HISTOLOGY AND ISOZYME ANALYSIS OF EARLY INFECTION OF NEARLY ISOGENIC SUSCEPTIBLE AND RESISTANT MAIZE CULTIVARS BY *PUCCINIA POLYSORA*

A Thesis

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 1989

Major Subject: Genetics

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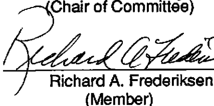
Clint W. Magill
(Chair of Committee)



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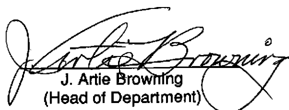

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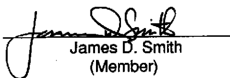

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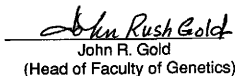

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ABSTRACT

Histology and Isozyme Analysis of Early Infection of Nearly Isogenic Susceptible and Resistant Maize Cultivars by *Puccinia polysora.* (May 1989)

Lynn Ann Hanson, B.S., University of Iowa

Chair of Advisory Committee: Dr. Clint W. Magill

Much attention has been concentrated on the study of phytoalexin production in plants in response to invasion by pathogens. There are many examples in the literature of fungitoxic compounds that are produced by plants upon infection. Many of these phenolic and related compounds are synthesized via the shikimic acid pathway.

Maize is an important crop plant worldwide, and many diseases caused by fungi occur on maize, reducing potential yield. Many of these diseases have already caused serious economic loss or have the potential to do so. However, in comparison to other plants, little is known about the resistance responses in maize to fungal or other pathogens. For this reason, the possible involvement of phenolic compounds in the resistance response of maize to *Puccinia polysora*, incitant of southern rust on maize, has been investigated.

Two nearly isogenic maize cultivars, B37 (susceptible) and B37R (resistant), were used to study the early histological events in the host-pathogen interaction between maize and *P. polysora*. Sectioning and staining of infected maize leaf tissues up to 48 hr after inoculation showed very slight differences between the compatible and incompatible reactions. There was considerably less penetration of the resistant cultivar (B37R) by *P. polysora*, but the pathogen was able to penetrate and colonize leaf tissue in both cultivars. There was a progressive lag in the growth of*P. polysora* in

B37R at all stages of the infection process up to 48 hr after inoculation. Host cell death did not occur rapidly in the resistant cultivar B37R in response to the pathogen. The type of hypersensitive response in the incompatible reaction appeared to be of a slow-reacting type. From macroscopic observations, it is proposed that the ultimate death of the fungus in the resistant B37R occurs much later toward the time of sporogenesis.

Analysis of isozymes for some enzymes involved in the shikimic acid pathway and other pathways linked to phytoalexin production showed that unique peroxidase isozyme bands were present in B37R. These as well as significantly higher levels of peroxidase activity in all isozyme bands distinguished the resistant cultivar, B37R , from the susceptible cultivar, B37. Shikimate dehydrogenase, chalcone isomerase, cinnamyl-alcohol dehydrogenase, and polyphenoloxidase did not show any distinctive isozyme bands or levels of enzyme activity that could be correlated to the resistance response in B37R within the first 48 hr after inoculation.

ACKNOWLEDGEMENTS

I would like to thank my committee chairman, Dr. Clint Magill for providing the opportunity for me to do this research and for his advice. Also, I would like to thank my other committee members, Dr. J. D. Smith and Dr. Richard Frederiksen for their advice and help in all aspects of this work.

I want to thank Dr. E. C. Bashaw for teaching me the histological techniques used and Dr. Charlie Biles for his help with the gel electrophoresis and enzyme staining techniques. I would also like to take this opportunity to sincerely thank Ethel Champaco, Zachee Ngoko, and Celsa Garcia for their assistance in preparing this document.

DEDICATION

I wish to dedicate this work to my Lord Jesus Christ, for without His creation to study and ponder and hold in awe, we would be bereft of even an inkling of His greatness and His glory.

I would also like to dedicate this to my parents, Robert L. and Gloria M. Hanson, for they are the greatest blessings given to me.

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CHAPTER I
INTRODUCTION

Plants have varied responses to potential pathogens. Some have pre-existent physical or chemical barriers, but many are induced to synthesize particular compounds upon infection. Changes in protein and RNA synthesis, production of proteins different from those in healthy plants, changes in enzyme systems, and shifts in metabolic pathways are all important factors in the resistance response (55).

In particular, much attention has been devoted to the study of certain fungitoxic compounds called phytoalexins and their role in resistance. Many infected plant tissues show an accumulation of secondary substances such as phenolics, flavonoids, coumarins, terpenoids, steroids, and also the activation of peroxidases and other phenoloxidizing enzymes (21).

The most important pathway involved in biosynthesis of phenols is the shikimic acid pathway. Shikimic acid is eventually converted to the aromatic amino acids phenylalanine or tyrosine. Phenylalanine is converted by action of phenylalanine ammonia-lyase (PAL) to cinnamic acid, which is converted to p-coumaric acid. Tyrosine, by action of tyrosine ammonia-lyase (TAL), is also changed to p-coumaric acid. From this point lignins are produced by action of peroxidases, and chalcone is made which gives rise to the synthesis of isoflavonoids and flavonoids. By the action of phenoloxidizing enzymes, flavonoids can be converted to quinones. Quinones are usually more fungitoxic than phenols. Many phenolic and related compounds of this pathway are synthesized in plants in response to infection. Chlorogenic and caffeic acid are produced in potato, pisatin in pea, phaseollin in green bean, gossypol in cotton,

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This thesis follows the style and format of *Phytopathology*.

isoflavones in red clover, and other phytoalexins are found in carrot, soybean, and other plants (36). The classic work of Walker and Link (62) was the first to show a direct correlation between phytoalexin accumulation and disease resistance responses. They showed that certain onion varieties resistant to *Colletotrichum circinans* (Berk.) Vogl. accumulate protocatechuic acid and catechol in the bulb scales that are yellow- and red-pigmented.

Since then many other examples have been described. Avenalumins are produced in oat in response to crown rust caused by *Puccinia coronata* Cda. f. sp. *avenae* Fraser et Led. Avenalumins have been linked to the resistance response (39). An application of PAL inhibitor, α-amino-oxyacetate, to incompatible oat leaves inoculated with rust greatly suppressed the rapid accumulation of the phytoalexin avenalumin (40). Work done in wheat showed that production and accumulation of lignin even in small quantities provided considerable resistance to fungal degradation of leaves. This lignification is induced almost exclusively by fungi. Enzymatic maceration of wheat leaves inoculated with*Septoria nodorum* Berk. showed rings of lignin around inoculation sites not present in water-inoculated controls (46, 50).

In addition to assaying for accumulation of the end products of the phenolic biosynthetic pathways, many workers have begun to look at the changes in activation of the enzymes involved. In pea pods, factors which stimulate pisatin formation also cause a rapid appearance of PAL activity (24). In diseased tobacco tissue there is a marked increase in the activity of PAL, which acts to produce cinnamic acid, a precursor to scopoletin (a phytoalexin) (47). Induction of PAL and chalcone synthase in response to a fungal elicitor has also been observed in cell suspension cultures of dwarf French bean (37).

In the *Graminaea,* and particularly in maize, PAL, TAL, and other

enzymes have not yet been studied intensively in connection with host-pathogen interactions. They may well prove to be involved in phenolic biosynthesis in resistance responses. One phytoalexin-like substance has been found and studied in maize. The glucoside 2,4-dihydroxy-7-methoxy - 1,4-benzoazin- 3-one (DIMBOA) has been shown to play a significant role in resistance to *Helminthosporium turcicum* Pass. (Syn. *Exserohilum turcicum* (Pass.) Leonard & Suggs), which causes northern corn leaf blight (12). Other phytoalexins have been partially characterized in maize (38).

More recent findings lend even more credence to the idea that the production of phenolics via the shikimic acid pathway is involved in the resistance response of maize to fungal pathogens. Hydroxycinnamic acid:CoA ligase activity in maize mesocotyls was found to increase in response to inoculation with *Helminthosporium maydis* Nisik. (Syn. *Bipolaris maydis* (Nisik.) Shoemaker). Both susceptible and resistant cultivars showed the increase. However, activity in susceptible cultivars ceased to increase approximately 12 hr after inoculation, whereas, in resistant cultivars, activity continued throughout a 48 hr period. No significant change was noted in PAL activity, suggesting the two enzymes are not coordinately regulated in this system (14). There is also evidence that isozymes of hydroxycinnamic acid:CoA ligase in maize mesocotyls are linked in their response to infection by *H. maydis* (62).

Southern rust, a damaging disease caused by *Puccinia polysora* Underw. on maize, has been devastating in Africa (9, 49). Epiphytotics have also occurred in the southern United States (20). Rodriguez-Ardon *et al* showed that average yield reductions of 4, 23, and 45% occurred due to southern rust in three biweekly plantings of susceptible maize hybrids in Mississippi (51). Resistance in maize (*Zea mays* L.) to southern rust occurs in at least two forms. One is thought to be a hypersensitive response conferred by a single dominant gene (Rpp9). The other is

dilatory and of unknown inheritance. However, it is presumed to be quantitative, since evaluation of 33 inbred lines showed continuous gradations of resistance ranging from extremely susceptible to highly resistant (3). The mechanisms of both forms of resistance remain obscure.

Resistance to *P. polysora* was incorporated into the maize inbred B37 by crossing with PI 186208 followed by seven backcrosses to B37 and then three generations of selfing. The resultant cultivar, B37R, carries the Rpp9 gene conferring monogenic resistance (51). In a previous study, these two near isogenic cultivars showed some changes in the overall profile of proteins synthesized when inoculated with uredospores of *P. polysora* as compared with controls. However, no conclusive results were obtained showing discreet differences between the susceptible B37 and the resistant B37R after inoculation (2).

The experiments outlined here combine the use of B37 and B37R nearly isogenic maize cultivars, *P. polysora,* and the findings from maize and other plants that indicate phenolic compounds (phytoalexins) are responsible for some of the resistance responses. B37(susceptible) and B37R (resistant) plants were inoculated with *P. polysora* uredospores and the histology of the infection process studied. Subsequently, both cultivars were inoculated and analyzed for the presence of isozymes. The study of isozymes of the phenolic pathway and their activities in the resistance response of maize to *P. polysora* seems well-suited to provide general information on resistance responses in maize and, more specifically, to address the southern rust problem.

To evaluate the role of phenolic biosynthesis in the resistance response in maize, the following objectives have been established:

1. Cytologically observe the early events in the infection process to determine how and when such events as penetration of the stomata,

vesicle formation, and tissue colonization by the fungus occur in the susceptible and resistant cultivars.

2. Determine if and when different forms (isozymes) of enzymes involved with phenolic biosynthesis (shikimate dehydrogenase, chalcone isomerase, cinnamyl-alcohol dehydrogenase, peroxidase, and polyphenoloxidase) are induced or repressed during infection of B37 and B37R as compared with uninoculated controls.
3. Determine the levels of isozyme activity by means of photometric scans of the electrophoretic gels.

CHAPTER II
EARLY CYTOLOGICAL EVENTS IN NEARLY ISOGENIC RESISTANT AND SUSCEPTIBLE MAIZE CULTIVARS INOCULATED WITH *PUCCINIA POLYSORA*

INTRODUCTION

To understand the interaction of host plant and pathogen, it is necessary to observe occurrences at the cellular interface of the two. Determining the events responsible for or resulting from the resistance response of the host plant, requires knowledge of cytological events of the infection process. Before assaying for the presence of phytoalexins, monitoring shifts in mRNA populations, or observing changes in protein production or any other metabolic events associated with resistance responses, we must know which physical events of infection might be correlated with these molecular phenomena. It is critical to understand the pathogen's mode of entry, penetration, colonization of host tissues, and its reproduction in the host plant. The best way to observe all of these events and when they occur after infection, is to use cytological techniques to section and study inoculated tissues.

Several studies have been made on the ultrastructure of host and parasite in interactions between *Z. mays* and *Puccinia sorghi* Schw., the causative agent of common maize rust. Hilu used nearly isogenic resistant and susceptible cultivars of maize to observe the pathological histology in compatible and incompatible reactions (26). Hilu reported that differential compatibility was manifested only after intimate association of the host and pathogen. Similar vesicles formed in both compatible and incompatible reactions. The difference was that fewer haustoria were present and those formed were encapsulated within a thick sheath, and many died prematurely in the incompatible reactions.

In addition to Hilu's work using light microscopy to view sectioned and mounted material, others have utilized the advances of electron microscopy to observe events at the host-pathogen interface. Van Dyke and Hooker used electron microscopy to study the interaction between *Z. mays* and *P. sorghi* (61). Later, Hughes used scanning electron microscopy to observe cellular events in the same system (30). All have observed that the incompatible reaction between host and pathogen involved a type of hypersensitive response of the host, which caused necrosis and death of mesophyll cells. This resulted in encapsulation and death of haustoria, subsequently delimiting growth of the fungus.

Similar studies of *P. polysora* histology have not been reported in the literature. The life cycle of *P. polysora* was first described by Cammack in West Africa (10). Cammack observed that six days after inoculation of a leaf surface with uredospores, faint, greenish yellow spots, 1mm in diameter, appeared at infection sites in a compatible reaction. These spots gradually yellowed toward the center. By the seventh day, these spots began to swell. The leaf epidermis ruptured eight to nine days after inoculation, usually by a longitudinal slit, revealing uredospores.

The uredosori (pustules) produced were small and circular. They were densely and uniformly distributed and cinnamon to yellowish brown in color. The uredospores were yellowish or golden, oval in shape, and usually 27-41 x 20-29 µm in size. The aecidial and pycnidial stages of *P. polysora* were apparently absent. Teliospores were rarely produced and did not germinate. Therefore, uredospores constituted both primary and secondary inoculum.

To identify the early cytological events in the infection process, segments of leaves from two nearly isogenic resistant and susceptible maize cultivars, B37 and B37R, were inoculated with uredospores of *P. polysora.* These leaf segments were subsequently cut, fixed, and stained for microscopic

examination.

MATERIALS AND METHODS

Biological Material

For all studies, kernels of B37 were initially from Iowa Foundation Seed, and B37R kernels were from Dr. Gene Scott at Mississipppi State University. Kernel stocks have been maintained by selfing in the field. A Texas isolate of *P. polysora* was used for inoculations in the cytological studies. The isolate was maintained in the greenhouse on susceptible maize inbred Tx5855.

Inoculations

Uredospores were prepared for inoculation as described by Bailey (2). Spores were collected 3-4 days after pustule eruption by gentle brushing of the leaves. Spores were then placed in a humidity chamber for 24 hr before use. After removal from the humidity chamber, about 0.25 g of uredospores were suspended in 1L of distilled water containing 0.001% polyoxyethylenesorbitan monolaurate (Tween 20). The spore solution was gently stirred on a stir plate for 45 min to enhance germination by removing germination inhibitors. Spores were collected by filtration onto Whatman No. 1 filter papers (25 ml spore suspension / paper) and then washed with 25 ml distilled water with 0.0001% Tween 20.

Maize plants were grown in 4-inch pots, 3 plants per pot, on greenhouse benches. Plants were inoculated at the 5- to 6-leaf stage. Small sections of Whatman No. 1 filter paper (1x3 cm) with uredospores were placed spore-side down on the fourth or fifth leaf of each plant. Before applying the papers, each leaf area to be treated was wetted by washing with distilled

water containing 0.0001% Tween 20 and rubbing with the thumb and fingers. Control plants were treated in like manner using filter papers with 0.001% Tween 20 without spores. Plants were placed in a dew chamber with 100% relative humidity at 26°C (29). After 16 hr in the dew chamber, all plants were removed and returned to greenhouse benches until samples from each designated time had been taken. Samples from leaf sections under control and treatment papers were taken at 4, 8, 16, 20, 22, 24, and 48 hr after inoculation.

Fixing, Dehydration, and Staining

After each treated leaf segment was cut out, it was immediately placed in a small vial containing FAA solution (32) for 24 hr to fix the tissue. After 24 hr, the samples were transferred to a saturated solution (5%) of erythrosin dye in 70% alcohol. Samples were evacuated by a vacuum system and then sequentially run through each step of a dehydration series (Table 1) until samples were embedded in Tissuemat (paraffin).

After sufficient cooling and hardening of the Tissuemat, blocks were cut with a rotary microtome, producing 12 µm-thick sections of leaf tissue. Rows of sequential sections were carefully affixed to glass slides with Haupt's adhesive (32). A few drops of 4% formalin were used to float the ribbons of sections for positioning. Excess formalin was blotted off with bibulous paper. Slides were placed on a warming plate at 50°C for 12 to 24 hr.

A modified safranin-fast green staining schedule (Dr. E. C. Bashaw, Texas A&M University, personal communication) was used to stain the sectioned material on the slides (Table 2). Coverslips were affixed to slides with Permount. Slides were examined and photographed using an Olympus light microscope.

Table 1. Dehydration series.

Step	Procedure	Time
1.	50ml distilled water 40ml 95% ethyl alcohol 10ml tertiary butyl alcohol (TBA)	2 hr
2.	30ml distilled water 50ml 95% ethyl alcohol 20ml TBA	6 hr
3.	15ml distilled water 50ml 95% ethyl alcohol 35ml TBA	1 hr
4.	45ml 95% ethyl alcohol 55ml TBA	1 hr
5.	25ml 95% ethyl alcohol 75ml TBA	1 hr
6.	Pure TBA	1 hr
7.	TBA	12 hr
8.	TBA	1 hr
9.	50-50 equal parts TBA and paraffin oil (place in oven at 56.5^{o}C)	24 hr
10.	Pour 50-50 mixture with sample into small vial containing melted Tissuemat. Place in oven.	24 hr
11.	Change Tissuemat	24 hr
12.	Change Tissuemat	6 hr
13.	Change Tissuemat	24hr
14.	Embed in Tissuemat using Tissue-Tec II embedding center.	

Table 2. Modified safranin-fast green staining schedule.

