, 2%, 5%, 10%, 20% levels of seed lot infection, respectively.

Comparison of the competitive PCR and visual disease assessment in infected barley seed

DNA extracted from seeds collected from different years were used for competitive PCR analysis using the RS1 and RS3 primer pair. The PCR product ratios between *R. secalis*-specific band and the internal control DNA band were converted to

DNA content (ng mg^{-1}). The resulting DNA contents for each sample were compared with the mean levels of DNA content calibrated according to the equation of the standard curve (Fig. 6-2b) to deduce seed infection levels in each sample. The PCR results obtained for seed samples (cv. Harrington) in 1995, 1996, 1997, 1998 and 1999 are shown in Fig. 6-3. The mean levels of DNA content for each sample showed poor relationship with an estimate of the mean DNA content extrapolated from the standard curve (Fig. 6-2b). However, DNA content of samples taken from 1995 correlated relatively well with those extrapolated from the standard curve. In contrast, the amounts of fungal DNA present in samples from 1999 were far less than the mean level of DNA content found in artificially mixed seed samples.

Cultural assay

Since *R. secalis* was also detected in seed samples without visible symptoms in the previous study (Lee *et al.*, 2001b), the conventional cultural assay was conducted to monitor the levels of symptomless infection of seed samples that were used for competitive PCR assay. A total number of 50 seeds from each year were initially assessed for the presence of external symptoms. Scald colonies that appeared on the LBA plates were then counted after 10 days incubation at 16 °C. Numbers of seeds with visible symptoms for each year were 2, 3, 0, 2, 5 for 1995, 1996, 1997, 1998 and 1999, respectively. In samples from 1996 and 1997, 3 and 2 seeds without symptoms gave rise to *R. secalis* colonies. No scald colonies were detected on the plates streaked from the seed of 1998 and 1999, although there were 2 seeds and 5 seeds with visible symptoms, respectively (Table 6-1).

6. 4 Discussion

In this study, we examined the potential of a competitive PCR assay for *R. secalis* detection and quantification in barley seed. Although the pathogen has no known sexual stage (Tekauz, 1991; Salamati *et al.*, 2000), *R. secalis* populations are highly variable resulting in the frequent breakdown of resistance of barley cultivars. In the absence of primary inoculum originating from barley stubble, seeds carrying

the pathogen may result in severe epidemics under favourable environmental conditions. This study was undertaken because a seedborne source of inoculum *of R. secalis* may have particular importance in the long-range dispersal of the pathogen to areas currently free of barley scald disease, and dispersal of new races of the pathogen that have repeatedly broken resistance of the previously resistant cultivars (Salamati *et al.*, 2000).

In an earlier study, we developed a PCR-based diagnostic method to detect *R. secalis* in infected barley seed using pathogen-specific primers derived from the internal transcribed spacer (ITS) regions of the pathogen (Lee *et al.*, 2001a). Although the assay allowed for the detection of 2% seed infection or less, it was impossible to obtain reliable quantitative data by comparing band intensity due to many factors affecting the amplification efficiency (Lee *et al.*, 2001a). Therefore, there has been a need for developing a reliable quantitative assay.

To do this a competitive assay was developed. Primer set RS1 and RS 3 was used to construct the internal control DNA, since it amplified a *R. secalis*-diagnostic band (375 bp) that could be easily differentiated from the internal control DNA band (445 bp) and showed a higher sensitivity at 55°C annealing temperature than other primer pairs tested in an earlier study (Lee *et al.*, 2001a). *R. secalis* DNA, over the range of 5 pg to 10 ng, was quantifiable in the presence of 5 pg of an internal control template DNA.

The utility of the competitive PCR assay for quantification of *R. secalis* in barley seed was demonstrated by using the artificially mixed samples, which had different levels of seed infection. The PCR assay is generally recognized to be highly sensitive and can detect the pathogen before symptoms appear (Doohan *et al.*, 1998). Symptomless infection of barley seed by *R. secalis* was reported by Kay and Owen (1973). We have also demonstrated the occurrence of symptomless infection using both PCR and an agar plate assay (Lee *et al.*, 2001b). For sampling infected seeds, naturally infected seeds of the cv. Harrington were carefully examined and seeds with typical scald symptoms were used for constructing the standard calibration curve. Although, there were slight differences in the size and shape of lesions when symptoms on lemma and palea were examined, the resulting standard curve (R^2 =

0.9401) appeared to be linear over the increasing levels of seed infection. However, higher variation was found in samples with higher levels of seed infection. Differences in inoculum loads may account for this variation.

In order to examine the potential use of the standard curve in quantifying seed infection, naturally infected seed samples were used for competitive PCR assay. Seed samples were taken from batches from different years and used for competitive PCR. The resulting PCR product ratios were converted into ng mg^{-1} plant material and compared with those resulting from the standard curve described above. Although, in the case of artificially constructed seed mixtures, relatively good correlation between visual disease assessment and the competitive PCR was found, there was poor correlation in the experiments using naturally infected seed samples. Factors contributing to this discrepancy could be due to the presence of symptomless infection reported elsewhere (Kay and Owen, 1973; Lee *et al.*, 2001b), some seeds with ambiguous symptoms mistaken as those caused by *R. secalis*, and natural variations of the disease loadings in the seed samples due to disease severity. In order to obtain accurate quantification results, further research should focus on application of appropriate statistical methods to seed sampling and size of samples.

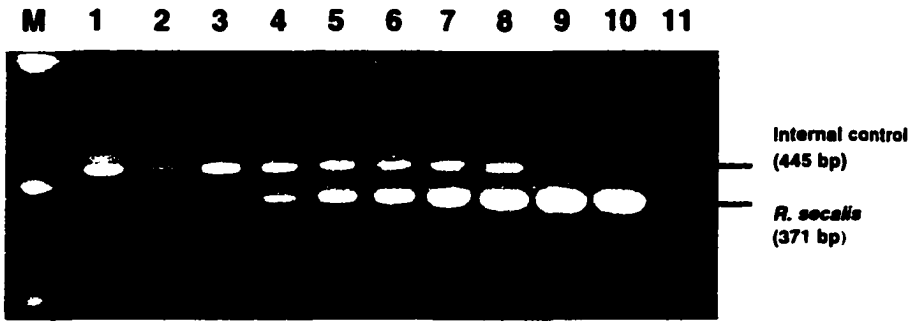
The competitive PCR approach has some drawbacks, such as laborious optimization procedures, a limited range of quantification, and the possibility of sample variation caused by increased handling. However our results suggest that the competitive PCR assay could provide an accurate means of quantifying inoculum in plants and predicting levels of seed infection, when optimized. As such it offers a reasonable alternative approach for seed health testing, as well as a sensitive tool in epidemiological studies of barley scald disease.

Recently, real-time quantitative PCR based on detection of a fluorescent signal produced proportionally during amplification of a PCR product has provided a rapid and accurate method for determination of the levels of specific DNA and RNA sequences (Grove, 1999; Zhang *et al.*, 1999). This technology is relatively expensive but it may offer a better alternative for routine seed health testing in future.

6. 5 Tables and Figures

Figure 6-1. Relationship between the amount of *Rhynchosporium secalis* DNA (RS006) and the resulting PCR product ratio. The heterologous internal control template was amplified in the presence of varying amounts of *R. secalis* genomic DNA (0.001 –10 ng). (a) Ethidium bromide stained gel of the PCR products (*R. secalis* DNA product 371 bp and internal control DNA product 445 bp). Lane M, DNA mass ladder; Lane 1, Internal control DNA only; Lanes 2-10, 0.001, 0 .005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 ng *R. secalis* DNA in the presence of a constant amount (5pg) of internal control DNA; Lane 11, negative control (lacking DNA). (b) Relationship between the amount of fungal DNA and the resulting PCR product ratio derived from the densitometric readings of the PCR products. The PCR product ratio (amount of *R. secalis* DNA product/internal control DNA product) was determined for each concentration and plotted against the concentration of *R. secalis* DNA.

(a)



(b)

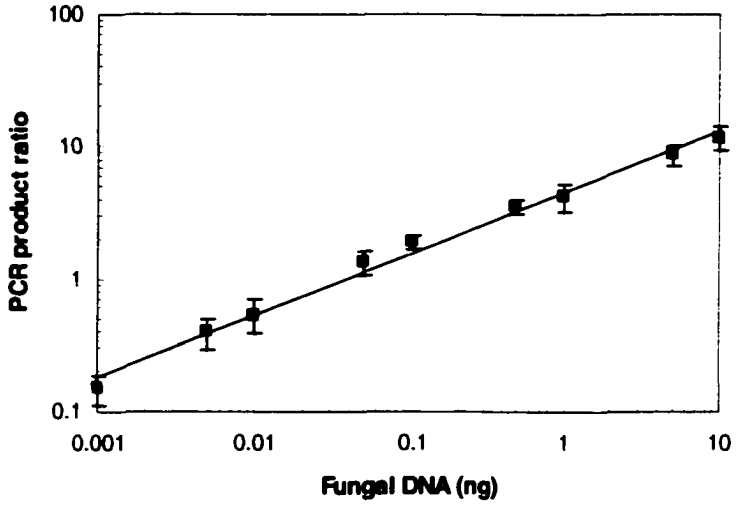
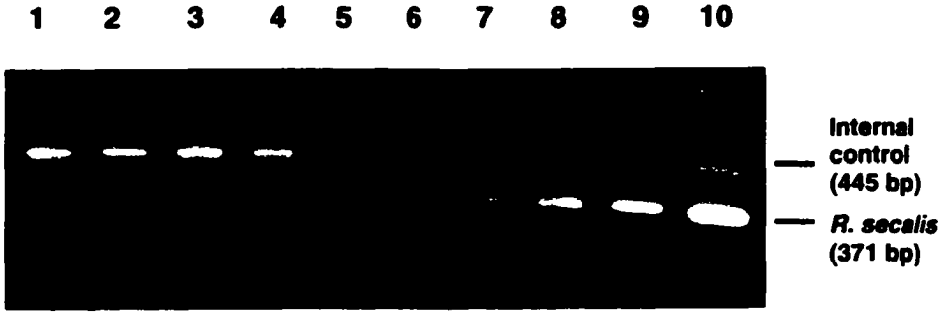


Figure 6-2. Relationship between the percentage of infected seeds and *Rhynchosporium secalis* DNA concentration after PCR reaction containing internal control DNA. (a) Ethidium bromide stained gel of the PCR products (*R. secalis* DNA product 371 bp and internal control DNA product 445 bp). Lanes 1 and 2 show 1% infection, Lanes 3 and 4 show 2% infection, Lanes 5 and 6 show 5% infection, Lanes 7 and 8 show 10% infection, Lanes 9 and 10 show 20 % infection. A constant amount of the internal control DNA (5 pg) was added to each reaction mixture containing DNA extract from seed samples with different levels of infection. (b) Relationship between the percentage of the infected seeds based on visual disease assessment (VDA) and the DNA concentration of *R. secalis* (ng fungal DNA/ mg seed material). Each point represents the mean level for ratios obtained from three replicates of each level of infection. The PCR product ratio (amount of *R. secalis* DNA product/internal control DNA product) was determined for each level of infection and plotted against it.

