

LITERATURE REVIEW

General Overview of Plant Phenolics

A phenol is an organic compound consisting of an hydroxylated benzene ring (66). The plant phenolic compounds can be subdivided into various classes depending on the functional groups attached to the phenol. Several classification systems have been reported based on structural differences and similarities while other systems have categorized phenolic compounds on their occurrence (44,92). The following classification divides plant phenols on the basis of their structure but also notes their relative occurrence (85).


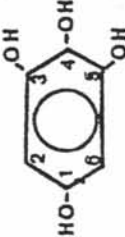
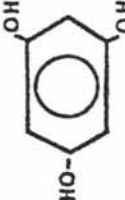


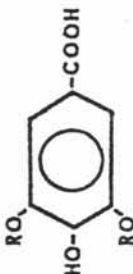

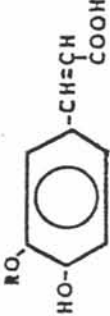
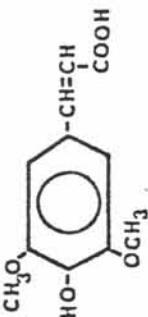
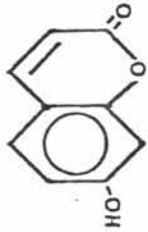

<u>Structure</u>	<u>Class of Phenols</u>	<u>Occurrence</u>
C_6	Simple Phenols	Rare to Common
C_6-C_1	Benzoic Acids and Related Compounds	Common
C_6-C_2	Acetophenones and Phenylacetic Acids	Rare
C_6-C_3	Cinnamic Acids and Related Compounds	Rare to Common
C_6-C_3	Coumarins	Common
$C_6-C_3-C_6$	Flavones	Common
$C_6-C_3-C_6$	Isoflavones and Isoflavonoids	Rare to Common
$C_6-C_3-C_6$	Flavonols	Common
$C_6-C_3-C_6$	Flavan-3 ols (catechins) and Flavan-3,4 diols (leucoanthocyanidins)	Common
$C_6-C_3-C_6$	Flavanones	Common
$C_6-C_3-C_6$	Anthocyanidins	Common
$C_6-C_3-C_6$	Chalcones, Aurones, and Dihydrochalcones	Common
C_{30}	Biflavonyls	Rare

<u>Structure</u>	<u>Class of Phenols</u>	<u>Occurrence</u>
$C_6-C_1-C_6$ and	Benzophenones, Xanthenes and	Rare
$C_6-C_2-C_6$	Stilbenes	Rare
$C_6, C_{10} +$	Quinones	Common
C_{14}		
C_{18}	Betacyanins	Rare
$(C_6-C_3)_n$	Lignin Polymer	Common
$(C_6-C_3-C_6)_n$	Tannin Polymer	Common to Rare

Within the class of simple phenols are catechol, phenol, hydroquinone, resorcinol, and phloroglucinol (85). These phenols are themselves uncommon plant constituents but phloroglucinol, resorcinol, and catechol may be found in combination with the cinnamic acids to form the various plant flavonoids.

Phenolic acids include the benzoic, phenylacetic, and cinnamic acids (85). The benzoic acids, C_6-C_1 structures, consist of p-hydroxybenzoic, protocatechuic, vanillic, gallic, syringic, salicylic, o-pyrocatechuic, and gentisic acids. Salicylic, gentisic, and o-pyrocatechuic acids differ from the other benzoic acids in that they are ortho hydroxylated. Reduction of the benzoic acids to their respective aldehydes and alcohols may occur. The phenylacetic acids are in the C_6-C_2 class of phenolic acids while the cinnamic acids are in the C_6-C_3 class. The four major cinnamic acids found in plants are p-coumaric, caffeic, ferulic, and sinapic acids (85). If the reduction of ferulic and sinapic acids to coniferyl and sinapyl alcohols occur, these alcohols may then be utilized in the synthesis of lignin. The phenolic acids will seldom be found in the free state but rather in a combined form as

Table 1. Four classes of naturally occurring phenols and the various phenolic compounds within each class with respect to the position and number of hydroxyl groups.

<u>CLASS OF PHENOLS</u>	<u>MONOHYDROXY</u>	<u>DIHYDROXY</u>	<u>TRIHYDROXY</u>
I. Simple phenols	 phenol	 3,4 dihydroxy-catechol 3,5 dihydroxy-resorcinol 1,4 dihydroxy-hydroquinone	 phloroglucinol
II. Benzoic acids	 p-hydroxybenzoic acid	 R=H, protocatechuic acid R=CH ₃ , vanillic acid	 R=H, gallic acid R=CH ₃ , syringic acid
III. Cinnamic acids	 p-coumaric acid	 R=H, caffeic acid R=CH ₃ , ferulic acid	 sinapic acid
IV. Coumarins	 umbelliferone	 R=H, aesculetin R=OCH ₃ , scopoletin	

esters. Other phenolic acids are the alkaloid, tyramine, and the amino acids, tyrosine and dehydroxyphenylalanine (44).

Coumarins, C_6-C_3 structures, are produced via the cyclization of the cinnamic acids (44,85). Cyclization of o-coumaric, p-coumaric, caffeic, and ferulic acids will result in coumarin, umbelliferone, aesculetin, and scopoletin, respectively.