Step	Procedure	Time
1.	Xylene	5 min
2.	Xylene	5 min
3.	Absolute alcohol : xylene (1:1)	5 min
4.	Absolute alcohol	5 min
5.	70% ethyl alcohol	5 min
6.	30% ethyl alcohol	5 min
7.	Distilled water	5 min
8.	Stain in aqueous safranin	3 min
9.	Rinse slides repeatedly in distilled water	10 min
10.	50% ethyl alcohol	5 min
11.	95% ethyl alcohol	5 min
12.	Fast green dye in 95% ethyl alcohol	20 sec
13.	95% ethyl alcohol	10 sec
14.	Absolute alcohol	1.5 min
15.	Absolute alcohol	1.5 min
16.	Absolute alcohol : xylene (1:1)	2 min
17.	Xylene	5 min
18.	Xylene	5 min

RESULTS

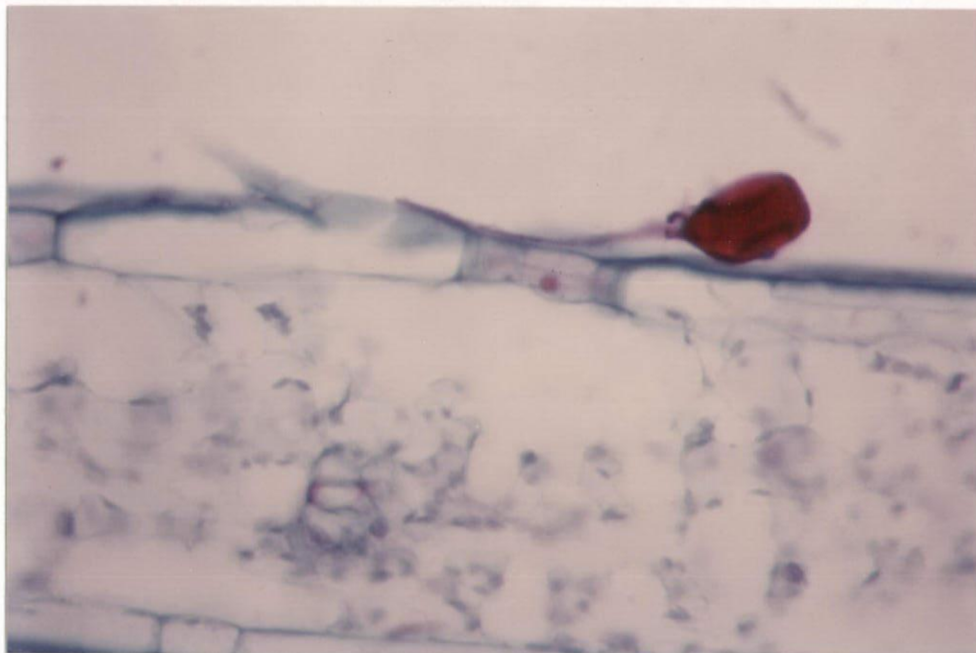
Uredospores were germinating by 4 hr after inoculation, usually by production of a single germ tube (Figs. 1-A and 1-B). Germination of uredospores was evident on both resistant (B37R) and susceptible (B37) cultivars of maize. More uredospores had germinated by 8 hr after inoculation and appressoria were appearing over stomata (Fig. 2). Some uredospores failed to germinate, and others, which possessed germ tubes, failed to penetrate the leaf.

Infection pegs developed from the appressoria and penetrated the stomata of the leaf by 16 hr after inoculation. The pegs enlarged to form substomatal vesicles usually 10-16 x 13-24 μm in size. Similar substomatal vesicles were formed in both resistant and susceptible maize cultivars (Figs. 3-A and 3-B). As reported by Hilu (26), when penetration failed the appressoria remained rigid and full of protoplasm. Figs. 3-A and 3-B show empty appressoria remaining on the outer surface of the stomate after successful penetration occurred. Fig. 4 shows appressoria still containing protoplasm, which failed to penetrate the leaf.

Although penetration of the fungus was successful in both the susceptible and resistant cultivars, the percentage of stomata penetrated was quite different in each (Table 3). In susceptible B37, the percentage of stomata penetrated on the inoculated side of the leaf was much higher than the percentage in resistant B37R at 16, 20, and 22 hr after inoculation. Of the stomata penetrated in both B37 and B37R, a greater percentage had just one substomatal vesicle as opposed to having two or more vesicles. The only exception was in B37 at 16 hr after inoculation, where the opposite was true. The presence of primary infection hyphae growing down from the substomatal vesicles into the mesophyll cells of the maize leaves was increasingly evident between 20 and 24 hr after inoculation. Visible at 20hr

Fig. 1. **A**. B37 at 4 hr after inoculation. Germ tube of uredospore visible. **B**. B37R at 4 hr after inoculation. Germ tube present. (Magnification ca. 300 X).

A.



B.

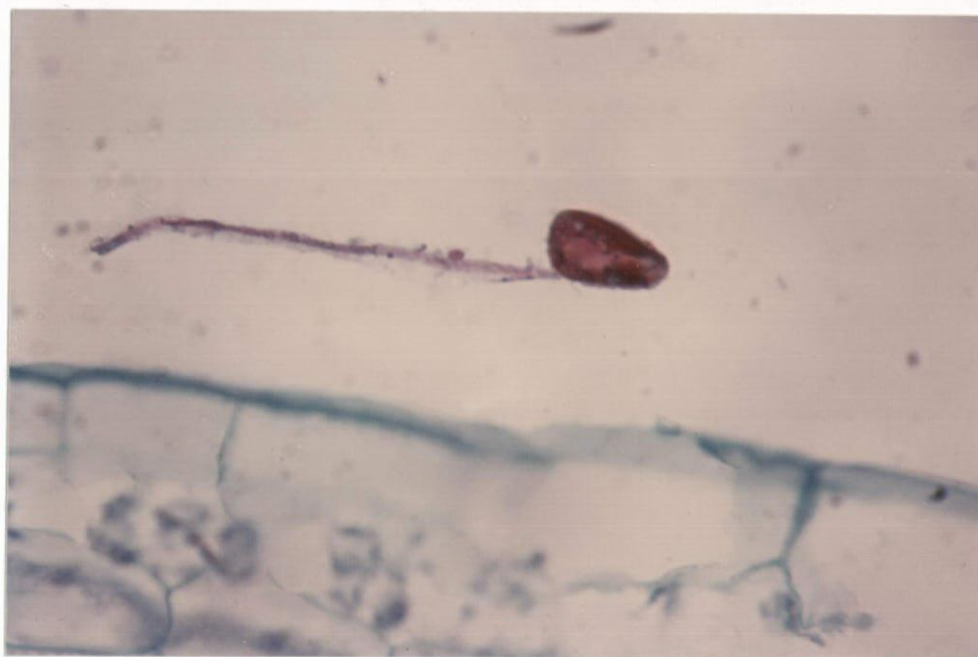


Fig. 2. B37 at 8 hr after inoculation showing formation of appressoria above the stomate. (Magnification ca. 300 X).

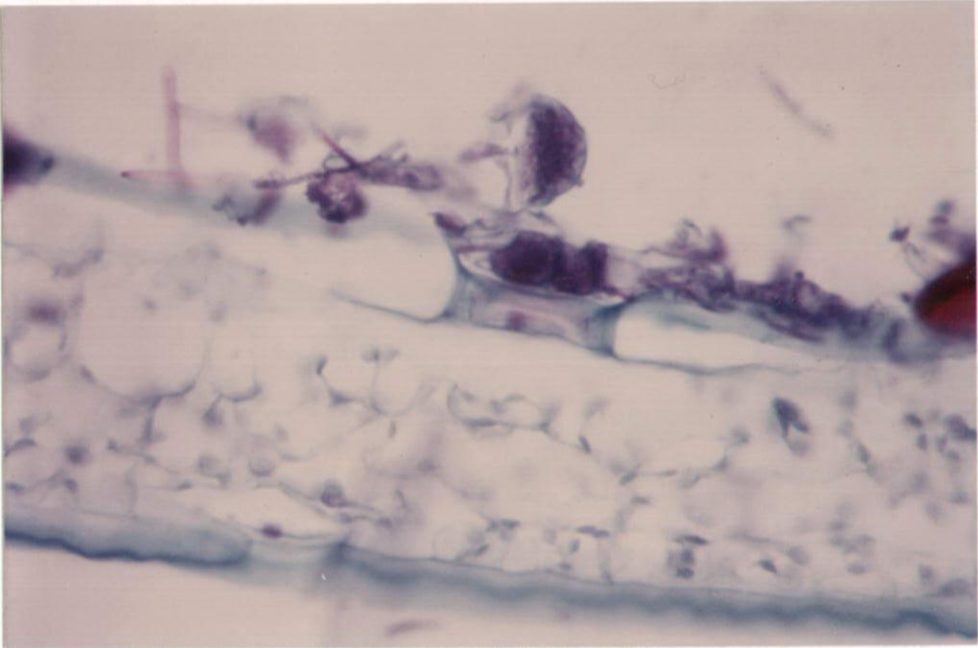
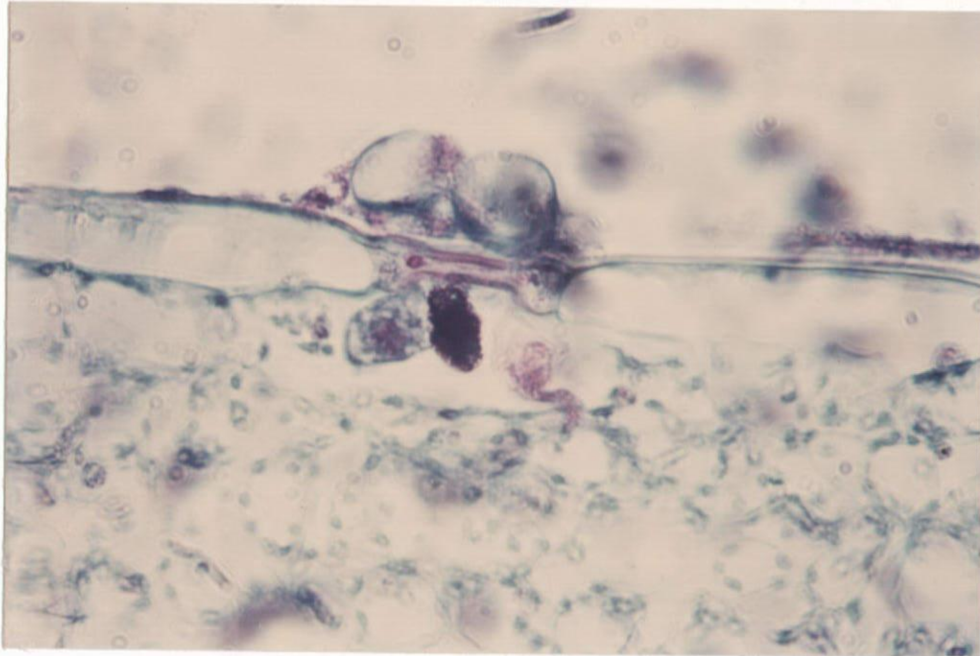


Fig. 3. **A.** B37 at 16 hr after inoculation. Substomatal vesiclesvisible. (Magnification ca. 300X). **B.** B37R at 16 hr after inoculation. Substomatal vesicles also formed in the resistant cultivar. (Magnification ca. 80X).

A.



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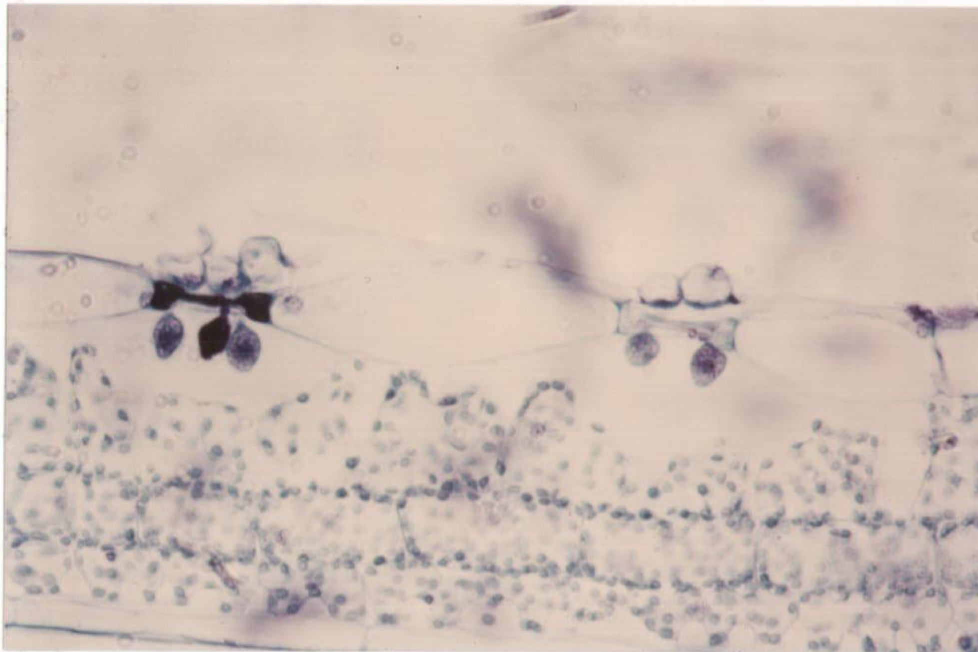


Fig. 4. B37R at 20 hr after inoculation. Appressoria, which failed to penetrate, are above the stomatal opening and still contain protoplasm. (Magnification ca. 300X).

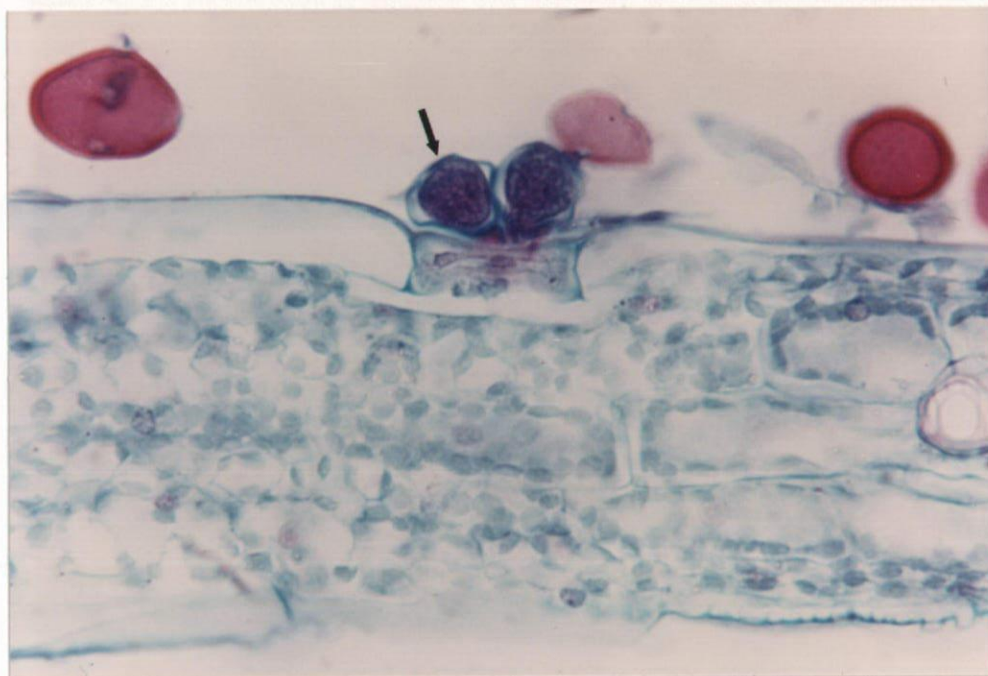


Table 3. Mean percentages of stomatal penetration and mean numbers of vesicles per stomate in B37 and B37R.

	Hours after inoculation	% stomata penetrated	% stomata with one vesicle	% stomata with >1 vesicle
B37	16	39.6	28.2	71.8
B37R	16	8.17	71.1	28.9
B37	20	29.4	58.6	41.4
B37R	20	9.4	69.0	31.0
B37	22	38.5	63.0	37.0
B37R	22	4.7	88.0	12.0

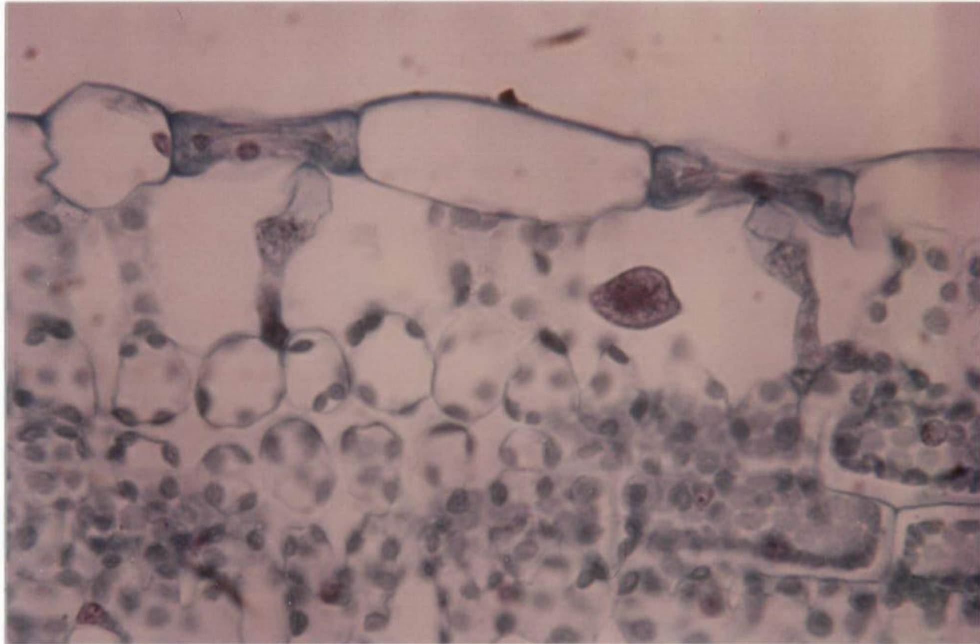
after inoculation, primary infection hyphae in B37 seem to already have progressed further in growth than those forming from vesicles in B37R (Figs. 5-A and 5-B). By 22 hr after inoculation, some infection hyphae have grown quite a distance through the intercellular spaces of the mesophyll cells (Fig. 6-A). Growth of hyphae in B37R lagged a bit behind B37 at 22 hr after inoculation (Fig. 6-B).

At 24 hr after inoculation, further growth of infection hyphae was visible in both maize cultivars (Figs. 7-A and 7-B). Of the substomatal vesicles present in B37 leaf sections at 24 hr, 28.4% of them had penetration hyphae, whereas, a slightly lower percent (21.2%) of the vesicles in B37R sections had hyphae.

