

PROTEIN PROFILES OVER THE TIME COURSE OF INFECTION OF
Triticum aestivum BY Puccinia recondita f. sp. tritici

by

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B.S. Kansas State University, 1984

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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1986

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INTRODUCTION

Parasite:host specificity is the basis for control of plant rust diseases and has been well documented. The genetics of interaction of Linum and Melampsora lini was investigated by Flor (1955); he found that for every gene in the host for reaction there is a corresponding gene for pathogenicity in the parasite. Loegering (1966) extended Flor's ideas and concluded that the association of parasite and host is a living entity, and named that entity the "aegricorpus." He formalized his conclusions in a two dimensional model in which the genotypic contributions from parasite and host make up an aegricorpus genotype (Loegering 1984).

Loegering (1978, 1984) used the term "definitive" to describe parasite- and host-genotypes that interact together to produce a unique aegricorpus phenotype and to describe the unique aegricorpus phenotype itself. Browder (1985) extended the concept of definitive to describe environments in which the definitive aegricorpus genotype can function. Genotypes and environments that do not produce the unique aegricorpus phenotype are termed "nondefinitive". The symbols 1 and 0 are used to denote the definitive and nondefinitive conditions, respectively. The

symbols p, h, e, ag, and ap are used to denote parasite genotype, host genotype, environment, aegricorpus genotype, and aegricorpus phenotype, respectively. Within this symbolization, $1p \times 1h \times 1e = 1ap$. All other interactions produce nondefinitive aegricorpus phenotypes. The concept of definitives applies to all systems, whether the definitive phenotypes result in "resistance" (Table 1) or "susceptibility" (Table 2).

Rowell et al (1963) proposed a "quadratic check" as an analytical tool to discern physiological or biochemical differences related to the unique (definitive) interaction within gene-for-gene relationships. In the wheat leaf rust system (Table 1), low infection type has been shown to be the definitive phenotype.

Ellingboe (1982) proposed models of molecular interaction between parasite and host. One of the models describes direct interaction of host and parasite proteins, leading to a dimer that results in the definitive phenotype. Although models of parasite:host associations have been presented by Ellingboe (1982), Loegering (1984), and Browder (1985); the identification of a biochemical entity that relates to gene-for-gene interaction has not been reported.

The definitive aegricorpus phenotype of corresponding gene pair 1 ($1p_{1r1} \times 1h_{1r1}$) in Puccinia recondita:Triticum is a very small, necrotic lesion with no sporulation. Definitive temperature for $1p_{1r1} \times 1h_{1r1}$ is 20 C. The active function of $1p_{1r1} \times 1h_{1r1}$ occurs during the first 48 hr after infection (Browder and Eversmeyer 1986). This suggests the function of the definitive genotype is specific to time as well as temperature.

This thesis reports efforts to describe the specific protein differences that occur in the association of P. recondita and Triticum entities having and not having definitive genotypes relating to the Lr1 locus. These materials constituted a quadratic check in which the two host cultivars differed by a single gene. The two cultures, although not isogenic, have different pathogenicity genotypes relating to Lr1. Sampling at intervals over the course of infection was used to identify the time at which the gene-for-gene event occurs.

MATERIALS AND METHODS

Biological materials. The Triticum aestivum L. cultivar Thatcher and a near-isogenic line derived from Thatcher having Lr1 (Samborski and Dyck, 1968; Dyck and Samborski, 1968) were used as hosts. Puccinia recondita Rob. ex Desm. cultures PRTUS3 and PRTUS21, abbreviated as C3 and C21, respectively, were used. In the framework of the quadratic check, C3 had the definitive parasite genotype, 1p, and C21 had the non-definitive parasite genotype, 0p. Thatcher had the nondefinitive genotype, 0h, and the near-isogenic line LR1(TC) had the definitive genotype, 1h (Table 1). An environment, 1e, that allowed for the expression of the definitive aegricorpus phenotype, 1ap, was held constant for all members of the study.