(a)



(b)

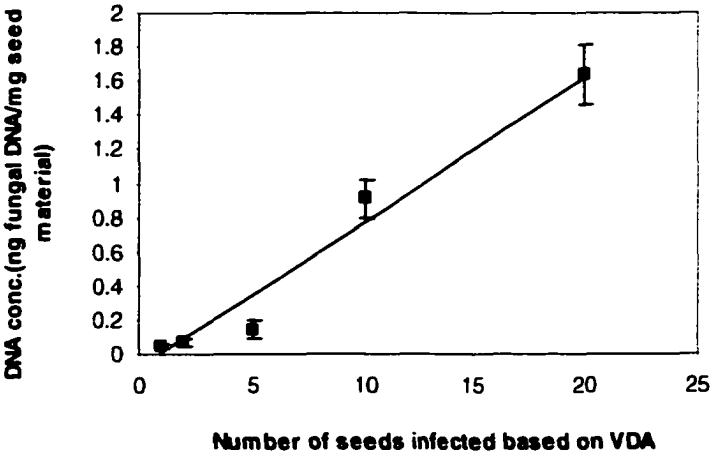
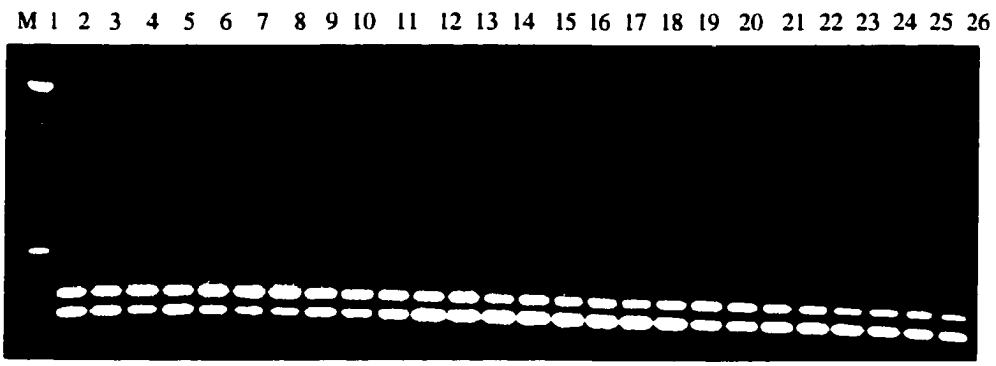
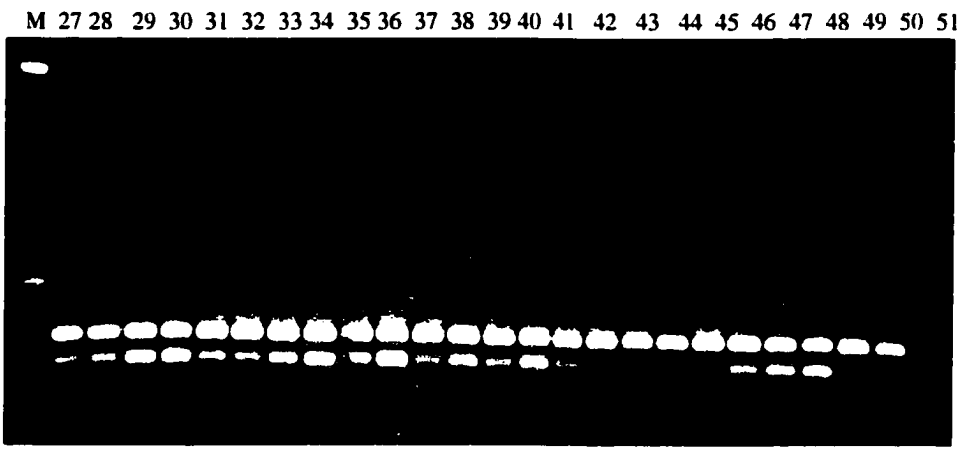


Figure 6-3. Relationship between visual disease score and DNA content of *Rhynchosporium secalis*; (a) Ethidium bromide stained gel of competitive PCR (*R. secalis* DNA product 371 bp and internal control DNA product 445 bp products) using DNA extracts of seed samples from different years (1995-1999) in the presence of a constant amount of the internal control DNA (5 pg). Lanes 1-10, Seeds from 1995; Lanes 11-20, Seeds from 1996; Lanes 21-30, Seeds from 1997; Lanes 31-40, Seeds from 1997; Lanes 41-50, Seeds from 1999; Lane 51, Negative control lacking DNA. (b) Each bar shows the mean levels of DNA content of two subsamples from each sample and the percentage of seed infection based on visual disease assessment. (□) Visual disease score; (■)DNA content.

(a)





(b)

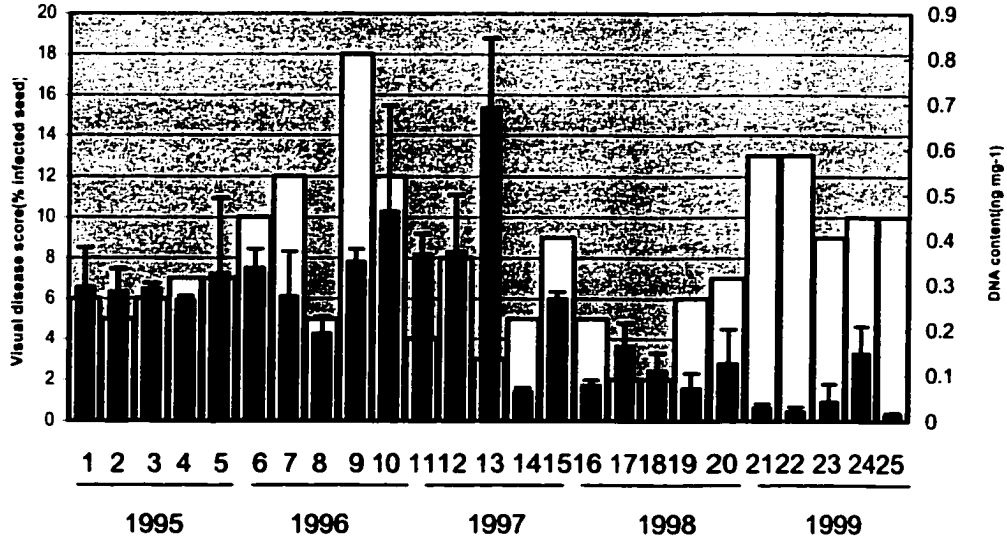


Table 6-1. Results of agar plate assay using 50 seeds from the batches of different years

Years	1995	1996	1997	1998	1999
Visual disease score	2	3	0	2	5
Number of seeds giving rise to scald colonies	2	6	2	0	0

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Chapter 7

Effects of the timing of Tilt® application and rotation trials on control of barley scald disease using quantitative PCR and VDA

7. 1 Introduction

Because barley scald is primarily stubble-borne and *Rhynchosporium secalis* may survive in infected residues for up to one or two years, control measures such as proper residue management and crop rotation practices have helped reduce or eliminate disease levels (Mayfield and Clare, 1984; Skoropad, 1965). Barley residues carrying sources of primary inoculum can be effectively destroyed through 1-2 year rotations with non-host crops (McLelland, 1989). However, the widespread use of conservation tillage and monoculture have increased the severity of the disease (Tekauz, 1991). Growing barley continuously generally results in significant yields and quality losses (Khan and Crosbie, 1988). Therefore, other control measures such as the use of resistant barley cultivars, application of foliar fungicide, and managing soil fertility should be taken into account in the areas where barley is grown year after year.

The foliar fungicide Tilt® (Propiconazole) has been used effectively to control scald (Scott *et al.*, 1992). However, its relatively high cost has limited use to high-priced malting barley cultivars seed production or areas where severe epidemics are expected. Precise timing of the application of a fungicide is vital importance for maximizing the desired effect.

Conventionally, field studies of barley scald and its control have relied on the assessment of the percentage of leaf area diseased (PLAD), yield losses mainly measured by thousand-kernel weight (TKW) and bushel weight (Xi *et al.*, 2000). However, head infection of barley has also been considered important as infected seeds carry new races and play an important role in long-range dispersal of the pathogen (Kay and Owen, 1973). In addition, kernel discoloration caused by scald infection also reduces the quality of malting barley (Khan and Crosbie, 1988).

Assessment of seed infection is made by determining the percentage of visibly diseased seeds. However, visual disease assessment (VDA) can have its own limitations such as underestimation, because of symptomless infection, confusion of ambiguous symptoms caused by other microorganisms, and the subjectivity of different observers (Kay and Owen, 1973; Lee *et al.*, 2001b). The currently developed quantitative PCR assay has shown the potential of an alternative seed health testing method, which may be useful especially in situations where quantification of the levels of infection is required (Lee *et al.*, 2002).

The present study was conducted primarily to investigate the effects of rotations involving combinations of barley cultivars with different genetic backgrounds of resistance and triticale on barley scald epidemics in subsequent barley cultivars and the effects of different fungicide regimes to control the disease. In addition, the quantitative PCR assay was used to determine its potential to assess the differential effects of fungicide timing and rotation sequences on head infection in comparison to the VDA. The relationship between other disease variables, yield loss, PCR and VDA were investigated.

7. 2 Materials and Methods

Field trials

Seed samples obtained from fungicide timing and rotation trials during the 1999 and 2000 growing seasons were provided by Dr. Turkington from the Agriculture and Agri-Food Canada, Lacombe Research Center, AB. For Tilt® timing trials, cv. Harrington barley was seeded into worked fallow on May 20 in a randomized complete block design (RCBD) with four replications. Plots were 4 rows, 5 in long with 2 rows of a non-host (wheat) between plots, all with 23 cm row spacing. Scald *(R. secalis)* infested straw and a 10^5 spore suspension/mL of *R. secalis* were applied to the plots on June 17 and June 21, respectively. Tilt® was applied in 200 L/ha water at the rates and times as noted in Tables 7-1 and 7-2. The full rate of Tilt® was applied at 125 g ai/ha and the half rate (1/2 Tilt®) at 62.5 g ai/ha. The flag leaf application was

done at Zadoks growth stage GS 37 on July 9 and the heading application at GS 65 on July 26. Four treatments with four replications were used for the 1999 trial: a single application of Tilt® at the flag-leaf stage (GS 37), a single application of ½ dose of Tilt® at the flag-leaf stage, a double application of ½ dose of Tilt® at the flag-leaf and heading stages (GS 37 and GS 65), and an untreated control. Two additional treatments were added in the 2000 trial: a single application of Tilt® at the heading stage (GS 65) and a single application of ½ dose of Tilt® at the heading stage (GS 65).

