Comparative virulence of Pyrenophora graminea Ito et Kurib isolates and the inheritance of resistance to P. graminea in barley

by Richard Lee Ruff

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
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Abstract:
Studies were continued to identify genes for resistance in barley to barley leaf stripe disease (causal organism : Pyrenophora graminea Ito et Kurib.). Because of the variability of infection, inoculation techniques were studied. Eight media were developed to compare substrate effects upon infection of a barley cultivar's seedlings by a P. graminea isolate. Although infection mean differences of the cultures were not significant, cultures grown on diffusate from germinated barley seed had slightly increased infectivity.

Virulence of 24 P. graminea isolates was tested to determine the spectrum of resistance in three barley cultivars. Artificial inoculation of seeds was by the layered mycelium method. Both of the highly susceptible cultivars, 'Lami' and 'Summit', were found to have a low level of resistance to some of these isolates. 'Betzes' seeds inoculated with 18 of the isolates resulted in plants without symptoms. However, up to 16% symptomatic seedlings were found after inoculation with six other isolates. Specific resistance in these cultivars to the isolates was inferred.

The Turkish cultivars, Tokak and Yesilkoy, were each reciprocally crossed to Betzes to study the inheritance of resistance in barley to leaf stripe disease. Tokak and Betzes are highly resistant to infection, and Yesilkoy has a low level of resistance. Seeds of the crosses were produced through the F3 and BC F2 generations and inoculated by the layered mycelium method. Seedlings were grown for six weeks and divided into a class with disease symptoms or a class without symptoms.

Seedlings' reactions from the F1 and F2 generations of the Betzes X Tokak crosses fit models indicating that a single dominant gene was inherited from each parent. The lack of fit of the data in the BC F2 families indicated a modification of the dominant genes models. Seedlings' reactions indicated that a recessive gene was inherited from Yesilkoy. Qualitative gene action with multiple genes or quantitative gene action was inferred from the results of seed inoculations. A method to calculate the effects of penetrance and expressivity on expected gene ratios was discussed.

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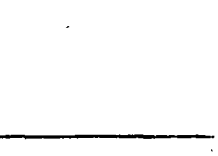
Signatue 

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CHAPTER 1

INTRODUCTION

Barley leaf stripe disease is seed-borne and systemic in barley, Hordeum vulgare L. The fungus, Pyrenophora graminea Ito et Kurib. is the causal organism. The anamorph stage is Drechslera graminea (Rab. ex Schlect.) Shoem., synonym Helminthosporium gramineum Rab. ex Schlect. Barley leaf stripe is a potentially threatening disease in barley growing areas thoughout the world. Early in this century, leaf stripe was one of the most significant, yield reducing diseases of barley. In the 1930's, the introduction of organic mercury seed treatments decreased the commercial importance of leaf stripe. Since the 1970's restricted use of these seed treatments, due to their long term effects in nature, has led to increased disease incidence.

The Pathogen. Ito (1930) described Pyrenophora graminea as the teleomorph stage of Drechslera graminea on barley in Japan. The description was based on

cultural characters and on the pathogenicity of the fungus. Alexopoulos and Mims (1979) placed the genus in the family Pleosporaceae, subclass Loculoascomycetidae. Pseudothecia of *P. graminea* are rarely reported in the literature. Smedegaard-Petersen (1973) described sclerotium-like bodies on barley straw collected from leaf stripe infected fields. The sclerotium-like bodies were superficially submerged and elongated with dark, rigid setae on the surface. Asci and ascopsores were seldom found, and no physical characteristics were given. Using inoculum from ascospore cultures, typical leaf stripe symptoms apparently developed on plants grown from inoculated seed (Smedegaard-Petersen, 1973).

Shoemaker (1962) described the conidial characteristics of the anamorph, *D. graminea*. Conidia were light green to medium yellowish brown. Cells within the conidia were shorter in length than width. Conidia measured 55-85 X 16-20 microns. Secondary conidiophores regularly formed on both apical and basal cells (Shoemaker, 1962).