The flavonoids comprise the entire class of phenolic compounds having the $C_6-C_3-C_6$ structure (44,85). This structure consists of two benzene rings, ring A and ring B, which are attached by a three carbon chain (C-3 unit) (Table 2). Differentiation among the flavonoids is based on the structure of the C-3 unit and its level of oxidation (44, 85). If the C-3 unit is an oxygen heterocycle the phenolic classes will consist of the flavones, isoflavones, flavonols, flavan-3 ols, flavan-3,4 diols, flavanones, and anthocyanidins (Table 3). Differences within this group are dependent on the level of oxidation of the oxygen heterocycle. The second group of flavonoids consist of the chalcones and dihydrochalcones (Tables 2-3). In this group of flavonoids the C-3 unit is non-heterocyclic and the difference between the chalcones and dihydrochalcones is also dependent on the level of oxidation of the C-3 unit. The aurones comprise the last group of flavonoids and are distinguished by their pentacyclic C-3 unit (Table 3). The flavonoids occur in the plant in a combined form as glycosides. Geissman (36) has listed the classes, structures, and names of the principal plant flavonoids (Table 3).

Table 2. The numbering system and structure for (I) the flavonoids having an oxygen heterocycle (II) the chalcones and dihydrochalcones and (III) the aurones.

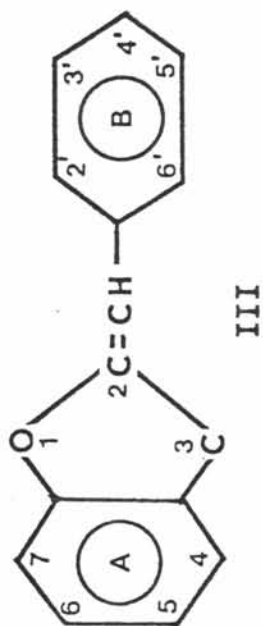
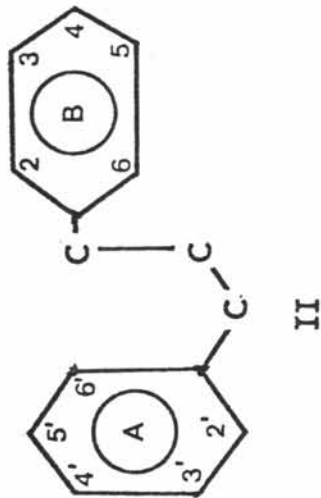
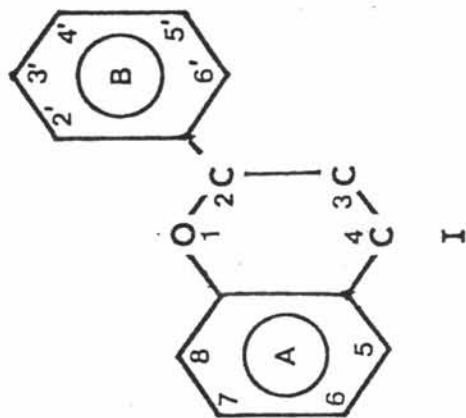
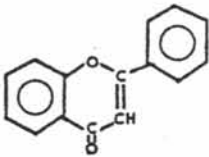
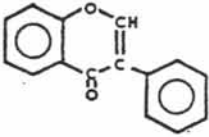
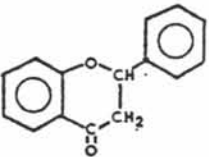
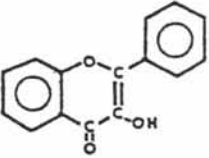
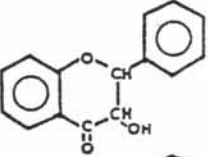
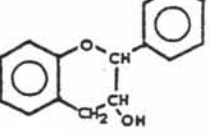
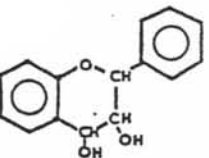
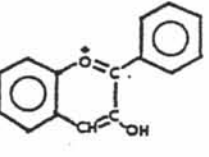
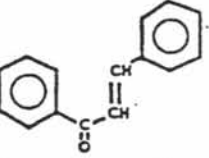
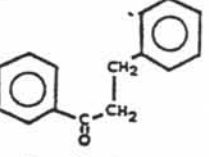
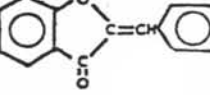


Table 3. The 11 principal flavonoid classes and structures. Differences in hydroxylation pattern will result in various flavonoid compounds within each class.

Class of Flavonoids	Structure	Hydroxylation Sites	Name
Flavones		5,7,4'	apigenin
		5,7,3',4'	luteolin
Isoflavones		7,4'	daidzein
		7	formononetin
Flavanones		5,7,4'	naringenin
		7,3,4'	butin
Flavonols		5,7,4'	kaempferol
		5,7,3',4'	quercetin
Flavanonols		7,3',4'	fusitin
		5,7,3',4'	taxifolin
Flavan-3-ols		5,7,3',4'	catechin
		5,7,3',4',5'	gallocatechin
Flavan-3,4-diols		5,7,3',4'	leucocyanidin
Anthocyanidins		5,7,3',4'	cyanidin
Chalcones		3,4,2',4'	butein
Dihydrochalcones		4,2',4',6'	phloretin
Aurones		6,3',4'	sulphuretin

The less common phenols include the biflavonyls, benzophenones, xanthenes, stilbenes, quinones, and betacyanins. The biflavonyls are formed by the condensation of two units of the flavone, apigenin, and this group of phenols is believed to be confined to the gymnosperms (85). The benzophenones and xanthenes consist of a $C_6-C_1-C_6$ structure while the stilbenes have a $C_6-C_2-C_6$ structure. These phenols are heartwood constituents. The class of quinones include the benzoquinones, naphthaquinones, and anthraquinones. Ubiquinone, a benzoquinone, is not only found in the plant kingdom but also in the animal kingdom. It functions as an electron transport agent in the electron transport system (57). Betacyanins are often referred to as nitrogenous anthocyanidins since they are nitrogen containing phenols having an absorption spectra resembling that of the anthocyanidins (85).