Sections taken at 48 hr after inoculation showed further intercellular mycelial growth in both maize cultivars. Substomatal vesicles were no longer present in these leaf sections. Mycelia up to 578 µm in length, and perhaps greater, were seen growing parallel to and beneath the leaf epidermis (Figs. 8-A and 8-B). Staining a bright red in color as did the uredospores, these mycelia most likely have lignified or suberized cell walls, since safranin-o dye specifically stains chromatin and lignified or suberized cell walls. Mycelial growth was much more prolific in susceptible B37 than in resistant B37R (Figs. 9-A and 9-B). Cammack (10) reported that each pustule was formed from an individual infection of *P. polysora*, and the mycelia were restricted to the host tissue immediately beneath the pustule. He stated that there was no spread of the original mycelium. However, the mycelia in these leaf sections appeared to grow beyond the area directly beneath the stomata where penetration occurred. It appeared that the mycelial growth at 48 hr after inoculation was beginning to cause some loss of integrity of the mesophyll cells in the maize leaves of both cultivars compared to sections at earlier times. Figures 8-A and 9-B showed loss of cell wall integrity, and chloroplasts were not as numerous (Figs. 8-A, 8-B, 9-A, and 9-B) as in earlier sections (Figs. 4 and 5-A).

Fig. 5. **A.** B37 at 20 hr after inoculation. Penetration hyphae developing. **B.** B37R at 20 hr after inoculation showing penetration hypha just beginning to develop. (Magnification ca. 330X).

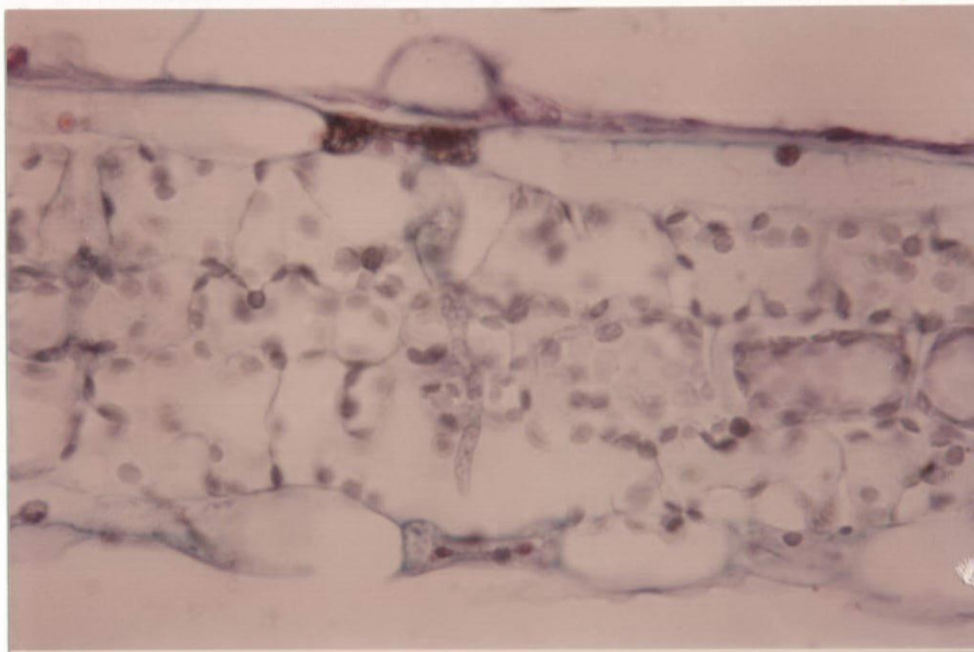
A.



B.

Fig. 6. **A.** B37 at 22 hr after inoculation. Infection hypha growing intercellularly. **B.** B37R at 22 hr after inoculation, showing infection hypha lagging in development. (Magnification ca. 300X).

A.



B.

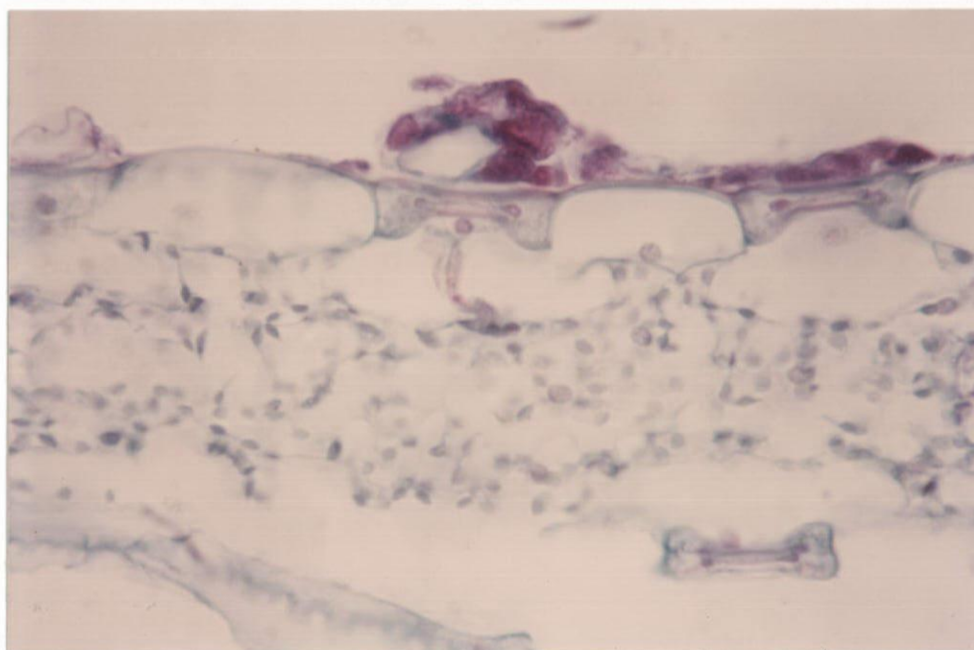
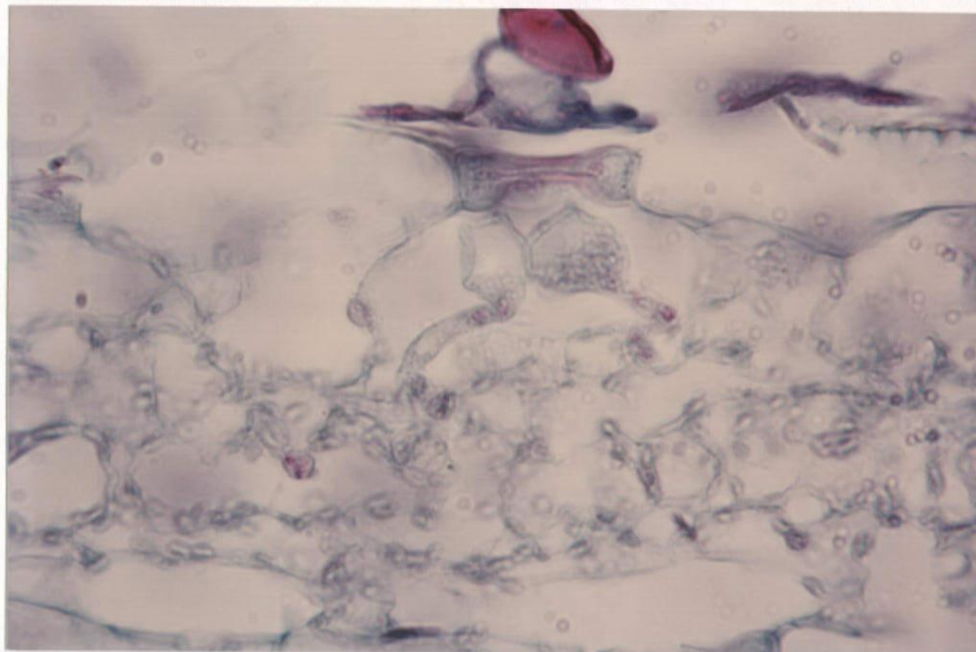


Fig. 7. **A.** B37 at 24 hr after inoculation. Infection hyphae growing intercellularly. **B.** B37R at 24 hr after inoculation, also *showing infection hypha.* (Magnification ca. 300X).

A.



B.

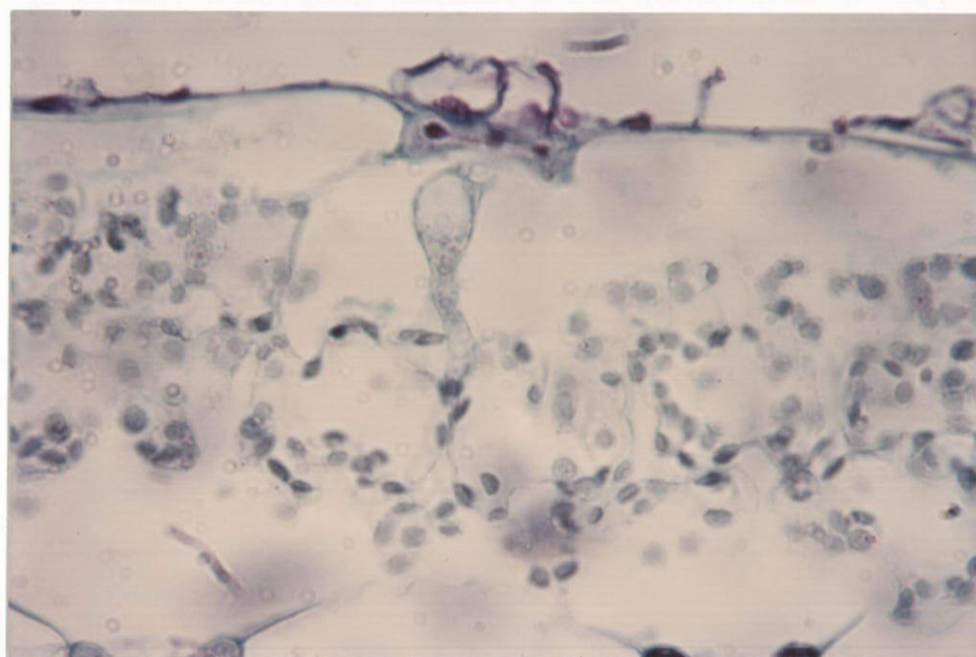
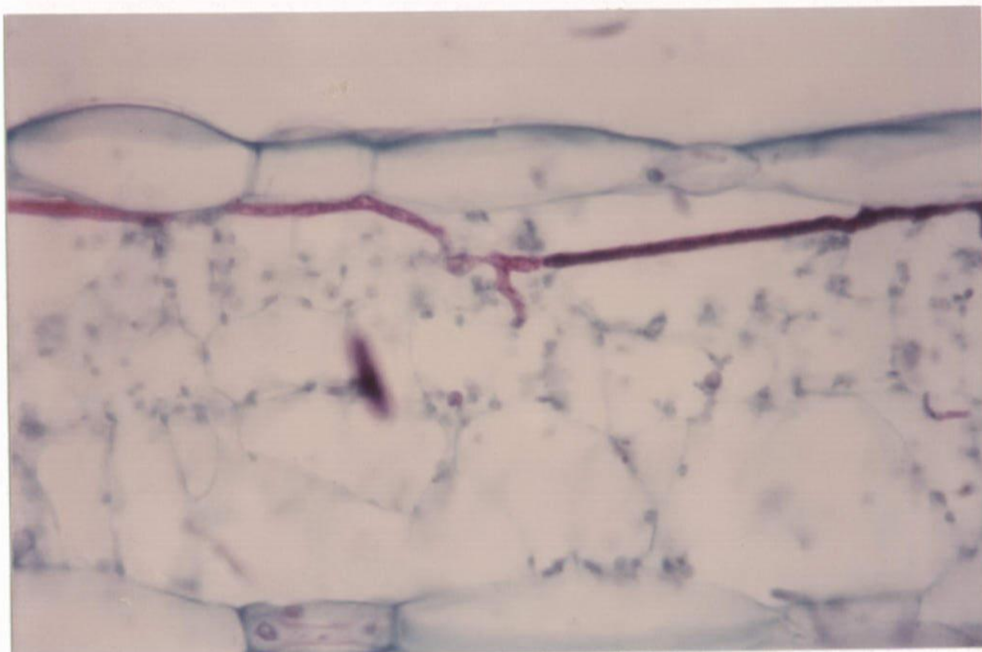


Fig. 8. **A.** B37 at 48 hr after inoculation. Intercellular mycelial growth. **B.** B37R at 48 hr after inoculation. Mycelial growth. (Magnification ca. 300X).

A.



B.

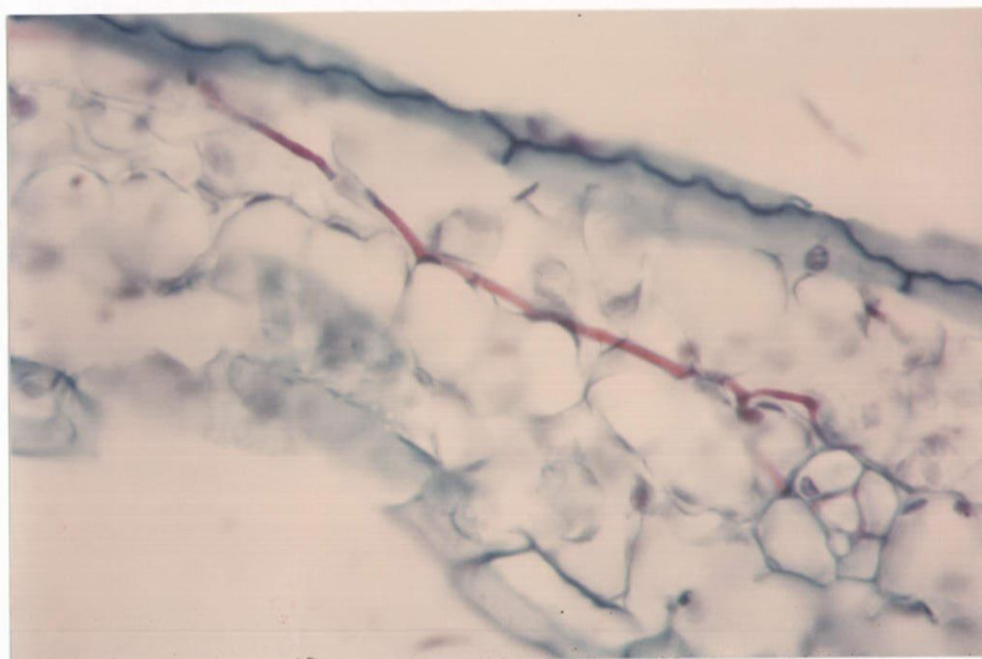
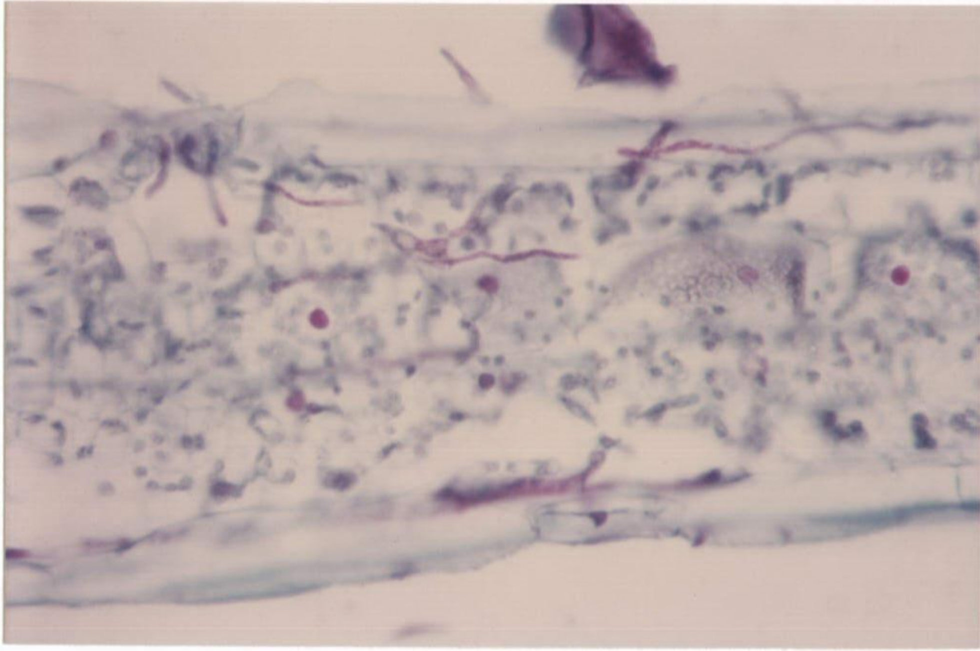
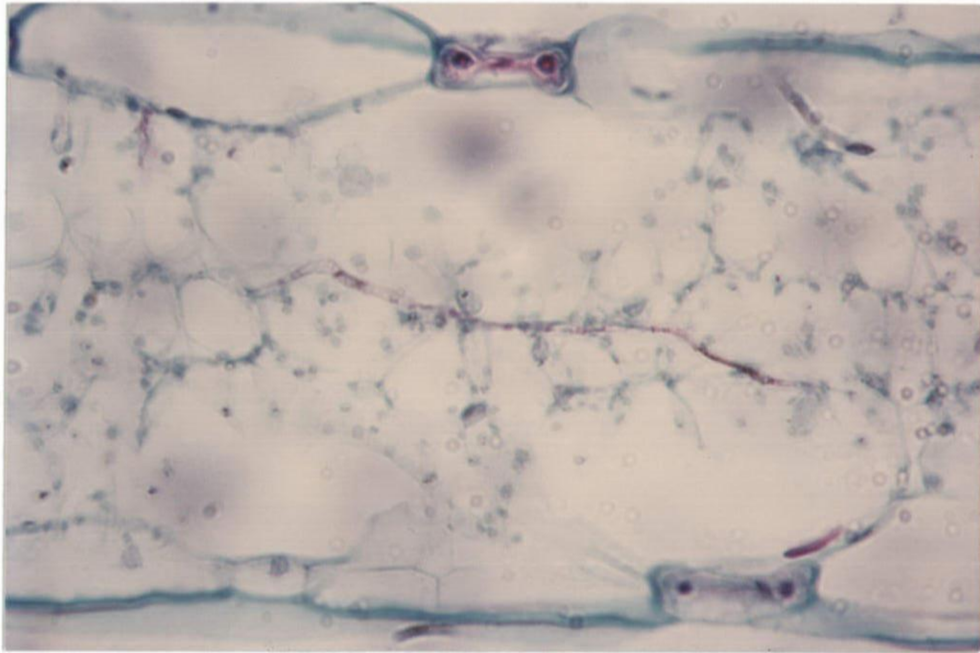


Fig. 9. **A.** B37 at 48 hr after inoculation. Prolific growth of mycelia. **B.** B37R at 48 hr after inoculation. Mycelial growth less than in B37. (Magnification ca. 300X).