For the rotation trials, four barley cultivars, cvs. Harrington, CDC Earl (susceptible), cv. AC Lacombe (intermediate) and cv. Kasota (resistant) and cv. Wapiti (triticale) were used. Three-year rotation sequences were carried out during the 1998 to 2000 growing seasons as noted in Table 7-5. The plot set up was similar to the Tilt® trial. The experiments were conducted using a four-replicate RCBD.

Disease and yield assessments

Data of disease and yield assessments were kindly provided by Dr. Turkington at the Agriculture and Agri-Food Canada, Lacombe Research Center, AB. Scald severity in each plot was assessed at GS 83 based on % leaf area diseased for scald on the flag (PLAD) and flag-1 (PLAD-1) leaves of 20 randomly selected plants per plot. At maturity, the entire plot was harvested, dried and thousand-kernel weight (TKW) was calculated. Percent thins (THINS %) were determined by placing 100 g of seed on a 2.4 min screen, shaking for 1 minute on a Ro-Tap seed shaker (W.S. Tyler, Inc., Gastonia, NC, USA) and weighing the grain that passed through the screen.

Visual disease assessment

To determine the presence and levels of scald infection on barley seeds, seeds were randomly taken from batches of each replicate of the treatment and one hundred seeds were counted and examined for the presence of scald symptoms. Scald symptoms were recognized as elliptical to irregular lesions with pale centers surrounded by dark margins.

Quantitative PCR amplification

The levels of seed infection were quantified using a previously developed competitive PCR assay (Lee *et al.*, 2002; see Chapter 6). Plant DNA was extracted from visually assessed seed samples and used for the PCR reaction, the DNA equivalent of 0.1 mg dry seed weight in 1 µL of sterilized distilled water was used as described by Lee *et al.* (2001a; see Chapter 4). PCR product ratios were converted to ng fungal DNA mg^{-1} plant material using the *R. secalis* standard calibration curve (Lee *et al.*, 2002; see Chapter 6). Each PCR result was based on three replicates of each reaction.

Statistical analysis.

The square roots of quantitative PCR and VDA results were used in order to approximate normal distributions of the data for statistical analysis. Analysis of treatment differences based on VDA scores and PCR results consisted of one-way analysis of variance (ANOVA) incorporating Tukey's pairwise comparison test (at a 5% level of significance). The relationships between pairs of disease variables and yield parameters were determined using Pearson's product moment correlation coefficients based on the means of the data. All analyses were performed using the SAS system 8.01.

7. 3 Results

Tilt® -timing trials

The effects of Tilt® -timing

Tilt® application reduced the severity of scald disease significantly for both 1999 and 2000 trials compared to the untreated plots. In 1999, application of 1/2 dose of Tilt® at the flag-leaf and heading stages led to an effective control of scald disease on leaves (Table 7-1). However, the VDA and quantitative PCR assay showed that Tilt® sprayed at the flag-leaf stage resulted in better control of head infections in the 1999 trial (Fig. 7-1 and Table 7-1). Statistical analysis of VDA results showed that the levels of head infection of Tilt®-treated plots were not statistically significant when compared with the untreated plot. In the case of quantitative PCR analysis, applications of Tilt® resulted in significant decreases of *R. secalis* DNA content of seeds. Mean reductions in DNA content by a single application of Tilt® at the flag-leaf stage and double applications of a 1/2 dose of Tilt® at the flag-leaf and heading stage were greater than that by a single application of a 1/2 dose of Tilt® at the flag-leaf stage.

In the 2000 Tilt® -timing trial, two additional treatments were added: a single application of Tilt® at the heading stage and a single application of a 1/2 dose of Tilt® at the heading stage. All Tilt® applications resulted in significant decreases in the percentage of leaf area diseased, scores of VDA and *R. secalis* DNA contents of barley seed when compared with an untreated plot (Table 7-2). Unlike the 1999 trial, statistical analysis of VDA scores showed that a single application of Tilt® at the flag-leaf stage and a single application of a 1/2 dose of Tilt® at the flag-leaf stage had no significant effect on *R. secalis* infection in barley heads compared with untreated plots. The most significant decrease in head-infection was observed in seeds obtained from plants that received Tilt® application at the heading stage. In contrast to the VDA results, the quantitative PCR results showed that all Tilt®-applied treatments led to a significant reduction of the *R. secalis* DNA content compared with an untreated plot. The differences among plants that received different timings of Tilt® applications

were not statistically significant. However, as found in the 1999 trial, double applications of ½ dose of Tilt® at the flag-leaf stage and heading stage appeared to have a greater effect on the control of head-infection by *R. secalis* than other treatments (Fig.7-2, Table7-2).

Relationship between pairs of disease variables and yield

In 1999, leaf disease severity (PLAD, PLAD-1) were significantly correlated with TKW, VDA, and THINS %, but not with PCR, whereas they were significantly correlated with TKW, PCR, and THINS %, but not with VDA in 2000 (Tables 7-3 and 7-4). The correlations between VDA and PCR were not significant for both years but the correlations between VDA and PCR results were closer for the 1999 trial (r=0.8044) than for the 2000 trial (r=0.6295).

Rotation trial

The effects of rotation sequences

The effects of rotation sequences on the severity of barley scald were evaluated using combinations of four different barley cultivars with different genetic backgrounds of resistance and with one triticale for a three-year period from 1998 to 2000. Analyses of variance were conducted over two years (Table 7-6 and Table 7-7). In general, the levels of leaf infection (PLAD, PLAD-1) and head infection (VDA, PCR) were significantly influenced by rotation sequences of cultivars.

In 1999, although different degrees of disease severity were observed at sites with rotations involving combinations of different barley cultivars, triticale followed by any barley cultivar appeared to be the most effective in reducing the severity of the disease. In general, rotation sequences involving the use of resistant or intermediate cultivars after intermediate, susceptible, resistant cultivars or triticale (cv. Wapiti) resulted in reductions of PLAD, PLAD-1, VDA, and PCR. Of two susceptible cultivars (cv. Harrington and cv. CDC Earl), any rotation sequences involving cv. CDC Earl resulted in higher levels of scald infection on leaf as well as head than cv. Harrington. Continuous cropping of the same cultivar generally led to severe scald

infection and two years of cv. CDC Earl resulted in the most severe incidence of scald disease on leaf as well as head. The disease on leaf and head was less severe in any barley cultivars after triticale when compared to other rotation sequences. Although results of head infection measured by PCR broadly agreed with those of leaf infection, statistical analysis showed that VDA scores were not significantly different among rotation treatments. However, significant differences in *R. secalis* DNA content were evident in this trial among rotation treatments (Table 7-6 and Fig. 7-3).

In 2000 rotation sequences resulted in significant differences in PLAD, PLAD-1, VDA, and PCR. Barley scald severity was lower following combinations of resistant cultivar in the first year, resistant, intermediate, susceptible cultivars or triticale in the second year and resistant cultivar in the final year compared to the continuous cropping of single cultivars or other rotation sequences (Table 7-7 and Fig. 7-4). Three-year rotations ending with the same cultivar of the first year revealed that one year rotation with tritcale had no significant effect in reduction of disease severity on leaf and head compared to the rotations involving resistant barley cultivar (cv Kasota). Disease severity on leaf was higher in rotation treatments ending with cv. CDC Earl than in those ending with cv. Harrington. However, the severity of head infection measured by VDA and PCR showed that rotations ending with cv. Harrington resulted in higher head infection than rotations ending with cv. CDC Earl. whereas the continuous cropping of the susceptible cv. CDC Earl (CCC) led to the most severe leaf infection among rotation treatments, disease severity was not significantly increased by continuous growing of susceptible cv. Harrington (HHH) compared to the rotation treatments of continuous cropping of intermediate cv. AC Lacombe (AAA) and cv. Kasota (KKK). There were no significant differences of the levels of head infection measured by PCR in the rotation treatments of continuous cropping of the same cultivar.

Relationship between pairs of disease variables

Results of correlation analysis between head infection measured by PCR and VDA and leaf infection (PLAD, PLAD-1) in the 1999 trial are shown in Table 7-8. Leaf-infection measured by PLAD and PLAD-1 had a significant relationship with the

head infection measured by quantitative PCR, but not by VDA. Results of the 2000 rotation trial were also subjected to the correlation analysis (Table 7-9). A significant relationship was found between the results of PCR, PLAD and PLAD-1. The correlation between VDA and PCR was closer for the 2000 trial (r=0.6893) than for the 1999 trial (r=0.2427) and the correlation between VDA and PCR for the 2000 trial was highly significant.

7. 4 Discussion

Application of a foliar fungicide, Tilt® and crop rotation have been effectively practiced to control barley scald in combination with other control measures as parts of the integrated crop management practice. The effects of fungicides and rotation practices on control of barley scald caused by *R. secalis* have been investigated in several countries (Khan, 1986; Martin and Ssanderson, 1988; Scott *et al.*, 1992). Because the fungus primarily attacks leaves, thus decreasing photosynthetic areas and resulting in poor filling of kernels, field studies of the disease have been focused on assessment of leaf infection and various yield parameters. However, seeds are also attacked by the pathogen and seedborne inoculum is of particular importance in epidemiological respects. Conventionally, the assessment of head infection has been based on examining external symptoms of harvested seeds. Since VDA has been proven to have some drawbacks for assessment of seed infection, a recently developed quantitative PCR assay (Lee *et al.*, 2001b; Lee *et al.*, 2002) was used to assess the levels of head infection in studies of the effects of fungicide timing and rotation on control of the disease. These were compared with VDA.