Historically, the form genera *Drechslera* and *Bipolaris* were in the form genus *Helminthosporium*. *Drechslera* and *Bipolaris* form species were later removed from the *Helminthosporium* form genus. These two form

genera were differentiated from each other essentially by two characters, conidium shape and germination pattern. Alcorn (1983) questioned the validity of separating the two form genera by the germination of conidia. He reported wide variation in the number of cells which germinated in each conidium. Alcorn (1983) proposed the point of origin of the germ tube from the basal cell and the germ tube's direction of growth in relation to the long axis of the conidium as distinguishable characteristics. D. graminea produces germ tubes growing laterally from the basal cell of the conidium.

The Host. Research suggests that the progenitors of barley grew in western Asia (U. S. Department of Agriculture, 1979). The world center for diversity in barley is Ethiopia. Barley, H. vulgare, belongs to the grass family, Gramineae, tribe Hordeae. Cultivated barley is diploid with a basic chromosome number of seven.

The barley kernel consists of the caryopsis, lemma, palea, and rachilla. The caryopsis consists of the pericarp, integuments, endosperm, and embryo. As the kernel germinates, the embryonic root, or radicle, first emerges through the coleorhiza. Later, the primordial shoot, or epicotyl, emerges.

Barley grows best in well-drained, loam and clay-loam soils with a pH of at least 6.0. It does not tolerate flooded soils. Barley grows best where the ripening season is long and cool, and rainfall is moderate. In the lower latitudes or the temperate zones, barley is often grown during the cool season. Where drought, summer frost, or alkaline soils are encountered, barley is one of man's most dependable cereal crops. In recent years an average of 91 million hectares per year have been devoted to barley production worldwide (U. S. Department of Agriculture, 1979; Mathre, 1982).

The Host-Pathogen Relationship. Barley kernels become infected with the leaf stripe organism during development. The embryo is not infected. Platenkamp (1976) found mycelium in the pericarp of ungerminated kernels. She described infection of the coleorhiza as the kernel germinates. From the coleorhiza, the mycelium grew into the apical meristem. Teviotdale and Hall (1976a), using naturally infected seed, found mycelium in the seedling apex after 21 days of germination at 6 C. Platenkamp (1976) found hyphae thoughout the leaves and stems of 12-day-old plants naturally infected and grown at 20 C.

Once the mycelium invades the culm, rapid elongation of the internodes breaks the hyphal strands, and infection loci are established in the elevated plant tissues. The stem apex is invaded just before the spike emerges from the boot (Skoropad and Arny, 1956). With high humidity at the time of heading, conidia are produced on leaves of infected plants. These conidia are windblown to nearby heads where infection of the developing kernels occurs.

Low soil temperatures (6-14 C) during germination and early growth of seedlings enhance disease development. Prasad, Leonard, and Murphy (1976) found higher disease incidence with an intermediate soil moisture (-7.1 bars) compared with a wet soil (-1.0 bar) or a dry soil (-12.9 bars). Irrigation near heading enhanced kernel infection in the field (Metz and Scharen, 1979).

Disease symptoms may appear soon after emergence of seedlings, but they are often not evident until six weeks post-planting. One or more long, chlorotic stripes develop parallel to the leaf ribs, often extending from the base to the tip of the leaf. Later the yellow stripes turn brown or grey as the tissue becomes necrotic. The leaf blade may split and have a frayed

appearance. Growth of infected seedlings is often retarded, and plant death may occur at an early stage. In the field, heavy attacks arrested spike emergence, and barren spikes or improperly developed kernels resulted (Smedegaard-Petersen, 1976). Yield reductions of 0.6% to 1.0% for each percent of leaf stripe infected plants are reported (Richardson, Whittle, and Jacks, 1976; Tekauz, 1983).

Early attempts to control leaf stripe involved cultural practices and seed treatments. The hot water seed soak was an early control method. With the advent of organic mercury seed treatments, the disease was effectively controlled. Organic mercury is not currently available for seed treatments. Johnston, Metz, and Riesselman (1982) found promising results in tests of leaf stripe control with systemic chemicals. The use of these chemicals in many areas of continued leaf stripe disease occurrence, for example North Africa and the Near East, is not practical due to infrastructure problems. Control of leaf stripe disease by increasing host resistance is a viable alternative to chemical control.

