/LIGNIN AS A MECHANISM OF FIELD RESISTANCE TO PHYTOPHTHORA ROT IN SOYBEANS/

by

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DISEASE REACTION AND GENETICS

Phytophthora root and stem rot of soybeans (Glycine max (L.) Merr.) is a very destructive soilborne disease incited by Phytophthora megasperma Drechs. f.sp. glycinea Kuan and Erwin (Pmg)(32). The disease was first observed in Indiana in 1948 (44), and in 1955 Suhovecky (47) implicated a species of Phytophthora as the causal agent of the disease. The disease appears more severe on poorly drained clay soils but can be a problem on any soil that is wet for several days (44). Phytophthora rot attacks susceptible soybean plants at all stages of development causing conditions from seedling damping-off to mature plant wilting and death. Symptoms seen on seedlings include water soaked lesions on stems and wilting of leaves until the seedling turns brown and dies (24). Characteristic symptoms on older plants include wilting of leaves and interveinal and leaf margin chlorosis while secondary roots are nearly destroyed by the disease and primary roots and stems are discolored (44). Dead and dying plants are usually found in clusters along a row or in adjacent rows.

Pmg survives in soil for long periods of time as thick walled oospores. These oospores germinate producing either hyphae or sporangia with the optimum temperature for this activation being 20-24 C (23). Sporangia in turn produce zoospores which are the primary inoculum of Pmg with zoospore production being optimum at 20 C (13). After dispersal from sporangia the zoospores are probably attracted to soybean roots chemotaxically accumulating along

the length of the roots, with the highest accumulation just behind the root cap (20). Zoospores encyst and germinate on the root surface leading to colonization of root tissue with hyphae and subsequent production of oospores. These oospores produce exogenous sporangia which are involved with the production of secondary inocula (46). Klarman and Corbett (31) showed that during colonization by Pmg hyphae host plant tissue became disorganized with disrupted cells being devoid of cytoplasm and cell walls being broken or collapsed. Ultrastructural evidence indicates that haustoria-like structures and/or hyphae penetrate the host cells after the cell walls have been degraded or dissolved (31,45).

Many techniques for artificial inoculation have been developed and published and include soil infestation (22,24), liquid or solid plant growth medium infested with zoospores or mycelium (26,53) and direct hypocotyl inoculation with either mycelia (14) or zoospores (43). Keeling (25) tested various techniques and concluded that the direct hypocotyl inoculation procedures were most effective for differentiating resistant from susceptible soybean lines.

This technique was used in the initial studies of the inheritance of resistance to Phytophthora rot by Bernard et al. (6). The inoculated F2 of a cross between resistant and susceptible lines segregated in a ratio of three resistant to one susceptible which indicated a single gene with the resistance trait dominant. A total of nine major resistance genes have been identified which occupy six different loci. One locus contains a series of four

alleles for resistance while the other five loci have single resistance alleles (4).

The backcross procedure has been used in breeding for resistance by making use of lines with major resistance alleles as the donor parents. Wilcox et al. (55) evaluated the efficiency of this technique and concluded that seven backcrosses "followed by elimination of plant rows that did not visually conform to the phenotype of the recurrent parent" would be the most efficient method of transferring resistance into susceptible lines. One problem with this breeding scheme is the lack of durability of resistance due to the buildup of new pathogenic races of Pmg. These pathogenic races have been determined by using a uniform set of soybean differential genotypes consisting of Harosoy, Harosoy 63, Sanga, Mack, Altona, P.I. 171442, and P.I. 103091. Using this set of soybean differentials and the hypocotyl inoculation technique, 22 pathogenic races of Pmg have been identified (34).

Keen et al. (28) and Yoshikawa et al. (56) reported evidence that antifungal compounds are produced in response to Pmg infection in resistant soybean tissues. These compounds have been identified as hydroxy pterocarpan phytoalexins. An inducer of these phytoalexins, discovered by Frank and Paxton (15), is found in culture filtrates of Pmg. This inducer apparently only elicits phytoalexin production in incompatible reactions which may explain the mechanism of race specificity in the soybean-Pmg system (27).

