Histopathological Study of Barley Cultivars Resistant and Susceptible to *Rhynchosporium secalis*

K. Xi, P. A. Burnett, J. P. Tewari, M. H. Chen, T. K. Turkington, and J. H. Helm

First and sixth authors: Alberta Agriculture, c/o Lacombe Research Centre, 6000 C & E Trail, AB, Canada T4L 1W1; second author: Agriculture & Agri-Food Canada, Lethbridge Research Centre, P.O. Box 3000, Lethbridge, AB, Canada T1J 4B1; third author: Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada T6G 2P5; fourth author: Medicine/Dentistry Electron Microscopy Unit, Surgical-Medical Research Institute, University of Alberta, Edmonton, AB, Canada T6G 2N8; and fifth author: Agriculture & Agri-Food Canada, Lacombe Research Centre, 6000 C & E Trail, Lacombe, AB, Canada T4L 1W1.

Accepted for publication 4 October 1999.

ABSTRACT

Xi, K., Burnett, P. A., Tewari, J. P., Chen, M. H., Turkington, T. K., and Helm, J. H. 2000. Histopathological study of barley cultivars resistant and susceptible to *Rhynchosporium secalis*. *Phytopathology* 90:94-102.

Differences in the penetration process by *Rhynchosporium secalis* were compared in resistant and susceptible barley cultivars at the seedling stage. Percent penetration and percent host cell wall alteration (HCWA) differed significantly among cultivars and isolates as revealed by light microscopy. Based on these two variables, the cultivars were statistically separated into two groups that corresponded to their disease reactions. The resistant cultivars, Johnston and CDC Guardian, showed 81.2 to 99.4% HCWA and 0.1 to 20.1% penetration at encounter sites, whereas the susceptible cultivars, Harrington, Argyle, and Manley, had 30.1 to 78.3% HCWA and 31.8 to 81.8% penetration. In the current study, cv. Leduc, which is susceptible at the seedling stage and resistant at the adult stage, showed the same percent HCWA and penetration as did susceptible cultivars. A significant negative correlation ($P < 0.01$) was found between percent penetration and percent HCWA for cultivars inoculated with two isolates of

the pathogen. Isolate 1 was less virulent than isolate 2 with respect to percent penetration and induced significantly fewer HCWA. Scanning electron microscopy showed various shapes of fungal appressoria but no apparent difference in host reaction between resistant and susceptible cultivars. Transmission electron microscopy revealed interactions between the host and pathogen at various stages of penetration. The resistant cv. Johnston responded by producing appositions, as evidenced by a layer of compact osmiophilic material deposited on the inner side of the cell wall. Infection pegs produced by conidia were unable to penetrate the cuticle where an apposition had formed inside. When penetration occurred in the susceptible cv. Argyle, cytoplasmic aggregates and separation of the plasmalemma were visible from the host cell wall, but the layer of compact osmiophilic material was not always present. Data based on light microscopic observations suggested that HCWA may be one of the mechanisms responsible for resistance that is characterized as penetration prevention rather than as a slow rate of mycelial growth after successful penetration. HCWA occurred in response to attempted cuticle penetration, suggesting that HCWA may produce chemical barriers that help to prevent penetration.

Scald of barley (*Hordeum vulgare* L.), caused by *Rhynchosporium secalis* (Oudem.) J.J. Davis, occurs worldwide (26). This disease has caused considerable yield and quality losses in central Alberta, Canada (19). The prevalence of this disease in this region is due to environmental conditions conducive to disease development and to intensive barley production. Scald of barley has been primarily controlled by the use of resistant cultivars (19). It is unclear, however, how resistant cultivars interact with the scald pathogen at the histological level.

The infection process in compatible interactions between *R. secalis* and barley has been extensively studied (3,6,11,14). Fungal development has been characterized by the growth of subcuticular hypha at an early stage, followed by penetration of epidermal cells and invasion of the mesophyll at later stages of infection (3). Histological information about potential mechanisms of resistance is limited. Inhibition of conidial germination (16) and increase in papilla size (15) have been suggested to be associated with cultivar resistance. Ayesu-Offei and Clare (3) observed that halos were more pronounced where penetration was unsuccessful compared with successful penetration within a susceptible cultivar. However, the extensive presence of cell wall appositions in susceptible cultivars led Lehnackers and Knogge (16) to conclude that resistance mechanisms were not related to apposition formation. Scanning electron

microscopy (SEM) has been successfully applied by several workers (12-14,23) to reveal events including conidial germination, appressorium formation, and sporulation of *R. secalis*. Due, in part, to the low probability that an ultrathin section encounters penetration sites, transmission electron microscopy (TEM) has not been successfully used in revealing the penetration process. As a result, host-pathogen interactions taking place at early stages of infection have not been thoroughly studied.

The objective of the current work was to compare the histological differences between resistant and susceptible cultivars when challenged with *R. secalis*, with emphasis on the penetration process and quantitative analysis of host reactions.