This study was initiated to determine available genes in barley for resistance to leaf stripe disease. A preliminary objective was to improve the artificial

inoculation techniques of the disease organism onto barley kernels. This preliminary study is discussed in chapter one. In chapter two, data are presented on the variation in virulence of *P. graminea* isolates from several sources. Any attempts to develop resistant barley cultivars will have to take this variation into account. In the third Chapter of this thesis, experiments are discussed on the determination of genes for resistance to the disease. Two reciprocal crosses were made, and progeny were tested for resistance in the backcross (BC) and F_1 to F_3 generations. Results of inoculation tests indicated dominant and recessive gene action; however, these effects were not confirmed in BC F_2 and F_3 generations of crossed material.

CHAPTER 2

MEDIA EFFECTS ON INFECTION STABILITY OF *PYRENOPHORA GRAMINEA* ISOLATES WHEN ARTIFICIALLY CULTURED

Introduction

One problem with artificial inoculation of *P. graminea* is the variability in infection. *P. graminea* does not readily sporulate in culture, although researchers report various methods to induce sporulation of the fungus in culture (Paxton, 1922; Houston and Oswald, 1946; Teviotdale and Hall, 1976b; Sengupta and Singh, 1979; Tekauz and Chiko, 1980; Grbavac, 1981). Even with these methods, conidia are not produced in enough abundance for use as an inoculum source. In the literature (Arny and Shands, 1942; Houston and Oswald, 1948; Nilsson, 1975; Metz, 1978; Smedegaard-Petersen and Jorgensen, 1982; Konak, 1983; Tekauz, 1983) mycelia, with or without conidiospores, are reported to be the predominant inoculum either grown on a cereal kernel substrate or on artificial media.

Shands and Dickson (1934) reported variation in hyphal-tip cultures of *P. graminea*. They reported differences in culture morphology on PDA (potato dextrose agar), in pathogenicity tests, and in symptoms when single spore cultures were transferred by hyphal-tips. Shands and Arny (1944) and Arny (1945) used hyphal-tip cultures of *P. graminea* that had been maintained on artificial media for over twelve years. These cultures were stable and virulent on a large number of barley varieties.

Nilsson (1975) compared the virulence of newly isolated *P. graminea* cultures and old cultures grown on PDA. Virulence of the stored cultures was determined after two and nine months. Nilsson showed that some cultures lost little virulence, while others completely lost the ability to induce disease symptoms on plants. Metz and Scharen (1979) identified barley cultivars with various reactions to different *P. graminea* isolates. The cultivars varied in their disease reaction among replications. They proposed genetic factors, environmental conditions, or the inoculation technique as causes for the variations in reaction.

Konak (1983) reported a dramatic loss in virulence (from 95% to 2% seedling infection) of specific *P.*

graminea isolates inoculated to a single barley cultivar. J.G.N. Davidson (personal communication) found culturing pure isolates of P. graminea impossible. Therefore, fresh isolates were obtained from infected leaves for each experiment. However, different isolations made from a single leaf at the same time in the same way differed in virulence (0%-100% infection). Such variability between experiments produces results that are not comparable.

As a preliminary task to my inheritance study, I attempted to devise a method to decrease the infection variability of the barley leaf stripe disease organism. I concentrated on the growth substrate used to culture P. graminea prior to inoculation. With the cooperation of other researchers, eight media were developed for comparing the effects of the substrate upon infection of a specific barley cultivar. After culturing a P. graminea isolate on these media for eight months, only small differences were detected in infection percentages.

Materials and Methods

A single conidiospore isolate of P. graminea was taken from naturally infected leaf tissue grown from breeder's barley seed, RPB 43971 (obtained from Mr. V.