Alternative methods for controlling disease losses include breeding for tolerance or field resistance (11,22,41,42,51). Tolerance was defined by Schmitthenner and Walker (42) as "the ability to survive infection without severe symptom development (killing, stunting, or yield loss)." Field resistance or "field tolerance" has been described by Jimenez (22): "such varieties lack major genes for resistance to Phytophthora root rot and give a susceptible (kill) reaction by hypocotyl inoculation yet survive and yield better than susceptible varieties in the field."

These types of resistance are most likely not race specific in conditioning ability and hence would be more durable over time due to the increased complexity one would suspect in these resistance types as compared to major gene race specific resistance (38). Buddenhagen (10) has suggested that the cultivars or lines being tested for tolerance or field resistance should not have major gene resistances for the races that comprise the inoculum as this would obfuscate any tolerance or field resistance reaction.

LIGNIFICATION

Plant cell walls are complex yet ordered structures involved in plant morphology, growth, and development and in the interaction of plant cells and pathogens. Cellulose, hemicellulose, pectic substances, protein, and lignin are the major constituents of cell walls (48). The three functional regions of a cell wall consist of primary wall, secondary wall, and middle lamella. The formation of the primary cell wall begins at cell division whereas

the secondary wall is not formed until cell elongation has stopped (17). The role of the middle lamella is to cement the cells together into a cohesive tissue.

Lignins are a class of polyphenolic polymers that are integral components of all three functional regions of the plant cell wall. Lignin arises from the dehydrogenative polymerization of coniferyl, sinapyl, and p-coumaryl alcohols. These aromatic macromolecules are associated covalently with hemicelluloses and are embedded in the crystalline microfibrillar structure of the cell wall (39) and are known to be very resistant to enzymatic degradation by most microorganisms (30).

Vance et al. (52) reviewed the role of lignin in disease resistance and listed five ways lignification of cell walls could hinder fungal growth. In short these mechanisms include impediments to mechanical penetration and enzymatic dissolution, restriction of enzyme and nutrient diffusion, active phenolic antifungal activity, and lignification of fungal hyphae. Lignification induced by pathogens has been observed in many host-pathogen systems and appears to be race specific in many of these systems (16,52) and to be localized to small areas around the infection site (18,52). Asada et al. (3) proposed that the induction of lignification in Japanese radish roots (Raphanus sativus) by infection with Peronospora parasitica was a delayed resistance-type would response to infection.

Lignin that is not induced by a pathogen but is a component of the cell wall prior to infection could act as a physical

preformed deterrent to colonization of the host tissues (1). This would be an example of a passive resistance mechanism and should be race non-specific since it is doubtful that simple race differentiation would be enough to enable a pathotype to produce the necessary enzymes to break through the lignin barrier.

DETERMINATION OF LIGNIN

Various methods for the isolation and quantitative determination of lignin are available and have been reviewed by Browning (9). The most widely used direct chemical methods are modifications of the 72% sulfuric acid (Klason) procedure. With the Klason method lignin is defined as the residue remaining after vigorous acid hydrolysis and is measured gravimetrically (52). A typical example of the Klason procedure is the TAPPI standard method T-13 which includes extractions with 95% ethanol, ethanol-benzene (1:2), and hot water previous to the sulfuric acid treatment (33). Difficulties encountered with this basic sulfuric acid procedure include degradation of the lignin, production of breakdown products capable of condensing with the lignin or forming an insoluble residue itself, and contamination with tannins, waxes, oils, and protein (50). Many modifications have been suggested for minimizing these sources of error. Degradation of the lignin material was shown to be dependent upon the length and the temperature of the acid hydrolysis. Treatment with 72% sulfuric acid for 2 hours at 20 C is considered satisfactory for most analyses (9). Preextraction with hot water (100 C) or dilute mineral acids has been suggested as a way to remove polysaccharide materials