In both the 1999 and 2000 Tilt®-timing trials, fungicide application resulted in significant reductions in scald infection on leaf and head. Although application of a ½ dose of Tilt® each at the flag-leaf and heading stages led to the most effective control of leaf infection for both yearly trials, the results of head infection measured by VDA or PCR showed different treatment effects for the 1999 and 2000 trials. In the case of the 1999 trial, head infection measured by VDA and PCR indicated that a single application of Tilt® at the flag-leaf stage was more effective in controlling head

infection than application of a ½ dose of Tilt® each at the flag-leaf and heading stages. However, the results were not significantly different. A single application of Tilt® at the flag-leaf stage may have reduced the upwards spread of disease thus decreasing head infection. Unfavorable environmental conditions during the heading stage, such as prolonged dry weather conditions, might also contribute to a reduction in head infection. Although the PCR results agreed with the VDA results in 1999, PCR and VDA results revealed differences between treatment effects in the 2000 trial. VDA results indicated that a single application of Tilt® at the heading stage was most effective in reducing head infection. The second most effective treatment was application of a ½ dose of Tilt® each at the flag-leaf and heading stages. However, PCR results showed significant reductions of head infection across all treatments compared to the untreated plots. In the case of PCR results measured by the DNA contents of *R. secalis* in seeds, applications of a ½ dose of Tilt® each at the flag-leaf and heading stages appeared to be most effective in head infection. Unlike the 1999 trial, a single application of Tilt® at the flag-leaf stage appeared ineffective in reducing the levels of head infection. This result suggested that head infection was influenced not only by the application of fungicide but also by the environmental conditions prevailing during the heading stage. In general, application of a ½ dose of Tilt® each at the flag-leaf and heading stages appeared to be effective in controlling foliar infection as well as head infection since it protects barley heads from fungal infection even under favorable environmental conditions. Khan (1986) and Scott *et al.* (1992) also reported similar results that repeated fungicide applications resulted in almost complete control of the leaf infection and yield increases. Although, significant pairwise correlations among the levels of PLAD, PLAD-1, TKW, and THINS % were found, the levels of head infection were not significantly correlated with TKW except for the PCR results of the 2000 Tilt® trial. Since flag leaves are known to be responsible for the head filling and maturation, the levels of leaf infection appeared to affect yield components.

No matter what tillage system is used, a one- to two-year crop rotation with non-host crop may provide good control of residue-borne diseases such as barley scald or net blotch because the disease organisms in the residues usually break down before

barley is grown again (Mayfield and Clare, 1984). However, rotating cultivars with different genetic backgrounds of resistance may also play an important role in reducing the severity of disease in areas where barley is grown year after year. Overall, rotation treatments involving combinations of barley cultivars with different genetic backgrounds of resistance and with one triticale significantly affected the levels of leaf infection (PLAD, PLAD-1) and head infection (VDA, PCR).

In 1999, rotation sequences involving the use of the resistant or intermediate cultivars after intermediate, susceptible, resistant cultivars or triticale (cv. Wapiti) resulted in reductions of PLAD, PLAD-1, VDA, and PCR. Of rotation treatments, growing a non-host crop, triticale followed by barley showed effective reductions of scald infection on the leaf and head. A reduction in disease severity was also found on rotation treatments ending with resistant cv. Kasota. Statistical analysis of the levels of head infection measured by PCR and VDA showed that VDA scores did not reveal significant differences among rotation treatments. In contrast, PCR results generally agreed with the results of leaf infection measured by PLAD and PLAD-1.

In the 2000 rotation trial, rotation sequences resulted in significant differences in the results of PLAD, PLAD-1, VDA, and PCR. Barley scald severity on the leaf and head was lower following combinations of a resistant cultivar in the first year, resistant, intermediate, susceptible cultivars or triticale in the second year and a resistant cultivar in the final year when compared to the continuous cropping of single cultivars or other rotation sequences. While rotation treatments ending with cv. CDC Earl resulted in higher levels of disease on flag leaves, rotation treatments ending with cv. Harrington led to higher disease ratings on barley heads. A one year rotation with triticale had no significant effect on reduction of scald disease when compared with the rotation sequence involving the resistant barley cv Kasota. Although there were differences in cultivar reactions to scald infection on barley heads, the results for levels of head infection within rotation treatments ending with the same cultivar appeared to be insignificant.

Correlation between *R. secalis* DNA content measured by PCR and VDA scores appeared to be insignificant except for the 2000 rotation trial. This discrepancy between the PCR and VDA results may be attributed to two factors. First, to the

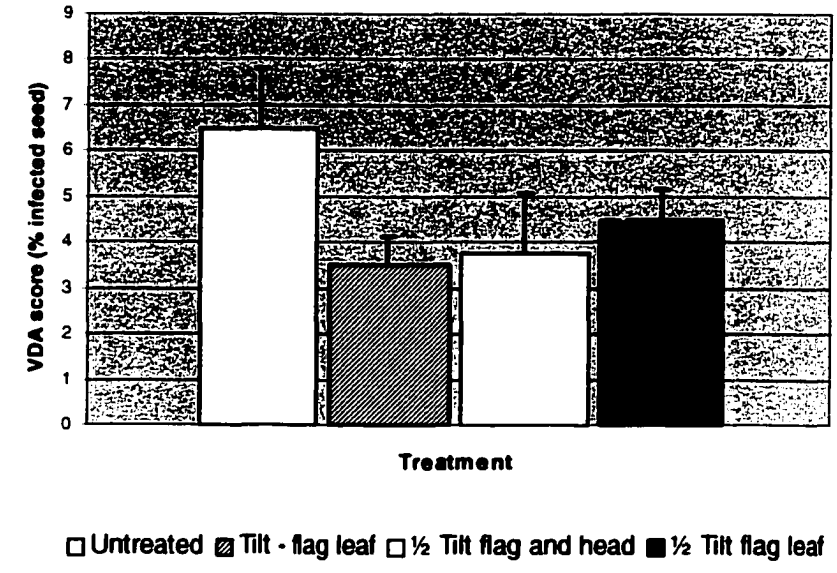
presence of symptomless infection caused by unfavorable environmental conditions when the barley head was most susceptible to *R. secalis* infection, and secondly, to overestimated seed infection levels due to some ambiguous symptoms caused by other saprophytes or pathogens. Incidence of seed infection may be dependent on environmental conditions at the flowering and seed maturation phases of plants, cultural practices, cultivar susceptibility, and the severity of scald infection in the fields (Skoropad, 1959). Of these factors contributing to head infection, unfavorable environmental conditions during flowering and seed maturation stages and infection late in the season may not allow for the development of typical scald symptoms thus leading to symptomless infection of seeds (Kay and Owen, 1973; Lee *et al.*, 2001b; Skoropad, 1959).

Correlation analysis for both of the Tilt®-timing and rotation trials revealed that the results of PCR and VDA were inconsistently associated with the levels of leaf infection (PLAD, PLAD-1). This discrepancy might be attributed to the unfavorable environmental conditions during the stage when barley heads were susceptible to scald infection. Kay and Owen (1973) also reported that the amount of visible infection of grains and the amount of leaf infection in parent crops were not correlated. However, overall PCR results had more significant relationships to the levels of leaf infection (PLAD, PLAD-1).

In general, PCR analysis allowed for discrimination of treatment effects in the Tilt® -timing and rotation trials and correlated better with the levels of leaf infection. In fungicide trials for Fusarium ear blight, a PCR-based assay had better correlations with yield losses compared with VDA (Doohan *et al.*, 1999). Quantitative PCR will be useful in epidemiological studies of plant diseases as it would enable detection, differentiation and quantification of the levels of fungal biomass under field conditions.

7. 5 Tables and Figures

(a)



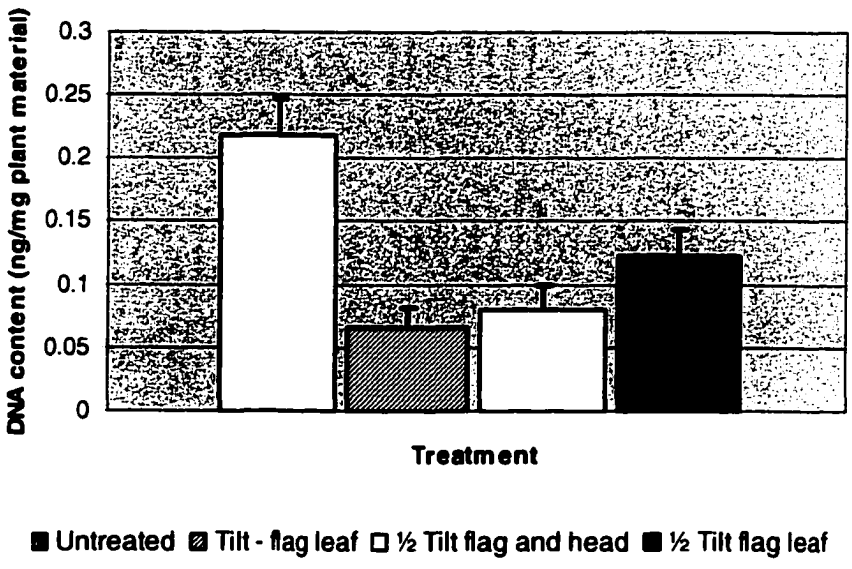
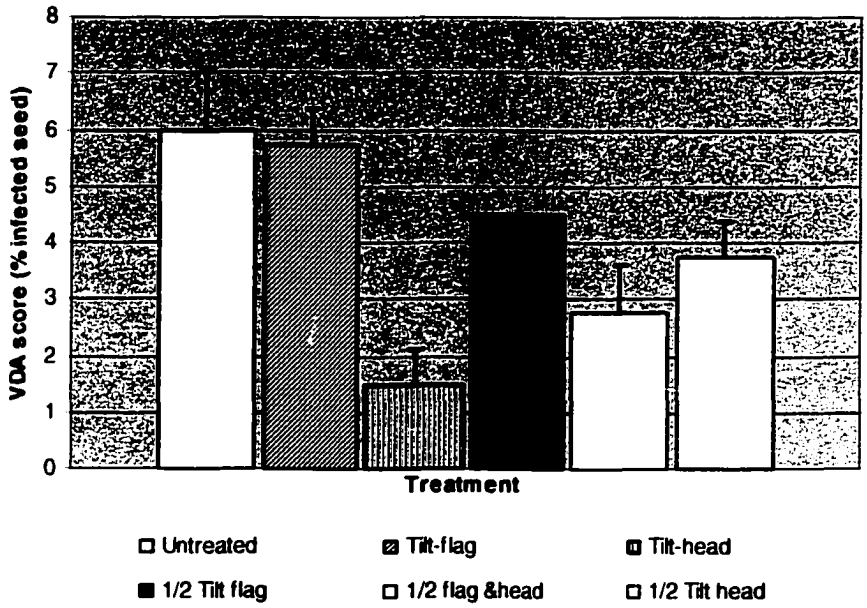


Figure 7-1. Analysis of the effect of Tilt® -timing on *Rhynchosporium secalis* infection of barley heads in the 1999 trial. Disease scores based on (a) visual disease assessment (VDA) and (b) quantitative PCR analysis. Bars indicate standard error of the means.