MATERIALS AND METHODS

General description. Appositions and halos differ in morphology but are commonly produced in plant cell walls in response to fungal invasion. An apposition is visualized as a circular or semicircular ridge on the cell wall at the site of attempted or successful penetration. The ridge is formed by the continuing deposition of wall structural materials on the existing cell wall (25). A halo is characterized by a circular area at the center of the apposition and is the result of a change in cell wall chemistry (25). The occurrence of appositions and halos in barley epidermal cell walls is considered to be closely associated with the response of the host to *R. secalis* attack (15). As a result, host cell wall alteration (HCWA) is an appropriate term to describe the events that change cell wall physical and chemical characteristics and is applied to host reac-

Corresponding author: K. Xi; E-mail address: Kequan.Xi@agric.gov.ab.ca

Publication no. P-1999-1122-01R

© 2000 Department of Agriculture and Agri-Food Canada, Government of Canada

tions that include apposition and halo formation in the current study. For quantitative assessment of the interaction between barley leaves and the pathogen using light microscopy (LM), the occurrence of appositions and halos was grouped together under the term HCWA. Thus, HCWA refers to the occurrence of apposition and halo formation, either individually or together.

Pathogen and host reaction. *R. secalis* was isolated from the infected leaves of cv. Harrington (*H. vulgare*) that were collected from the Agriculture and Agri-Food Canada (AAFC), Lacombe Research Centre, Alberta, Canada. A single-spore isolate was produced from this collection and designated isolate 1. Another single-spore isolate was obtained from infected leaves of cv. Summit collected from the AAFC, Lacombe, and designated isolate 2. The fungus was cultured on Difco lima bean agar (Difco Laboratories, Detroit) at 15 to 20°C for 2 weeks. Conidia were harvested in sterile distilled water and filtered through two layers of cheese-cloth. Tween 20 was added to give a concentration of 0.1%, and the spore suspension was adjusted to 5×10^5 conidia per ml for inoculation.

Two to three cultivars were grown in a 14-cm-diameter plastic pot containing a soilless potting mix, and for each cultivar, there were four replicate pots. Each cultivar was in a separate clump produced from seven to eight seeds, and each clump was thinned to five plants after emergence. The ingredients of the mix were as follows: peat moss, 107 liters; vermiculite, 60 liters; perlite, 30 liters; superphosphate, 600 g; dolomite, 3,000 g; potassium nitrate, 500 g; calcium nitrate, 500 g; iron chelate, 16 g; fritted trace element, 28 g; slow-release fertilizer, 560 g (16-10-10 Type 70 Nutricote [Plant Products Co., Ltd., Brampton, Ontario, Canada]); and sodium borate, 14 g. The pots were in a phytochamber programmed at 20°C with a daily 14-h photoperiod at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ and watered twice weekly from the bottom. Plants at Zadoks growth stage 13 (33), usually 14 days after seeding, were used for inoculation. Plants were sprayed with the conidial suspension until runoff and were then kept in a mist chamber at room temperature to maintain relative humidity at 100% for 48 h. The inoculated plants were kept in a growth chamber at 20°C for 12 days before disease assessment using a 0 to 3 scale. The virulence of the two isolates was determined based on lesion development on the leaves of eight barley cultivars at the seedling stage. Isolate 2 was found to be more virulent than isolate 1 on several cultivars (Table 1), and the former sporulated about five times as much as the latter in vitro. As a result, the two isolates represented a range of virulence on the cultivars tested and were selected for use in the current study.

LM examination. Six barley cultivars were chosen for LM examination because they represent two-row malt barley and six-row general purpose barley varieties that have been extensively grown in western Canada (Table 1). In the first experiment, cvs. Argyle, Harrington, CDC Guardian, and Johnston were inoculated separately with isolates 1 and 2. In a second experiment, cvs. Leduc and Manley were inoculated with isolate 1 only. Plants were inoculated in the same way as described above, except for using a suspension of 6×10^6 conidia per ml to facilitate the location of conidia for microscopic examination. Second leaves were sampled 24, 48, 72, and 96 h after inoculation for subsequent examination.

Whole-mount preparations were used because they permitted sufficient differentiation between subcuticular hypha and leaf tissue. Leaf blade pieces of approximately 6×6 mm were cleared in a mixture of lactophenol/methanol/chloroform (5:3:2, vol/vol) for 24 h at room temperature. The leaf pieces were then stained for 1 h in 0.5% trypan blue in lactophenol (4). Leaf blade pieces were also cleared and stained with 1% potassium metabisulfite after basic fuchsin was used, to obtain sufficient differentiation. The cleared specimens were examined for pathogen penetration in relation to HCWA, including the formation of both cell wall appositions and halos in the host, with an Olympus microscope (model BX50; Olympus Optical Co., Ltd., Tokyo) using bright field microscopy. Penetration was recorded when a hypha was observed beneath the

encounter site. An encounter site was defined as an unsuccessful penetration (potential site) (1), as evidenced by the presence of an apposition without penetration, or as a successful penetration (actual penetration site) (1), as visualized by the presence of a cuticular hypha regardless of the presence of HCWA. At least 15 leaf sections with a total of 50 to 130 host cell-pathogen encounter sites for each treatment were examined. Subcuticular hyphal length, defined as the distance from the penetration site to the hyphal tip, of isolate 1 was measured for cvs. Johnston and Argyle 24, 48, 72, and 96 h after inoculation. About 20 measurements of hyphal length were taken from each combination of time and cultivar, and mean lengths were calculated and presented. The test for examination of encounter sites and for the length of hyphae was repeated once.