The phenolic compounds present in polymeric form include the condensed tannins and lignins (85). The condensed tannins are the polymers of the flavan-3 ols and flavan-3,4 diols. Lignin synthesis is believed to occur via the polymerization of coniferyl and sinapyl alcohols (31,33).

Role of Plant Phenolics

Since plant phenolics are secondary metabolites the relative importance of these compounds to the plant has been an area of extensive research. The probable role of the phenols encompasses such topics as waste-storage areas, structural support, respiratory substrates, plant growth regulators, plant coloration components, and plant disease resistance (18,23,37,86,93,110).

Since plants lack an efficient excretory system, waste products are stored within the plant. The presence of phenolic compounds may therefore be attributed to their role as waste - storage

compounds (110). In addition these compounds may have been and may still be of some survival value to the plant, therefore, permitting the differential accumulation of phenolic compounds through the process of natural selection.

Polymers of lignin have been shown to be involved in structural support in many plant species (110). In woody plants lignin concentration is believed to be between 18-35%. Two precursors in lignin synthesis are coniferyl and sinapyl alcohols; the reduced state of the phenolic ferulic and sinapic acids.

The possibility that phenols may be utilized as respiratory substrates has been reported (109,110). Radioisotope labelled catechin was shown to be oxidized when introduced into tea plants (109). Also o-diphenols and phenoloxidases may function together as a terminal oxidase system (110).

The implication of phenols as plant growth regulators centers on their regulation of indoleacetic acid oxidase (37,110). An in vitro study with pineapple indoleacetic acid oxidase revealed that o- and

p-dihydric phenols inhibit the oxidase enzyme while monohydric phenols activate this enzyme (37).

Various plant colors have been attributed to the water-soluble flavonoids (86). The anthocyanidins are responsible for the red and blue colors. The different hues of each color are dependent on the level of hydroxylation to ring B of the respective flavonoid. The chalcones and aurones may also be responsible for the yellow pigment present in certain flowers while the flavones and flavonols dictate the different hues of white (86).

Phenolic Compounds Implicated in Disease Resistance

Preformed Phenols

The phenolic compounds present in the plant before infection will often be in a combined form with sugars, quinic acid, or tartaric acid to form an ester or a glycoside (85). Many of the flavonoids are joined to sugars via a glycosidic bond while the cinnamic and benzoic acid derivatives and the coumarins may be linked to yield an ester or a glycoside. The glycoside or ester may be hydrolyzed by enzymatic action to produce an aglycone (the phenolic compound) and its respective constituent (sugar, etc.). Investigations of various host-parasite interactions have shown that the aglycone of the ester or glycoside may be fungitoxic or that the glycoside and/or ester forms are themselves fungitoxic (53,56,65,80,81,87). Also the polymerization products of phenolic compounds, such as the tannins, have been implicated in disease resistance (71).

Research on potato scab, Streptomyces scabies, indicated that the concentration of chlorogenic acid, an ester of caffeic and quinic acids, was inversely proportional to disease incidence (52,53,87). Resistant varieties exhibited higher concentrations of chlorogenic acid than did susceptible varieties. The analysis for chlorogenic acid was performed by the iron chloride test (FeCl_3) which was shown to be an accurate and quick method of estimating the concentration of chlorogenic acid (52,65). The basis of the test is that FeCl_3 will react with o-dihydroxy phenols to give a green reaction mixture with the intensity of the color increasing with higher concentrations of o-dihydroxy phenols. Two other important aspects of the disease resistant response was the localization of high concentrations of chlorogenic acid in the periderm

of the potato tuber and the oxidation of chlorogenic acid to a quinone (52). In a subsequent report (53) in vitro results suggested that the quinone structure was the toxic principle preventing growth of S. scabies.

More extensive and conclusive work clarifying the role of chlorogenic acid in disease resistance was reported on potatoes for the fungal pathogen Verticillium albo-atrum (56,65). Analysis of twelve types of tissue from six varieties of potatoes revealed that the three resistant varieties had a higher concentration of chlorogenic acid in their underground vascular tissues than did the susceptible varieties (65). A time course study of chlorogenic acid for both resistant and susceptible varieties demonstrated that phenolic concentrations decreased more rapidly and to a lower level in susceptible than in resistant varieties over the growing season. Coinciding with this above phenomenon, the pathogen proliferated and caused greater damage to the susceptible varieties than to the resistant varieties.

An in vitro study (56) demonstrated that chlorogenic acid had only a slight effect on the growth of V. albo-atrum at concentrations as high as 1,200 ppm at pH 4.5. However, if the pH of the medium was increased to 8.5, a believed auto-oxidation occurred to chlorogenic acid resulting in the production of a quinone with subsequent inhibition of the pathogen. Further in vitro studies (65) indicated that chlorogenic acid at 1,000 ppm in a medium of pH 5 was fungistatic to V. albo-atrum while mycelial growth was hindered at 500 ppm and spore germination inhibited at 50 ppm. The results from these in vitro studies have even greater significance when compared to the in vivo concentration of 7,500-10,000 ppm of chlorogenic acid in the vascular

system of the potato (65).

In several comprehensive and well documented studies over a period of ten years, the relationship of a phenolic glycoside, phloridzin, to the resistance of young apple leaves to Venturia inaequalis was clarified (54,72,73,80,81,82). Phloridzin is present in apple leaves at a relatively high concentration. Early studies first suggested that phloridzin or possible by-products were responsible for resistance (54,72,73). Phloridzin concentration was found not to be the key to resistance since both resistant and susceptible varieties contained the same levels of phloridzin and under in vitro conditions both varieties were capable of reducing pathogen sporulation (54,72,73). A puzzling yet interesting question remained concerning the lack of resistance in vivo of the susceptible varieties when it appeared that they had the potential to inhibit V. inaequalis. Subsequent research not only addressed itself to the preceding question but also to the elucidation of the exact fungitoxic component.