(a)



(b)

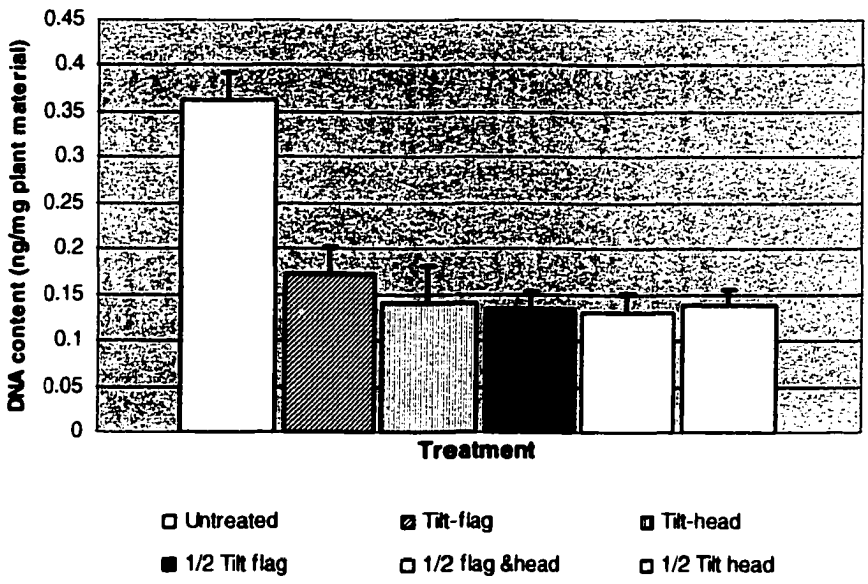


Figure 7-2. Analysis of the effect of Tilt® -timing on *Rhynchosporium secalis* infection of barley head in the 2000 trial. Disease scores based on (a) visual disease assessment (VDA) and (b) quantitative PCR analysis. Bars indicate standard error of the means.

Table 7-1. The percentage of leaf area diseased on flag and flag-1 leaves (PLAD, PLAD-1), thousand kernel weight (TKW), THINS %, scores of visual disease assessment (VDA), *Rhynchosporium secalis* DNA contents based on quantitative PCR analysis (QPCR) for Tilt® - timing trial in 1999.

SPRAY TIMING	PLAD	PLAD-1	TKW	THINS %	VDA	QPCR
Untreated	26.2 a	36.1 a	33.5 b	46.8 a	6.5 a	0.2187 a
Tilt –Flag[a]	2.8 b	6.6 b	37.5 a	24.9 b	3.5 a	0.0662 b
½ Tilt –Flag and Head[b]	2.5 b	4.9 b	39.4 a	19.1 b	3.8 a	0.0790 b
½ Tilt –Flag[a]	5.1 b	7.8 b	37.9 a	29.4 b	4.5 a	0.1220 ab

Note: Values followed by different letters are significantly different according to Tukey's pairwise comparison test at p=0.05.
[a] Single application of Tilt® (GS 37)
[b] Split application of Tilt® (GS 37 and GS 65)

Table 7-2. The percentage of leaf area diseased on flag and flag-1 leaves (PLAD, PLAD-1), thousand kernel weight (TKW), THINS %, scores of visual disease assessment (VDA), *Rhynchosporium secalis* DNA contents based on quantitative PCR analysis (QPCR) for Tilt® -timing trial in 2000.

SPRAY TIMING	PLAD	PLAD-1	TKW	THINS %	VDA	QPCR
Untreated	6.9 a	16.9 a	37.7 b	24.4 a	6.0 a	0.3623 a
Tilt –Flag[a]	2.2 b	5.4 bcd	41.7 ab	12 b	5.8 a	0.1732 b
Tilt –Head[a]	3.8 b	12.7 abc	41.1ab	13.6 b	1.5 b	0.1417 b
½ Tilt Flag[a]	3 b	4.3 cd	43 a	12.7 b	4.5 a	0.1333 b
½ Tilt –Flag and Head[b]	2 b	3.1 d	44.2 a	9.2 b	2.8 ab	0.1307 b
½ Tilt-Head[a]	3 b	13.3 ab	40.8 ab	14.6 b	3.8 ab	0.1376 b

Note: Values followed by different letters are significantly different according to Tukey's pairwise comparison test at p=0.05.
[a] Single application of Tilt® (GS 37 or GS 65)
[b] Split application of Tilt® (GS 37 and GS 65)

Table 7-3. Pearson's correlation coefficients among leaf infection (PLAD, PLAD-1), head infection (QPCR, VDA), thousand-kernel weight (TKW), and THINS % of cv. Harrington obtained from the 1999 Tilt® - timing trial, Lacombe, Alberta.

	PLAD	PLAD-1	TKW	QPCR	VDA
PLAD-1	0.9987**				
TKW	-0.9547*	-0.9639*			
QPCR	0.7765	0.7540	-0.5538		
VDA	0.9749*	0.9631*	-0.9066	0.8044	
THINS %	0.9624*	0.9608*	-0.9760*	0.6228	0.9625*

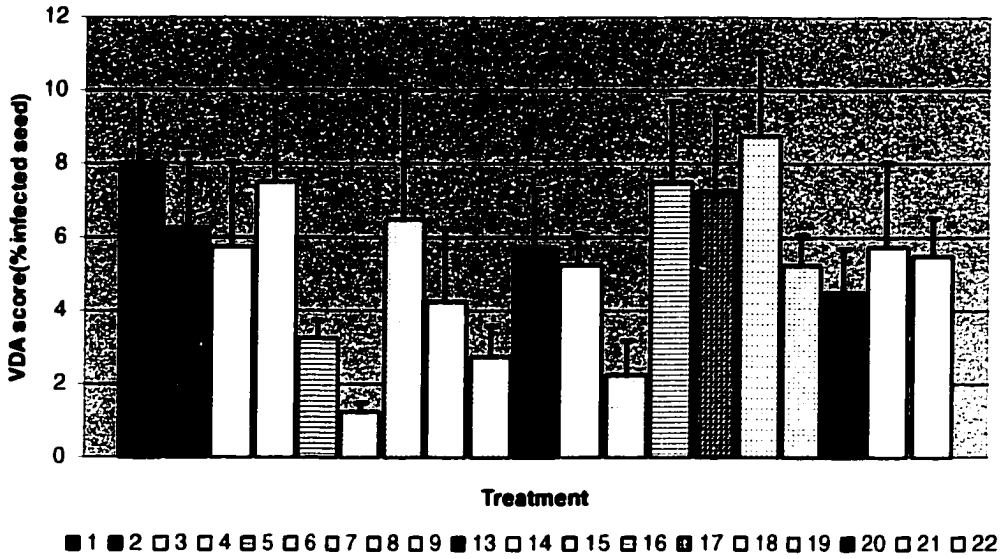
* Significant at P = 0.05
** Significant at P = 0.01

Table 7-4. Pearson's correlation coefficients among leaf infection (PLAD, PLAD-1), head infection (QPCR, VDA), thousand-kernel weight (TKW), and THINS % of cv. Harrington obtained from the 2000 Tilt® -timing trial, Lacombe, Alberta.

	PLAD	PLAD-1	TKW	QPCR	VDA
PLAD-1	0.8131*				
TKW	-0.8934*	-0.9158**			
QPCR	0.8991*	0.6355	-0.8442*		
VDA	0.3509	0.0833	-0.4420	0.6295	
THINS %	0.9694**	0.8267*	-0.9442**	0.9296**	0.5172

* Significant at P = 0.05
** Significant at P = 0.01

(a)



(b)

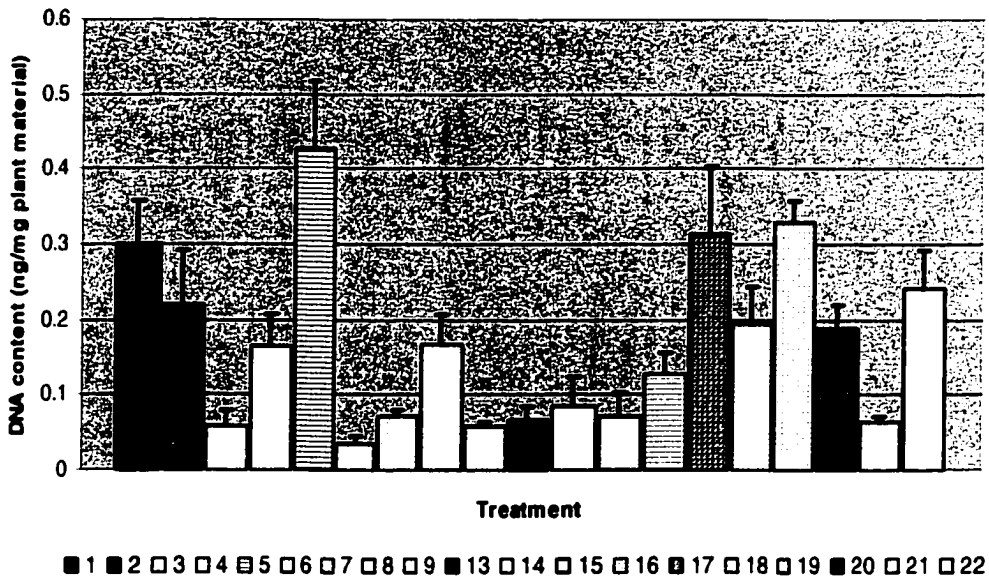
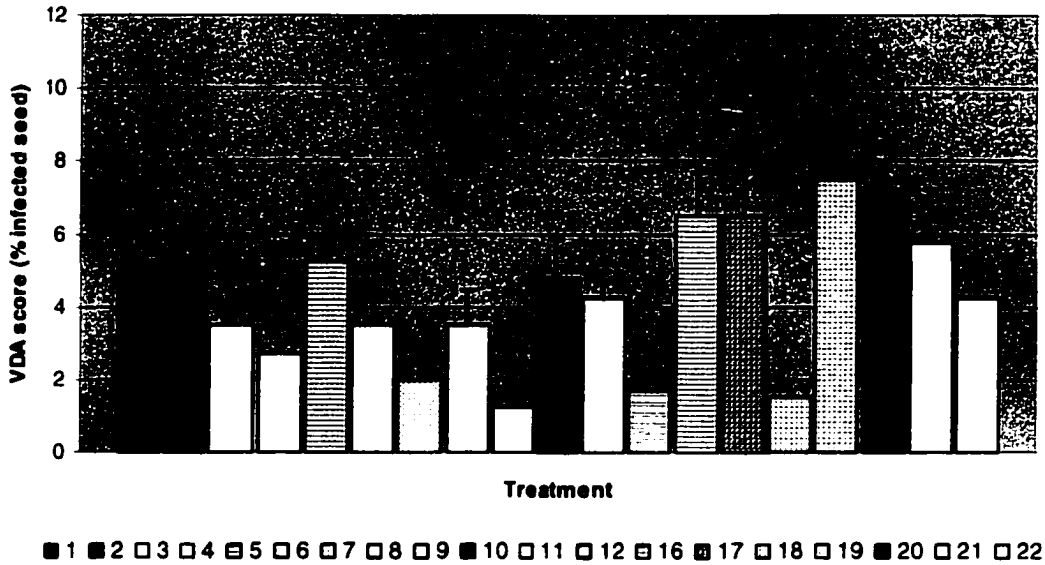


Figure 7-3. Analysis of the effect of rotations involving combinations of 4 different barley cultivars and one triticale on *Rhynchosporium secalis* infection of barley heads in the 1999 trial. Disease scores based on (a) visual disease assessment (VDA) and (b) quantitative PCR analysis. Bars indicate standard error of the means.