Planting and sampling. Twelve seeds of each line were sown in 4 cm plastic pots for each culture and grown in a greenhouse for 7 days. Urediniospores of each culture were retrieved from liquid nitrogen storage and heat shocked at 40 C in H₂O for 5 minutes. The plants

TABLE 1. The Wheat Leaf Rust Quadratic Check

Environment	(20 C) 1e	
Parasite	Ip(C3)	Op(C21)
<u>Host</u>		
1h(LR1TC)	1ap "R"	0ap "S"
0h(TC)	0ap "S"	0ap "S"

TABLE 2. The Victoria Blight Quadratic Check

Parasite	Tox ⁺ (1p)	Tox ⁻ (0p)
Sens ⁺ (1h)	1ap "S"	0ap "R"
Sens ⁻ (0h)	0ap "R"	0ap "R"

were inoculated by dipping them into H₂O, on which about 0.2 gm of urediniospores were suspended (Browder 1971). Both inoculated and uninoculated plants were placed in a mist chamber at 100% relative humidity and 15 to 20 C for 8 hr. The inoculated and uninoculated plants were placed in separate growth chambers at 20 C.

Sampling began at the time of inoculation and continued until sporulation resulted from nondefinitive genotypes or necrotic flecks resulted from the definitive genotype. Four leaves, at least 7.5 cm long, were sampled from each treatment. One hundred and fifty mg aliquots were weighed in the first experiment; 400 mg aliquots were weighed in the second experiment. The sample material was ground with a mortar and pestle with 500 μ l of extraction buffer: 50 mM tricine, 25 mM KCl, 1 mM benzimidazole, 1 mM norleucine, 1 mM phenyl methyl sulfonyl-fluoride. The solution was centrifuged in an Eppendorf centrifuge for 3 minutes at 15,000 x g and the supernatant transferred to another container and recentrifuged for 10 minutes at 15,000 x g, the supernatant was then stored in a freezer at -80 C.

Fungal extractions. Puccinia recondita cannot easily survive and reproduce without living host tissue, which makes mycelial fungus extractions difficult. Urediniospores were germinated and analyzed for soluble protein profile in order to relate their contribution to the aegricorpus proteins. Germinated spores were scraped from the surface of the agar plates and mixed with the protein extraction buffer described above. The spore-buffer suspension was placed into containers and 0.05 mm zircon beads were added. The containers were then placed in a mini-bead beater (Biospecs Products, Inc.). The spore walls were

mechanically disrupted for 3 minutes, placed in an Eppendorf centrifuge and centrifuged at 15,000 x g for 3 minutes. The supernatant was decanted and stored at -80 C. Protein analysis was performed on 10% - 20% gradient polyacrylamide gels.

Gel preparation. Electrophoresis of the proteins in the samples was performed by using 10% - 20% gradient polyacrylamide slab gels (Guikema and Sherman 1981).

Sample preparation. Protein concentration of the samples was determined by the procedure of Bradford (1976). Aliquots of the samples were mixed with a sample buffer which contained (in equal volumes) 60% sucrose, 90% b-mercaptoethanol, and 20% lithium dodecyl-sulfate. The proteins were solubilized in a 70 C H₂O bath for 20 minutes.

Gel operation. The gels were exposed to constant power of 2.5 watts at 5 C. Fifty ug samples were loaded into each lane; fifty µg of molecular weight markers in 10 µl of buffer were loaded into check lanes. Current was applied to the gels until the dye-front reached the bottom edge of the gel (approximately 8 hr).

Staining. Gels were stained with 0.1% Coomassie-Blue stain in H₂O and methanol for 45 minutes and then destained overnight. Ten ml glacial acetic acid was added to each 200 ml of stain immediately before staining to fix proteins and thus decreased distortion in the bands of the gel. The gels were destained in a 200 ml portion from 1.4 liters distilled H₂O, 500 ml methanol and 100 ml glacial acetic acid. Photographs were taken with Kodak 2415 high contrast film.

Analytical design. Experiment 1 was used to correct inconsistencies in the extraction procedure. The results from experiment 2 were compared in two ways. First, samples of each aegricorpus genotype taken at the same time were compared on a single gel. Second, samples from all time increments of each aegricorpus genotype were compared in adjacent lanes. Electrophoresis of each sample in each organization was done twice.

RESULTS

In experiment 1, symptoms appeared 116 hr after inoculation for both host cultivars inoculated with culture C21 and 128 hr for Thatcher inoculated with C3. Necrotic flecks did not appear on LR1(TC) infected with C3 until 154 hr after inoculation (Table 3). In experiment 2, (Table 4), culture C21 sporulated with both hosts at 122 hr. Culture C3 with Thatcher began sporulating at 146 hr, and with LR1(TC) produced necrotic flecks at the same time.