Since phloridzin itself was not responsible for resistance, an enzymatic study was undertaken in order to identify the fungitoxic component (80,81). The results suggested that V. inaequalis was not inhibited by phloridzin or phloretin but by the oxidation of phloretin and phloridzin to produce a polymeric compound. Enzyme and oxygen uptake studies support the following scheme (Figure 1).

Phloridzin may be converted to phloretin by action of β -glycosidase or it may remain as a glycoside. At this stage both compounds may be hydroxylated at carbon-3 to give 3-hydroxyphloridzin or 3-hydroxyphloretin. Further oxidation by the phenolase complex will produce the respective quinones for each compound. The enzyme, β -glycosidase, may

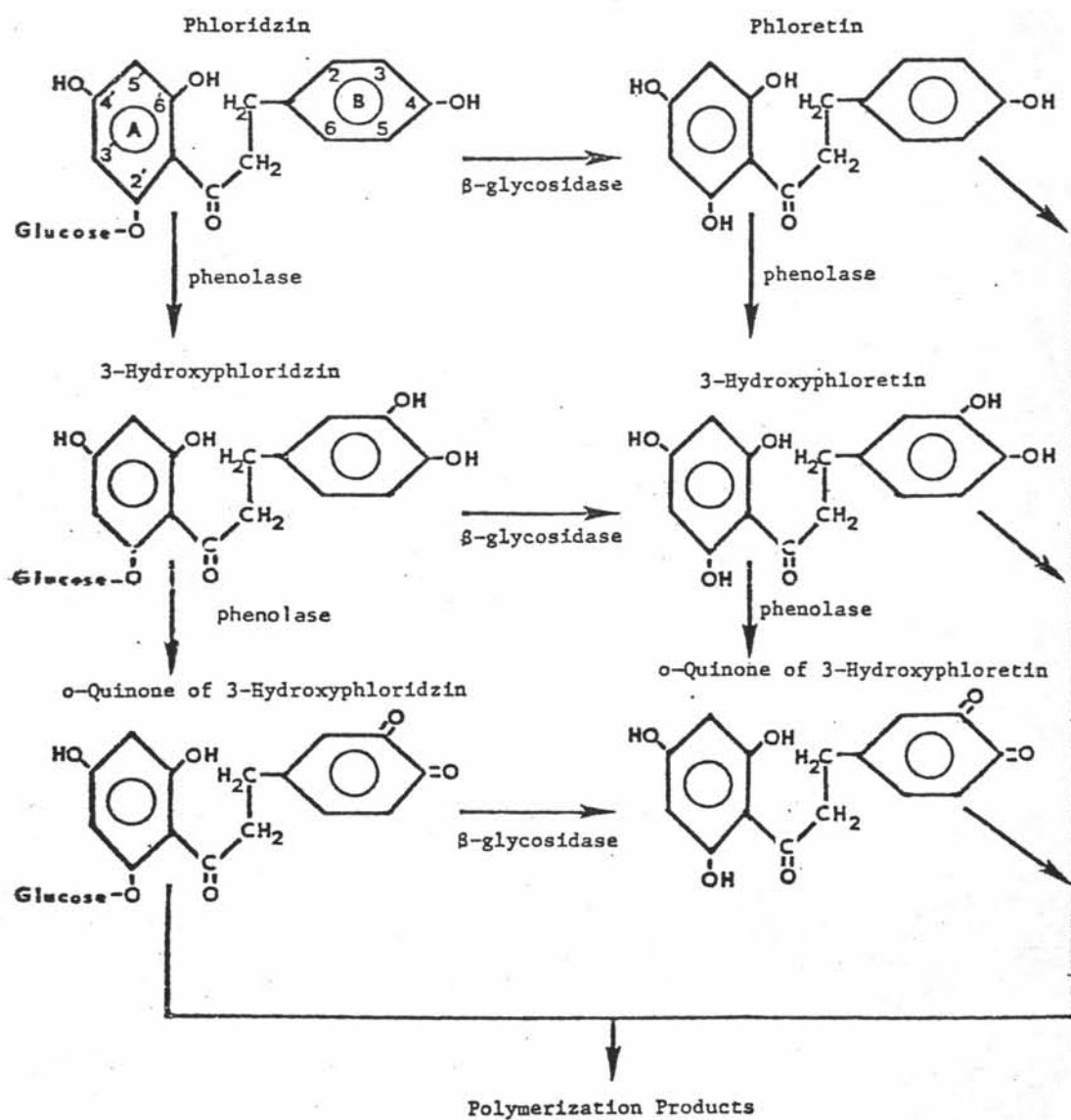


Figure 1. The polymerization scheme for the oxidation of phloridzin and phloretin by phenolase.

be active in transforming 3-hydroxyphloridzin or the quinone of 3-hydroxyphloridzin to their respective aglycones. The quinone structures, when polymerized, are believed to be the principal fungitoxic components.

Pectinase is the extracellular enzyme of V. inaequalis which is assumed to be responsible for the pathogenicity of this organism (80). It was revealed that pectinase, when combined with the phloridzin-apple leaf enzyme complex, was inactivated by the polymeric or oligomeric oxidation products of phloridzin (80). This phloridzin-enzyme complex was also differentially toxic to various morphological stages of the pathogen. Germinating conidia of V. inaequalis were extremely sensitive while ungerminated conidia were tolerant (80).