(a)



(b)

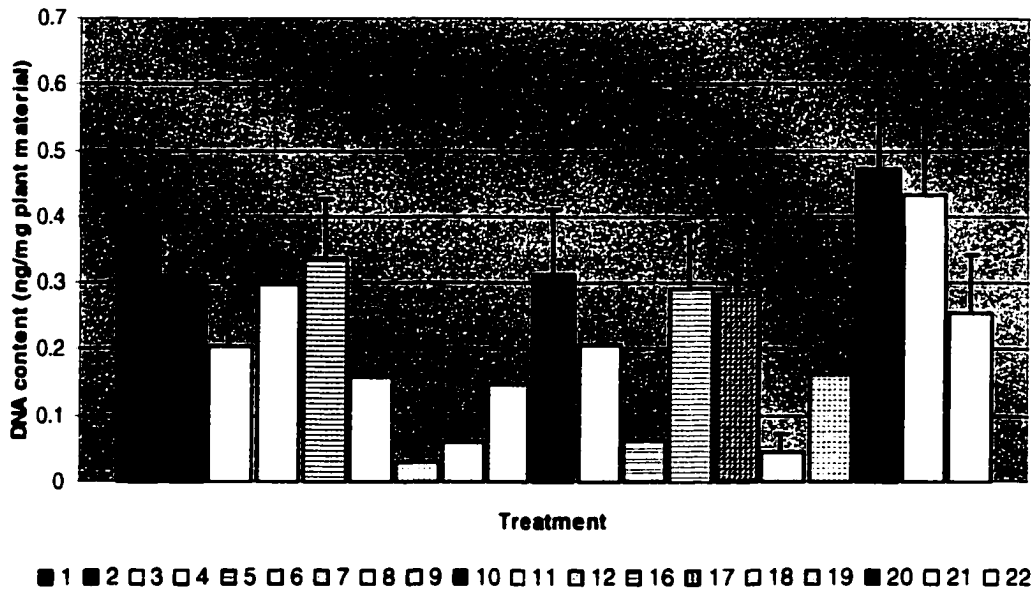


Figure 7-4. Analysis of the effect of rotations involving combinations of 4 different barley cultivars and one triticale on *Rhynchosporium secalis* infection of barley heads in the 2000 trial. Disease scores based on (a) visual disease assessment and (b) quantitative PCR analysis. Bars indicate standard error of the means.

Table 7-5. Rotation sequence

Treatment	Cultivars		
	Year 1(1998)	Year 2(1999)	Year 3(2000)
1	AC Lacombe	AC Lacombe	AC Lacombe
2	AC Lacombe	CDC Earl	AC Lacombe
3	AC Lacombe	Kasota	AC Lacombe
4	CDC Earl	AC Lacombe	CDC Earl
5	CDC Earl	CDC Earl	CDC Earl
6	CDC Earl	Kasota	CDC Earl
7	Kasota	AC Lacombe	Kasota
8	Kasota	CDC Earl	Kasota
9	Kasota	Kasota	Kasota
10	AC Lacombe	Wapiti	AC Lacombe
11	CDC Earl	Wapiti	CDC Earl
12	Kasota	Wapiti	Kasota
13	Wapiti	AC Lacombe	Wapiti
14	Wapiti	CDC Earl	Wapiti
15	Wapiti	Kasota	Wapiti
16	AC Lacombe	Harrington	AC Lacombe
17	CDC Earl	Harrington	CDC Earl
18	Kasota	Harrington	Kasota
19	Harrington	CDC Earl	Harrington
20	Harrington	Harrington	Harrington
21	Harrington	Kasota	Harrington
22	Harrington	Harrington	Harrington

Table 7-6. The percentage of leaf area diseased on flag and flag-1 leaves (PLAD, PLAD-1), scores of visual disease assessment (VDA), *Rhynchosporium secalis* DNA contents based on quantitative PCR analysis (QPCR) for the rotation trial in 1999.

TRT. #	ROTATION SEQUENCE 1998/1999	PLAD	PLAD-1	VDA	QPCR
1	AA	5.2 bcde	31.7 bc	8.0 a	0. 30 ab
2	AC	10.5 bc	33.9 bc	6.3 a	0.22 abc
3	AK	0 e	0.2 e	5.8 a	0.06 cd
4	CA	4.7 cde	24.1 bcd	7.5 a	0.17 abcd
5	CC	32.6 a	62.0 a	3.3 a	0.43 a
6	CK	0.2 e	0.5 e	1.3 a	0.03 d
7	KA	0.5 e	8.5 de	6.5 a	0.07 cd
8	KC	6.5 bcde	24.7 bcd	4.3 a	0.17 abcd
9	KK	0.1 bce	0.6 e	2.8 a	0.06 cd
10	AW	-	-	-	-
11	CW	-	-	-	-
12	KW	-	-	-	-
13	WA	0.4 e	6.1 de	5.8 a	0.07 cd
14	WC	1.3 de	12.2 cde	5.3 a	0.08 cd
15	WK	0 e	0.1 e	2.3 a	0.07 cd
16	AH	6.8 bcde	41.4 ab	7.5 a	0.12 bcd
17	CH	8.9 bcde	31.9 bc	7.3 a	0.31 ab
18	KH	2.9 cde	11.7 cde	8.8 a	0.19 abcd
19	HA	4.6 cde	38.6 b	5.3 a	0.33 ab
20	HC	12.8 b	33.2 bc	4.5 a	0.19 abcd
21	HK	0.2 e	0.5 e	5.8 a	0.06 cd
22	HH	6.0 bcde	34.7 b	5.5 a	0.24 abc

Note: Values followed by different letters are significantly different according to Tukey's pairwise comparison test at p=0.05
A= AC Lacombe, C= CDC Earl, H= Harrington, K= Kasota, W=Wapiti

Table 7-7. The percentage of leaf area diseased on flag and flag-1 leaves (PLAD, PLAD-1), scores of visual disease assessment (VDA), *Rhynchosporium secalis* DNA contents based on quantitative PCR analysis (QPCR) for the rotation trial in 2000.

TRT. #	ROTATION SEQUENCE 1998/1999/2000	PLAD	PLAD-1	VDA	QPCR
1	AAA	7.3 d	11.9 efghi	5.3 abc	0.39 ab
2	ACA	7.3 d	22.2 cdefgh	5.3 abc	0.31 abc
3	AKA	2.9 d	6.3 ghi	3.5 abc	0.20 abc
4	CAC	27.9 ab	47.1 ab	2.8 abc	0.29 abc
5	CCC	39.3 a	64.1 a	5.3 abc	0.34 abc
6	CKC	15.3 bcd	29.2 bcdef	3.5 abc	0.16 abc
7	KAK	0.4 d	1.5 hi	2.0 abc	0.03 c
8	KCK	1.1 d	1.4 hi	3.5 abc	0.06 bc
9	KKK	4.1 d	7.9 fghi	1.3 c	0.15 abc
10	AWA	4.1 d	8.5 efghi	4.8 abc	0.31 abc
11	CWC	14.8 bcd	20.9 defghi	4.3 abc	0.21 abc
12	KWK	0 d	0.3 i	1.8 abc	0.06 bc
13	WAW	-	-	-	-
14	WCW	-	-	-	-
15	WKW	-	-	-	-
16	AHA	10.4 cd	27.8	6.5 ab	0.29 abc
17	CHC	24.7 abc	43.6 abc	6.5 abc	0.28 abc
18	KHK	0.6 d	1.9 hi	1.5 bc	0.04 c
19	HAH	11.7 bcd	29.7 bcde	7.5 a	0.16 abc
20	HCH	15.3 bcd	34.6 bcd	7.0 a	0.47 a
21	HKH	6.1 d	12.8 efghi	5.8 abc	0.43 ab
22	HHH	5.0 d	10.4 efghi	4.3 abc	0.25 abc

Note: Values followed by different letters are significantly different according to Tukey's pairwise comparison test at p=0.05
A= AC Lacombe, C= CDC Earl, H= Harrington, K= Kasota, W=Wapiti

Table 7-8. Pearson's correlation coefficients among leaf infection (PLAD, PLAD-1) and head infection (QPCR, VDA) of four barley cultivars and tritcale obtained from the 1999 rotation trial, Lacombe, Alberta.

	PLAD	PLAD-1	QPCR
PLAD-1	0.8375 **		
QPCR	0.7705 **	0.8777 **	
VDA	-0.0738	0.2245	0.2427

* Significant at P = 0.05
** Significant at P = 0.01

Table 7-9. Pearson's correlation coefficients among leaf infection (PLAD, PLAD-1) and head infection (QPCR, VDA) of four barley cultivars and triticale obtained from the 2000 rotation trial, Lacombe, Alberta.

	PLAD	PLAD-1	QPCR
PLAD-1	0.9727 **		
QPCR	0.4491 *	0.5065 *	
VDA	0.386	0.5163 *	0.6893 **

* Significant at P = 0.05
** Significant at P = 0.01

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Chapter 8

8. 1 General Discussion and Summary

Leaf scald of barley caused by *Rhynchosporium secalis* (Oud.) J. J. Davis (syn. *Marssonia secalis* Oudem.) is a major foliage disease throughout the cooler, semi-humid barley growing areas of the world. Although the fungus predominantly overwinters in infected-barley residues in a field, in the absence of residue-borne inoculum, infected seeds play an important role in distributing random infection foci at the beginning of the season (Caldwell, 1937; Kay and Owen, 1973; Reed, 1957; Skoropad, 1959). Seed transmission of the pathogen is important because it efficiently transfers the pathogen both spatially and temporally (Baker, 1980). Control of seedborne diseases can be achieved through seed treatment in combination with seed health inspection programs.

Since no resistant malting barley varieties have been registered in Canada and there is extreme variability of the pathogen, protection of the crop from seedborne sources of inoculum is of particular importance in terms of developing an integrated crop management program. In particular, understanding of seedborne phase of the disease and the rapid and reliable diagnosis of seed infection by *R. secalis* would allow for protection of disease-free areas and help make disease management decision. Therefore, present research was conducted to investigate infection of barley head by *R. secalis,* develop a cultural assay for pathological study, develop a reliable PCR-based seed health testing assays for *R. secalis* and examine the potential of the assays for quantification of the levels of *R. secalis* infection in naturally infected barley seed. Finally, molecular seed health testing assay developed was used to investigate the effects of Tilt® -timing and rotation on the control of head infection of barley scald in comparison to conventional visual disease assessment (VDA).