Protein profiles were examined and data are presented in Figs. 1 through 9. Consistency of the number of clearly defined protein bands in all experimental treatments in all trials is indicated in Figs. 1 and 2. Thirty-five bands were resolved in each treatment, although at low protein concentrations some of those bands were not as clear as at higher concentrations. Results from samples representing all aegricorpus genotypes collected at 0 hr postinoculation are compared in Fig. 3. Results from the first experiment are shown on the left and results from the second experiment are shown on the right side of Fig. 3. The

TABLE 3. Time Intervals for protein extraction and symptom appearance: Experiment 1.

Interaction Member	0	8	20	32	44	56	68	80	92	104	116	128	140	154	
Thatcher (Control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lr1(TC) (Control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thatcher :C3	-	-	-	-	-	-	-	-	-	-	-	S	+	+	Chlorosis at 128 hr Sporulation at 132 hr
Lr1(TC) :C3	-	-	-	-	-	-	-	-	-	-	-	-	-	F	Necrotic fleck at 154 hr.
Thatcher :C21	-	-	-	-	-	-	-	-	-	-	S	+	+	+	Chlorosis at 116 hr. Sporulation at 120 hr.
Lr1(TC) :C21	-	-	-	-	-	-	-	-	-	-	S	+	+	+	"

TABLE 4. Time Intervals for protein extraction and symptom appearance: Experiment 2.

Interaction Member	0	8	14	20	26	32	38	50	74	98	122	146
Thatcher (Control)	-	-	-	-	-	-	-	-	-	-	-	-
Lr1(TC) (Control)	-	-	-	-	-	-	-	-	-	-	-	-
Thatcher :C3	-	-	-	-	-	-	-	-	-	-	-	S
Lr1(TC) :C3	-	-	-	-	-	-	-	-	-	-	-	F
Thatcher :C21	-	-	-	-	-	-	-	-	-	-	S	+
Lr1(TC) :C21	-	-	-	-	-	-	-	-	-	-	S	+