The enzymatic and bioassay studies have substantiated that the fungitoxic component of the phloridzin complex is its polymeric oxidation products but the lack of control in vivo of susceptible varieties to V. inaequalis is believed to be closely associated with the hypersensitive response. Histological examination of the tissue at the host-parasite interaction site indicated that V. inaequalis will germinate, penetrate the cuticle, and establish itself in a subcuticular position in both susceptible and resistant apple leaves (75). However, upon further growth in resistant apple leaves an immediate collapse of surrounding host cells occurs (hypersensitive response) resulting in the enzymatic oxidation of phloridzin while susceptible plants fail to undergo the hypersensitive response but rather permit the continued proliferation of the pathogen.

An explanation for the lack of the hypersensitive response was proposed (82). The explanation for this response relies heavily on the

gene-for-gene relationship as explained by Flor (32). It has been shown that the pathogen, V. inaequalis, produces peptides or small proteins (isotoxins) by the action of avirulent alleles of the pathogen. If the host has corresponding alleles (resistant alleles), it will be able to recognize the isotoxins of the parasite thus causing a hypersensitive response with subsequent accumulation of fungitoxic components. However, if the pathogen has one or more than one allele which does not correspond to the resistant alleles of the host, the inability of the host to recognize the respective isotoxin will not result in the hypersensitive response nor rapid accumulation of fungitoxic materials. Susceptible varieties may, therefore, have the potential to form fungitoxic compounds but the triggering mechanism (hypersensitive response) is not activated thus resulting in disease development.

Evidence supporting a glycoside as a fungitoxic compound and not its aglycone was demonstrated for the pathogen Ophiobolus graminis and the glycoside avenacin (105). Ophiobolus graminis will damage several of the small grains and other grasses but will not affect oats. Extracts from oats were found to inhibit the pathogen in vitro and subsequent isolation attempts resulted in the identification of avenacin. Avenacin consist of two molecules of glucose, two molecules of a pentose sugar and an aglycone. A race of the pathogen, O. graminis avenae, was found and shown to be capable of causing damage to oats. Since avenacin was present in the host, a study was performed to elucidate the mechanism of disease incidence. It was revealed that the pathogen had the ability to degrade avenacin via the enzyme avenacinase. This enzyme, a glycosidase, hydrolyzed the glycoside into products of lower toxicity thus resulting in susceptibility of the host.

The hydrolyzable tannins, polymers of gallic acid, were suggested to be a factor in disease resistance of chestnuts (Castanea spp.) to the pathogen Endothia parasitica (71). A positive correlation was found between the water-soluble tannins and disease resistance of three species of chestnut. The resistant Chinese chestnut had the highest concentration of the pyrogallol (gallic acid) tannin when compared to the susceptible Japanese and American chestnuts.

Phenolic Compounds Increasing After Infection

Extensive research for the purpose of establishing a correlation between increased phenolic synthesis and disease resistance has been conducted. The results indicate that a relationship may exist for specific host-parasite interactions. However, this is only one aspect of many in disease resistance. The term phytoalexin will also be discussed under this topic, however, not all phytoalexins are phenolic compounds. For example, certain terpenoid compounds are regarded as phytoalexins since they have been found to increase to fungitoxic levels in response to infection (24,25).

Phenolic compounds, tentatively identified as flavonoids, accumulated to approximately twice the level of controls following inoculation with Helminthosporium carbonum race 1 to soybean (Glycine max) hypocotyl (9). Coinciding with increased phenolic synthesis a hypersensitive response occurred. The total phenols in soybean hypocotyls began to increase shortly after inoculation and peaked at approximately 24-29 hours later. By 60 hours two of the three major phenols, which had increased earlier, decreased by 50 percent. Phenylalanine ammonia lyase activity was also examined and shown to increase to a level of five

times that of the control eleven hours after inoculation. The product resulting in this reaction was identified as trans-cinnamic acid. The enzyme activity after 22 hours decreased to a level of roughly twice that of the control. The phenolic compounds which did increase, as indicated by the diazotized sulfanilic acid test, were those of the host and not the parasite. The data suggest that an alteration of plant metabolism occurs due to the presence of the pathogen which results in an increase of phenols via the shikimic acid pathway. The decline of the phenols after 60 hours is attributed to the oxidation and polymerization reaction of the phenolic compounds (9). In this study it was also noted that the type of phenol produced was dependent on the host and not the pathogen. However, the concentration of the respective phenols synthesized was dependent on the pathogen.

The fungitoxic component of the Glycine max-Helminthosporium carbonum interaction was further investigated (10). Bioassay results indicated that germ tube growth of five fungi were inhibited 75-90 percent after incubation with tissue extracts of the G. max-H. carbonum interaction. Thin layer chromatography using 30 percent glacial acetic acid as the solvent revealed a phenol (flavanoid) having an R_f of .77-.84 which was inhibitory to fungal growth. Extracellular pectinolytic enzymes of the pathogen were also found to be inhibited by the tissue extract from this host-parasite interaction (10).

Previous investigations of tomato plants infected by Fusarium oxysporum f. sp. lycopersici indicated that resistant varieties are able to substantially increase their phenolic constituents more quickly

than susceptible varieties following inoculation (11,22). A study was conducted to determine the effect of races of F. oxysporum on phenol levels in tomato plants (62). The different form species of F. oxysporum used, were diantha from carnations, callistephi from aster, lupini from lupine and lycopersici from tomato. After inoculation the non-pathogenic form species of F. oxysporum; diantha, callistephi and lupini, initiated a significant increase of phenols in tomato plants while the pathogenic form, lycopersici, did not. Further tests with diantha and lycopersici on tomato were evaluated for a period of 18 days (63). The form species, diantha, caused a 250 percent increase of phenols in the stem and a 50 percent increase in the leaves of the tomato plant three days after inoculation while lycopersici did not substantially incite the phenolic content of either the stem or leaf within this same time period. By the fifth day the phenols in diantha infected plants had decreased to 25 percent and 0 percent for the stem and leaf, respectively. However, the phenolic compounds in lycopersici infected plants did not decrease but continued to accumulate for the period of the study. At the eighteenth day the phenolic content of the diantha infected plants was 175 percent and 135 percent that of the control for the stem and leaf tissue, respectively. The results suggest that resistance is conferred to plants which are capable of a rapid and substantial increase in phenolic compounds after infection by a pathogen.