Stewart, Northwest Agricultural Research Center, Kalispell, Montana). The isolate was first grown on water agar medium. Two weeks later, a single 6 mm plug of mycelia and medium from the perimeter was transferred to the eight media that follow: 1. water agar; 2. barley leaf piece agar (Teviotdale and Hall, 1976a); 3. potato dextrose agar (PDA) amended with 2 grams spring wheat bran per liter of water; 4. PDA amended with 20 grams spring wheat bran per liter of water; 5. PDA amended with germinated barley seed diffusate (Appendix Table 12); 6. PDA amended with germinated barley seed extract (Appendix Table 13); 7. V8 juice; and, 8. barley leaf extract (as in Konak [1983] but with 30 g/l fresh barley leaves). Individual cultures were transferred by a single, 6 mm, mycelial bore to their respective media every two weeks from January to August 1983. A single conidiospore was again isolated in August and transferred to the same media. These newly isolated cultures were compared to the above cultures in an inoculation test.

Inoculum for the inoculation tests was prepared by autoclaving 30 g 'Ingrid' barley, C. I. 10083, with 30 cc distilled water in 1 pt Mason jars for 30 minutes at 121 C (Arny and Shands, 1942; Metz and Scharen, 1979; Konak, 1983). The jars were shaken within two to three hours

after removing from the autoclave to loosen the seed mats, and then they were allowed to cool overnight. The sterile barley kernels were inoculated with three 6 mm bores of mycelium and medium per jar. At this time approximately 4 cc sterile, distilled water was added to the jars, and the substrate was mixed well. Noninoculated checks were included in each experiment. The autoclaved barley kernels in jars were treated the same; however, the kernels were not inoculated with P. graminea mycelia. The jars containing inoculated and noninoculated kernels were incubated at 12-14 C (12 h light/dark cycles) for five to seven days.

'Summit' seed used in the experiments was obtained from Dr. E.A. Hockett, USDA, Montana State University. Prior to inoculating the 60-seed lots with P. graminea mycelia, the seeds were surface disinfected for three to five minutes in 10% Clorox (0.5% sodium hypochlorite) + 2% ETOH. Surface disinfected seed lots were allowed to dry on paper towels over night in a Microvoid IIC clean air chamber. The seed lots were then mixed with the previously prepared barley kernel inoculum.

One pt Mason jars of P. graminea mycelia on kernel substrate and a Summit seed lot were then placed in a refrigerator at 4 ± 2 C. The barley seeds were allowed to

germinate in this substrate for 12-14 days at which time most seeds had noticeable shoots. The entire mixture of germinated kernels, mycelia, and substrate was then planted in a 1:1 sand:soil mixture in 12 cm plastic pots. The pots were placed in a growth chamber at 16 C / 2 C ± 1 C (12 h light/dark cycle). Emergence of the seedlings was evaluated at 10-14 days after planting. Number of symptomatic plants was read at three, four, and six weeks.

The disease readings taken six weeks after planting were recorded as percent symptomatic plants of total emerged plants in each replication. Analyses of variance were calculated for each experiment and for the four experiments conducted over time, including the reisolation. Analyses were conducted on the Superbrain II microcomputer with statistics programs written by R. E. Lund (1983).

Results and Discussion

Table 1 lists the infection means (two replications each) of *P. graminea* grown on each medium for the four experiments. No significant differences (LSD 0.05) between means of the cultures grown on different media were noted in any of the planting times. In both 29

August plantings no significant differences (LSD 0.05) were calculated between any of the means of the cultures and the noninoculated check. These data indicate that the substrates did not affect the stability of virulence of this P. graminea isolate when serially cultured over eight months.

Table 1: Per cent of barley seedlings infected as a result of inoculation with cultures of Pyrenophora graminea isolate RPB grown on different media.

Media	Planting Date 3 Mar	5 Jun	29 Aug	29 Aug[a]
Noninoculated	00.0	00.0	00.0	00.0
Water agar	30.0	38.0	2.0	1.0
B L P A[b]	20.0	44.0	1.2	2.0
PDA + 1[c]	20.0	32.0	00.0	2.0
PDA + 10[d]	35.0	28.0	3.9	3.9
PDA + seed dif.[e]	38.0	51.0	2.0	2.8
PDA + seed ext.[f]	35.0	32.0	1.3	1.0
V 8 juice	30.0	44.0	2.9	0.93
B L Ext.[g]	24.0	45.0	00.0	3.6
Means[h]	25.0	35.0	1.5	1.5

[a] Trial using reisolated P. graminea cultures.
[b] Barley Leaf Piece Agar (Teviotdale and Hall, 1976a).
[c] PDA amended with 2 g/l spring wheat bran.
[d] PDA amended with 20 g/l spring wheat bran.
[e] PDA amended with germinated seed diffusate (see appendix Table 12).
[f] PDA amended with germinated seed extract (see appendix Table 13).
[g] Barley leaf extract medium (Konak, 1983, but with 30 g/l fresh barley leaves).
[h] Means of symptomatic seedlings per total emerged seedlings for each planting date.