that could form complexes with lignin or form insoluble residues during strong acid hydrolysis (33). Lyness and Schenker (35) have shown, however, that the hot water extract contains significant amounts of lignin and should be avoided. The dilute acid extraction on the other hand seems to remove much of the polysaccharides but the extract contains some lignin. Browning (9) makes the point that any advantage gained by removing the polysaccharides is offset by loss of lignin. Preextraction with dilute mineral acid would be inappropriate if it was employed merely to remove polysaccharide material. The use of this preextraction is advantageous, however, in the removal of protein which can be a great source of error in young herbaceous tissues (36,37,50). The dilute acid treatment is not effective in removing all of the protein present in the sample. Digestion of protein by enzymes has been utilized and appears to be efficacious in removing a considered fraction of the protein when coupled with the dilute acid treatment (50). Even with these protein extraction steps, samples from young tissues still contain a significant amount of protein that should be corrected by determining the nitrogen content and multiplying that by 6.25 to obtain an estimate of crude protein left after digestion (12,33). The use of a sodium carbonate extraction for further removal of protein has been published (49).

Two important criteria in selecting between the various lignin determination methods available, for studies other than structural, are the speed and the convenience of the method. Thacker (49) published a method that introduced the use of an

autoclave in place of refluxing. The procedure is based on the method of Ellis et al. (12) and considerably shortens the determination time. The modified method also reduces the number of transfers from vessel to vessel making the method attractive for studies with a large number of samples.

OBJECTIVES

The objectives of this study were to measure the amounts of lignin in eight soybean cultivars that had been previously rated for field resistance or tolerance. The means of the cultivar lignin yields were then compared to the field ratings (high, medium, and low). A laboratory test for these resistance types would be helpful in screening soybean germplasm and would also be instrumental in a study of the physiological mechanism involved.

MATERIALS AND METHODS

SOYBEAN CULTIVARS AND CULTURE

The eight soybean cultivars used in this study were Asgrow 3127, Sparks, Williams, Williams 82, Soybean Research Foundation 307P, Cumberland, Amsoy 71, and Ring Around RAX88. The cultivars were grouped into three tolerance or field resistance rankings which included high tolerance, medium tolerance, and low tolerance from field data gathered in Kansas (19,40) and Ohio (7,8, 41). Table 1 summarizes this data and shows the relative ranking of the cultivars.

Seeds were germinated at 30 C on moist germination paper until seedlings were approximately 10 cm. in length. The seedlings

were transferred to 2 liter black plastic pots 16 cm. in diameter. The covers of the pots had six holes 1.5 cm. in diameter with the seedlings being supported by drilled corks and glass wool. All seedlings were grown in Hoagland's nutrient solution (21) with FeSO4-EDTA as the iron source and adjusted to a pH of 5.9 to 6.1. The pots were aerated continuously throughout the experiment by an air pump and tubing system. The nutrient solution was changed weekly and distilled water was added when needed. Plants were grown in a greenhouse with 1,000 watt high pressure sodium lamps as supplemental lights and a photoperiod of 14 hours.

Table 1. Field response of soybeans to Phytophthora rot.

Relative Ranking	Cultivar	Kansas *		Ohio **		Ohio #
		81	82	81	82	
		----------score----------				
High	A3127	0	0	2.8	1.9	2.2 (2)
	Sparks	-	0	2.5	1.5	2.5 (2)
	Williams	1	0	2.2	2.6	2.1 (6)
	Williams 82	1	0	-	-	-
Medium	SRF 307P	-	-	-	-	3.4 (2)
	Cumberland	1	0	2.9	3.1	2.9 (2)
Low	Amsoy 71	4	1	2.9	-	3.9 (8)
	RAX88	-	3	-	-	-
	LSD (0.05)	1.5	0.7	-	0.6	-

*From Kansas soybean performance tests (18,40): 0=no plants killed, 1=1-9% plants killed, 2=10-19%, 3=20-29%, 4=30-39%, 5=40-50%.

**From Ohio soybean performance trials (7,8): 1=no dead plants or no stunting, 2=no dead plants and slight stunting or few dead plants and no stunting, 3=few dead plants and moderate stunting or several dead plants and slight stunting, 4=up to 50% dead plants and moderate stunting, 5=over 50% dead plants and severe stunting.

#From Schmitthenner et al. (41), tolerance ratings same as 7 and 8 but averaged over the number of years tested (in parentheses).

The experiment consisted of four treatments: 1) plants were grown for two weeks after transplanting to pots and percent lignin determined, 2) plants were grown for three weeks and percent lignin determined, 3) plants were grown for four weeks and percent lignin determined, 4) pots were inoculated with Pmg zoospores at two weeks and plants allowed to grow to four weeks and percent lignin determined.