Figure 1. Complete profile at 0 and 8 hours postinoculation,
experiment 1

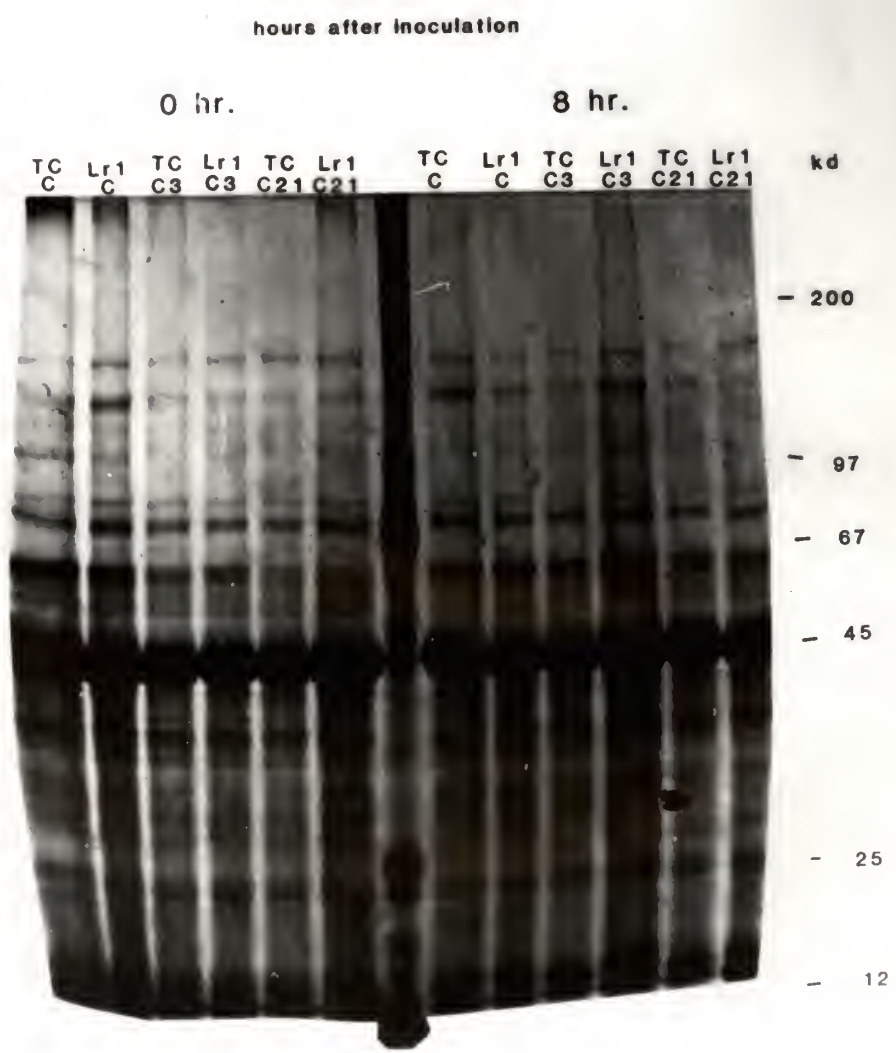


Figure 1

Figure 2. Complete profile at 38 and 50 hours postinoculation,
experiment 2

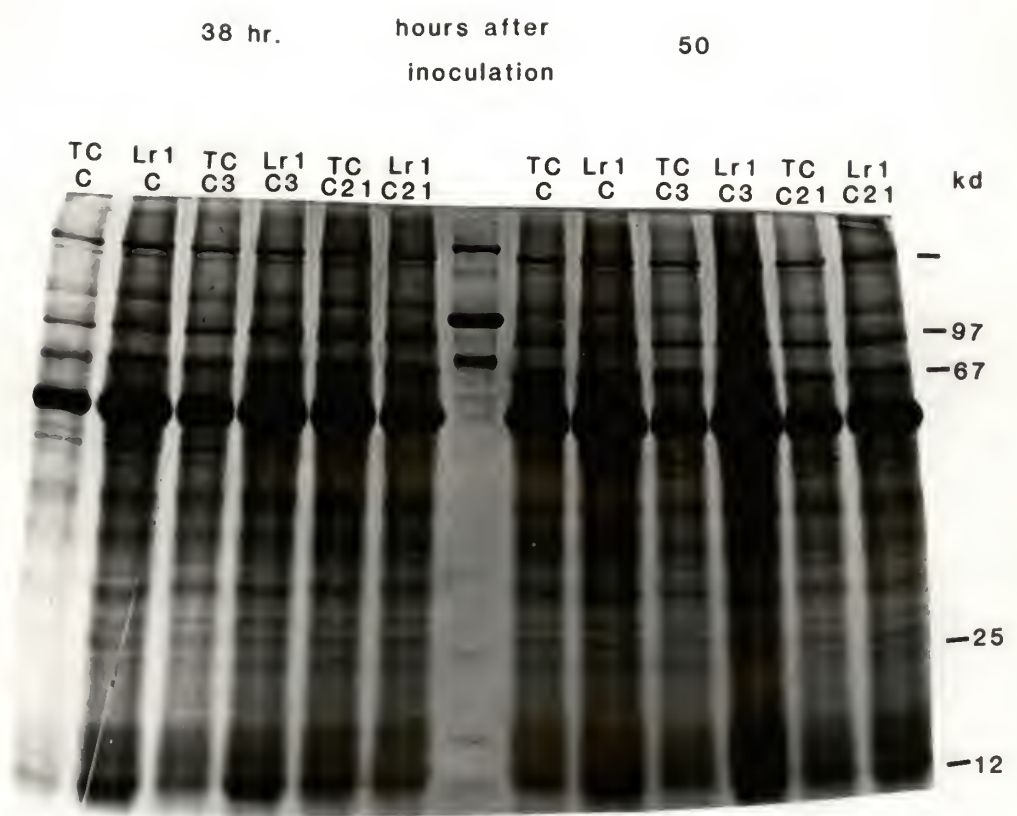


Figure 2

Figure 3. Comparative between experiments 1 and 2, at 0 hours
postinoculation

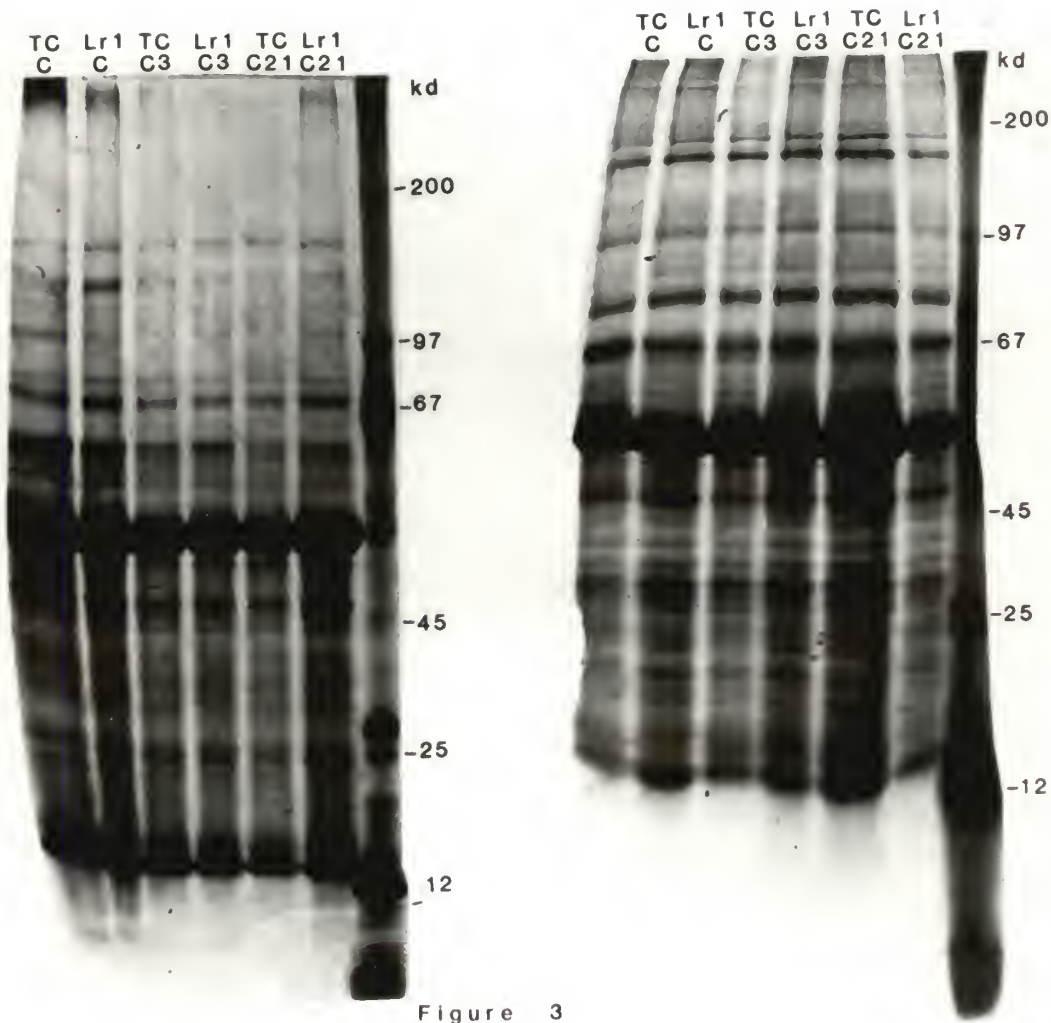


Figure 3

bands were consistent; not only within Fig. 3, but with Figs. 1 and 2 as well. Differences in resolution of protein sizes between Fig. 1 and Fig. 2 are evident, but the relation of distinct bands to the molecular weight markers was similar in all cases. This indicates reproducibility of the clearly defined protein profiles, even though protein concentration differences were present.