Sections of the sampled second leaves were also stained with 0.1% neutral red to examine the density and distribution of conidia. Staining was done by adding one drop of neutral red to leaf pieces (5 to 10 mm²) on a slide. Leaf areas with a high conidial density were excised and fixed in 3% glutaraldehyde in 0.01 M phosphate buffer at pH 6.8. The leaf sections were vacuumed, rinsed, and postfixed in 1% OsO₄ in Millonig's phosphate buffer and dehydrated in an ethanol series from 50 to 100%. The specimens were stained with 2% uranyl acetate in 70% ethanol for 0.5 h during dehydration. They were then embedded in Spurr's medium and sectioned at approximately 1 to 2 μm thickness with a glass knife using an ultramicrotome (Sorvall Porter-Blum MT-2; DuPont Co., Newton, CT). The sections were stained with 1% methylene blue in 1% borax and examined with an Olympus BX50 microscope.

Statistical analysis for LM examination. The interactions between barley leaves and pathogen encounter sites, observed with LM, were classified into one of three categories: HCWA without successful penetration, HCWA with successful penetration, and successful penetration without HCWA (25). Percent HCWA for each cultivar or isolate was obtained by dividing the number of HCWA by the total number of encounter sites, regardless of penetration. Percent penetration for each cultivar or isolate was obtained by dividing the number of sites when penetration occurred by the total number of encounter sites, regardless of HCWA. Percent HCWA and percent penetration were found to be similar at all sampling times (24 to 96 h after inoculation) for individual cultivars; therefore, data from samples taken 48 h after inoculation from repeated experiments were combined and subjected to analysis of variance. Leaf sections were considered to be the experimental unit. An arcsine transformation was applied to percent HCWA and percent penetration to equalize variance (29). Means were compared using Duncan's multiple range test. The relationship between percent HCWA

TABLE 1. Field reactions of barley cultivars (*Hordeum vulgare*) to *Rhynchosporium secalis* and disease severity at the seedling stage when inoculated with two pathogen isolates

Cultivar	Spike type ^x	Field assay ^y	Seedling assay ^z			
			Isolate 1		Isolate 2	
			Severity	Classification	Severity	Classification
CDC Guardian	2	R	1.1	MR	1.4	MR
Johnston	6	MR	1.0	R	1.0	R
CDC Earl	6	R	1.1	MR	1.4	MR
Diamond	6	R	1.4	MR	1.8	MS
Leduc	6	R	1.6	MS	1.6	MS
Manley	2	S	1.8	MS	2.1	S
Argyle	6	S	2.5	S	2.5	S
Harrington	2	S	2.6	S	2.8	S

^x Number indicates two-rowed or six-rowed barley in terms of spike type.

^y Reactions of adult plants to scald in the field according to Tekauz (31) and Xue et al. (32).

^z Scald severity was based on the average diseased area of 10 leaves of each cultivar using a 0 to 3 scale, in which 0 = no symptoms; 1 = 1 to 10% diseased area; 2 = 11 to 40% diseased area; and 3 = >40% diseased area. A rating of 0 to 1.0 was considered resistant (R), 1.1 to 1.4 was moderately resistant (MR), 1.5 to 2.0 was moderately susceptible (MS), and >2.1 was susceptible (S).

and percent penetration 48 h after inoculation was evaluated using Pearson's correlation analysis. All analyses were carried out using the Statistical Analysis System version 6.10 (SAS Institute, Cary, NC).

Electron microscope (EM) examination. For EM examination, cvs. Johnston and Argyle were used to detect differences in interactions to the fungus at the histological level. Under field con-

ditions, cv. Argyle is very susceptible and the leaves exhibit distinctive lesions, whereas cv. Johnston is resistant to most pathogen isolates. The plants were grown according to procedures described for LM examination. The leaves or leaf sheaths of inoculated plants of both cultivars were sampled 24 to 96 h after inoculation. For SEM preparations, the plants were sprayed with a conidial suspen-

Fig. 1. Light micrographs of responses of barley leaf cells to penetration by *Rhynchosporium secalis* 48 h after inoculation. **A**, A semicircular apposition (a) on the groove between adjacent epidermal cells of resistant cv. Johnston. The apposition was formed at the point of an attempted penetration by an appressorium (ap) from a *R. secalis* conidium (c). The section was stained in trypan blue. Bar = 20 µm. **B**, A circular apposition (a) on the cell wall of resistant cv. Johnston. An appressorium (ap) from a *R. secalis* conidium (c) is found at the point of an attempted penetration. The section was stained with trypan blue. Bar = 20 µm. **C**, A large halo (h) formed on the cell wall of resistant cv. Johnston in response to an attempted penetration by a *R. secalis* conidium (c). The section was stained with basic fuchsin. Bar = 5 µm. **D**, Responses from susceptible cv. Argyle. A successful penetration evidenced by a subcuticular hypha (s) from a *R. secalis* conidium (c) without a halo or visible apposition and an unsuccessful penetration with a halo (h). The section was stained with basic fuchsin. Bar = 5 µm. **E**, Responses from susceptible cv. Argyle. A subcuticular hypha (s) is present at the site of penetration with a small diameter halo (h1); an unsuccessful penetration is associated with a larger diameter halo (h2). The section was stained with basic fuchsin. Bar = 5 µm. **F**, A subcuticular hypha (s) in susceptible cv. Argyle after successful penetration. An apposition (a) is present at the site of penetration. The section was stained in trypan blue. Bar = 5 µm.

sion of isolate 1 and incubated in a mist chamber for 48 h at room temperature. Leaf pieces of 3 to 4 mm² were fixed in 3% glutaraldehyde and in 1% OsO₄ in 0.01 M phosphate buffer at pH 6.8 and dehydrated in an ethanol series. Samples were then CO₂ critical point-dried at 31°C for 5 to 10 min, mounted on stubs, and coated with gold in a sputter coater (Edwards S150B; Edwards, West Sussex, England). Specimens were observed using a HITACHI S-2500 (Hitachi, Tokyo) SEM.