Cross-protection studies were shown to be effective in conferring resistance to susceptible host (21,62). The phenomenon is accomplished by inoculating the host with a non-pathogenic race of the parasite which will cause a substantial increase in fungitoxic phenols within the host. Evidence for the cross-protection phenomenon was initially demon-

strated for tomato plants (21). When a susceptible host was inoculated beforehand with the non-pathogenic form species, diantha or callistephi, the host became resistant to the pathogenic form species lycopersici for a period of several days (21).

The delay in immediate phenolic synthesis of susceptible varieties of tomato plants to F. oxysporum f. sp. lycopersici was theorized to depend on the absence of an unspecified stimuli in lycopersici to induce the hypersensitive reaction or the ability of lycopersici to inhibit or delay the hypersensitive response (63). Results tend to indicate that the second explanation may be the case. A susceptible variety of tomatoes to lycopersici was inoculated with lycopersici, diantha, and a combination of both form species. Diantha, when inoculated by itself, was able to stimulate phenolic synthesis within 24 hours while lycopersici and the combination inoculum did not. It is therefore believed that lycopersici in some manner inactivates the hypersensitive response thus prohibiting the rapid increase in phenolic compounds (63).

Several fungal pathogens of white clover (Trifolium repens) were capable of increasing phenolic compounds in both field and greenhouse-grown clover plants (103). The phenolic compounds which were isolated and found to increase after disease infection were the flavones; luteolin, 4',7-dihydroxy flavone, 3',4',7-trihydroxy flavone and geraldone; and the coumestans, 7,10,12-trihydroxy coumestan (repensol), 12-o-methyl-coumestrol, coumestrol, and trifoliol.

When clover leaves were infected by Pseudopeziza trifolii and separated into lesion and non-lesion areas, phenolic compounds were found to be concentrated in the lesion areas while the non-lesion areas were substantially lower in phenolic compounds. Another study was

conducted to determine the source of the phenols (103). Urediospores from rust (Uromyces trifolii) infected clover were found to contain the full array of phenolic compounds previously identified in the diseased samples; however, when Cymadothea trifolii, another pathogen, was cultured on artificial medium none of the recognized phenolic compounds were found.

An investigation of the fungitoxicity of these phenolic compounds was not conducted but it was mentioned that the coumestans are structurally similar to the phytoalexin pterocarpan, phaseollin and pisatin. It is believed that a pterocarpan phytoalexin may be present but as yet not isolated since the coumestans are structurally and biogenetically related to the pterocarpan (103,104).

Phytoalexins. The origin of the term, phytoalexin, was used to describe the fungitoxic component identified by Müller and Börger for the Solanum tuberosum-Phytophthora infestans interaction (18). The name phytoalexin was derived from two Greek words "phyton" meaning plant and "alexin" referring to a warding off compound (18). The results obtained from this host-parasite interaction led Müller and Börger to propose the Phytoalexin Theory (18). The theory is as follows:

- (a) A principle, designated as "phytoalexin," which inhibits the development of the fungus in hypersensitive tissue, is formed or activated only when the host cells come into contact with the parasite.
- (b) The defensive reaction occurs only in living cells.
- (c) The inhibitory material is a chemical substance and may be regarded as the product of necrobiosis of the host cell.
- (d) This phytoalexin is non-specific in its toxicity towards fungi; however, fungal species may be differentially sensitive to it.

(e) The basic response that occurs in resistant and susceptible hosts is similar. The basis of differentiation between resistant and susceptible hosts is the speed of formation of the phytoalexin.

(f) The defense reaction is confined to the tissue colonized by the fungus and its immediate neighborhood.

(g) The resistant state is not inherited. It is developed after the fungus has attempted infection. The sensitivity of the host cell that determines the speed of the host reaction is specific and genotypically determined.

The term phytoalexin, as defined by Müller and Börger, has come under criticism concerning several of its assumptions. The induction of phytoalexins has been accomplished by agents other than parasitic fungi. The list of elicitors includes cell free extracts, heavy metals, specific environmental conditions, metabolic inhibitors, various fungicides, ultra-violet irradiation, mechanical injury, and viral agents (18,23,55, 84,106). Compounds identified as phytoalexins have been found in healthy plants (23) and at least two phytoalexins have been shown to be systemic (23,106). The term phytoalexin has therefore become ambiguous and in an attempt to clarify this term several authors have accepted a more general definition of phytoalexins (23,55). A phytoalexin is now thought of as any anti-microbial substance formed in sufficient concentrations which may be induced by pathogenic organisms or other agents.

The principal phytoalexins according to Kuć (55) are chlorogenic and caffeic acids, α -solanine, α -chaconine, rishitin, and phytuberin from potatoes (Solanum tuberosum), pisatin from peas (Pisum sativum), phaseollin from green bean pods (Phaseollus vulgaris), hydroxyphaseollin

from soybeans (Glycine max), chlorogenic acid and 6-methoxy mellin from carrot (Daucus carota), chlorogenic, isochlorogenic, and caffeic acids, scopoletin, umbelliferone, and ipomeamarone from sweet potato (Ipomoea batatas), orchinol from orchids (Orchis spp.), and formononetin, biochanin A, coumestans, trifolirhizin, maackiain, daidzein, 7,4'-dihydroxy flavone, 7,3',4'-trihydroxy flavone, and medicarpin from the clovers (Trifolium spp.).