Some differences were observed in banding patterns in samples from different aegricorpus genotypes. These differences, however, occurred where bands were indistinct. These differences were difficult to reproduce. Results from the definitive aegricorpus genotype, represented by LR1(TC)/C3, at 14 and 20 hr postinoculation are compared to a nondefinitive aegricorpus genotype, represented by TC/C3, at the same times in Fig. 4. A 13 kd protein associated with the definitive aegricorpus genotype was observed in the profile at 14 hr but not at 20 hr. This change was observed only in the first trial. A 13 kd protein (illustrated in Fig. 4) was observed in the profile again at 38 hr but not at 50 hr after inoculation (Fig. 5). This change was also difficult to substantiate. A 45 kd protein was observed in the sample from LR1(TC)/C3 taken at 14 hr postinoculation, but not in samples from the other treatments (Fig. 6). This band was very thin and was not noticed until photographic enlargements were made. Results from LR1(TC)/C3 at 0 hr and LR1(TC)/C3 at 8 hr postinoculation are shown in Fig. 7. A 42 kd protein was observed in the 0 hr sample, but not in the 8 hr sample. The need for caution in making determinations from gels is demonstrated in Figs. 8 and 9. A 60 kd protein was present in TC/C3 samples at 0 hr and at 8 hr postinoculation, but was not present in the uninoculated

Figure 4. Comparative profiles between LR1(TC)/C3 and TC/C3
at 20 hours postinoculation

Figure 5. Comparative profiles between LR1(TC)/C3 and TC/C3
at 50 hours postinoculation

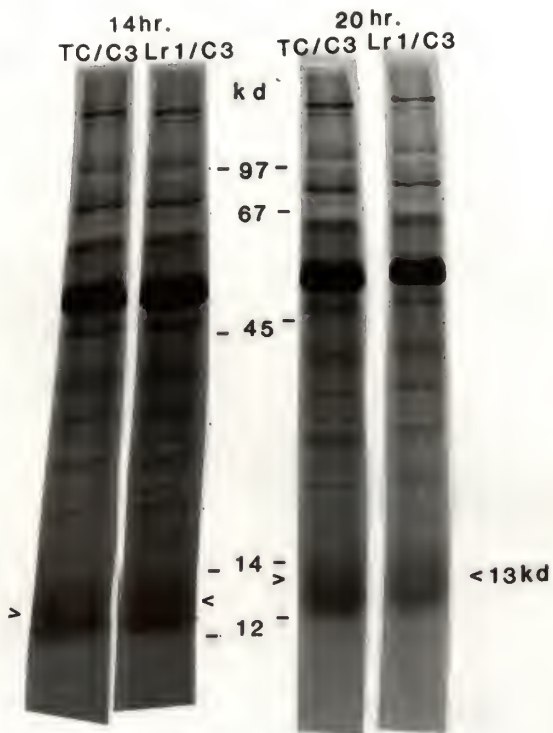


Figure 4

hours after inoculation



Figure 5

Figure 6. Comparative profiles between LR1(TC)/C3 and TC/C3
at 14 hours postinoculation

Figure 7. Comparative profiles between LR1(TC)/C3 at 0 and 8
hours postinoculation



Figure 6

hours after inoculation



Figure 7

Figure 8. Comparative profiles between TC/C and TC/C3
at 0 and 8 hours postinoculation

Figure 9. Comparative profiles between TC/C3 and TC/C21
at 0 and 8 hours postinoculation



Figure 8



Figure 9

Thatcher check (Fig. 8). The 60 kd protein was not observed in that position in TC/C3 samples at 0 hr or 8 hr postinoculation, even though the tests were made with aliquots of the same sample.

No comparison of fungal proteins to aegricorpus proteins was possible because of low protein concentrations in the fungal samples. The amount of fungal proteins for a given sample was one-tenth to one one-hundredth the amount of the aegricorpus proteins. If the fungal protein profiles were the same between the parasite:host association and fungal extractions, the fungal proteins from the leaf preparations were in such low concentration that resolution was not possible.

DISCUSSION

The clearly defined bands in the electrophoretic profiles did not differ, either between aegricorpus genotypes, within the same aegricorpus at different times during the association, or in different assays of the same samples. This was the most important result of these experiments. The technique did not resolve the presence of a protein (or proteins) that could be related to the function of the definitive aegricorpus genotype. Differences in some indistinct bands did occur in samples from different aegricorpus genotypes and from the same aegricorpus genotype sampled at different times, but this technique did not accurately reproduce them. Obviously, a different approach must be used to characterize differences and changes in the indistinct protein constituents of the aegricorpus.