For preparation of specimens for TEM, 12 µl of the conidial suspension was dropped in sheaths of the second leaves or leaves were spray-inoculated. Drop and spray-inoculated plants were grown in a pot using procedures similar to those previously described. The pot was then kept under a glass bell jar and incubated at 15 to 20°C for 48 h. Water was sprayed to the inner side of the bell jar to maintain relative humidity at 90 to 100%. After sampling, dehydration and embedding procedures were the same as those for the LM study. The procedures described by Zeyen and Bushnell (35) were followed to locate desirable areas for sectioning the embedded blocks. Ultrathin sections were stained with lead citrate for 5 min and washed using the procedures described by Chen (5). The specimens were observed with a HITACHI H-7000 TEM.

RESULTS

LM examination—Leaf sections. Failure of penetration was evidenced by the cessation of germ tube growth at the site of HCWA in the resistant cv. Johnston (Fig. 1A). The morphology of appositions was found to be variable among tissues, but not among cultivars. Semicircular appositions were usually observed on the anticlinal wall where an attempted penetration took place. Appositions as a complete circle were common on the periclinal wall (Fig. 1B). The differences in morphology of the apposition, however, may depend on the angle of viewing or the stage of fungal development. Aist (1) indicated that semicircular appositions result from the absence of or slight intracellular fungal development at an en-

counter site. When more advanced penetration has occurred, an apposition may form a complete circle, encasing the fungal infection structure that is present, such as an infection peg. For a substantial number of encounter sites, a halo was also observed at the point of attempted penetration in cv. Johnston (Fig. 1C). In the susceptible cv. Argyle, successful penetrations were observed with the presence of a subcuticular hypha growing out from the germ tube and no halo forming around the point of penetration (Fig. 1D). The failure of penetration with a different germ tube, but the same conidium, was associated with the formation of a halo (Fig. 1D). Occasionally, successful penetration also occurred despite halo (Fig. 1E) or apposition formation (Fig. 1F). However, successful penetrations were usually associated with the absence of appositions or with poorly developed appositions. In response to penetration, relatively larger halos were found in cv. Johnston compared with cv. Argyle. When transversely sectioned, the apposition was found to be a thickening on the inner side of the cell wall at the point of attempted penetration. The wall thickening observed in the current study using LM closely resembled the one described by Jorgensen et al. (15), although the dyes used for staining were different.

LM examination—Whole mounts. There were significant differences in percent penetration and percent HCWA among the six cultivars tested (Table 2). When inoculated with isolate 1, cvs. Harrington and Argyle were significantly different from cvs. CDC Guardian and Johnston. The four cultivars were separated into two groups that corresponded to disease reactions observed at the seedling and adult stages (Table 1). When inoculated with isolate 2, cvs. CDC Guardian and Johnston were significantly different with regard to percent penetration and HCWA. Differences in percent HCWA or penetration between cvs. Manley and Leduc inoculated with isolate 1 were not significant. There were significant differences ($P < 0.05$), on average, between the isolates; inoculation with isolates 1 and 2 resulted in 16.6 and 41.3% penetration and induced 87.9 and 67.8% HCWA, respectively. Although susceptible cultivars responded to inoculation with a considerable amount of HCWA, ranging

TABLE 2. Mean percent host cell wall alteration (HCWA) and percent penetration of untransformed data for four barley cultivars (*Hordeum vulgare*) 48 h after inoculation with two isolates of *Rhynchosporium secalis*

Cultivar ^y	Experiment 1				Experiment 2	
	Isolate 1		Isolate 2		Isolate 1	
	HCWA	Penetration	HCWA	Penetration	HCWA	Penetration
Argyle (S)	55.4 a ^z	52.4 a	40.0 a	73.1 a		
Harrington (S)	68.7 a	42.5 a	30.1 a	81.8 a		
CDC Guardian (R)	99.4 b	1.8 b	81.2 b	20.1 b		
Johnston (MR)	99.3 b	0.1 b	99.3 c	2.6 c		
Leduc (S)					73.2 a	33.6 a
Manley (S)					78.3 a	31.8 a

^y Letters in brackets indicate level of scald resistance in the field: S = susceptible, R = resistant, and MR = moderately resistant.

^z Numbers within a column followed by different letters are significantly different at $P = 0.05$ according to Duncan's multiple range test.

Fig. 2. Percent host cell wall alteration formation and penetration in cvs. Johnston and Argyle 24 to 96 h after inoculation with A, isolate 1 and B, isolate 2 of *Rhynchosporium secalis*.

from 30 to 78%, their level of penetration was always higher compared with resistant cultivars. For cv. Johnston, percent HCWA was consistently higher and percent penetration was consistently lower compared with cv. Argyle 24 to 96 h after inoculation with isolates 1 (Fig. 2A) and 2 (Fig. 2B). A significant negative correlation ($P < 0.01$) between percent HCWA and percent penetration 48 h after inoculation was found for all combinations of cultivar and isolate (Fig. 3). The average length of subcuticular hyphae observed for cv. Argyle (0.35 mm) was substantially longer than for cv. Johnston (0.01 mm) 96 h after inoculation (Fig. 4). The shorter subcuticular hyphae, on average, in cv. Johnston resulted from the fact that no hyphae were found at many encounter sites as a result of unsuccessful penetration.