A two-factorial analysis of variance for the experiments conducted over time was also calculated. Percentage of emerged plants that had symptoms was one factor, and the date that the experiments were conducted was the second factor. The results, including a mean comparison using a LSD of 0.05, are given in Appendix Table 14. The LSD (0.05) between culture means over time in this analysis indicated that the inoculum grown on PDA amended with germinated, barley seed diffusate had the highest percentage of infected plants. However, the eight month cultured test and the reisolation test (both planted on 29 August) resulted in extremely low infection levels on the susceptible cultivar, Summit. These low infection levels tended to increase the significant differences found between the cultures over the timed experiments as indicated by the relatively large F-value for planting dates in the analysis of variance.

Prasad, Leonard, and Murphy (1976) found that differences in soil water potential affected percent diseased plants grown from artificially inoculated seeds. Highest infections were in plants grown in soils of intermediate water potential (average of -7.1 bars). Infections decreased greatly in plants grown in soils with high water potentials. In my experiments water was

added to large pans containing up to 21 pots, and the water was absorbed by the soil. Without monitoring the soil water potential, high potentials were quite possible during emergence of the inoculated seeds. This may explain the overall low infection levels obtained in the plants in the last two planting dates.

Tekauz (1983) found varying levels of infection within replications of his tests with artificially inoculated barley seed. He proposed that the lack of good contact between the wheat kernel inoculum and the seed, due to clumping of the substrate, may have decreased infection levels. Also, he suggested minor environmental variations and possible partial contamination as causes for the variability in infection between replicates. In continuing experiments, I have isolated low levels of bacteria from the autoclaved barley kernels. Although mycelia were present on all the substrates at the time of planting in these experiments, partial contamination that would hinder infection is possible.

This comparison of culture media was begun with three P. graminea isolates. However, one isolate proved difficult to maintain on all the substrates. A second isolate gradually lost the ability to grow on some of the

substrates so that by October 1983, it was being maintained on only three of the substrates. Therefore, data from only one isolate were analyzed. No significant differences between the infection means of the cultures were detected for any of the isolates.

In the inoculation method used in this study, the inoculated seed was treated harshly. First, the seed was surface disinfected, then dried, and then placed with P. graminea inoculum growing on a kernel substrate. After germination, the seeds were tansplanted into a planting mixture. Any injury of these germinated seeds during transplanting resulted in decreased emergence. The production of inoculum on a substrate that can be mixed with the planting mixture would be beneficial. The surface disinfected seed could then be planted directly into the inoculum and planting mixture. Such an inoculum production method has been proposed recently (Miles and Wilcoxson, 1984).

The purpose of this study was to develop a more practical, efficient, and reliable method of artificially inoculating the leaf stripe organism onto barley seeds. Konak (1983) presented results indicating that P. graminea grew more profusely on a rich medium, his barley leaf extract medium. More recently, J.G.N. Davidson

(personal communication) stated that virulence was independent of the isolation method or the medium (V8 juice and water agar). My attempt was to extend the spectrum of substrates used. Since *P. graminea* becomes active and systemically invades the plant during germination, I amended a proven medium, PDA, with the diffusate and extract of germinated barley seed. Although mean differences of the cultures were not highly significant, an indication was obtained that diffusate from germinated barley seed did increase culture pathogenicity. These results may provide impetus for continued research into better inoculation methods with *P. graminea*.

CHAPTER 3

VIRULENCE OF *PYRENOPHORA GRAMINEA* ISOLATES ON SPECIFIC BARLEY CULTIVARS

Introduction

Christensen and Graham (cited in Stakman and Christensen, 1960) in a 1934 bulletin were the first to detail variation in virulence among single spore isolates of *Pyrenophora graminea* S. Ito & Kuribay. Virulence of 24 *P. graminea* isolates inoculated on 'Peatland', C. I. 2613, barley varied from almost 0% to 77% infection in a greenhouse experiment.