These phytoalexins can be classified into two major classes based on their structure. The principal group is the phenolic phytoalexins and for purposes of clarification they are subdivided as follows: chlorogenic, isochlorogenic, and caffeic acids are in the cinnamic acid derivative group; scopoletin, umbelliferone, 6-methoxy mellin, are in the coumarin group; formononetin, biochanin A, daidzein, (isoflavones) 7,4'-dihydroxy flavone, and 7,3',4'-trihydroxy flavone (flavones) are in the flavonoid group; pisatin, phaseollin, hydroxyphaseollin, trifolirhizin, maackiain, and medicarpin are in the pterocarpan group; and coumesterol, 12-o-methylcoumesterol, trifoliol, and repensol are in the coumestan group. In the second major class are the terpenoid derivatives and they include the following: rishitin (norsesquiterpene alcohol), phytuberin (aliphatic, unsaturated sesquiterpene acetate), α -solanine and α -chaconine (steroid glycoalkaloids) and ipomeamarone (furano terpenoid).

The biosynthesis of the terpenoid compounds is via the mevalonic acid pathway (58) while the phenolic phytoalexins are produced by way of the shikimic acid pathway and/or the acetate pathway (70,95). Pisatin, a phenolic phytoalexin, will be discussed more thoroughly.

Pisatin. After infection by Sclerotinia fructicola the fungitoxic component of peas, pisatin, was identified. The structure of pisatin was identified as 3-hydroxy-7-methoxy-4',5'-methylendioxy-chromanocoumarin and is classified as a pterocarpan (77,78). Pisatin may be produced by leaves, stems, or pods and it is not phytotoxic to the plant at the concentrations which are fungitoxic (106). The ED₅₀ (effective dose which is lethal to 50% of the microbial population) value of pisatin for 44 non-pathogenic fungi is between 25 to 75 µg/ml while for six pathogenic fungi it is greater than 100 µg/ml (106). Therefore, fungi pathogenic to pea are relatively insensitive to the amounts of pisatin accumulating after infection whereas non-parasitic fungi are extremely sensitive.

The conditions which affect the production of pisatin have been reported (19,41,61). The factors which influence the synthesis of pisatin are the physiological condition of the host, the amount and type of inoculum and the environmental conditions both before and during the host-parasite interaction. An inverse relationship exists between pod maturity and pisatin concentration (19). As pods mature pisatin concentration decreases. Pisatin was not detected in healthy or mechanically injured pea pods (19). The environmental conditions for optimum pisatin production are an aerobic atmosphere and temperatures ranging between 10-30°C. When pea pods were stored at 4°C and under well aerated conditions their capacity to produce pisatin increased as opposed to closed storage conditions at 20°C. Pisatin was not produced under anaerobic conditions (19). In general the higher the inoculum concentration the greater the pisatin production. Pisatin synthesis, however, was not only induced by fungal pathogens but also by

mercury, copper, ultraviolet irradiation, ethylene, low levels of metabolic inhibitors (actinomycin D, chloramphenicol), and cell free extracts of pathogens (19,20,41,88).

A time course study demonstrated that pisatin accumulation undergoes a 6-8 hour lag period and then increases linearly for the next 50 hours when treated with cupric chloride at 3×10^{-3} M (19). This response curve was similar to fungal induced pisatin except that a leveling out of pisatin occurred at 30 hours but then began to increase again at 48 hours. This second period of rapid increase in pisatin is believed to be due to the release of bound pisatin or a second cycle in synthesis of pisatin (19). It was also noted that bacteria failed to stimulate pisatin synthesis (19).

The possibility that pisatin is degraded by pathogenic fungi has been reported (100,101) and may explain the relative insensitivity of pisatin to pathogenic fungi. When the pathogenic fungi, Fusarium oxysporum f. sp. pisi race 1 and Mycosphaerella pinodes, were incubated for one week in shake cultures and then treated with pisatin and allowed to remain for an additional week, the results indicated that pisatin was degraded. However, when the non-pathogenic fungi; Cladosporium cucumerinum, Collectotrichum lindemuthianum, and Monilinia fructigina were incubated and analyzed as above, pisatin could not be recovered in the supernatant or from the mycelium of the fungi (100). A subsequent report using labelled pisatin confirmed that pisatin was degraded (100). Another consideration which was elucidated was that the carbohydrate source in the nutrient medium may also influence the breakdown of pisatin (102). It is suggested that the synthesis of pisatin degrading enzymes may be subject to catabolic repression.

The biosynthesis of pisatin is believed to occur by the combined action of the shikimic acid and acetate pathways to produce a chalcone followed by transformation to an isoflavone and eventually to a pterocarpan (78,104). Phenylalanine ammonia lyase activity was found to increase ten-fold when inoculated with fungal spore suspensions (40).

The induction of pisatin formation was proposed to occur by way of the derepression of certain genes by selectively inhibiting the negative gene control mechanisms (88). Several microbial metabolites, such as actinomycin D and chloramphenicol, were shown to be efficient elicitors of pisatin synthesis when administered at low concentrations. Since these metabolites are capable of arresting total protein synthesis at proper concentrations it is believed that at lower concentrations they selectively inhibit the action of the regulator gene in producing the repressor complex. Therefore, the structural gene is now capable of transcribing the enzyme responsible for pisatin production (88). This explanation also helps to clarify the biochemical mechanisms of the gene for gene disease interaction discussed earlier.