SEM examination. The process of attempted penetration began with or without a germ tube. A cuticular bulge near a conidium indicated a successful penetration over the periclinal cell wall, despite the lack of an apparent germ tube (Fig. 5A). The resulting subcuticular hypha grew close to the anticlinal wall between two adjacent epidermal cells. Typically, one or both conidial cells germinated and there were usually varying-shaped appressoria at the germ tube tip, depending on the area of leaf through which penetration was attempted. The appressorium was either triangular (Fig. 5B) or round (Fig. 5C), filling the groove area above the anticlinal walls between two adjacent epidermal cells. A germ tube branched before penetration (Fig. 5D) or grew from an appressorium to another penetration site (Fig. 5E). A possible penetration attempt with a germ tube and without an apparent appressorium was also observed (Fig. 5F). There were no apparent differences between the resistant cv. Johnston and the susceptible cv. Argyle in the morphology of fungal infection structures or host reactions.

TEM examination. We were unable to locate any penetration sites in cv. Johnston with TEM preparations, presumably as a result of the highly resistant nature of this cultivar, although subcuticular hyphae were occasionally found under LM. In all observed cells in cv. Johnston, attempted penetration sites responded by development of an apposition consisting of highly electron-opaque and compact osmiophilic material between the wall and plasmalemma of the epidermal cell (Fig. 6A). In contrast to the uninfected cell wall (Fig. 6B), the plasmalemma layer in an infected cell tended to be separated from the inner cell wall and cytoplasmic aggregates were also observed. The origin and nature of the osmiophilic material were not determined.

Penetration processes in the susceptible cv. Argyle were monitored 48 to 96 h after inoculation. Before a noticeable penetration occurred, cytoplasmic aggregates containing vesicles were visible beneath the penetration site, but the compact osmiophilic material was not always present (Fig. 6C). Partial penetration of the cuticle of cv. Argyle by an infection peg and extracellular sheaths (ES)

were visible (Fig. 6D). The osmiophilic material under the cell wall of cv. Argyle was found to be less dense compared with that of an unsuccessful penetration site for cv. Johnston (Fig. 6A) and a successful penetration site for cv. Argyle (Fig. 6F). Thus, osmiophilic material in the resistant cultivar was found to be consistently dense, while density in the susceptible cultivar varied. The ES appeared to coat the appressorium and established a close connection between the germ tube and the cuticle. An attempted penetration was observed from an appressorium that pushed itself into the groove region of cv. Argyle (Fig. 6E). As a result, host cell wall morphology changed at the point of attack, cytoplasm aggregated, and the plasmalemma separated from the wall. However, no apparent apposition developed in response to the attempted penetration. A specimen sampled 48 h after inoculation showed that a complete penetration of the cuticle was associated with a layer of compact osmiophilic material on the inner side of the cell wall (Fig. 6F). As a result of this, the cell wall was considerably thickened and the breach of the wall caused by penetration was healed. Subcuticular hyphae were capable of invading tissues by breaking through the inner anticlinal wall (Fig. 6G). The leaf sheath of cv. Argyle was heavily infected 96 h after inoculation (Fig. 6H). No stomatal penetration was observed using LM or EM throughout this investigation.

DISCUSSION

Previous studies suggested that attempted cuticular penetration by *R. secalis* was prevented in resistant cultivars (4,9) and that papilla formation was considered to be associated with the resistance mechanism responsible for preventing penetration (15). The current study demonstrated a significant negative correlation between HCWA and penetration 48 h after inoculation. It also showed that susceptible cultivars had significantly higher percent penetration and lower percent HCWA compared with resistance cultivars 24 to 96 h after inoculation. Furthermore, there was limited hyphal growth in the resistant cv. Johnston as opposed to substantial growth in the susceptible cv. Argyle 24 to 96 h after inoculation. HCWA may be the consequence of a series of host reactions at various genetic and biochemical levels. However, at the histological level, the negative correlation between HCWA and penetration and the significant differences for these two variables between susceptible and resistant cultivars suggested that HCWA is a resistance mechanism operating at the early stages of the infection process by *R. secalis*. Given that resistance in the host is expressed at the stage of attempted penetration, we consider limited hyphal growth in the resistant cv. Johnston to be a consequence of the prevention of penetration rather than a resistance mechanism itself. Thus, infection events in relation to host reactions, monitored primarily from 24 to 48 h after inoculation, were sufficient to differentiate resistant

Fig. 3. Correlation between percent penetration by two isolates of *Rhynchosporium secalis* and percent host cell wall alteration on the leaf blades of six barley cultivars examined 48 h after inoculation.

Fig. 4. Hyphal length in cvs. Argyle and Johnston 24 to 96 h after inoculation with isolate 1 of *Rhynchosporium secalis*.