PATHOGEN ISOLATES AND INOCULA

Cultures of Pmg used in the study were isolated from diseased soybean plants taken from a field in Pawnee county, Kansas. The isolates were grown in petri dishes containing 10 ml. lima bean agar (Difco Laboratories), maintained at 25 C and subcultured every three months. Races were determined on all isolates by using the differential soybean cultivars Harosoy, Harosoy 63, Sanga, Mack, Altona, P.I. 171442, P.I. 103091, and inoculating the hypocotyls with mycelium (34). Three isolates used in the experiment were determined to be races 3, 4, and 5.

Zoospores from the isolates were produced by the method of Eye et al. (13). The number of zoospores per milliliter was determined by counting them in a hemacytometer under a compound microscope. The inocula consisted of approximately equal proportions of all three isolates (races) used and was added directly into the hydroponic pots. Total inocula depended upon the replication but was between 1.9 and 2.1 million zoospores per pot. All cultivars used were tested for race specific reactions against the three isolates by direct inoculation of the hypocotyls with

mycelium. All cultivars except Williams 82 were fully susceptible to the isolates used; Williams 82 was resistant to all three isolates.

LIGNIN DETERMINATION

When plants reached the appropriate treatment age the roots and hypocotyls were cut off from the rest of the plants and air dried at room temperature (20 C) for one week. Plant material was then ground in a Wiley mill to pass a 40 mesh screen. Samples were weighed to approximately one gram and placed into a 250 ml wide-mouth Erlenmeyer flask. The samples were covered with 20 ml. of an ethanol-benzene mixture (32 parts 95% ethanol: 68 parts benzene). The mixture was allowed to stand for 30 minutes and then the supernatant was drawn off using Pyrex immersion fritted glass filter tubes (30 cm. diameter, medium porosity) under vacuum. The residue was washed with 95% ethanol, filtered, and the procedure repeated until the filtrate was clear. The samples were then covered with 40 ml. of 1% pepsin in 0.1N HCl and incubated 24 hours at 40 C. The pepsin solution was filtered off and the residue washed with warm water (45-50 C) and filtered. After the filtration, 100 ml. of 0.25% sodium carbonate was added to the flasks and incubated 24 hours at 40 C. After incubation, the sample and sodium carbonate solution was transferred into a 50 ml. Pyrex Gooch-type fritted disc bottomed crucible with the aid of a stream of warm water. The Erlenmeyer was thoroughly washed out with warm water and the crucible contents filtered by vacuum. The crucibles were placed into 150 ml.

glass beakers and 40 ml. of 5% sulfuric acid was added into the crucibles with enough added to the beakers to discourage gravity filtering from the crucibles into the beakers. The beakers were autoclaved at 20 lb. pressure for 10 minutes, allowed to cool, and filtered. The residue was washed with 95% ethanol, filtered, and 20 ml. of 72% sulfuric acid added to the crucibles and again extra into the beakers. The sample was subjected to the treatment for 2 hours then diluted with 30 ml. of warm water. Upon filtering the residue was washed with warm water and 40 ml. of 3% sulfuric acid was added to the crucibles, and extra beakers, then autoclaved for 10 minutes at 20 lb. pressure. After cooling the residue was washed with warm water and filtered. The crucibles were dried overnight at 100 C, weighed, and ashed at 500 C for 3 hours. When the crucibles had cooled they were weighed again and percent lignin was determined by the equation: % lignin= (100)(pre-ash wt.)-(post-ash wt.)/sample dry wt.

Nitrogen determinations were run for all varieties to estimate the contamination by protein. Samples were obtained for these determinations by pooling ground plant material from the three and four week treatments for each variety. After subjecting the samples to the extraction procedures mentioned above nitrogen was determined colorimetrically (2).

STATISTICAL ANALYSIS

The experimental design was a randomized complete block with blocks being replications over time. Four blocks were analysed, each with 32 observations (8 cultivars x 4 treatments) and each

observation was composed of 6 soybean roots and hypocotyls. Significant differences for percent lignin were estimated using Fisher's LSD test.