A.



B.



DISCUSSION

During the early events of the infection process of *P. polysora* in maize, there did not appear to be one particular event which clearly differentiated the compatible and incompatible reactions. Rather, there seemed to be a progressive lagging in the growth and development of *P. polysora* in the resistant B37R when compared to the susceptible cultivar, B37.

Uredospores were capable of germinating on and penetrating the stomata of both cultivars, however, the level of penetration was much higher in the susceptible B37. Evidently there must have been some conditions at this stage which first began to put *P. polysora* at a disadvantage in the incompatible reaction with B37R. Whether this was due to physical or chemical deterrants is not at all clear. Similar substomatal vesicles formed in both maize cultivars, and primary infection hyphae developed and began to display intercellular growth between 20 and 24 hr after inoculation. However, infection hyphae appeared slightly sooner and developed more rapidly in the compatible reaction. Not only is growth of *P. polysora* deterred at the time of penetration, but also here when the fungus began to colonize the leaf tissue. Again, nothing visible in the sectioned leaf material seemed to indicate a difference in cellular structure which would account for this difference between growth patterns. No evidence of cell collapse or death was observed in the areas of vesicle formation, and only a slight indication of loss of cellular integrity occurred later at the time of intercellular mycelial growth (48 hr after inoculation).

Even at 48 hr after inoculation mycelial growth was observed in both reaction types. However, the growth was more abundant in the susceptible B37. Still, the progress of *P. polysora* in B37R continued to be impeded.

It appeared that no single event in the early stages of infection stopped the growth of *P. polysora* in the resistant maize cultivar B37R. Instead, it seemed that a progression of events at each phase of early infection slowed

the growth and development of the pathogen.

Close to 10 days after inoculation of the resistant B37R, chlorotic flecks appeared on the leaves at the sites of infection, but no pustule development occurred. This same reaction has been previously described in other incompatible reactions between maize and *P. polysora* (58). Perhaps the resistance response in maize cultivar B37R involved a series of steps which progressively deterred and eventually terminated the growth of the pathogen closer to the time of uredospore production. Indeed, the death of the fungus in B37R did not occur within the first 48 hr after inoculation. It must be presumed, therefore, that the point of ultimate cessation of fungal growth in B37R occurred after 48 hr but before 9 or 10 days after inoculation, when pustule formation usually occurs. Haustoria formation could not be clearly studied in these leaf sections. Whether haustoria formation occurred in both cultivars by 48 hr after inoculation was not discerned. Inability to obtain nutrients from the host cells due to lack of haustoria may have been another factor involved in the progressive slowing of *P. polysora* growth in B37R. Perhaps use of a leaf-clearing technique and whole mounted tissue would have helped to determine whether or not haustoria had formed and when in each reaction.

Considering that the resistance to *P. polysora* in B37R is dominant monogenic resistance, it was expected by many that the Rpp9 gene would confer a type of resistance causing a hypersensitive response. However, this hypersensitive response needs to be more clearly defined. Here is evidence that the resistance in B37R is not a quickly enacted hypersensitive response involving early and limited host cellular death and resulting in the arrested growth and death of the fungus. Evidently, the Rpp9 gene product caused or interacted in a series of events occurring or conditions existing in the leaf cells which worked together to confer resistance. This resistance ultimately expressed itself at a time much later toward the time of uredosporogenesis. What may be involved in some of those cellular events

or conditions is explored next.

CHAPTER III
ISOZYME ANALYSIS OF ENZYMES IN SHIKIMIC ACID PATHWAY IN RELATION TO THE RESISTANCE RESPONSE OF *ZEA MAYS* TO *PUCCINIA POLYSORA*

INTRODUCTION

Many host resistance responses have been correlated to accumulation of phenolic compounds and increased activity of oxidizing enzymes (35). Benzoic and cinnamic acids, chlorogenic acid, coumarins, isocoumarins, anthocyanins, isoflavonoids, lignins, and other related phenolic compounds are products of the shikimic acid pathway (Fig. 10). In higher plants, this pathway not only leads to the aromatic amino acids but also to a variety of more complex aromatic compounds (43). In the hypersensitive or incompatible response to infection, many enzymes of the shikimic acid pathway and related pathways (phenylpropanoid pathway) have been observed to increase in activity. Some of these enzymes include phenylalanine ammonia-lyase (14), hydroxycinnamate:CoA ligase (14), chalcone synthase (37), polyphenoloxidase (13, 56), peroxidase (48), and shikimate dehydrogenase (42). The enzymes considered here are: cinnamyl-alcohol dehydrogenase, chalcone isomerase, polyphenoloxidase, peroxidase, and shikimate dehydrogenase.

Shikimate dehydrogenase (SKDH) (E. C. 1.1.1.25.) catalyzes the conversion of 5-dehydroshikimic acid to shikimic acid (Fig. 11). Few, if any workers have investigated SKDH isozymes and their possible role in the resistance response of maize to fungal infection. Since SKDH is active relatively early in the pathway, it may have a more significant regulatory role in the production of phenolics. Changes in SKDH isozyme patterns could give indication of this.

Chalcone isomerase (CHI) (E. C. 5.5.1.6.) is the second enzyme directly

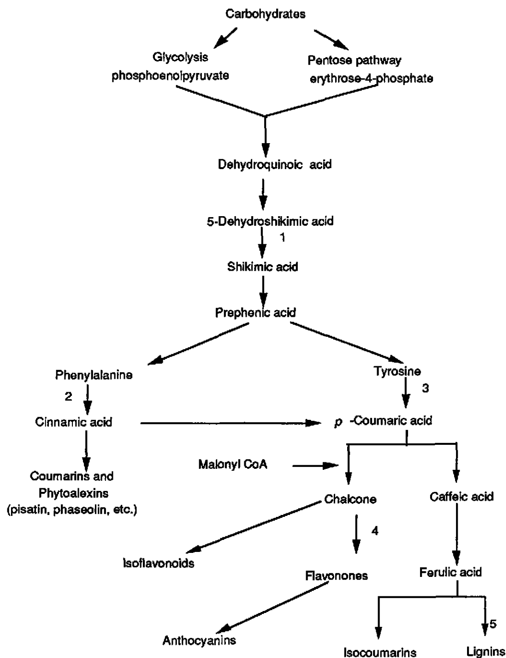


Fig. 10. Phenolic biosynthetic pathways.
1=shikimate dehydrogenase, 2= phenylalanine ammonia-lyase
3=tyrosine ammonia-lyase, 4=chalcone isomerase, and
5=peroxidase.

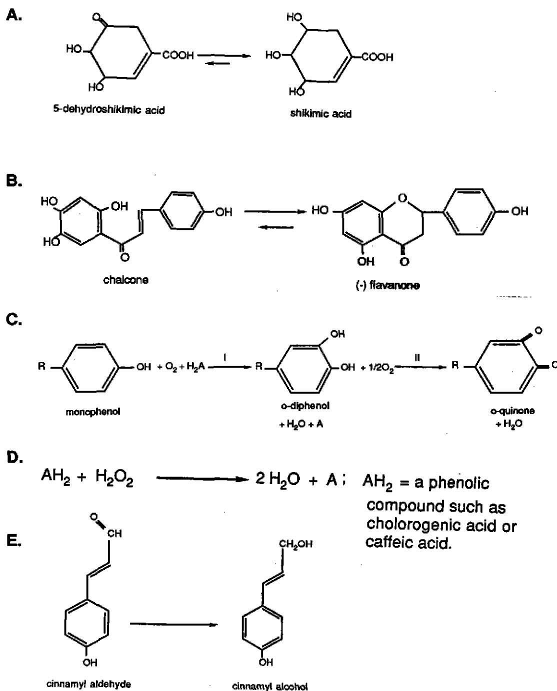


Fig. 11. Enzyme reactions. **A.** Shikimate dehydrogenase. **B.** Chalcone isomerase. Adapted from Mol *et al* (41). **C.** Polyphenoloxidase. Adapted from Conn (11). **D.** Peroxidase. Adapted from Goodman, Kiraly, and Wood (21). **E.** Cinnamyl-alcohol dehydrogenase. Adapted from Grand, Sarani, and Lamb (22).

involved in flavonoid biosynthesis, after chalcone synthase, in higher plants. It catalyzes the stereospecific conversion of chalcones to their corresponding (-) flavonones (Fig. 11). Considerable attention has been focused on chalcone isomerase, as well as other enzymes of phenylpropanoid metabolism, in view of their inducibility by agents such as UV light, fungal pathogens, and elicitor molecules isolated from fungal pathogens (16). Chalcone isomerase has been observed to increase in activity in *Glycine max* (L.) Merril following infection by *Phytophthora megasperma* f. sp. *glycinia* Kuan & Erwin (44) and in *Phaseolus vulgaris* L. cell suspension cultures treated with biotic (17) or abiotic (15) elicitors.

Polyphenoloxidase (PPO) (E. C. 1.10.3.2.) is a copper-containing protein that catalyzes the oxidation of mono- and ortho-dihydroxy-phenolic compounds, yielding quinones (35) (Fig. 11). In general, phenolic compounds are more toxic to plant pathogens in their quinone forms (35). Greater activity of this enzyme has been reported to occur in mechanically injured tissue (31), in virus-infected tissue (33), and in tissue infected by fungi and bacteria (31).

Observed increases in polyphenoloxidase activity in host-parasite interactions are not always the result of *de novo* synthesis of the enzyme. It seems likely that some increase is due to activation of latent enzyme or increased solubilization of polyphenoloxidase from particulate matter (35). The antimicrobial activity of quinones produced via polyphenoloxidase is attributed to their reaction with proteins or intracellular amino acids, alteration of the cellular redox potential, interference with cofactor and enzyme synthesis, and inhibition of specific enzyme systems (27, 65). It has been shown that products formed by oxidation of phenols inactivate enzymes including hydrolytic enzymes, such as polygalacturonase, produced by plant pathogenic fungi (45, 8).

Involved in the oxidation of a variety of compounds, including phenolic compounds, peroxidase (PER) (E. C. 1.11.1.7.), has been often correlated to

disease resistance (19). Peroxidase is a haemoprotein widely distrubuted in higher plants. It also catalyzes a H_2O_2-dependent oxidative polymerization of phenylpropane compounds, yielding lignin-like substances (6, 54) (Fig. 11). Potentially, lignin formation can act as a barrier to further spread of an invading pathogen (25, 60). Isozymes of peroxidase have been found to increase in number as a result of injury or disease (34, 53). Electrophoresis showed an increase in one of 14 peroxidase isozymes which was consistently associated with development of a resistant reaction in a study of near isogenic resistant and susceptible wheat lines to race 56 of *Puccinia graminis* f. sp. *tritici* Pers. (52).

Catalyzing the last step in a series of reactions leading from L-phenylalanine to substituted cinnamyl alcohols, cinnamyl-alcohol dehydrogenase (CAD) (E. C. 1.1.1.195.), is also involved in lignin biosynthesis (23). Found to occur widely in the plant kingdom, cinnamyl-alcohol dehydrogenase is a zinc-containing, $NADP^+$-dependent enzyme that catalyzes the last step in the reduction of cinnamoyl-CoA esters to cinnamyl alcohols (Fig. 11). Cinnamyl alcohols are the primary building blocks of lignin. Previous study has shown that CAD synthesis is stimulated in bean cell suspension cultures treated with fungal elicitor (22). This was correlated with a marked increase in extractable enzyme activity. In addition, it has been shown that there is a rapid but transient induction of CAD mRNA synthesis in bean cell suspension cultures treated with fungal elicitor (64). The presence of CAD isozymes has also been shown in cell suspension cultures of soybean (23).

Increases in the activity of these enzymes being correlated to phytoalexin accumulation in resistance responses has sparked interest in the study of their isozyme patterns. Isozymes are electrophoretically distinguishable forms of an enzyme which utilize the same substrate. Isozymes can differ in conformation, isoelectric point, or in molecular weight. Differences in the migration patterns of isozymes on an electrophoretic gel visibly show

evidence of differences between the proteins. Since proteins, or the subunits which comprise them, are products of particular genes, variation in gel migration patterns in isozymes show that products of different genes are being produced. From these visible banding patterns, information on the genetics of the system can be detected. Electrophoretic isozyme analysis has been used extensively in the study of fungal disease effects on general host metabolism (7). In the present study, SKDH, CHI, PPO, PER, and CAD isozyme patterns have been studied to detect possible genetic markers for the resistance response of maize to *P. polysora*. Distinct isozyme patterns correlated to the resistance response in maize could begin to shed light, in particular, on the function of the Rpp9 resistance gene as well as on the general resistance responses in maize.

MATERIALS AND METHODS

Biological Materials

Kernels of B37 and B37R were obtained and maintained as described previously (see Chapter 2). For analysis of isozymes and their estimated levels of activity, an isolate of *P. polysora* was provided by M. R. Bonde at the USDA, ARS, Foreign Disease-Weed Research Unit at Frederick, Mayland. This isolate was maintained in the greenhouse on susceptible maize inbred Tx5855. Inoculum was also increased in the field and subsequently stored in liquid nitrogen.

Inoculations

Uredospores were collected as previously described (Chapter 2) or were taken out of storage in liquid nitrogen and heat-shocked in a water bath at 40° C for 1-2 minutes. After collection, uredospores were allowed to air-dry for 24 hr in open petri dishes. Spore germinability and infectivity are

reduced at high storage relative humidity (28).

Uredospores (0.07 g) were suspended in 300 ml distilled water with 0.001% Tween 20. Spore concentration determined with a haemocytometer was 24,000±63.24 spores/ml. Larger sections of Whatman No. 1 filter paper were employed in this study. A small volume of spore suspension (15 ml) was filtered onto each filter paper, followed by a wash with 10 ml of distilled water with 0.0001% Tween 20. Each filter paper covered 9.62 cm^2 of leaf area. An inoculum density of 31,185 spores/cm^2 was applied with each paper. In order to position papers to one side or the other of the midrib, each paper was cut in half before applying to the leaf. Control papers were prepared in the same manner but with no spores.

Plants were once again treated at the 5-6 leaf-stage of growth. Afterwards, all plants were placed in a dew chamber with 100% relative humidity at 26°C to promote infection (29). Inoculated and control samples were taken at 12, 24, 36, and 48 hr after inoculation. After 16 hr all plants were removed from the dew chamber and returned to a greenhouse bench.

Protein Extraction and Quantification

For analysis of isozyme patterns and enzyme activity assays, control and inoculated leaf segments were taken at 12, 24, 36, and 48 hr after treatment. Leaf segments covered with filter paper were cut out and papers removed. Samples from each time period were immediately weighed and homogenized on ice with cold mortar and pestle (4°C), using cold Modified Calson's Buffer (0.1M Tris, 0.1M KCl, 0.04M 2-mercaptoethanol, 0.1M sucrose, pH 7.5). Buffer was added in a 2:1 ratio of µl buffer:mg leaf tissue for each sample. A small pinch of insoluble polyvinyl polypyrrolidone was also added to bind phenols. After homogenizing to a slurry, each sample

was transferred to a plastic microcentrifuge tube and centrifuged at 12,400 rpm at 4°C for 30 min using a Fisher Model 235A Microcentrifuge. The supernatant was then pipetted to a new tube and centrifuged again for 15 min. The resulting supernatant was transferred to a new tube and stored at -70°C.

Soluble protein content in each sample was determined according to Bradford (5) using bovine serum albumin as a standard. The standard protein concentration plot and sample determinations were done using a Beckman DU-40 spectrophotometer. Based on an analysis of variance, none of the samples' mean protein content was significantly different from the others. Therefore, no dilutions of the samples were made.

Electrophoresis

All electrophoresis was done on a PhastSystem from Pharmacia. Pre-made isoelectric focusing (IEF) gels and native polyacrylamide gradient gels were both used. IEF PhastGels were homogeneous (5% T, 3% C) polyacrylamide gels containing ampholytes which created a continuous pH range from 3 to 9. IEF gels allow proteins to migrate under an electric field to a point in the pH gradient that corresponds to their pI (isoelectric point). Approximately 1 µl of sample was loaded onto each lane. Samples were applied to both anode and cathode positions to insure that all isozymes reached their pI. The gel bed was maintained at 15°C throughout the run of each gel. The length of separation time and voltage applied are as prescribed in the PhastSystem owner's manual (1).

Native gradient polyacrylamide gel electrophoresis allowed separation of isozymes with intact conformation and biological activity. Gels had a gradient of 10-15% polyacrylamide. The buffer system in the gels was of 0.112M acetate and 0.112M Tris, pH 6.4. Buffer strips with 0.88 L-alanine

and 0.25M Tris, pH8.8 were used with these gels. The buffer strips are composed of 2% Agarose IEF. Approximately 1µl of sample per lane was applied to the cathode position of the gel. Again, the gel was maintained at 15°C. Lengths of running time (in volthours) and voltage used were as described in the PhastSystem manual (1). Gels stained for chalcone isomerase were run twice. All gels for the other enzymes studied were run and stained at least three times.

Staining Gels for Isozyme Analysis

Immediately after the gels were run on the PhastSystem separation unit, each was treated with a dye solution specific for the enzyme of interest. Peroxidase and polyphenoloxidase were stained by procedures described by Biles (4). A modification of a stain reported by Tanksley and Rick (57) was used for shikimate dehydrogenase. Chalcone isomerase was stained using modifications (59) of procedures by Eigen *et al* (18). Cinnamyl-alcohol dehydrogenase staining solution was that used by Biles (4). The small PhastGels were stained in disposable petri dishes and photographed atop a lightbox. However, a longwave UV light source (366nm) was used to photograph gels stained for chalcone isomerase, since a fluorescent dye system was used.