The infection route and location of inoculum on and in seeds are of primary importance in longevity of the pathogen during the seedborne phase, and ultimately,

their transmission from seeds to seedlings (Maude, 1996). Therefore, the present study was undertaken to examine scald symptoms on barley heads with respect to different barley cultivars using light microscopy as well as scanning electron microscopy (SEM). Scald-infected barley seeds were examined using seeds stored for two years and others collected in the same year from the barley fields in the 1997 to 2000 growing seasons.

External scald symptoms observed in the present study were generally similar to those described by Skoropad (1959) and Kay and Owen (1973). The scald symptoms were distributed over the lemmas, paleas, awns, glumes and rachises, but were most common on glumes and awns. However, unlike barley heads collected from the fields, there were difficulties in assessing the seed infection using stored seed samples when putative scald lesions were not present. These ambiguous lesions were confused with symptoms caused by other pathogens such as *Drechslera teres, Fusarium poae, Pseudomonas syringae* and kernel discololation caused by other microorganisms. Whole mount preparations of the infected lemmas and paleas showed the presence of abundant thick and short-celled dormant mycelium mainly restricted to the lesions under a light microscope. Scanning electron micrographs showed hyphal colonization and few detached conidia of *R. secalis,* which were confined to the inner surface of lesions. For seeds that exhibited ambiguous symptoms, typical *R. secalis* conidia or dormant mycelium were not always observed. Although lemmas and paleas used for the study confirmed the presence of putative scald symptoms using visual identification, some samples appeared to be heavily colonized by microorganisms other than *R. secalis,* such as *D. teres*, *Cladosporium* sp., and mycelia of unknown fungi. Microscopic study showed that in seeds (both stored and newly-collected from the barley fields) mycelia and conidia of the pathogen were primarily distributed between the pericarp and lemma or palea, and colonization of the pathogen was restricted to the inner surfaces of lesions. No lesions were observed near the embryo end. Most collected heads had heavy awn-infections. As indicated by Skoropad (1959), it is assumed that head infection occurs mainly when kernels, which are enclosed by floral bracts are half-filled. Rain-splashed water droplets containing conidia enter through the opening near the upper lemma-end where the infection is

initiated. The edges of the lemmas and glumes that form the groove appeared to be the most susceptible areas of the scald infection since they provide ideal incubators for infection and sporulation of the pathogen due to the accumulation of water. Rain-splashed conidia are known to be responsible for the dispersal of *R.secalis* (Reed, 1957; Shipton *et al.*, 1974). Mucilage surrounding many of the fungal pathogens belonging to the group Deuteromycetes protects and controls the release of conidia by rain splash (Maude, 1996). Scanning electron micrographs revealed that conidia of *R. secalis* present on the inner surfaces of lemmas were surrounded by material that may be mucilage. Barley heads of most cultivars examined in this study were susceptible to scald infection. However, some cultivar-related differences in overall seed infection by *R. secalis* were observed, which may be a reflection of differential host susceptibility.

In many plant-pathogen interactions, calcium sequestration from the host cell walls by oxalic and other organic acids produced by the pathogen has been recognized during pathogenesis (Rao and Tewari, 1987; Yang *et al.*, 1993). In the present study, rod-shaped calcium-containing crystals were found associated with conidia of *R. secalis* and were abundant on the inner surface of glume-lesions of the susceptible cv. Harrington, but rarely seen on the other parts of the heads or other resistant cultivars. Tewari *et al.* (1995) first observed the cation sequestration by *R. secalis* from scald-infected leaf lesions of the susceptible cv. Manley. Mukerji *et al.* (1998) showed that the levels of calcium of host plants were related to the levels of resistance of host plants to the barely scald disease. The calcium sequestration found in the present study suggests and supports an earlier report (Tewari *et al.*, 1995) that the pathogenesis of *R. secalis,* may partly involve sequestration of calcium ions from the host plant, as found in some other pathosystems.

Direct examination of seed samples and seed incubation tests are both important methods for detecting pathogens in general seed pathological research and in seed health testing. In particular, cultural methods have been used for routine seed health testing by hundreds of seed labs worldwide today, because they give information of viability of seedborne pathogen and are useful for studying the pathogen races carried on infected seeds. Therefore, the present study was conducted to develop a cultural

method that could be used for isolating *R. secalis* from seeds and which could be applied to seed health testing.

To develop a cultural method, the lemmas and paleas with lesions were incubated in antibiotic amended sterilized distilled water (SDW) for 0-48 hours at 16°C to enrich the scald fungus in relation to the contaminants present. Subsequently, *R. secalis* was successfully isolated using a streaking method. By this method, the number of seeds, lemmas and paleas from which gave rise to scald fungus colonies, did not vary much for the different incubation periods. However, as the incubation time was increased, contaminants often overwhelmed the scald colonies. Therefore, either no incubation or an incubation period of 12 hrs or less is recommended for isolating *R. secalis* using the cultural method described in the present study. This method could be useful for examining the distribution and viability of the seedborne inoculum of *R. secalis* as well as studying pathogen races carried with seeds. In our investigation, *R. secalis* could be consistently isolated after 7 to 10 days from colonies appearing on lima bean agar plates. This appears to be the first report on successful isolation of *R. secalis* from infected barley seed (Lee *et al.*, 1999).

Assessment of seed infection by *R. secalis* has relied on visual inspection of the external symptoms on seeds. However, scald symptoms can be confused with those caused by other microorganisms, and the actual number of symptomless seeds can be underestimated (Lee *et al.*, 1999; Lee *et al.*, 2001b). The cultural method developed in this study, which is based on the isolation of the scald pathogen, is another choice of diagnostic method. However, since the assay requires an incubation period for scald colony development, it has a limitation for routine seed testing when large-scale seed testing is required within a limited time span.

Recently various molecular approaches have been widely applied in the detection and identification of diverse phytopathogenic microbes. However, attempts to use molecular approaches for detecting seed infection have received less attention. In the present study, a polymerase chain reaction (PCR)-based assay was developed as a potential alternative diagnostic method since it is highly sensitive, rapid, and cost effective to develop and there is no need for an incubation period.

A series of *R. secalis*-specific primers were designed based on the sequence analysis of the internal transcribed spacer (ITS) regions of rDNA of two isolates of *R. secalis* and one isolate of *R. orthosporum*. The sequence comparison analysis revealed that DNA sequences of the two isolates of *R. secalis* from cvs. Harrington and Manley were identical over a 627-bp stretch from the 3' end of the 18S rRNA gene to the 5' end of the 28S rRNA gene (Lee *et al.*, 2001a). However, there was 93% homology in the ITS regions between *R. secalis* and *R. orthosporum*. Sequence divergence found in the ITS1 (95% identity) and ITS2 (86% identity) regions between the two species suggested the potential value of the ITS sequence as a molecular marker for *R. secalis*. Five sets of primers, constructed from the divergent sequences within the ITS1 and ITS2 regions of *R. secalis*, produced fragments of expected size from the DNA extract of *R. secalis*. Of these primer sets, primer set E was selected for this study due to its high specificity. All primer sets produced detectable fragments from as little as 1 to 10 pg *R. secalis* DNA.

Lemma and palea lesions have been the basis for visual disease assessment of scald infection in barley seed (Kay and Owen, 1973). Therefore, seeds bearing no symptoms on the lemma and palea and seeds with visible symptoms were separately sorted and used for PCR analysis. PCR amplification of fungal DNA in a crude DNA extract from 0.1 g of seed samples allowed for the detection of a presumably small biomass of *R. secalis*. Effects of inhibitors of the PCR reaction commonly found in the DNA extracts from plant materials were overcome by diluting the *R. secalis* DNA from 10- to 100-fold with sterilized distilled water. The PCR amplification revealed the presence of *R. secalis* in the series of *R. secalis*-infested powdered seed samples. Even though a difference in the band intensity for each level of *R. secalis*-infected samples was found, sample to sample variations were observed in different replications of samples prepared for the lower levels of *R. secalis* infestation (<2%). The variability in PCR results of the five independent experiments may be attributed to variations in inoculum loads in the samples. In some cases, the DNA extracts from seed powder that had 0% visual infestation with *R. secalis* produced a faint positive band, while the negative control samples without *R. secalis* DNA did not produce any bands. The result suggested that high sensitivity of the PCR assay allowed for the

detection of symptomless infection of barley seeds, as reported by Kay and Owen (1973).

In this study, an attempt to construct *R. secalis*-specific primers to detect the pathogen from barley seed, which was conventionally assayed by visual disease assessment, was successful. In comparison to those PCR-based assays used for the detection of other seedborne pathogens, which require long incubation periods for completion of the assay (Smith *et al.*, 1996; Taylor, 1993), the methodology described here only takes about one working day from DNA extraction to PCR detection, without a pathogen enrichment period. In addition, the method described is more sensitive and less laborious compared to the cultural method, which takes about 7-10 days for incubation (Lee *et al.*, 1999).

Following initial infection by *R. secalis* at early stage of the seed development, the pathogen continues the infection process during the seed maturation stage thus giving rise to typical scald symptoms. However, if seeds are infected at later stage of seed maturation, they may remain symptomless. The incidence of seedling infection may be dependent upon the spread of inoculum from the residue, as well as from both the visible and latent (symptomless) seedborne inoculum (Jackson and Webster, 1976). In U. K., Habgood (1971) and Kay and Owen (1973) reported the presence of symptomless infection. Since then, despite the prevalence of seedborne infection as a source of inoculum, latent infection of seeds has rarely been reported. Therefore, the present study was conducted to demonstrate the presence and extent of symptomless infection in barley seeds by using and comparing a cultural method and a PCR-based diagnostic method.

Seed samples of the susceptible cv. Harrington, collected from Calmar, Alberta in 1996 were used for the study. Twelve seeds were taken and inspected visually for the presence of symptoms and used for the cultural assay according to the method described by Lee *et al.*(1999). Six batches of seeds were tested. The PCR-based assay was also used to monitor the symptomless infection of barley seed. Five sets of 100 seeds of susceptible cv. Harrington and two sets of 100 seeds of resistant cv. Seebe were individually examined for the presence of lesions, followed by DNA extraction according to the method by Lee *et al.* (2001a).

As a result of six independent trials of the cultural assay, an average of 14% of the seeds without symptoms gave rise to scald colonies. This incidence of symptomless infection of seeds was somewhat higher than reported earlier, which ranged from 6% to 10% by Kay and Owen (1973). The use of susceptible cv. Harrington, which had higher seed infection rates, may account for this result.

When seed samples without visible symptoms taken from cv. Harrington were used for PCR amplification using the pathogen-specific primers, the expected size of a single fragment was produced in all sub-samples tested, whereas no PCR products were amplified in DNA extracts from cv. Seebe. The results of the present study suggest that conventional visual assessment of the seeds underestimates the extent of seed infection due to latent infections (Lee *et al.*, 2001b).