RESULTS AND DISCUSSION

Nitrogen determinations were made for all cultivars and multiplied by 6.25 to correct for residual protein after digestion. Residual protein ranged from a high of 7.5% of the digested sample for Williams to a low of 6.2% for A3127. Statistical analysis of the data corrected for residual protein showed no significant differences among cultivar means for percent lignin averaged across all treatments. Analysis of the data without correction for residual protein again showed no significant differences among cultivar means for percent lignin averaged across all treatments (Table 2). The means for the cultivars were, in general, about one percent lower in the protein corrected analysis. The relative ranking of the cultivar means averaged across all treatments was the same as in the protein corrected analysis with the exception that SRF 307P and A3127 switched places in the ranking. Because of this small difference all further analysis was performed on data not corrected for protein.

Significant differences were found among cultivar means for lignin yield within the two week, three week, and inoculated treatments (Table 3). No straightforward comparisons was possible between field ratings and cultivar treatment means because of a cultivar by treatment interaction. An example of this inconsistency in cultivar means across treatments can be found between the Williams 82 means. In the two week treatment the Williams 82 mean ranked highest, but in the three week treatment the mean ranked lowest and then ranked highest again the four week treatment.

Table 2. Cultivar percent lignin means averaged across all treatments.

Field rating	Cultivar	Lignin	Ranking for means
		--- % ---	
High	A3127	15.32	7
	Sparks	16.37	3
	Williams	15.79	5
	Williams 82	16.12	4
Medium	SRF 307P	15.42	6
	Cumberland	16.86	1
Low	Amsoy 71	15.22	8
	RAX 88	16.76	2
	LSD (0.05)	N.S.	

Table 3. Cultivar percent lignin means for treatments.

Field rating	Cultivar	Treatment			
		2 Week	3 Week	4 Week	Inoc.
		---------- % ----------			
High	A3127	13.30	15.95	19.23	12.81
	Sparks	14.67	19.62	15.98	15.21
	Williams	14.51	19.61	14.61	14.44
Medium	SRF 307P	10.46	21.87	14.91	14.47
	Cumberland	15.99	17.62	17.51	16.30
Low	Amsoy 71	13.06	15.22	19.55	13.07
	RAX 88	13.36	16.90	18.51	18.27
	LSD (0.05)	5.64	6.89	N.S.	5.38

Other inconsistencies, although not as drastic, were found between treatment means for SRF 307P, Amsoy 71, and Williams. Because the two week and the four week treatments were more consistent with each other in the relative ranking of the cultivar means they were analyzed separately. No treatment by cultivar interaction

was found and Williams 82 was significantly different than SRF 307P.

Significant differences were found among treatment means for percent lignin across all cultivars (Table 4). The two week and inoculated treatment means were significantly lower than the three week and four week treatment means. This difference suggested that in the inoculated treatment the diseased plants had lower rates of lignin biosynthesis over the next two weeks than did the plants in the three and four week treatments (Figure 1).

No conclusions could be made as to the relationship between cultivar lignin yields and field ratings for tolerance to Pmg. Inconsistent results for lignin yields could possibly be improved through more replications, however, due to the variability observed within the experimental procedure it is doubtful that more replications would be itself solve the problem. Much variability was observed between the fritted glass immersion tube filters for the time needed in drawing off an equal volume of filtrate. This may have been the result of differences in porosity between filters which would have affected the determination in increasing the weight of the residual material after filtration in the flasks with slower filters. Another source of variability was observed during the 72% sulfuric acid digestion. Some of the plant material floated to the top of the acid solution during the digestion reducing the surface area available for hydrolysis. The effect of this problem on percent lignin would have been an apparent increase due to the presence of polysaccharides that

Table 4. Treatment lignin means averaged across all cultivars.

Treatment	Lignin
	---%--
2 Week	14.26
3 Week	17.30
4 Week	17.59
Inoculated	14.77
LSD (0.05)	2.39

remained unhydrolysed.

Another determination technique that offered less experimental variability would of course be the more satisfactory way of approaching the problem. One technique that could be an improvement over the direct chemical methods is that of determining lignin spectrophotometrically (9). This method measures the absorbtivity of the samples in the ultraviolet spectrum. The only hindrance for this technique is the need for a lignin standard that would be measured along with the samples. If this standard was readily available this spectrophotometric technique could be of value in lignin studies with a large number of small sized samples.