From the data of Browder and Eversmeyer (1983a, 1983b, 1984, 1986) the active function in the wheat leaf rust system must exist in the definitive aegricorpus genotype residing in a definitive environment. Because the models of Loegering and Browder are supported by the genetic data, I conclude that the molecule(s) of interaction is (are) in low concentrations or exist only for short periods during the association. Membrane-bound proteins were not investigated in this study; therefore the possibility of a protein-protein interaction at the membrane interface between the parasite haustorium and the host plasmalemma was not eliminated. Ehrlich and Ehrlich (1963) demonstrated an encapsulating sheath around the fungal haustorium. The sheath keeps the host and pathogen membrane surfaces separated by a distance of 800 - 3400 angstroms. This would preclude the membrane-bound proteins of parasite and host from interacting directly, but does not disallow the interaction of secondary products from those proteins across the boundary of the encapsulating sheath.

Ellingboe's (1982) models to describing intracellular communication considered proteins, nucleic acids and carbohydrate groups. His first model describes the direct interaction of a host and a parasite protein to produce a dimer with a specific function. The second model describes a protein-glycoprotein (or complex carbohydrate) interaction mediated by gene-product enzymes. The third model describes the interaction of gene products from both host and parasite that activate other genes in the host, which stimulate the synthesis of antimicrobial compounds, phytoalexins. The fourth model, a variation of the third

model, assumes phytoalexin synthesis to be constitutive, but controlled by the gene products at a certain point in the synthetic process.

There is some evidence of the involvement of these types of molecules in the event that determines specificity. Hadwiger and Loschke (1981) demonstrated the involvement of a hexosamine (chitosan). Some studies have implicated oligosaccharides; Albersheim and Darvill (1985) have summarized current information from these studies. Holden and Rohringer (1985) concluded there were differences in glycoproteins between *P. graminis* and *P. recondita*, using inoculated and noninoculated samples, but did not relate this to parasite:host specificity. Rohringer (1974) suggested the involvement of nucleic acids in the determination of specificity. Thus, it is possible that the mechanism of specificity involves the interaction of proteins, carbohydrates, glycoproteins, nucleic acids, or any combination of them.

Other techniques must be used before proteins are unequivocally implicated in determining specific aegricorpus phenotypes. Some of these are immuno-precipitation of irrelevant parasite- and host-proteins that interfere with the sensitivity of electrophoresis (e.g. ribulose biphosphate carboxylase) and the use of radio-labeling during the course of infection to follow de novo protein synthesis.

Thus, the mechanism of specificity is unknown and will remain so until the information and ideas of subdisciplines of plant pathology, especially physiology and genetics, are coordinated. It is clear that whoever finds the answer to this incredible mystery must consider and integrate the ideas of many who have gone before him.

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ACKNOWLEDGMENTS

I wish to thank and express deep gratitude to the comajor professors of this work, Dr. Lewis E. Browder and Dr. James A. Guikema, this was definitely a cooperative project and their patience and understanding were extraordinary. This thesis research was financially supported by USDA-ARS, and my appreciation extends to that agency. There were many people who contributed their ideas and encouragement to this study. I would like to thank Dr. Jan E. Leach, Thomas May, Ralph Henry, T. H. McGrath, Dr. Tag EIDin Mohammed Shehab EIDin, Dr. Peter P. Wong, Mrs. Cathy Bolte, and C. Elburn Parker. The person I am most indebted to for her support, is my wife, Teresa. Without her love, understanding and patience this thesis would never have been completed.

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ABSTRACT

The interaction between two Triticum aestivum L. cultivars and two Puccinia recondita Rob ex Desm. cultures was investigated, using gel electrophoresis to examine soluble protein profiles. The quadratic check, designed to produce one incompatible interaction that could be compared with three compatible interactions, was used. Four parasite:host genotypes were sampled at time intervals from inoculation to appearance of symptoms.

Two extraction experiments were performed, using different time intervals, in the different experiments. Soluble proteins were extracted from whole leaf preparations. One dimensional polyacrylamide gels were made such that the gels were in a gradient of 10% at the top to 20% at the bottom. The protein fractions were organized in two different patterns, all interaction members for a single time increment and all time increments for a single interaction member, on individual gels.

No major changes were found, either between members of the quadratic check or during the course of infection for any of the interaction members. Minor changes were seen in certain gels but could not be reproduced.