Fig. 5. Scanning electron micrographs showing the location of penetration on the host and the morphology of fungal structures for penetration. **A**, A conidium (c) of *Rhynchosporium secalis* on susceptible cv. Argyle 96 h after inoculation. A subcuticular hypha was indicated by superficial bulges (b) under the cuticle. Bar = 0.5 μ m. **B**, A triangular appressorium (ap) at the groove of susceptible cv. Argyle 48 h after inoculation. Bar = 0.2 μ m. **C**, A round appressorium (ap) in the groove of resistant cv. Johnston 48 h after inoculation. Bar = 0.5 μ m. The micrograph at lower magnification shows the conidium and germ tube. Bar = 1 μ m. **D**, A germ tube (g) of a conidium on cv. susceptible Argyle 48 h after inoculation. The tip of the germ tube was branched for penetration. Bar = 0.2 μ m. **E**, A germ tube (g) growing from an appressorium (ap) in the groove of susceptible cv. Argyle 48 h after inoculation. Bar = 0.5 μ m. **F**, An attempted penetration in resistant cv. Johnston. There is no apparent appressorium formation at the tip of the germ tube (g) 72 h after inoculation. Bar = 0.5 μ m.

Fig. 6. Transmission electron micrographs showing the development of infection structures of *Rhynchosporium secalis* after penetration and host responses. **A**, Cell wall reactions to penetration in resistant cv. Johnston 48 h after inoculation. There was an apposition formation in which deposition of highly electron-opaque osmiophilic material (om) between the wall (w) and plasmalemma (p) of the epidermal cell was observed. Bar = 0.5 μ m. **B**, Cell wall with attached plasmalemma (p) of resistant cv. Johnston from an uninfected cell. Bar = 1 μ m. **C**, Cell wall reactions in susceptible cv. Argyle 48 h after inoculation. Note cytoplasmic aggregates containing vesicles (v) at the site of penetration by *R. secalis* conidia (c) without apparent compact osmiophilic materials. Bar = 0.5 μ m. **D**, Initial penetration of susceptible cv. Argyle 48 h after inoculation. An infection peg (ip) from the appressorium that nearly breached the cuticle and an extracellular sheath (es) surrounding the appressorium (ap) were visible. There was less dense osmiophilic material (om) under the cell wall than that observed in **A** and **F** in response to penetration. Bar = 0.5 μ m. **E**, Attempted penetration in susceptible cv. Argyle 48 h after inoculation. The fungal germ tube pushed into the groove. Less electron-dense, amorphous materials (am) were deposited at both sides of the groove underneath the epidermal cell wall. Bar = 0.5 μ m. **F**, Successful penetration at the groove of cv. Argyle 48 h after inoculation. The cuticle and most parts of the epidermal wall were penetrated. An apposition consisting of a layer of compact osmiophilic material (om) was visible and cytoplasmic aggregates (ca) are present at the attempted penetration site. Bar = 0.5 μ m. **G**, Penetration in the groove of cv. Argyle 72 h after inoculation. A hypha (hy) penetrating the mesophyll cells was observed. Bar = 1 μ m. **H**, Heavy infection of the leaf sheath of susceptible cv. Argyle 96 h after inoculation. Many hyphae (hy) are seen under the cuticle (cu) and in the epidermis (e). Bar = 1 μ m.

from susceptible reactions. The inhibition of conidial germination was suggested to be a mechanism of resistance (16,26). However, we failed to observe differences in conidial germination between resistant and susceptible cultivars, as did Fowler and Owen (8). The discrepancy with previous studies might have been due to differences in the expression of resistance mechanisms among different fungal isolates and host cultivars. The suggestion that HCWA may be a resistance mechanism does not exclude other means of resistance. Reduced sporulation and increased latency period have been found to be associated with race-nonspecific resistance to *R. secalis* (8,9).

Field observations at AAFC, Lacombe, supported the conclusion from the current study that HCWA may be a mechanism that prevents penetration by *R. secalis* (K. Xi, A. G. Xue, P. A. Burnett, J. H. Helm, and T. K. Turkington, *unpublished data*). Field trials over a 3-year period showed that susceptible cultivars including Harrington and Argyle had significantly more lesions per leaf than the resistant cvs. Johnston, Leduc, and CDC Guardian. Moreover, the susceptible cultivars had a significantly larger lesion size compared with that of the resistant cultivars in only one of three seasons. These results and a previous study (9) suggest that lesion size was more associated with environmental conditions than with genotype and that an increase in susceptibility measured based on diseased leaf area was due primarily to an increase in lesion number. Consistent significant differences in lesion number suggested that resistance was expressed before successful penetration occurred. Cultivar resistance or susceptibility to scald is generally consistent between the seedling and adult stages (8), except for a few cultivars (32). The current results showed that disease reactions of the cultivars tested at the seedling stage were consistent with those reported at the adult stage, except for cv. Leduc. Although considered to be resistant in the field, cv. Leduc was moderately susceptible based on symptoms at the seedling stage and had the same magnitude of HCWA and penetration as the susceptible cv. Manley in the current study. It is noteworthy that Xue et al. (32) reported susceptibility of cv. Leduc at the seedling stage based on disease severity.

The process of appositional cell wall formation as a general mechanism of nonhost resistance to fungal penetration in the suborder Gramineae has been proposed (25). However, in the current study, the differences in percent penetration and percent HCWA between resistant and susceptible cultivars can only be determined quantitatively. Resistance and susceptibility were statistically differentiated based on observations using LM, but not EM. For TEM studies, it was impossible to generate a sufficient number of sections for quantitative analyses. Although there were quantitative differences in penetration and HCWA between the two isolates with LM, a negative correlation between percent HCWA and percent penetration occurred for both isolates, indicating a similar nature of reaction in the cultivars toward both isolates. Thus, HCWA may be considered a resistance mechanism in the barley-*R. secalis* system under investigation. *R. secalis* is characterized by extreme variability in virulence (31), rendering cultivar resistance ineffective. When resistance is overcome, it will be worthwhile to determine whether this is associated with a reduced percent or intensity of HCWA.