Evaluation of Isozyme Gel Banding Patterns

Isoelectric points of isozymes on the IEF gels were determined using Sigma IEF markers (Kit No. IEF-MI). Bands on the polyacrylamide gradient gels were evaluated in terms of Rf values. Rf values were determined by dividing the distance a band had migrated down the gel from the origin of sample application (cathode position) by the total distance of the lanefront

from the origin.

Photometric Scanning of Gels

After staining, gels were dried and then analyzed by a Beckman DU-40 spectrophotometer with a gel scanning program. Each gel was inserted into the gel holder and scanned lane by lane. Ten absorbance readings per millimeter were read down each lane of the gel. From these values, a plot was created. The peak absorbance of each band and the area of each peak was calculated as well as plotted.

Both IEF and gradient gels stained for shikimate dehydrogenase were scanned for absorbance values with visible light at 340 nm. Gels stained for chalcone isomerase were scanned using UV light at 366 nm. Visible light at 330 nm was used for IEF gels stained for CAD, and 360 nm was the wavelength used on CAD native gradient gels. Peroxidase-stained gels were scanned for absorbances at 430 nm. Visible light at 340 nm gave absorbance readings for PPO gels.

Analysis of Gel Scans

Each lane in each gel was scanned twice, resulting in a mean peak absorbance for each band detected by the spectrophotometric gel scanner. Graphic outputs showing the absorbance profile of each gel were studied and compared to the actual gel's banding patterns. This comparison helped determine the number of peaks (representing bands) for which mean absorbances should be tested and compared for significant differences. After an analysis of variance, a Tukey multiple comparison was run on the mean peak absorbances for each band in a given gel. Comparisons were made between treatments across all of the times and between times across all of the treatments.

RESULTS

Shikimate Dehydrogenase

Shikimate dehydrogenase (SKDH) IEF gels showed one band in all lanes at an approximate pI of 7.15. No differential bands appeared in these gels, and differences in band intensities were slight and difficult to visually decipher (Fig. 12). The photometric scan of the gel indicated by absorbance values the activity of each isozyme band. The resultant absorbance means for each sample at each time are given in Table 4. A plot of these mean values (Fig. 13) showed that there was interaction between the different plants (treatments) and time. All samples except B37R inoculated showed an increase in SKDH activity by 48 hr after inoculation. B37R inoculated seemed to peak in SKDH activity at 24 hr after inoculation. At 12 hr B37R control had higher SKDH activity than the B37 control. The same was true at 48 hr. However, at 24 and 36 hr SKDH activity in B37R control dropped below that in B37 control.

When inoculated, the two cultivars began at 12 hr with about the same level of SKDH activity. The activity in B37R peaked at 24 hr, but by 48 hr after inoculation, B37 had a much higher SKDH activity than B37R did. In comparing B37 inoculated to its control, it was seen to have a lower level of enzyme activity until 36 hr after inoculation, when it surpassed the level in the B37 control. B37R inoculated began with a much lower level of SKDH activity than its control. By 24 hr after inoculation, SKDH activity in B37R inoculated was much higher than in its control. At 36 hr they both had about the same level of activity and then by 48 hr B37 control had greatly increased, whereas activity in B37R inoculated continued to decrease.

Across all times, there were no significant differences between the mean levels of SKDH activity in any of the samples. The great amount of fluctuation in the controls over time may have been due to changes in

enzyme levels related to physiological changes in the plants through development. The fluctuations may also have occurred due to the conditions in the dew chamber putting a unique type of stress on the plants.

Table 4. SKDH IEF (3-9) gel mean band absorbance values at 340 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inocul.	B37R inocul.	B37R control
Band 1 pI=7.15				
12	0.4280	0.3525	0.3530	0.5175
24	0.4885	0.2850	0.4825	0.1730
36	0.4250	0.4945	0.3700	0.3445
48	0.5810	0.6860	0.3120	0.7080
Means	0.4806a	0.4545a	0.3794a	0.4358a

Within a band, means that have the same letter are not significantly different at the 0.05 level of probability.

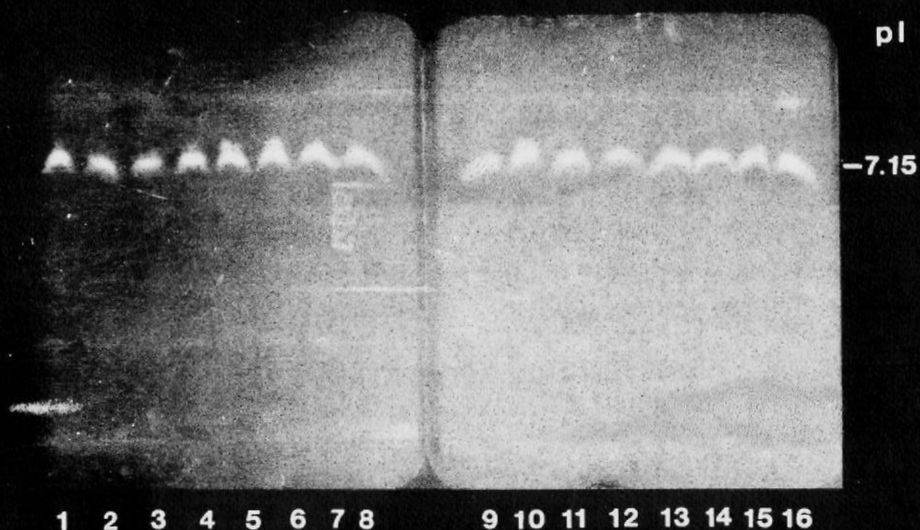


Fig. 12. Shikimate dehydrogenase IEF gels with pH range of 3-9. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7 = B37R I 24 hr, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

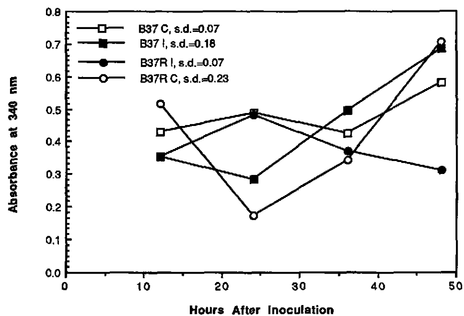


Fig. 13. Plot of SKDH IEF gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation.

The native gradient (10-15%) gels stained for shikimate dehydrogenase showed five bands of isozyme activity. These bands were at approximate Rf values of 0.00, 0.190, 0.340, 0.598, and 1.00 (Fig. 14). The mean absorbance values for these bands are in Table 5. Band 2 did not give consistent results in the gel scan, so only plots of bands 1, 3, 4, and 5 are given (Fig. 15). In band 1 at Rf 0.00, SKDH activity in B37 control is much higher than in B37 inoculated at 12 hr but falls below that of B37 inoculated from 24 to 36 hr. At 48 hr SKDH activity in both is at about the same level. B37R control also begins with a higher level of SKDH activity than B37R inoculated at 12 and 24 hr. At 36 hr B37R inoculated began to increase in level of enzyme activity, however, at 48 hr the level of SKDH activity in B37R control rose dramatically above that in B37R inoculated. SKDH activity in B37 inoculated began at a higher level than in B37R inoculated and peaked early at 24 hr after inoculation. Enzyme activity in B37R (inoc.) rose slowly but steadily until it surpassed that in B37R control. At 12 and at 48 hours the control samples had higher levels of SKDH activity than their respective inoculated samples. At 12 hr B37 had the higher values but at 48 hr, B37R did. Over time, however, no significant differences in mean SKDH activity was seen in any of the samples in band 1 (Table 5).

In band 3 at Rf 0.340, B37 SKDH activity in B37 control steadily decreased over time. The level of activity of shikimate dehydrogenase in B37 inoculated peaked at 24 hr and then dramatically decreased by 36 hr. B37R control started with a lower level than B37 control but gradually increased until 36 hr, after which it decreased. B37R inoculated followed the same pattern as did B37 inoculated, peaking at 24 hr. Again, in band 3 there were no significant differences in mean SKDH activity over time in any of the samples (Table 5).

SKDH activity in band 4, Rf = 0.590, was higher in B37 inoculated than in B37 control throughout the 48 hr. By 36 hr the level of enzyme activity in B37R inoculated had also surpassed that in B37R control. B37R inoculated

peaked in activity at 36 hr, later than the other samples.

All samples seemed to peak at 24 hr in band 5 at Rf=1.00, but B37R inoculated had the highest peak. Both B37 and B37R inoculated samples had higher values than their respective controls until 48 hr when the levels of enzyme activity were just slightly higher in the control samples than in the inoculated. However, even in bands 4 and 5 in the SKDH native gels, the level of enzyme activity did not significantly differ in any of the samples.

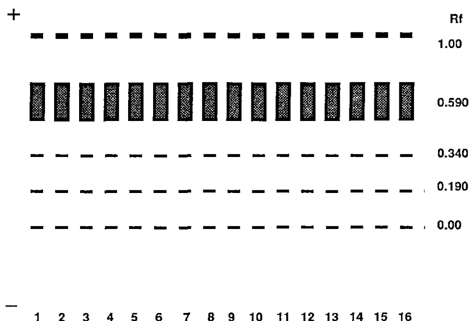


Fig. 14. Shikimate dehydrogenase native gradient (10-15%) gels. The bottom of the gel is the loading end (cathode position). 1 = B37 C 12 hr, 2 = B37 I 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7 = B37R I 24 hr, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 =B37R C 48 hr.

Table 5. SKDH native gradient (10-15%) gel mean band absorbance values at 340 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 Rf=0.00				
12	0.3605	0.2215	0.1500	0.1890
24	0.1985	0.3575	0.1540	0.2275
36	0.1160	0.1775	0.2165	0.0730
48	0.2280	0.2135	0.2610	0.4565
Means	0.2258a	0.2425a	0.1954a	0.2365a
Band 2 Rf=0.190				
12	-	-	-	0.0230
24	-	0.0340	0.0690	0.0345
36	-	-	-	0.0155
48	0.0250	0.0145	0.0450	-
Means[a]				
Band 3 Rf=0.340				
12	0.1395	0.0895	0.0715	0.1145
24	0.0675	0.2375	0.1470	0.1385
36	-	0.0170	0.0445	0.1490
48	0.0390	0.0575	0.0875	0.0940
Means	0.0820a	0.1004a	0.0876a	0.1240a
Band 4 Rf=0.590				
12	0.7840	1.0450	0.5955	0.7370
24	0.8735	1.2765	0.6040	0.8470
36	0.8060	1.1565	1.3895	0.5360
48	0.7250	1.4205	1.3070	1.4195
Means	0.7971a	1.2246a	0.9740a	0.8849a
Band 5 Rf=1.00				
12	0.1275	0.2345	0.1665	0.1535
24	0.1420	0.2525	0.4095	0.2085
36	0.1400	0.1665	0.1940	0.1385
48	0.1970	0.1500	0.1725	0.1835
Means	0.1518a	0.2009a	0.2356a	0.1710a

Within a band, means that share the same letter are not significantly different at the 0.05 level of probability.

Table 5. (continued)

[a] Based on a Tukey studentized range test of confidence intervals of the means, none of the means were significantly different from each other.

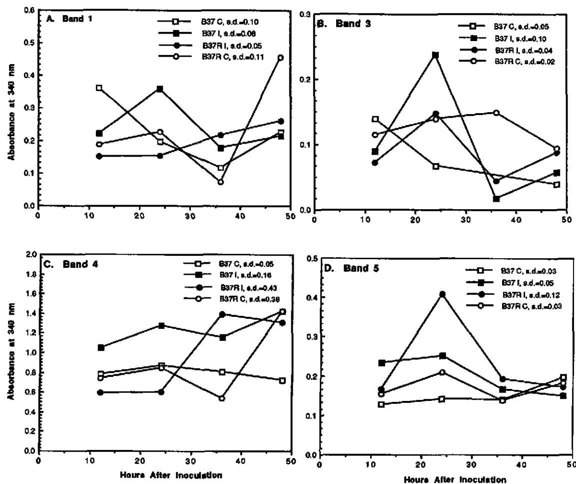


Fig. 15. Plots of SKDH native gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 Rf = 0.00. **B.** Band 3 Rf = 0.340. **C.** Band 4 Rf = 0.590. **D.** Band 5 Rf = 1.00.

Chalcone Isomerase

Neither the IEF gels nor the native gradient gels stained successfully for chalcone isomerase (CHI) activity. Bands could be detected neither visually nor photometrically with the gel scanner using a UV light source. It is presumed that the staining procedure as reported (57) was not suitable for work with the system used in this study or else CHI activity was at an undetectable level.

Cinnamyl-alcohol dehydrogenase

IEF gels stained for cinnamyl-alcohol dehydrogenase (CAD) displayed two isozyme bands in each lane (Fig. 16). One band was at pI 7.50 and the other at pI 8.68. These bands were consistent across all lanes of the gel; no unique bands were present in any of the samples. Mean absorbance values from the photometric scans are in Table 6. The plot of absorbance values for band 1 at pI 7.50 showed a general decrease in CAD activity in B37 control over 48 hr (Fig. 17). The same trend was basically followed by B37 inoculated. B37R inoculated CAD activity also decreased over time, although its level of activity began above that of the B37 samples. CAD activity in B37R control began at the same level as B37R inocualted and B37 inoculated, but it dramatically peaked at 24 hr and then fell off drastically by 36 hr. Again, by 48 hr, it rose way above the other samples.

CAD activity in band 2 at pI 8.68 also decreased over time in most of the samples. Only B37R control showed an increase in activity from 24 through 48 hr. Initially, B37 control had the highest level of enzyme activity at 12 hr, and B37R control had the lowest level. By 48 hr, however, that situation was completely reversed. For both band 1 and band 2, none of the samples showed significantly different mean levels of SKDH activity over time (Table 6).

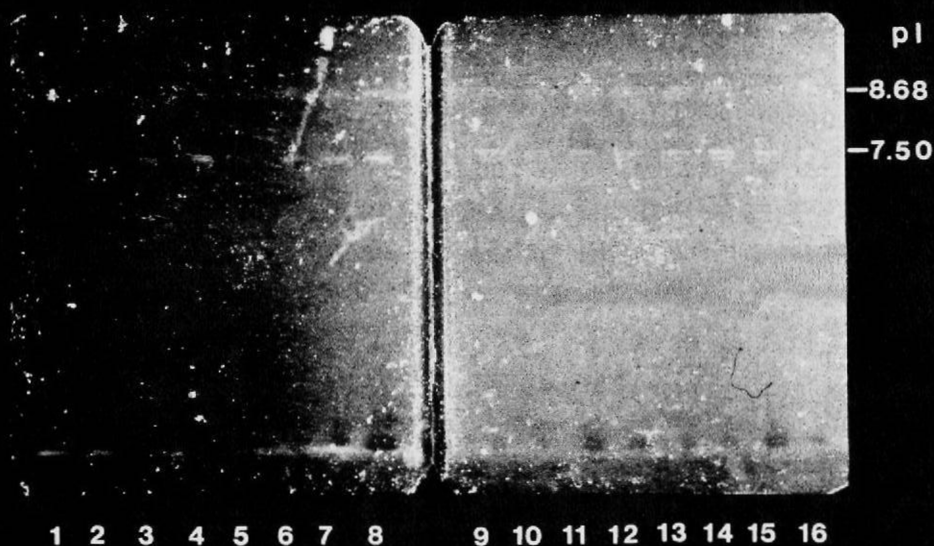


Fig. 16. Cinnamyl-alcohol dehydrogenase IEF (3-9) gels with pH range of 3-9. 1 = B37 C(control) 12 hr (hours), 2 = B37 I(inoculated) 12 hr, 3 =B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr,7= B37R I 24 hr, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, 16 = B37R C 48 hr.

Table 6. CAD IEF (3-9) gel mean band absorbance values at 330 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 pI=7.50				
12	0.2540	0.3150	0.3270	0.3230
24	0.1720	0.2130	0.2990	0.4145
36	0.1180	0.0450	0.0640	0.0340
48	0.1190	0.1115	0.0930	0.3175
Means	0.1658a	0.1711a	0.1958a	0.2723a
Band 2 pI=8.68				
12	0.2500	0.1990	0.2300	0.1800
24	0.1600	0.1505	0.1940	0.1070
36	0.0665	0.0130	0.0715	0.1260
48	0.0375	0.0670	0.0375	0.1670
Means	0.1285a	0.1074a	0.1332a	0.1450a

Within a band, means that have the same letter are not significantly different at the 0.05 level of probability.

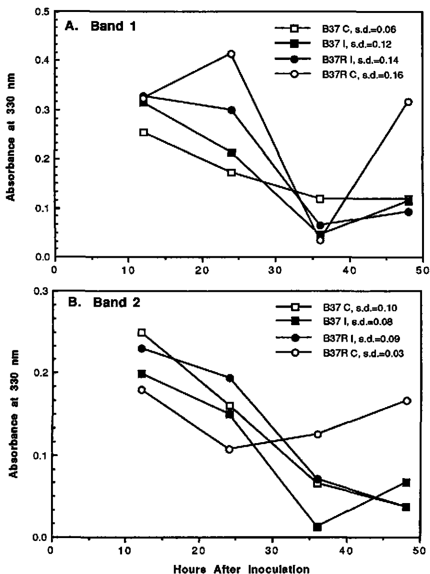


Fig. 17. Plots of CAD IEF gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 pI = 7.50. **B.** Band 2 pI = 8.68.

Native gradient gels stained for CAD showed 6 bands of activity at Rf values of 0.243, 0.393, 0.557, 0.864, 0.920, and 0.989 (Fig. 18). The mean absorbance values for the bands in all lanes are given in Table 7. The first band at Rf 0.243 showed a general decrease over time in both B37 and B37R controls. However, isozyme activity peaked slightly at 24 hr in B37 (inoc.) and gradually increased up to 48 hr in B37R (inoc.) (Fig. 19). By 48 hr, B37R inoculated had the highest level of CAD activity. The isozyme band at Rf 0.393 was not consistently recorded across all lanes of the gel. Although visible, this second band did not register on the photometric scan in B37R samples until 36 hr and then, only in the control (Table 7). This band finally registered in B37R inoculated samples at 48 hr. In B37, the peak absorbance values in both inoculated and controls were at 36 hr, the increase in B37 control being much more dramatic than in the B37 inoculated sample.

The third band (Rf=0.557) showed a general decrease in activity between 12 and 48 hr for every sample (Fig. 19). B37 inoculated began with the highest level of CAD activity at 12 hr and ended with the second highest at 48 hr. Both B37 and B37R inoculated samples began and ended the time course with higher levels of CAD activity than their respective control samples.