Arny (1945) detailed physiologic specialization among three *P. graminea* isolates tested on five barley cultivars. The results showed that one isolate was pathogenically distinct from two other isolates.

Kline (1971) reported resistance levels to barley leaf stripe disease in 82 winter barley cultivars. Kline wrote "Isolates did not differentially attack cultivars, and there was no evidence of pathogenic specialization among these isolates on these cultivars."

Several researchers have hypothesized physiologic specialization in pathogenic isolates of P. graminea. These researchers include Nilsson (1975) in Sweden; Metz & Scharen (1979) in Montana, USA; Tekauz and Chiko (1980) in Canada; and, Knudsen (1980) in Denmark. Knudsen (1980) stated that resistance in barley to his population of P. graminea was mainly determined by quantitative factors which would support the hypothesis of physiological specialization in the fungus.

Smedegaard-Petersen and Jorgensen (1982) conducted experiments on 28 barley cultivars using 15 P. graminea, single spore isolates collected in Denmark. Artificial inoculation of seeds was by the layered mycelium technique. They found considerable variation in virulence of P. graminea isolates on specific barley cultivars. The isolates also showed variation in virulence to all cultivars. They proposed that the separation of P. graminea isolates into different pathogenic races was indicated by these data.

Konak (1983) reported on virulence tests utilizing the barley cultivar 'Summit' with 27 P. graminea isolates obtained from Montana, USA, Turkey, and Ethiopia. Inoculation of seed was by a modified barley kernel

inoculum method. Variation in virulence of these isolates was 0% to 95.3% infection.

Virulence studies on *P. graminea* reported in this paper describe the variation in virulence of 24 *P. graminea* isolates from several sources on three barley cultivars that exhibit varying degrees of resistance.

Materials and Methods

Leaf samples from plants symptomatic of barley leaf stripe disease were collected in Tunisia, Egypt, Syria, Turkey, and Montana, USA. Leaf pieces were placed on moistened filter paper or water agar in Petri dishes after surface sterilization in 10% Clorox solution (approximately three minutes). After conidiophore and conidia production, masses of spores were transferred to water agar. Then, after 16-24 hours germination at approximately 15 C, a single conidium was transferred to barley leaf piece agar (BLPA) (Teviotdale and Hall, 1976a). Cultures were allowed to grow on BLPA for 12-16 days at 15 C, after which a single, six mm bore from the perimeter of growing mycelia was placed in autoclaved barley kernels (30 g 'Ingrid', C. I. 10083, barley + 36 cc distilled water). Mycelia grew on the barley kernels for 9-12 days at 15 C. Then, a single barley kernel with

mycelia was placed on PDA + 4 (4 g/l wheat bran) medium in each plastic Petri dish. Cultures were again allowed to grow at approximately 15 C for 12-15 days.

Three barley cultivars were used in these experiments: 'Betzes', C. I. 6398, originally obtained from Dr. E.A. Hockett, was increased at the Post Farm, Bozeman, Montana, and harvested in September, 1982; Summit seed, originally from North American Plant Breeders, was increased at Mesa, Arizona and harvested in April, 1983; and, 'Lami' seed, obtained from Ms. Sally Metz, was increased at Mesa and harvested in April, 1983. Forty-seed lots were surface sterilized for 4-5 minutes in 10% Clorox plus 2% ETOH, after which the seeds were dried in a Microvoid IIC clean air chamber for eight to twelve hours.

Inoculation was by the layered mycelium method (Shands, 1934; Houston and Oswald, 1948; Nilsson, 1975). Individual seed lots (40 seeds each) were placed on top of a layer of actively growing mycelia on PDA + 4 medium. Another layer of mycelia growing on the medium was placed over the top of the seeds. Petri dishes were sealed with Parafilm strips and placed in a growth chamber at approximately 8 C (12 h light/dark cycle) for seven days. At that time the seeds had germinated, and radicles were