Our study supports the finding by Jorgensen et al. (15) that resistance is related to the size of apposition. The larger size of apposition appears to act primarily as a chemical barrier preventing the fungus from penetrating (15), since an apposition is formed on the inner side of the cell wall, whereas fungal infection structures were observed outside the cuticle (Fig. 6A). Aist (2) indicated that both apposition and halo formation were structural responses that may confer resistance by establishing a permeability barrier. Protection from fungal toxins and blocking the flow of nutrients and water to lesions are considered major functions of the permeability barrier. The current study also showed that, 48 h after inoculation, the susceptible cv. Argyle may or may not respond by producing appositions when penetration occurred. HCWA in susceptible cul-

tivars may not play a significant role in preventing penetration. A smaller size of halo (Fig. 1D and E) and a lower percent HCWA 24 to 96 h after inoculation (Fig. 2A and B) were demonstrated in the susceptible cultivar compared with those in the resistant cultivar (Fig. 1C). Zeyen and Bushnell (34) indicated that a successful penetration by *Erysiphe graminis* was likely to be associated with the inability of barley epidermal cells to complete the entire sequence of events in papilla deposition. The less dense osmiophilic material in the susceptible cv. Argyle compared with that in the resistant cv. Johnston may reflect such an inability of the cells in the susceptible cultivar to complete this sequence of events before attempted fungal penetration.

The groove between epidermal cells appears to be a common site for the fungus to initiate penetration. This area was reported to be rich in pectic substances (17), and the pathogen presumably produced pectic enzymes for penetration and subsequent growth. A phytotoxic glycoprotein isolated from *R. secalis* was found to elicit the formation of plant cell wall material (18). There is a need to identify host compounds that accumulate at the infection site to establish their role in resistance. Hydrolytic enzymes in the larger-sized papillae of a barley cultivar were suggested to confer resistance to *E. graminis* f. sp. *hordei* (30). Ride (22) suggested that cessation of fungal growth during unsuccessful penetration attempts may result from a growth inhibitor. It remains to be determined if HCWA are elicited by chemical stimuli from appressoria or germ tubes with the host responding by producing compounds that restrict fungal penetration around the HCWA. Callose was considered a major component of papilla (24), and the role of callose deposition in the prevention of penetration has been intensively studied. *E. graminis* was found to be involved in the induction of various compounds such as callose formation in the papillae of barley (28). Callose deposition was also observed in the form of collar formation (27) or encasements of the invading structures at the point of rust fungus penetration in cowpea (10). Thus, HCWA is closely associated with callose deposition in response to infection. Successful penetration by appressoria depends on the type of plant exudates (7) that may act as a signal. Nicole et al. (21) indicated that ES from fungi may play an important role in specific attachment in plant-fungus systems, allowing wall degradation and penetration. McRae and Stevens (20) found that ES contain specific proteins for recognition that may result in penetration. However, no substantial differences in morphology of the ES in the scald fungus were found between resistant and susceptible cultivars in the current study. It is unclear if the ES play a role in determining resistance or susceptibility to *R. secalis*. Further studies are needed to identify interactions involving the infection sequence from signaling, recognition, and successful host penetration.

ACKNOWLEDGMENTS

We thank the Alberta Barley Commission and the Alberta Agricultural Research Institute for financial support. We also thank D. Orr for providing a pathogen isolate and technical assistance.

LITERATURE CITED

1. Aist, J. R. 1976. Papillae and related wound plugs of plant cells. *Annu. Rev. Phytopathol.* 14:145-163.
2. Aist, J. R. 1983. Structural responses as resistance mechanisms. Pages 33-70 in: *The Dynamics of Host Defense*. J. A. Bailey and B. J. Deverall, eds. Academic Press, Sydney, Australia.
3. Ayesu-Offei, E. N., and Clare, B. G. 1970. Processes in the infection of barley leaves by *Rhynchosporium secalis*. *Aust. J. Biol. Sci.* 23:299-307.
4. Ayres, P. G., and Owen, H. 1971. Resistance of barley varieties to establishment of subcuticular mycelia by *Rhynchosporium secalis*. *Trans. Br. Mycol. Soc.* 57:233-240.
5. Chen, M. H. 1993. A modified autowasher device for rapidly washing larger numbers of EM-grids. *Microsc. Res. Tech.* 26:177-179.
6. Döken, M. T. 1988. Some aspects of the host-pathogen interaction in leaf scald of barley caused by *Rhynchosporium secalis* (Oudem.) J.J. Davis. *J. Turk. Phytopathol.* 18:9-17.