Band 4 at Rf=0.864 showed a steady increase in absorbance value up to 48 hr in both B37 and B37R inoculated samples, which began and ended at about the same levels as each other. The level of CAD activity just increased sooner in B37 inoculated. In the controls of both cultivars, absorbance values peaked at 36 hr, the level of enzyme activity in B37 being higher than in B37R. The bands of highest intensity, and therefore highest enzyme activity, were 4 and 5. The fifth band (Rf=0.920) showed a steady increase in activity in both B37 and B37R inoculated samples. B37R inoculated had a higher level of CAD activity than B37. The level of CAD

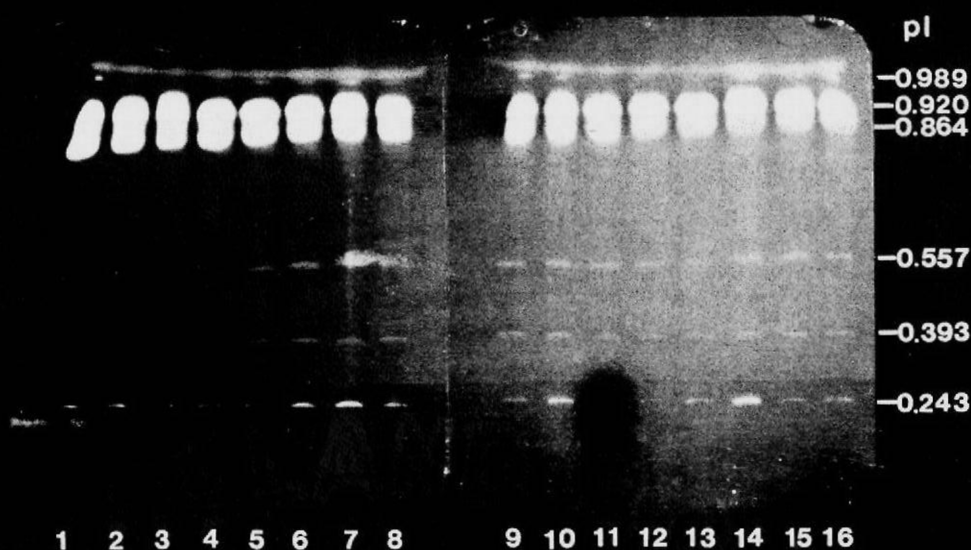


Fig. 18. Cinnamyl-alcohol dehydrogenase native gradient (10-15%) gels. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7=B37R I 24h, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

Table 7. CAD native gradient (10-15%) gel mean band absorbance values at 360 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 Rf=0.243				
12	0.2530	0.1540	0.1980	0.2435
24	0.2180	0.1820	0.1645	0.2005
36	0.1385	0.0455	0.1720f	0.1870
48	0.1240	0.1105	0.2450	0.0180
Means	0.1909a	0.123a	0.1949a	0.1622a
Band 2 Rf=0.393				
12	0.0145	0.2390	-	-
24	0.0105	0.2275	-	-
36	0.1255	0.2445	-	0.0125
48	0.0130	0.1945	0.1245	0.2315
Means	0.0409bc	0.2264a	0.1245ac	0.1220ac
Band 3 Rf=0.557				
12	0.0810	0.1400	0.0775	0.0585
24	0.0460	0.0785	0.0495	0.0380
36	0.0470	0.0390	0.0295	0.0465
48	0.0420	0.0555	0.0615	0.0175
Means	0.0540a	0.0782a	0.0545a	0.0401a
Band 4 Rf=0.864				
12	1.0730	1.0675	1.1345	1.1305
24	1.2760	1.2070	1.0625	1.1430
36	1.8260	1.4715	1.1220	1.5415
48	1.4910	1.5005	1.5200	1.2065
Means	1.4040a	1.3116a	1.2098a	1.2629a
Band 5 Rf=0.920				
12	0.8110	1.2980	2.0070	1.9730
24	2.0320	1.6005	1.9955	1.9935
36	0.7725	1.7510	2.0205	2.2705
48	2.2460	2.2150	2.3390	2.1210
Means	1.4654a	1.8222a	2.0905a	2.0895a

Table 7. (continued)

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 6 Rf=0.989				
12	0.2650	0.2640	0.2355	0.2240
24	0.2905	0.2950	0.2715	0.3790
36	0.1535	0.2680	0.2200	0.1955
48	0.1850	0.2225	0.2730	0.2535
Means	0.2235a	0.2624a	0.2500a	0.2630a

Within a band, means that have the same letter are not significantly different at the 0.05 level of probability.

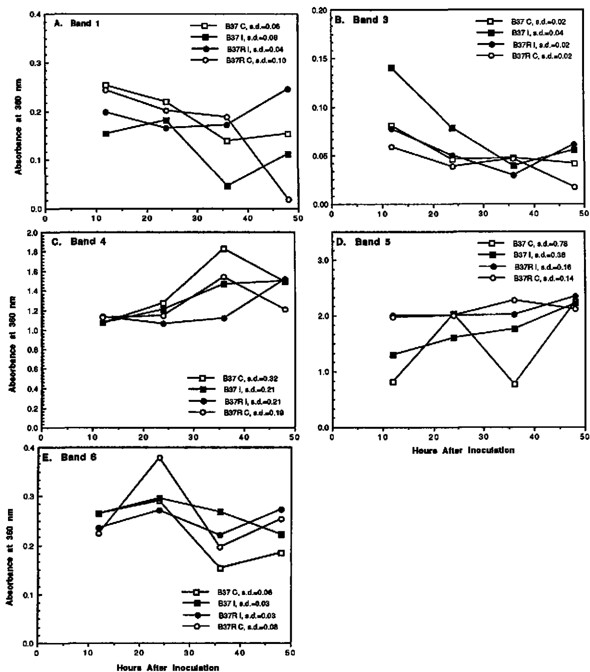


Fig. 19. Plots of CAD native gradient gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 Rf = 0.243. **B.** Band 3 Rf = 0.557. **C.** Band 4 Rf = 0.864. **D.** Band 5 Rf = 0.920. **E.** Band 6 Rf = 0.989.

activity in B37R control also showed a steady increase over time, with peak activity occurring at 36 hr. However, B37 control displayed erratic changes in CAD activity over time (Fig. 19). Activity in B37 control peaked at 24 hr and again at 48 hr. Although its enzyme activity level was the lowest at 12 hr, by 48 hr its level was about the same as the other samples.

CAD activity in band 6 (Rf = 0.989) was at about the same level in both B37 control and inoculated at 12 hr and then peaked at 24 hr in both and from there decreased gradually in B37 inoculated, but quite drastically in B37 control. In B37R samples, the level of CAD activity was also on equal par at 12 hr. Then activity increased in B37R inoculated but decreased in B37R control at 24 hr. CAD activity in B37R control increased from there through 36 and 48 hr. In B37R inoculated CAD activity fell at 36 hr but rose again by 48 hr. Both B37 and B37R inoculated samples had higher levels of enzyme activity than their respective controls over time. B37R samples began at a lower level of enzyme activity then B37 but by 48 hr they had the higher levels.

Peroxidase

Peroxidase-stained IEF gels displayed many isozyme bands (Fig. 20). Most bands consistently apppeared in all samples, but some differential bands were present. The additional bands of peroxidase activity appeared in lanes 3, 4, 7, 8, 11, 12, 15, and 16, which were B37R inoculated and B37R controls at 12, 24, 36, and 48 hr. Table 8 shows the absorbance values of the isozyme bands, and these are plotted in Fig. 21. The band nearest the cathode position at pI 2.75 was evident in all the lanes. Both B37R control and inoculated samples increased in PER activity over time. The level of activity in B37R was higher than in B37 throughout the 48 hr. B37 control and inoculated started at about the same level of PER activity at 12 hr, but B37 inoculated decreased by 24 hr. By 24 hr, B37 control had peaked in

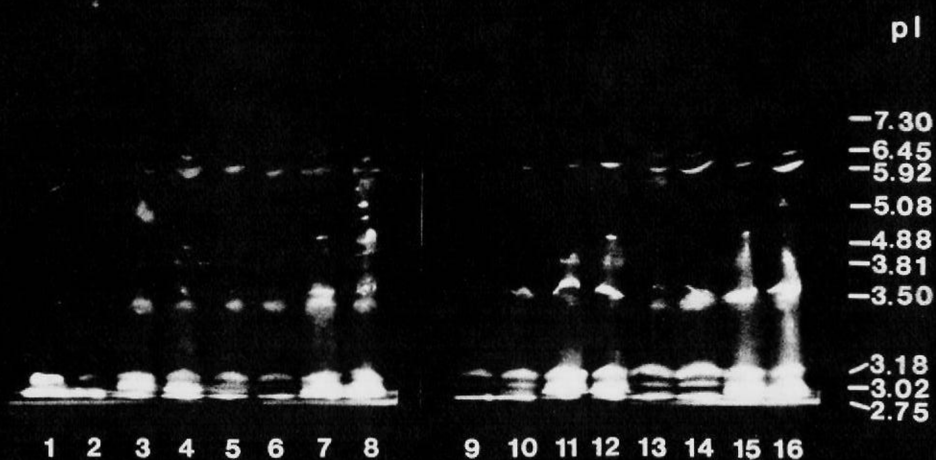


Fig. 20. Peroxidase IEF gels with pH range 3-9. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7=B37R I 24h, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

Table 8. PER IEF (3-9) gel mean band absorbance values at 430 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 pI=2.75				
12	1.0135	0.9715	1.0965	1.4420
24	1.2510	0.5770	1.2030	1.8200
36	0.7335	1.0475	1.8495	1.8660
48	1.0040	1.3285	2.0745	2.1955
Means	1.0005b	0.9811b	1.5559ab	1.8309a
Band 2 pI=3.02				
12	-	-	-	-
24	0.4595	-	-	-
36	0.2030	0.6170	-	-
48	0.3985	0.6620	-	-
Means	0.3537a	0.6395a		
Band 3 pI=3.18				
12	1.2595	0.2465	1.1100	0.7410
24	0.0830	0.3390	-	-
36	0.3855	0.6790	1.7000	0.7710
48	0.5515	0.7975	0.9285	1.0340
Means	0.5699a	0.5155a	1.2462a	0.8487a
Band 4 pI=3.50				
12	0.2320	0.0305	0.6355	0.2080
24	0.0190	0.0580	0.3500	0.2955
36	0.0595	0.5645	1.1700	0.7420
48	0.2880	0.4840	1.3335	1.5755
Means	0.1496a	0.2842a	0.8722a	0.7052a
Band 5 pI=5.92				
12	0.2245	0.0955	0.1770	0.2780
24	0.1290	0.1315	0.3595	0.4285
36	0.2980	0.3710	0.5000	0.2460
48	0.5960	0.4685	0.1970	1.0310
Means	0.3119a	0.2666a	0.3084a	0.4959a

Table 8. (continued).

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 7 pI=7.30				
12	0.0950	0.1005	0.1465	0.1755
24	0.0355	0.0495	0.0890	0.1565
36	0.3515	0.2680	0.1975	0.1715
48	0.3105	0.3500	0.2880	0.5115
Means	0.1981a	0.1920a	0.1802a	0.2538a

Values within one band that share the same letter are not significantly different at the 0.05 level of probability.

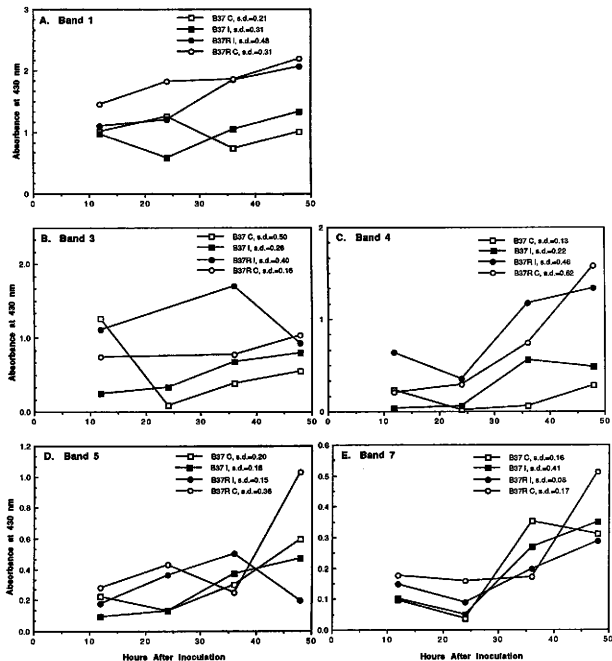


Fig. 21. Plots of PER IEF gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 pI=2.75. **B.** Band 3 pI=3.18. **C.** Band 4 pI=3.50. **D.** Band 5 pI=5.92. **E.** Band 7 pI =7.30.

enzyme activity. Overall, B37R control had the highest levels of PER activity over the 48 hr. B37R inoculated had slightly less, and B37 control and B37 inoculated showed much less PER activity (Table 8).

The second band at pI 3.02 was evidently present in all the lanes (Fig. 20), however, it was often scanned as part of the activity in band 1. Absorbance values which did record were in B37 control and inoculated samples only (Table 8). According to a test of the means' intervals, B37 inoculated and B37 control means were not significantly different for band 2.

The third band at pI 3.18 showed a decrease in PER activity over time in B37 control, whereas, B37 inoculated increased in PER activity over time (Fig. 21). B37R control stayed at about the same level of activity until 36 hr, after which there was a dramatic increase in PER activity to 48 hr. B37R inoculated had a very high peak of PER activity at 36 hr and then decreased in activity by 48 hr. Across time B37 control and B37 inoculated showed no significant difference in their levels of PER activity in this isozyme band. B37R inoculated had the higher overall mean level of PER activity, but none of the means were significantly different (Table 8).

Both B37R control and inoculated increased steadily in PER activity in band 4 (pI=3.50), with B37R inoculated being higher. B37 control also increased in activity after 36 hr. However, B37 inoculated appeared to peak in enzyme activity at 36 hr and then decreased. Overall, the means appeared to be quite different, yet due to the degree of variability, none of the means were actually significantly different (Table 8).

B37 control and B37 inoculated samples gradually increased in PER activity in band 5 (pI=5.92) over time. B37R control had a rather erratic pattern of absorbance readings. PER activity peaked slightly at 24 hr, then dropped by 36, but rose again dramatically by 48 hr. B37R inoculated gradually increased in PER activity to a peak at 36 hr and then decreased by 48 hr. In this band, B37R control had the highest level of PER activity over time, B37 control had the next highest, B37R inoculated had slightly lower

activity, and B37 inoculated had the lowest level of enzyme activity (Table 8). However, none of the means were significantly different.

The next band to occur in all lanes was at pI=6.45. This band did not give reliable readings on the gel scan, therefore analysis of the band intensities was impossible to perform.

The last band which appeared in all lanes was at pI 7.30. PER activity in both B37 and B37R inoculated samples decreased between 12 and 24 hr, but then gradually increased after 24 hr. The control samples had very different patterns of PER activity. B37 control peaked at 36 hr. B37R control PER activity remained at about the same level until 36 hr, after which enzyme activity dramatically increased. A Tukey test of the means showed that none of the means were significantly different.

Lane 3 (B37R inoculated, 12 hr) showed a band at a pI of 5.08. Lanes 7 and 8 (B37R inoculated and control at 24 hr), lane 12 (B37R control at 24 hr), and lanes 15 and 16 (B37R inoculated and control at 48 hr) also showed a band at this position (Fig. 20). Lane 4 (B37R control at 12 hr) had a band of peroxidase activity at pI 4.88 as did lanes 7, 8, 11, 12, 15, and 16. These respresent B37R inoculated and control samples at all of the times in the study. Lanes 11, 12, 15, and 16 (B37R inoculated and control samples at 36 and 48 hr) also had a different band at pI 3.81, which was not evident in any of the other lanes (Fig. 20).

Five bands appeared consistently in all lanes of the native gradient gels stained for peroxidase activity (Fig. 22). These bands were located at Rf values of 0.098, 0.157, 0.274, 0.470, and 0.824 (Fig. 22). A band was also visible on the gels in lanes 9, 10, 11, and 15 at an Rf of 0.916. This band was only very faintly suggested in the other lanes (Fig. 22). The mean absorbance values for these bands is given in Table 9. Plots of the means of each band are shown in Figure 23.

In addition to the bands in common to all lanes, there were unique bands of activity faintly distinguishable in lanes 6, 10, 11, 14, and 15. Located at Rf

values of 0.686, 0.627, and 0.549, these bands were only seen in B37 and B37R inoculated samples but not in any controls (Fig. 22).

In band 1 at Rf=0.098, B37 and B37R controls had opposite trends in PER activity. B37 control gradually decreased in PER activity over time, whereas, B37R control greatly increased over time. B37 inoculated gradually increased in PER activity until 36 hr, after which the activity leveled out. B37R inoculated had a tremendous peak of peroxidase activity at 36 hr after inoculation. For band 1, B37R inoculated had the highest mean level of PER activity over time (Table 9). B37R control had slightly less overall activity. B37 control and B37 inoculated had levels of enzyme activity significantly lower than B37R inoculated (Table 9).

In band 2 at Rf=0.157, B37 control and B37 inoculated both displayed the same pattern of change in PER activity (Fig. 23). Both had quite low levels of activity which peaked slightly at 36 hr. PER activity in B37R control changed only slightly during the 48 hr, bur activity in B37R inoculated showed a very dramatic increase after 24 hr. B37R inoculated had the highest level of PER activity over time followed by B37R control. B37 control and B37 inoculated had significantly lower mean absorbance readings than B37R inoculated (Table 9).

In band 3 at Rf=0.274, the same types of trends as in band 2 occurred (Fig. 23). Both B37 control and B37 inoculated had very low levels of PER activity. B37R inoculated had the highest levels.

Peroxidase activity in B37 control for band 4 (Rf=0.470) decreased slightly over time (Fig. 23). The same was true for B37R control. B37 inoculated showed somewhat of an increase after 36 hr, and B37R inoculated increased in PER activity dramatically after 24 hr. Once again, B37R inoculated had the highest mean level of PER activity over time. Although B37R control, B37 control, and B37 inoculated were all at lower levels of activity, these were not significantly different than B37R inoculated (Table 9).