One of the major disadvantages of PCR-based diagnostic methods in seed health testing is to quantify the levels of infection in seed samples. Methods of competitive PCR have been developed which take into account the intrinsic unsuitability of this technique in quantitative studies.

In an earlier study, a series of *R. secalis*-specific primer sets were designed from the internal transcribed spacer (ITS) regions of ribosomal RNA gene of the pathogen and used for detection of the pathogen in barley seed. Although the assay described above allowed for the detection of 2% seed infection or less, it was difficult to obtain reliable quantitative data by comparing band intensity as a result of several factors affecting PCR amplification efficiency. Therefore, the present study was carried out to adapt a qualitative PCR assay for quantitative analysis by introducing an internal control and examining the potential of the assay for quantification of *R. secalis* infection in barley seed.

In order to make the assay quantitative, a primer set RS1 and RS 3 was used to construct the internal control DNA, which competes for the same primer set during the PCR reaction thus allowing for quantification of the initial DNA concentration of the target DNA. Primer set RS1 and RS 3 amplified a *R. secalis*-diagnostic band (375 bp) that could be easily differentiated from the internal control DNA band (445 bp) and showed a higher sensitivity at 55°C annealing temperature than other primer pairs

tested in an earlier study. *R. secalis* DNA, over the range of 5 pg to 10 ng, was quantifiable in the presence of 5 pg of an internal control template DNA.

The utility of the competitive PCR assay for quantification of *R. secalis* in barley seed was demonstrated by using the artificially mixed samples, which had different levels of seed infection. Although, there were slight differences in the size and shape of lesions when symptoms on lemma and palea were examined, the competitive PCR assay using different levels of seed infection generated the standard calibration curve ($R^2 = 0.9401$), which appeared to be linear over the increasing levels of seed infection. However, a higher variation was found in samples with higher seed infection. Differences in loads of fungal biomass may account for this variation.

In order to examine the potential use of the standard curve in quantifying seed infection, seeds were taken from each yearly batch of field-infected seed from 1995-1999 and used for competitive PCR assay. The resulting PCR product ratios were converted into ng mg^{-1} plant material and compared with those resulting from the standard curve described above. Although, in the case of artificially constructed seed mixtures, relatively good correlation between visual disease assessment and the competitive PCR was found, there was poor correlation in the experiments using naturally infected seed samples. Factors contributing to this discrepancy could be due to the presence of symptomless infection reported elsewhere (Kay and Owen, 1973; Lee *et al.*, 2001a), some seeds with ambiguous symptoms mistaken as those caused by *R. secalis*, and natural variations of the disease loadings in the seed samples due to disease severity. Therefore, in order to help obtain accurate quantification results, application of appropriate statistical methods to seed sampling and the size of samples will need to be investigated in future.

The competitive PCR approach has some drawbacks such as laborious optimization procedures, a limited range of quantification and sample variation caused by increased handling. However, our results suggest that this competitive PCR assay, when optimized, would provide an accurate means of quantifying *R. secalis* infection in plants and predicting levels of seed infection. Therefore, this method offers a reasonable alternative to conventional seed health testing and also provides a sensitive tool in epidemiological studies of barley scald.

Because the causal fungus of barley scald is primarily stubble-borne, control measures such as proper residue management and crop rotation practices have been effectively used in reducing the levels of primary inoculum in a field (Mayfield and Clare, 1984; Skoropad, 1965). However, the widespread use of conservation tillage coupled with continuous cropping of a single cultivar have resulted in increased disease severity and significant yield and quality losses (Khan and Crosbie, 1988). Therefore, other disease management strategies such as the use of resistant barley cultivars, application of foliar fungicide, and managing soil fertility should be considered in areas where barely is grown year after year.

This study was conducted primarily to investigate the effects of rotations involving combinations of barley cultivars with different genetic backgrounds of resistance and triticale on barley scald epidemics in subsequent barley cultivars and the effects of different fungicide regimes to control the disease. In addition, the quantitative PCR assay was used to determine its potential to assess the differential effects of fungicide timing and rotation sequences on head infection in comparison to the VDA. The relationship between other disease variables, yield loss, PCR and VDA were investigated.

Seed samples obtained from fungicide timing and rotation trials during the 1999 and 2000 growing seasons were provided by Dr. Turkington from the Agriculture and Agri-Food Canada, Lacombe Research Centre, AB. In both the 1999 and 2000 Tilt®-timing trials, fungicide application resulted in significant reductions in scald infection on leaf and head. Although application of a ½ dose of Tilt® each at the flag-leaf and heading stages led to the most effective control of leaf infection for both yearly trials, the results of head infection measured by VDA or PCR showed different treatment effects for the 1999 and 2000 trials. In the case of the 1999 trial, head infection measured by VDA and PCR indicated that a single application of Tilt® at the flag-leaf stage was more effective in controlling head infection than application of a ½ dose of Tilt® each at the flag-leaf and heading stages. However, for this trial, only results of PCR discriminated the treatment effects. Although the PCR results agreed with the VDA results in 1999, PCR and VDA results revealed differences between treatment effects in the 2000 trial. VDA results indicated that a single application of Tilt® at the

heading stage was most effective in reducing head infection. The second most effective treatment was application of a ½ dose of Tilt® each at the flag-leaf and heading stages. However, PCR results showed significant reductions of head infection across all treatments compared to the untreated plots. In the case of PCR results measured by the DNA contents of *R. secalis* in seeds, applications of a ½ dose of Tilt® each at the flag-leaf and heading stages appeared to be most effective in head infection. Unlike the 1999 trial, a single application of Tilt® at the flag-leaf stage appeared ineffective in reducing the levels of head infection. This result suggested that head infection was influenced not only by the application of fungicide but also by the environmental conditions prevailing during the heading stage. In general, application of ½ dose of Tilt® each at the flag-leaf and heading stages appeared to be effective in controlling foliar infection as well as head infection since it protects barley heads from fungal infection even under favorable environmental conditions. Khan (1986) and Scott *et al.* (1992) also reported similar results that repeated fungicide applications resulted in almost complete control of the leaf infection and yield increases. Although, significant pairwise correlations among the levels of PLAD, PLAD-1, TKW, and THINS % were found, the levels of head infection were not significantly correlated with TKW except for the PCR results of the 2000 Tilt® trial. Since flag leaves are known to be responsible for the head filling and maturation, the levels of leaf infection appeared to affect yield components.

For rotation trials, in 1999, rotation sequences involving the use of the resistant or intermediate cultivars after intermediate, susceptible, resistant cultivars or triticale (cv. Wapiti) resulted in reductions of PLAD, PLAD-1, VDA, and PCR. Of rotation treatments, growing a non-host crop, triticale followed by barley showed effective reductions of scald infection on the leaf and head. A reduction in disease severity was also found on rotation treatments ending with resistant cv. Kasota. Statistical analysis of the levels of head infection measured by PCR and VDA showed that VDA scores did not reveal significant differences among rotation treatments. In contrast, PCR results generally agreed with the results of leaf infection measured by PLAD and PLAD-1.

In the 2000 rotation trial, rotation sequences resulted in significant differences in the results of PLAD, PLAD-1, VDA, and PCR. Barley scald severity on the leaf and head was lower following combinations of a resistant cultivar in the first year, resistant, intermediate, susceptible cultivars or triticale in the second year and a resistant cultivar in the final year when compared to the continuous cropping of single cultivars or other rotation sequences. While rotation treatments ending with cv. CDC Earl resulted in higher levels of disease on flag leaves, rotation treatments ending with cv. Harrington led to higher disease ratings on barley heads. A one year rotation with triticale had no significant effect on reduction of scald disease when compared with the rotation sequence involving the resistant barley cv Kasota. Although there were differences in cultivar reactions to scald infection on barley heads, the results for levels of head infection within rotation treatments ending with the same cultivar appeared to be insignificant.

Correlation between *R. secalis* DNA content measured by PCR and VDA scores appeared to be insignificant except for the 2000 rotation trial. This discrepancy between the PCR and VDA results may be attributed to two factors. First, to the presence of symptomless infection caused by unfavorable environmental conditions when the barley head was most susceptible to *R. secalis* infection, and secondly, to overestimated seed infection levels due to some ambiguous symptoms caused by other saprophytes or pathogens. Incidence of seed infection may be dependent on environmental conditions at the flowering and seed maturation phases of plants, cultural practices, cultivar susceptibility, and the severity of scald infection in the fields (Skoropad, 1959). Of these factors contributing to head infection, unfavorable environmental conditions during flowering and seed maturation stages and infection late in the season may not allow for the development of typical scald symptoms thus leading to symptomless infection of seeds (Kay and Owen, 1973; Lee *et al.*, 2001b; Skoropad, 1959).

Correlation analysis for both of the Tilt®-timing and rotation trials revealed that the results of PCR and VDA were inconsistently associated with the levels of leaf infection (PLAD, PLAD-1). This discrepancy might be attributed to the unfavorable environmental conditions during the stage when barley heads were susceptible to

scald infection. Kay and Owen (1973) also reported that the amount of visible infection of grains and the amount of leaf infection in parent crops were not correlated. However, overall PCR results had more significant relationships to the levels of leaf infection (PLAD, PLAD-1).

Molecular and pathological diagnostic methods developed in the present study have successfully been used for detection, differentiation and quantification of the scald pathogen in barley seeds and shown potential as an alternative seed health testing method. Although the role of the seedborne phase of the disease has received less attention and much research still needs to be done, effective control of the seedborne source of inoculum through appropriate seed health tests will reduce the use of unnecessary chemicals and reduce the danger of introducing the disease or new races into uncontaminated areas.

8. 2 Future Considerations

1. Need for a multiplex PCR assay

Fungal pathogens compete for ecological niches and are often present in plants as complexes. Therefore, it is desirable to detect more than one economically important pathogen at a time to reduce the cost and time required for the assays. Barley seeds are attacked by several seedborne pathogens and each pathogen may serve as important primary inocula under favorable environmental conditions. Therefore, development of a multiplex PCR assay that allows for detection of several economically important seedborne pathogens will be beneficial.

2. Need for studies on the contribution of seedborne inoculum of *R. secalis* to the development of foliar disease

Although seed transmission of *R. secalis* has been demonstrated here amd elsewhere, further research is required to address such questions as the importance of the contribution of infected seed to the disease epidemics under field conditions, and the effect of particular levels of seed infection on subsequent yield losses.

3. Need to establish seed infection thresholds

Seed health testing is undertaken primarily to manage disease by inoculum thresholds, to determine the potential effect of seedborne inoculum on stand establishment in the planted field, and to meet requirements for phytosanitary certification of seed lots to be exported. Effective application of seed health testing data is dependent on knowledge of inoculum thresholds for transmission of pathogens. To be of value, the thresholds should be established in well-designed experiments.

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