7. Emmett, R. W., and Parbery, D. G. 1975. Appressoria. *Annu. Rev. Phytopathol.* 58:147-167.
8. Fowler, A. M., and Owen, H. 1971. Studies of leaf blotch of barley (*Rhynchosporium secalis*). *Trans. Br. Mycol. Soc.* 56:137-152.
9. Habgood, R. M. 1977. Resistance of barley cultivars to *Rhynchosporium secalis*. *Trans. Br. Mycol. Soc.* 69:281-286.
10. Heath, M. C. 1971. Haustorial sheath formation in cowpea leaves immune to rust infection. *Phytopathology* 61:383-388.
11. Hosemans, D., and Branchard, M. 1985. In vitro study of barley leaf scald: *Rhynchosporium secalis* asexual reproduction cycle—Host histology. *Phytopathol. Z.* 112:127-142.
12. Howlett, S. G., and Cooke, B. M. 1987. Scanning electron microscopy of sporulation in *Rhynchosporium secalis*. *Trans. Br. Mycol. Soc.* 88:547-557.
13. Howlett, S. G., and Cooke, B. M. 1992. More scanning electron micrographs of conidial production in *Rhynchosporium secalis* on barley. *Mycologist* 6:16-17.
14. Jones, P., and Ayres, P. G. 1974. *Rhynchosporium* leaf blotch of barley studied during the subcuticular phase by electron microscopy. *Physiol. Plant Pathol.* 4:229-233.
15. Jorgensen, L. H. J., Neergaard, E. D., and Smedegaard-Petersen, V. 1993. Histological examination of the interaction between *Rhynchosporium secalis* and susceptible and resistant cultivars of barley. *Physiol. Mol. Plant Pathol.* 42:345-358.
16. Lehnackers, H., and Knogge, W. 1990. Cytological studies on the infection of barley cultivars with known resistance genotypes by *Rhynchosporium secalis*. *Can. J. Bot.* 68:1953-1961.
17. Martin, J. T., and Junpier, B. E. 1970. *The Cuticles of Plants*. Edward Arnold, London.
18. Mazars, C., Lafitte, C., Marquet, P. Y., Rossignol, M., and Auriol, P. 1990. Elicitor-like activity of the toxic glycoprotein isolated from *Rhynchosporium secalis* (Oud.) Davis culture filtrates. *Plant Sci.* 69:11-17.
19. McLelland, M. B. 1989. Barley production in Alberta. Agdex 114/20-I. Alberta Agriculture, Food and Rural Development, Lacombe, Alberta, Canada.
20. McRae, C. F., and Stevens, G. R. 1990. Role of conidial matrix of *Colletotrichum orbiculare* in pathogenesis of *Xanthium spinosum*. *Mycol. Res.* 94:890-896.
21. Nicole, M., Ruel, K., and Ouellette, G. B. 1994. Fine morphology of fungal structures involved in host wall alteration. Pages 13-30 in: *Host Wall Alterations by Parasitic Fungi*. O. Petrini and G. B. Ouellette, eds. The American Phytopathological Society, St. Paul, MN.
22. Ride, J. P. 1978. The role of cell wall alterations in resistance to fungi. *Ann. Appl. Biol.* 89:302-306.
23. Ryan, C. C., and Grivell, C. J. 1974. An electron microscope study of the outer layers of barley leaves infected with *Rhynchosporium secalis*. *Aust. J. Plant Physiol.* 1:313-317.
24. Sherwood, R. T., and Vance, C. P. 1976. Histochemistry of papillae formed in reed canarygrass leaves in response to noninfecting pathogenic fungi. *Phytopathology* 66:503-510.
25. Sherwood, R. T., and Vance, C. P. 1980. Resistance to fungal penetration in Gramineae. *Phytopathology* 70:273-279.
26. Shipton, W. A., Boyd, W. J. R., and Ali, S. M. 1974. Scald of barley. *Rev. Plant Pathol.* 53:839-861.
27. Skalamera, D., and Heath, M. C. 1996. Cellular mechanisms of callose deposition in response to fungal infection or chemical damage. *Can. J. Bot.* 74:1236-1242.
28. Stanbridge, B., Gay, L. J., and Wood, R. K. S. 1971. Gross and fine structural changes in *Erysiphe graminis* and barley before and during infection. Pages 367-379 in: *Ecology of Leaf Surface Microorganisms*. T. F. Preece and C. H. Dickson, eds. Academic Press, London.
29. Steel, R. G. D., and Torrie, J. H. 1980. *Principles and Procedures of Statistics. A Biometrical Approach*, 2nd ed. McGraw-Hill, New York.
30. Takahashi, K., Aist, J. A., and Israel, H. W. 1985. Distribution of hydrolytic enzymes at barley powdery mildew encounter sites: Implications for resistance associated with papilla formation in a compatible system. *Physiol. Plant Pathol.* 27:167-184.
31. Tekauz, A. 1991. Pathogenic variation in *Rhynchosporium secalis* on barley in Canada. *Can. J. Plant Pathol.* 13:298-304.
32. Xue, G., Burnett, P. A., Helm, J., and Rossnagel, B. 1995. Variation in seedling and adult-plant resistance to *Rhynchosporium secalis* in barley. *Can. J. Plant Pathol.* 17:46-48.
33. Zadoks, J. C., Chang, T. T., and Konzak, C. F. 1974. A decimal code for growth stages of cereals. *Weed Res.* 14:415-421.
34. Zeyen, R., and Bushnell, W. R. 1979. Papilla response of barley epidermal cells caused by *Erysiphe graminis* and method of deposition determined by microcinematography and transmission electron microscopy. *Can. J. Bot.* 57:898-913.
35. Zeyen, R., and Bushnell, W. R. 1980. An in-block, light microscope viewing procedure for botanical materials in plastic embedments; with emphasis on location and selection of host cell-microbe encounter sites. *Can. J. Bot.* 59:397-402.