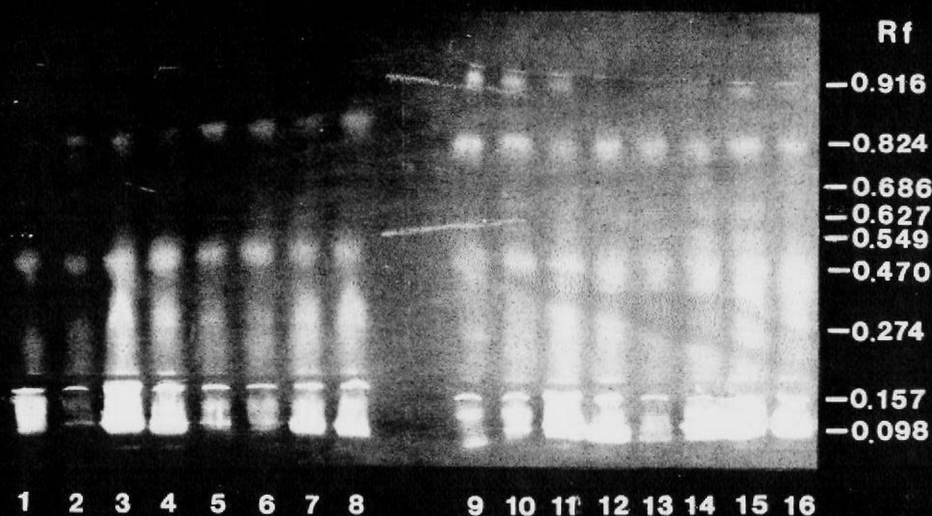


Fig. 22. Peroxidase native gradient (10-15%) gels. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7=B37R I 24h, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

Table 9. PER native gradient (10-15%) gel mean band absorbance values at 430 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 Rf=0.098				
12	0.7540	0.2810	1.7210	0.8600
24	0.6020	0.3675	1.3580	1.1090
36	0.5560	0.7970	2.9155	1.7165
48	0.2985	0.7265	1.7565	2.1030
Means	0.5510b	0.5430b	1.8702a	1.4471ab
Band 2 Rf=0.157				
12	0.4330	0.3265	1.4730	0.6160
24	0.4525	0.3015	0.6975	0.7410
36	0.7465	0.5635	1.0825	0.4645
48	0.3635	0.1865	2.0645	0.8230
Means	0.4989b	0.3445b	1.3294a	0.6611ab
Band 3 Rf=0.274				
12	0.0795	0.0490	0.3320	0.1680
24	0.0730	0.0525	0.1645	0.1990
36	-	-	0.2250	0.0510
48	0.0165	0.0475	0.1480	0.1510
Means	0.0762b	0.0497b	0.2174a	0.1422ab
Band 4 Rf=0.470				
12	0.1150	0.0420	0.1515	0.0850
24	0.0335	0.0420	0.0360	0.0865
36	0.0410	0.0280	0.1390	0.0575
48	0.0390	0.0980	0.2675	0.0710
Means	0.0571a	0.0525a	0.1485a	0.0750a
Band 5 Rf=0.824				
12	0.0740	0.0340	-	-
24	-	-	-	0.0120
36	0.0815	-	-	-
48	-	-	-	-
Means[a]				

Within a band, means that share the same letter are not significantly different at the 0.05 level of probability.

[a] Means could not be generated due to lack of values in most samples.

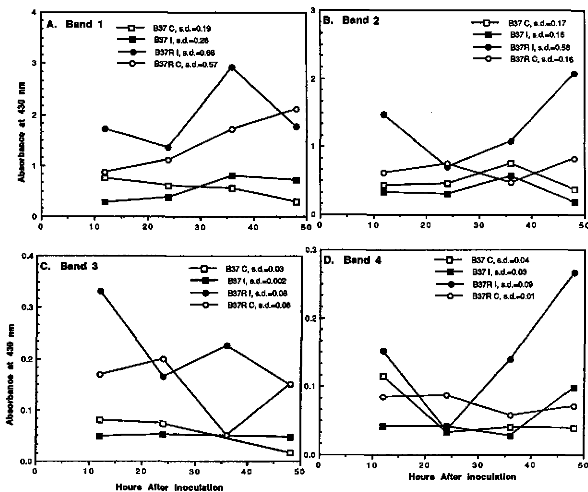


Fig. 23. Plots of PER native gradient gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 Rf=0.098. **B.** Band 2 Rf=0.157. **C.**Band 3 Rf=0.274. **D.** Band 4 Rf=0.470.

Polyphenoloxidase

Polyphenoloxidase (PPO) IEF gels displayed 2 main bands of enzyme activity at pI 5.10 and pI 7.40. Both of these isozyme markers were apparent in every lane (Fig. 24). Absorbance values for the two bands are shown in Table 10. Absorbance values were very erratic in pattern (Fig. 25). The absorbance values in band 1 (pI=5.10) across the lanes of the gels produced no clear pattern of change over time. B37R (inoc.) started high and decreased over time, whereas B37 (inoc.) band 1 had peak activity at 36 hr. Both controls showed an overall increase in absorbances over time, although the pattern was quite irregular. A test of the means showed none of them to be significantly different from each other (Table 10).

In band 3 (pI=7.40) both B37 and B37R inoculated samples increased in PPO activity over time. B37R control did not display much change in activity, and B37 control had quite erratic changes in level of PPO activity over time (Fig. 25). An analysis of the absorbance means over time for band 3 showed that no means were significantly different from each other (Table 10).

Staining for PPO activity with native gradient gels produced bands of very weak intensity. There were 3 visible bands of isozyme activity at Rf 0.182, 0.424, and 0.788. A diagrammatic representation of the gels is given (Fig. 26). Only the first and third bands (Rf=0.182 and Rf=0.788) gave consistent results in the photometric scans for enzyme activity (Table 11 and Fig. 27). B37 and B37R inoculated samples both had peak enzyme activity at 36 hr in band 1 at Rf=0.182. Both of the control samples showed a general pattern of increase in PPO activity over time. B37R inoculated had the highest mean level of PPO activity over time, however none of the means were significantly different from each other (Table 11). In band 3 at Rf=0.788, the same peak in activity occurred in B37 and B37R inoculated samples. However, in band 3 the controls had rather irregular patterns of

change in PPO activity (Fig. 27). Overall, B37 inoculated had the highest PPO activity in band 3. The levels in B37R control, B37R inoculated, and B37 control were less but not significantly different from each other or from that of B37 inoculated (Table 11).

Table10. PPO IEF (3-9) gel mean band absorbance values at 340 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 pI 5.10				
12	0.1470	0.0735	0.3540	0.1645
24	0.1200	0.1270	0.1495	0.1700
36	0.1220	0.1420	0.1345	0.0795
48	0.1675	0.1315	0.1355	0.2350
Means	0.1389a	0.1185a	0.1934a	0.1622a
Band 3 pI 7.40				
12	-	0.0135	0.0485	0.0140
24	0.0970	0.0290	0.0605	0.0420
36	0.0320	0.0290	0.0435	0.0565
48	0.0915	0.0845	0.0955	0.0430
Means	0.0735a	0.0390a	0.0620a	0.0389a

Within a band, means that share the same letter are not significantly different at the 0.05 level of probability.

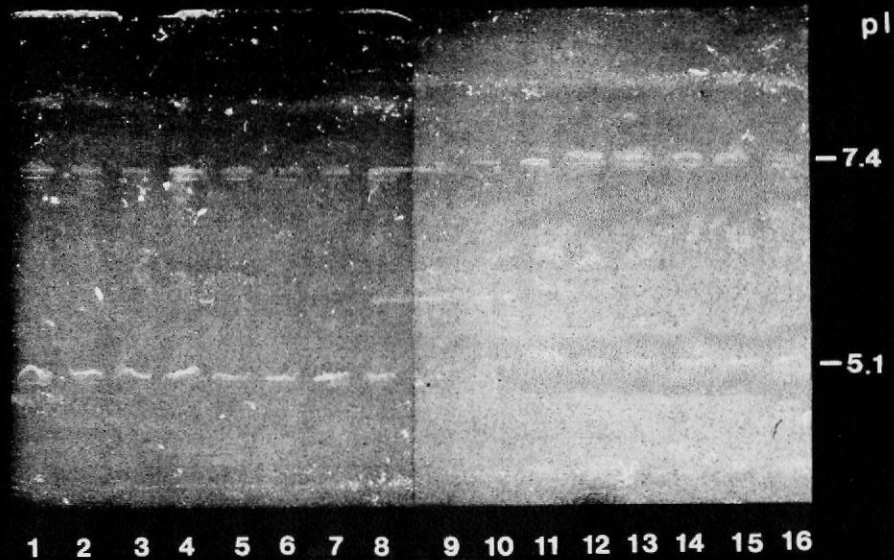


Fig. 24. Polyphenoloxidase IEF gels with pH range of 3-9. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7=B37R I 24h, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

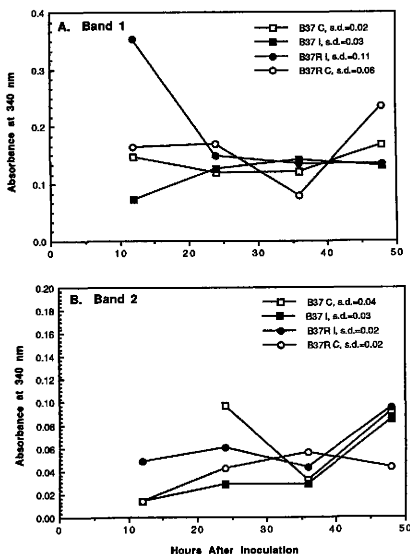


Fig. 25. Plots of PPO IEF gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 pI=5.10. **B.** Band 2 pI=7.40.

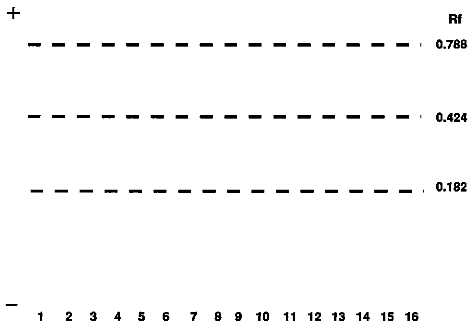


Fig. 26. Polyphenoloxidase native gradient (10-15%) gels. The bottom of the gel is the loading end (cathode position). 1 = B37 C (control) 12 hr (hours), 2 = B37 I (inoculated) 12 hr, 3 = B37R I 12 hr, 4 = B37R C 12 hr, 5 = B37 C 24 hr, 6 = B37 I 24 hr, 7=B37R I 24h, 8 = B37R C 24 hr, 9 = B37 C 36 hr, 10 = B37 I 36 hr, 11 = B37R I 36 hr, 12 = B37R C 36 hr, 13 = B37 C 48 hr, 14 = B37 I 48 hr, 15 = B37R I 48 hr, and 16 = B37R C 48 hr.

Table 11. PPO native gradient (10-15%) gel mean band absorbance values at 340 nm for control and inoculated B37 and B37R 12-48 hr after inoculation.

Hr after inoculation	B37 control	B37 inoc.	B37R inoc.	B37R control
Band 1 Rf=0.182				
12	0.2625	0.2155	0.3230	0.1860
24	0.2085	0.2100	0.2985	0.2360
36	0.3080	0.4460	0.4220	0.3500
48	0.4495	0.3285	0.3740	0.3225
Means	0.3071a	0.3000a	0.3544a	0.2736a
Band 3 Rf=0.788				
12	0.0880	0.0990	0.0690	0.0550
24	0.0540	0.1375	0.1210	0.1245
36	0.1940	0.1545	0.1545	0.1550
48	0.1260	0.1375	0.1295	0.1420
Means	0.1155a	0.1321a	0.1185a	0.1191a

Within a band, means that share the same letter are not significantly different at the 0.05 level of probability.

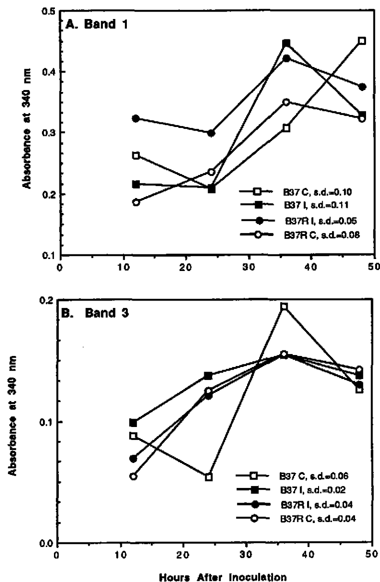


Fig. 27. Plots of PPO native gradient gel mean absorbance values for control (C) and inoculated (I) B37 and B37R 12-48 hr after inoculation. **A.** Band 1 Rf=0.182. **B.** Band 3 Rf=0.788.

DISCUSSION

In an effort to correlate the presence of isozymes with the resistance response of maize to *P. polysora*, several enzymes involved in the shikimic acid pathway of phenolic production were studied in near isogenic resistant and susceptible maize cultivars after inoculation. Both isoelectric focusing and native gradient polyacrylamide gel electrophoresis were used to determine the presence of isozymes of shikimate dehydrogenase, chalcone isomerase, cinnamyl-alcohol dehydrogenase, peroxidase, and polyphenoloxidase. In general, native gradient gel electrophoresis allowed for the distinction of more isozyme bands than did the IEF gels.

Shikimate dehydrogenase is involved early on in the shikimic acid pathway leading to the production of phenolic phytoalexins. Although it may be assumed that changes in the level of activity of this enzyme would be primary early indicators of increased phenolic production, this did not occur in this system. Both IEF and gradient gels showed the same isozyme banding patterns in all of the samples, regarless of cultivar type or treatment. No differential bands appeared which could be correlated with a resistance response. In the SKDH IEF gel, the pattern of change in enzyme activity in the inoculated samples was exactly opposite of what would be expected. B37 inoculated continued to increase in SKDH activity over time, whereas B37R inoculated peaked at 24 hr, and then decreased in SKDH activity. If phenolic production is part of the resistance response, then it would be expected that SKDH activity would continuously increase over time in the resistant cultivar but would peak early and then decrease in the susceptible cultivar. This was not the case.

In the native gradient gels stained for SKDH activity, most of the bands showed no consistent patterns in the changes in enzyme activity that could be correlated to the resistance response in B37R. B37R inoculated had the

highest levels of SKDH activity over time in band 2 at Rf=0.190 and in band 5 at Rf-1.00. However, B37 inoculated had the highest levels of activity in band 1 at Rf=0.00 and in band 4 at Rf=0.590 (Table 5). However, since none of these means were significantly greater than the others, shikimate dehydrogenase did not appear to be a good marker for studying the resistance response in maize to southern rust.

Gels stained for cinnamyl-alcohol dehydrogenase activity also showed rather homogenous banding patterns across the samples. As for SKDH, the CAD native gradient gels were able to distinguish more isozyme bands than IEF gels. Cinnamyl-alcohol dehydrogenase is involved in the steps preceding lignin synthesis. If lignin deposition is involved in the resistance response, then its isozyme patterns may more directly indicate a difference between the compatible and incompatible reactions between maize and *P. polysora*. The IEF gels indicated that over time, neither of the maize cultivars, and neither control or inoculated, showed a significantly greater level of CAD activity (Table 6).

In band 1 (Rf=0.243) of the native gradient gels, the level of CAD activity in B37R inoculated continued to rise after that in B37 inoculated began to decrease after 24 hr. A similar trend occurred in the activity levels in isozyme band 6 at Rf 0.989. After 24 hr, the level of CAD activity in B37 inoculated decreased but that in B37R inoculated increased again after 36 hr. These two isozyme bands may be indicators of an enzymatic difference in the response of B37 and B37R to the pathogen. Perhaps particular forms of the cinnamyl-alcohol dehydrogenase enzyme are involved more directly in the response of the plant to the pathogen, while other isozymic forms of CAD are involved in other metalbolic processes. These may or may not be directly related to the events of resistance.

Both IEF and native gradient gels displayed many isozymes of peroxidase. Both types of gels also allowed for the detection of differential banding patterns in different samples at different times. Peroxidase

isozymes at pl 3.81, 4.88, and 5.08 were only seen in B37R inoculated and control samples (Fig. 20). Although these PER isozymes are not unique to the B37R inoculated samples but are in the controls as well, their significance cannot be overlooked since they only occurred in B37R, the resistant cultivar. Isozyme bands were much more intense in the B37R samples than in B37 throughout the time course. Evidently B37R produces more (and unique) peroxidase isozymes in response to stress, as in a pathogenic invasion than does its susceptible counterpart, B37.

The native gradient gels showed the same trend of more intense banding patterns in B37R samples than in B37. In band 1 at Rf 0.098, in band 2 at Rf 0.157, in band 3 at Rf 0.274, and in band 4 at Rf 0.470, B37R inoculated always had the highest mean PER activity over time. B37R control also had quite high levels of PER activity over time. The B37R cultivar may produce higher levels of peroxidase constitutively, and also have the ability for greater levels of induced activity.

Like shikimate dehydrogenase, polyphenoloxidase activity did not provide a very distinct marker for differentiating the compatible and incompatible reactions either. In the IEF gels, B37R inoculated had slightly but not significatly higher mean PPO activity over time, but the pattern of change in enzyme activity did not follow any coherent scheme that could be correlated with a resistance response. The same was basically true for the results from the native gradient gels. Both B37R and B37 inoculated showed peaks in PPO activity at the same time (36 hr) after inoculation.

Only peroxidase isozymes, and to a lesser extent CAD isozymes, proved to be somewhat correlated to or indicative of a difference between the compatible and incompatible reactions between B37 and B37R maize cultivars and *P. polysora* in the first 48 hr after inoculation. Since both of these enzymes are involved in lignin synthesis, increased lignin production and deposition in the resistant B37R may be one of the lines of defense against pathogenic invasion or a response to other forms of stress. This

correlation between higher peroxidase and cinnamyl-alcohol dehydrogenase activity in all or certain isozymes and the resistance of B37R maize cultivar does not imply a causal relationship. Indeed, polyphenoloxidation is associated with many necrotic reactions that may occur as a result of stress to the plant tissue. The hypersensitive type of resistance response involves a quick reaction of the host plant to the pathogen resulting in host cell death and also subsequent death of the pathogen. This type of reaction may cause the activity of enzymes such as peroxidase to increase simply due to the necrotic reactions occurring. Therefore, whether peroxidase activity is a cause or result of a resistance response is still unclear. However, the higher levels of peroxidase in the resistant cultivar (B37R) as compared to the near isogenic susceptible cultivar (B37), would seem to suggest that in some respect peroxidase is an indicator for resistance in this system.