In summary, field tolerance to Phytophthora rot appears to be a more durable type of resistance in that the race specific nature of major gene resistance is missing or not as prominent. Selection for field tolerance has generally been done in naturally infested fields but this scheme has posed problems because of climatic effects on disease incidence and development and uneven pathogen dispersion throughout the field. An understanding of

the mechanism(s) of field tolerance would be helpful in developing a controlled test for selection of highly tolerant lines. In evaluating lignin content in soybean cultivars as a possible mechanism of field tolerance no conclusive results were obtained. Lignin cannot be ruled out as a mechanism of field tolerance as the determination technique formulated in this study was insufficient for the purpose of consistently finding true genetic differences.

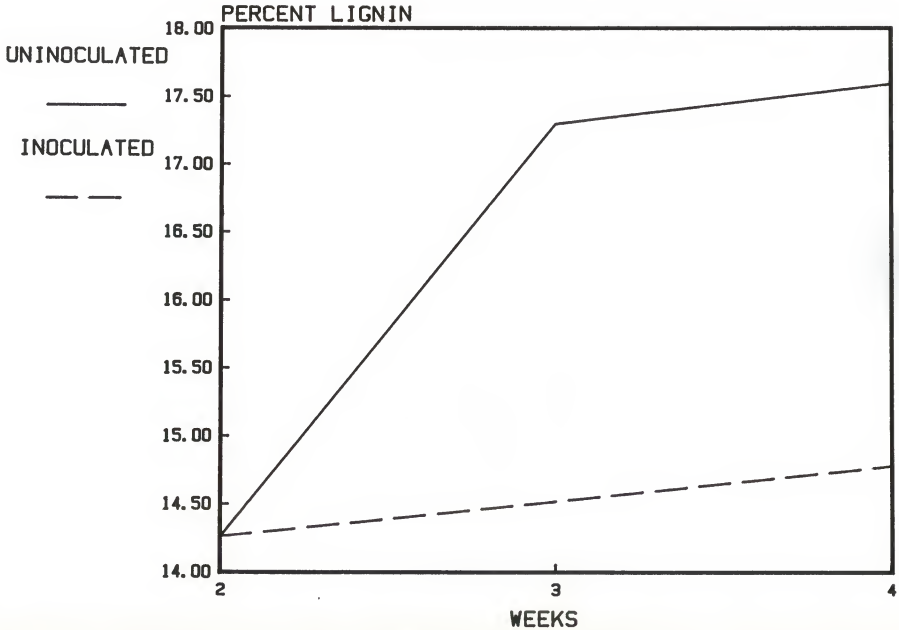

Figure 1. Treatment percent lignin averaged across all cultivars.

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LIGNIN AS A MECHANISM OF FIELD RESISTANCE TO PHYTOPHTHORA ROT IN SOYBEANS

by

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ABSTRACT

Breeding for resistance to root and stem rot incited by Phytophthora megasperma f.sp. glycinea has, in the past, utilized backcrossing to transfer single resistance genes into agronomically superior lines. These genes confer total resistance to specific physiologic races of the fungus. This breeding scheme has proved inadequate to control the disease over time due to the ever increasing number of races.

An alternative method would be to breed for field tolerance or field resistance as these resistance types do not appear to be race specific in conditioning ability. The mechanism(s) for these resistance types have not been determined and no reliable laboratory tests have been developed to aid in the selection of field tolerant lines.

The objective of this study was to determine the amounts of lignin as percentage of dry weight for eight varieties of soybean with known field tolerance ratings. Ground root and hypocotyl material was extracted with solutions of ethanol-benzene, pepsin-HCl, sodium carbonate, and sulfuric acid (5%, 72%, and 3%). Treatments consisted of lignin determinations at plant ages of two, three, and four weeks, and inoculated at two weeks with lignin determined at four weeks of age.

No significant differences were found for percent lignin between varieties. This technique, as it is currently formulated, does not hold promise as a laboratory test for field tolerance to Phytophthora rot in soybeans.