CHAPTER IV
CONCLUSIONS

Understanding the mechanisms of host plant resistance is important not only to plant pathologists, but to molecular biologists, geneticists, plant breeders, and the growers of crop plants as well. As important as discovering what physiological events are involved, is learning how to use this information on a practical level in agriculture to facilitate successful crop production. The first step, however, is to discover what molecular events are involved in resistance responses in order to understand how to utilize them for the increased protection of our crop plants against the devastation of disease.

Many investigators have studied the role that phytoalexin production may play in the response of host plants to potential pathogens. Phytoalexins have proven to be fungitoxic and their production related to events leading to a resistance response to disease in many cases. Therefore it was undertaken to see what role, if any, the production of these phenolic compounds has in the resistant response of maize to *P. polysora*, the causative agent of southern rust disease.

First, a histological study of the infection process in two near isogenic resistant and susceptible maize cultivars was done. Differences in the pathogen's success of penetration of the host, subsequent growth in the host tissue, and eventual reproduction in the two cultivars could give clues as to what physiological events may be concurring with the resistance response. Increases in enzymes' activities, induction of mRNA's, and other phenomena are not totally meaningful outside of an understanding of the cytological events involved in infection.

In the first 48 hr after inoculation with uredospores of *P. polysora,* there was no great distinctive event marking a difference in the pathogen's growth in the two maize cultivars. The uredospores successfully germinated on

both cultivars and also successfully penetrated the stomata of both. Substomatal vesicles of the same size and characteristics developed in both cultivars and infection hyphae also developed from these. Like histological studies of the closely related organism, *P. sorghi*, on near isogenic maize cultivars (26), the study here showed that fungal growth may proceed equally well in susceptible and resistant plants early in the infection process. However, penetration of stomata was much greater in the susceptible, B37. The main difference was in the rate of growth of the fungus in each cultivar. Growth and development in the resistant B37R seemed to lag behind that in the susceptible B37. Within the course of this study no evidence of ensheathment and death of fungal haustoria was seen as that reported by Hilu (26) in incompatible reactions.

Hilu reported several types of incompatible reactions occurring in inbreds resistant to *P. sorghi* (26). Some were very rapid hypersensitive-type reactions, whereas others were much slower, in which much more fungal growth and development occurred before eventual death of the pathogen. This variation in reactions is due to differences in the interaction of the pathogen's genotype with that of varied genotypes in different maize cultivars. Resistance to *P. polysora* in maize appears to be of a slower reactive type. No evidence of a quick hypersensitive response within the first 48 hr after inoculation was seen. Rather, the resistance response seems to be the outcome of a sequence of events occurring over time, presenting a series of hurdles which the fungus is faced with overcoming or succumbing to.

To more completely understand the mode of resistance in maize cultivar B37R, it is necessary to also study events later than 48 hr after inoculation. The ultimate manifestation of the resistance response of the host, resulting in death of the pathogen, must occur later toward the stage of sporogenesis. It may also be beneficial to specifically stain embedded inoculated tissues to detect the presence of such compounds as lignin, which may contribute

to the defense of the host. Until further cytological work is done on this system, the total picture of the defense response in maize remains grossly incomplete.

Since the production of many phytoalexins proceeds via the shikimic acid pathway for synthesis of aromatic amino acids, enzymes in this and related pathways were assayed for isozymes which might be correlated to the defense response in maize. As reported by Biles in the study of the induced resistant response in watermelon to *Fusarium oxysporum* (4), shikimate dehydrogenase displayed no isozymes useful for differentiating between compatible and incompatible reactions. Isozymes of polyphenoloxidase were also not useful markers for a resistant response. Only isozymes of cinnamyl-alcohol dehydrogenase and peroxidase gave results which could be correlated to the resistance response of B37R to southern rust.

Cinnamyl-alcohol dehydrogenase is involved in the production of cinnamyl alcohols, building blocks of lignin. Two isozymes of CAD had high levels of activity in B37R inoculated samples. The activity of this isozyme increased steadily throughout the first 48 hr after inoculation, whereas levels of the same isozyme peaked and decreased in B37 inoculated samples. These results may suggest a possible role of lignin synthesis in the resistance response.

In further support of this idea were the results of the assay for peroxidase isozymes. Differential isozyme bands appeared only in the B37R samples. The levels of PER activity were highest in B37R inoculated samples as shown in photometric scans of the bands in the native gradient gels. Also evident is the intensity of banding visible on the PER IEF gels in B37R samples as compared to both control and inoculated B37 samples. In agreement again with the results of Biles on watermelon (4), peroxidase may prove to be a isozyme marker for differentiating susceptible and resistant plants.

Further work needs to be done to address this issue in more detail. As well as the cytological studies, the analysis of isozymes, should be carried out at times later in the infection process. Perhaps, isozymes of these enzymes which are involved in the resistance response come into play at a later time. There may be a period beyond 48 hr after inoculation which would be much more ammenable to studying the events involved in the resistance response. In addition to enzymatic studies, genetic studies on the Rpp9 gene responsible for conferring resistance to *P. polysora* in maize are necessary. The isolation of the gene and its product would extremely facilitate the investigation of this system. Not only would it provide knowledge about this specific host-pathogen system, but perhaps for host-pathogen interactions in general. In order to successfully address the problems posed by plant pathogenic fungi, as well as other pathogenic agents on important crop plants, information about modes of resistance must be obtained. These answers will come, however, only after the questions have actively been asked through study of host-pathogen interactions at all levels.

LITERATURE CITED

1. Anonymous. PhastSystem owner's manual. Pharmacia LKB Biotechnology, Inc. Piscataway, NJ.

2. Bailey, B. A. 1987. Resistance to *Puccinia polysora* in *Zea mays* L.: Identification, genetic transmission (linkage), and induced molecular responses. Ph. D. Dissertation. Texas A&M University.

3. Bailey, B. A., W. Schuh, R. A. Frederiksen, A. J. Bockholt, and J. D. Smith.1986. Identification of slow-rusting resistance to*Puccinia polysora* in maize inbreds and single crosses. Plant Dis.71(6): 518-521.

4. Biles, C. L. 1988. An isozyme analysis of the induced resistance response to fusarium wilt of watermelon incited by incompatible vascular wilt fusaria. Ph. D. Dissertation. Texas A&M University.

5. Bradford, M. M. 1976. A repid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

6. Brown, S. A. 1966. Lignins. Ann. Rev. Plant Physiol. 17:223-244.

7. Burdon, J. J., and D. R. Marshall. 1983. The use of isozymes in plant disease research. p. 401-412. In S. D. Tanksley, and T. J. Orton, (ed.), Isozymes in plant genetics and breeding, Part A. Elsevier Science Publishers B. V., Amsterdam.

8. Byrde, R. J. W. 1963. Natural inhibitors of fungal enzymes and toxins in disease resistance. p. 31-41. In S. Rich, (ed.), Perspectives of biochemical plant pathology, Conn. Agr. Expt. Sta. Bull. Vol. 663. New Haven, CN. 191 p.

9. Cammack, R. H. 1953. Observations in *Puccinia polysora* Underwood in West Africa. West Africa Maize Rust Res. Unit Annu. Rep. 1:16-31.

10. Cammack, R. H. 1959. Studies on *Puccinia polysora* Underw. III. Description and life cycle of *P. polysora* in West Africa. Trans. Brit. Mycol. Soc. 42(1):55-58.

11. Conn, E. E. 1964. Enzymology of phenolic biosynthesis. p. 399-435. In J. B. Harborne, (ed.), Biochemistry of phenolic compounds. Academic Press, New York. 619 p.

12. Couture, R. M., D. G. Routley, and G. M. Dunn. 1971. Role of cyclic hydroxamic acids in monogenic resistance of maize to *Helminthosporium turcicum.* Physiol. Plant Path. 1:515-521.

13. Deverall, B. J. 1961. Phenolase and pectic enzyme activity in the chocolate spot disease of bean. Nature 184:311.

14. Dickerson, D. P., S. F. Pascholati, A. E. Hagerman, L. G. Butler, and R. L. Nicholson. 1984. Phenylalanine ammonia-lyase and hydoxycinnamic acid:CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum.* Physiol. Plant Path. 25:111-123.

15. Dixon, R. A., and D. S. Bendall. 1978. Changes in the levels of enzymes of phenylpropanoid and flavonoid synthesis during phaseollin production in cell suspension cultures of *Phaseolus vulgaris.* Physiol. Plant Path. 13:295-306.

16. Dixon, R. A., P. M. Dey, and C. J. Lamb. 1979. Phytoalexins: Enzymology and molecular biology. p. 1-136. In A. Meister, (ed.), Advances in enzymology. Vol. 55. John Wiley & Sons, Inc., New York.

17. Dixon, R. A., and C. J. Lamb. 1979. Stimulation of de novo synthesis of L-phenylalanine ammonia-lyase in relation to phytoalexin accumulation in *Colletotrichum lindemuthianum* elicitor-treated cell suspension cultures of french bean (*Phaseolus vulgaris*). J. Biochim. Biophys. Acta. 586:453-463.

18. Eigen, E., M. Blitz, and E. Gunsberg. 1957. The detection of some naturally occurring flavanone compounds on paper chromatograms. Arch. Biochem. Biophys. 68:501-502.

19. Fehrmann, H., and A. E. Dimond. 1967. Peroxidase activity and *Phytophthora* resistance in different organs of the potato plant. Phytopath. 57:69-72.

20. Futrell, M. C. 1975. *Puccinia polysora* epidemics on maize associated with cropping practice and genetic homogeneity. Phytopath. 65:1040-1042.

21. Goodman, R. N., Z. Kiraly, and K. R. Wood. 1986. The biochemistry and physiology of plant disease. University of Missouri Press, Columbia. 433 p.

22. Grand, C., F. Sarni, and C. J. Lamb. 1987. Rapid induction by fungal elicitor of the synthesis of cinnamyl-alcohol dehydrogenase, a specific enzyme of lignin synthesis. Eur. J. Biochem. 169:73-77.

23. Grisebach, H. 1981. Lignins. p. 457-478. In E. E. Conn, and P. K. Stumpf, (ed.), The biochemistry of plants: A comprehensive treatise. Vol. 7: Secondary plant products. Academic Press, New York. 798 p.

24. Hadwiger, L. A. 1968. Changes in plant metabolism associated with phytoalexin production. Neth. J. Plant Path. 74(1):163-169.

25. Hijwegen, T. 1963. Lignification, a possible mechanism of active resistance against pathogens. Neth. J. Plant Path. 69:314-317.

26. Hilu, H. M. 1965. Host-pathogen relationships of *Puccinia sorghi* in nearly isogenic resistant and susceptible seedling corn. Phytopath. 55:563-569.

27. Hoffman-Ostenhof, O. 1963. Enzyme inhibition by quinones. p. 145-159. In R. M. Hochster, and J. H. Quastel. (ed.), Metabolic inhibitors. Vol. 3. Academic Press, New York. 1028 p.

28. Hollier, C. A. 1985. Effects of temperature and relative humidity on germinability and infectivity of *Puccinia polysora* uredospores. Plant Dis. 69:937-939.

29. Hollier, C. A., and S. B. King. 1985. Effect of dew period and temperature on infection of seedling maize plants by *Puccinia polysora*. Plant Dis. 69:219-220.

30. Hughes, F. L., and F. H. J. Rijkenberg. 1985. Scanning electron microscopy of early infection in the uredial stage of *Puccinia sorghi* in *Zea mays*. Plant Path. 34:61-68.

31. Hyodo, H., and I. Uritani. 1965. O-Diphenol oxidases in sweet potato infected by the black rot fungus. J. Biochem. (Japan). 57:161-166.

32. Johansen, D. A. 1940. Plant microtechnique. McGraw-Hill Book Company, Inc., New York. 523 p.

33. John, V. T., and M. Weintraub. 1967. Phenolase activity in *Nicotiana glutinosa* infected with tobacco mosaic virus. Phytopath. 57:154-158.

34. Kanazawa, Y., H. Shichi, and I. Uritani. 1965. Biosynthesis of peroxidases in slices of black rot-infected sweet potato roots. Agr. Biol. Chem. 29:840-847.

35. Kosuge, T. 1969. The role of phenolics in host response to infection. Ann. Rev. Phytopath. 7:195-222.

36. Kuc, J. 1972. Phytoalexins. Ann. Rev. Phytopath. 10:207-232.

37. Lawton, M. A., R. A. Dixon, K. Hahlbrock, and C. J. Lamb. 1983. Rapid induction of the synthesis of phenylalanine ammonia-lyase and of chalcone synthase in elicitor-treated cells. FEBS 129:593-600.

38. Lim, S. M., A. L. Hooker, and D. J. Paxton. 1970. Isolation of phytoalexins from corn with mongenic resistance to *Helminthosporium turcicum*. Phytopath. 60:1071-1075.

39. Mayama, S., S. Hayashi, R. Yamamoto, and T. Tani. 1982. Effects of elevated temperature and α-amino-oxyacetate on the accumulation of avenalumins in oat leaves infected with *Puccinia coronata* f. sp. *avenae*. Physiol. Plant Path. 20:305-312.

40. Mayama, S., Y. Matsura, H. Iida, and T. Tani. 1982. The role of avenalumin in the resistance to oat crown rust, *Puccinia coronata* f. sp. *avenae*. Physiol. Plant Path. 20:189-199.

41. Mol, J. N., M. P. Robbins, R. A. Dixon, and E. Veltkamp. 1985. Spontaneous and enzymic rearrangement of naringenin chalcone to flavanone. Phytochem. 24(10):2267-2269.

42. Montalbini, P., and V. Ragge. 1974. Alteration of shikimate dehydrogenase and phenylalanine ammonia-lyase activities in tomato plants infected by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. et Hans. Phytopathol. Medit. 13:124-127.

43. Neish, A. C. 1964. Major pathways of biosynthesis of phenols. p. 295-359. In J. B. Harborne, (ed.), Biochemistry of phenolic compounds. Academic Press, New York. 618 p.

44. Partridge, J. E., and N. T. Keen. 1977. Soybean phytoalexins: Rates of synthesis are not regulated by activation of initial enzymes in

flavonoid biosynthesis. Phytopath. 67:50-55.

45. Patil, S. S., and A. E. Dimond. 1967. Inhibition of *Verticillium* polygalcturonase by oxidation products of polyphenols. Phytopath. 57:492-496.

46. Pearce, R. B., and J. P. Ride. 1980. Specificity of induction of the lignification response in wounded wheat leaves. Physiol. Plant Path. 16:197-204.

47. Pegg, G. F., and L. Sequeira. 1968. Stimulation of aromatic biosynthesis in tobacco plants infected by *Pseudomonas solanacearum*. Phytopath. 58:476-483.

48. Reuveni, R., and J. F. Ferreira. 1985. The relationship between peroxidase activity and the resistance of tomatoes (*Lycopersicum esculentum*) to *Verticillium dahliae*. Phytopath. Z. 112:193-197.

49. Rhind, D., J. M. Waterston, and F. C. Deighton. 1952. Occurrence of *Puccinia polysora* Underw. in West Africa. Nature 169:631.

50. Ride, J. P. 1980. The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. Physiol. Plant Path. 16:187-196.

51. Rodriguez-Ardon, R., G. E. Scott, and S. B. King. 1980. Maize yield losses caused by southern corn rust. Crop Sci. 20:812-814.

52. Seevers, P. M., J. M. Daly, and F. F. Catedral. 1971. The role of peroxidase isozymes in resistance to wheat stem rust disease. Plant Physiol. 48:353-360.

53. Solymosy, F., J. Szirmai, L. Beczner, and G. L. Farkas. 1967. Changes in peroxidase isozyme patterns induced by virus infection. Virology 32:117-121.

54. Stafford, H. A. 1964. Comparison of lignin-like products found naturally or induced in tissues of Phleum, Elodea, and Coleus, and in a paper peroxidase system. Plant Physiol. 39:350-360.

55. Stahmann, M. A. 1967. Influence of host-parasite interactions on proteins, enzymes, and resistance. p. 357-369. In C. J. Mirocha, and I. Uritani, (ed.), The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society. St. Paul, MN. 372 p.

56. Sutton, D. C., and B. J. Deverall. 1982. Liberation of antifungal activity during the extraction of soybean leaves. Physiol. Plant Path. 20:365-367.

57. Tanksley, S. D., and C. M. Rick. 1980. Isozymic gene linkage map of tomato: Applications in genetics and breeding. Theor. Appl. Genet. 57:161-170.

58. Ullstrup, A. J. 1965. Inheritance and linkage of a gene determining resistance in maize to an American race of *Puccinia polysora.* Phytopath. 55:425-428.

59. Vallejos, C. E. 1983. Enzyme activity staining. p. 469-512. In S. D. Tanksley, and T. J. Orton, (ed.), Isozymes in plant genetics and breeding, Part A. Elsevier Science Publishers B. V., Amsterdam.

60. Vance, C. P., T. K. Kirk, and R. T. Sherwood. 1980. Lignification as a mechanism of disease resistance. Ann. Rev. Phytopath. 18:259-288.

61. Van Dyke, C. G., and A. L. Hooker. 1969. Ultrastructure of host and parasite in interactions of *Zea mays* with *Puccinia sorghi.* Phytopath. 59:1934-1946.

62. Vincent, J. R., and R. L. Nicholson. 1987. Evidence for isoenzymes of 4-hydroxycinnamic acid:CoA ligase in maize mesocotyls and their response to infection by *Helminthosporium maydis* race 0. Plant Path. 30:121-129.

63. Walker, J. C., and K. P. Link. 1935. Toxicity of phenolic compounds to certain onion bulb parasites. Bot. Gaz. 96:468-484.

64. Walter, M. H., J. Grima-Pettenati, C. Grand, A. M. Boudet, and C. J. Lamb. 1988. Cinnamyl-alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor. Proc. Natl. Acad. Sci. USA. 85:5546-5550.

65. Webb, J. L. 1966. Quinones. p. 421-594. In Enzymes and metabolic inhibitors. Vol. 3. Academic Press, New York. 753 p.

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