2

Poa pratensis L. and Helminthosporium spp. Interaction

Leaf spot or melting-out of Kentucky bluegrass (Poa pratensis L.) was first reported in 1922 by Dreschsler (26) who identified the causal organism as a Helminthosporium species. He later named the pathogen Helminthosporium vagans. Since that time several Helminthosporium species (H. sativum=H. sorokinianum, H. dictyoides, and H. triseptatum) have been identified as causal organisms in the United States (5,7,76,98). This disease has two distinct phases (17). The leaf spotting phase occurs predominantly in the spring and fall months and is generally localized as lesions on the leaves of Kentucky bluegrass (17). The melting-out phase is associated with the warmer periods of the year, such as the summer months, where the majority of lesions are found on the lower sheath area, the crown area, and in the root and rhizome area (17). Also severe leaf blighting has been associated with this phase of the disease.

The factors affecting this host-parasite interaction which have been investigated are environmental conditions, varietal differences of the host and pathogen, and cultural practices.

The environmental conditions that have been examined are temperature, moisture, light intensity, and seasonal variation (5,6,28,33,34,42,48, 98). When common Kentucky bluegrass was inoculated with H. sativum and incubated at 100 percent relative humidity at 20, 25, 30 and 35° C for a period of seven days, the following symptoms developed (98). At 20°C only leaf spotting was present while at 25°C leaf blighting began to occur but leaf spotting still predominated. Leaf blighting increased as the temperature regime increased to 30° and 35 °C. Field observations in eastern Nebraska during mid-summer

also revealed that leaf blighting was associated with moist conditions and high temperatures (98). In a study conducted in West Virginia H. vagans was shown to be most destructive during the month of May while the least destructive time period was during the warm and dry periods of August and September (28). A correlation between disease incidence of several foliar pathogens to temperature and rainfall data was found not to be significant (28). However, a relationship between environmental conditions and Helminthosporium leaf spot did seem to be present but no statistical support of the data was provided. The optimum temperature for growth of H. sativum and H. dictyoides determined by in vitro studies was 27°C and the optimum pH occurred between 5.5 and 6.2 (5). High relative humidities have been reported to promote conidia germination for Helminthosporium spp. (5,98). Greenhouse studies indicated that for substantial infection to occur bluegrass plants and conidia must be subjected to 100 percent relative humidity for a period of 24-36 hours (5).

Alternate cycles of wetting and drying have been reported to increase the incidence of leaf spot due to H. sativum (30). Spores of H. sativum did not germinate on grass clippings that were kept moist but if the clippings were allowed to air dry and then remoistened the conidia germinated.

Merion Kentucky bluegrass, when exposed to high and low light intensities followed by inoculation with H. dictyoides, resulted in different lesion development patterns (34). Under the low light treatment lesion size was significantly greater than under the high light intensity treatment. It was also noted that the number of infections for both treatments did not differ (34).

The effect of seasonal variation on the distribution patterns of Helminthosporium spp. was observed (7,67). In New York during the spring and fall months H. vagans was the principal species isolated, however, in the summer H. sativum was the predominant species isolated (67). In the Washington, D.C. area H. triseptatum was the causal organism found most often from November 1963 to February 1964 while H. dictyoides was present throughout the spring and summer months of 1964 (7). However, other reports indicate that the ratio of Helminthosporium species or the occurrence of an individual species remains the same throughout the year (8,98).

The second area of investigation is the varietal difference of the host and the parasite. Excellent resistance of varieties, such as Merion and Fylking, to Helminthosporium spp. has been cited numerous times (34,35,42,48). However, the resistant response is believed to be modified by environmental conditions and/or the presence of other species or races of the pathogen (48).

The first extensive histological study performed to examine the differences in the host-parasite interaction due to varietal and species differences in the host and pathogen was reported by Mower (67). Selected for study was the resistant variety, Merion, and the susceptible variety, common Kentucky bluegrass, while the pathogens used for the evaluation were H. sativum and H. vagans.

When H. sativum was inoculated on detached leaves of common Kentucky bluegrass approximately 80-90 percent of the conidia germinated within 18 hours while on Merion Kentucky bluegrass only 60-70 percent of the conidia germinated. During this time period disintegration and irregular growth of germ tubes was observed on Merion but not on

common bluegrass. Also at the end of 18 hours, 45 percent of the germ tubes developing on common Kentucky bluegrass had formed appressoria while only 15-25 percent of the germ tubes formed appressoria on Merion.

The principal mode of ingress by the parasite was dependent on the host variety. Direct penetration, at the juncture of lateral epidermal cell walls occurred 40-60 percent of the time in common while in Merion only 1-4 percent of the successful penetrations developed in this manner. In Merion the predominant form of ingress was through the stomates. This type of entry accounted for approximately 60-70 percent of the penetrations in Merion while in common only 5-10 percent of the penetrations occurred in this manner.

Once penetration was complete in both common and Merion there was no appreciable difference in the colonization of the hosts by the parasite. Primary and secondary hyphae grew intercellularly and intracellularly. Correlated to secondary hyphal development at approximately 40-54 hours after inoculation was the appearance of macroscopic symptoms which were small circular brown to purplish lesions. After approximately 60 hours the lesions expanded both in width (1-3 mm) and in length (2-8 mm). The center of the lesions turned light brown to tan while the margins remained dark brown to purplish. The leaf tissue area adjacent to the lesions often appeared chlorotic and chloroplast disintegration was also observed to be occurring at this time.

When Mower (67) tested H. vagans on the two bluegrass varieties the morphological symptoms produced were very similar to those associated with H. sativum. However, the sub-epidermal cells within the lesion area differed. In H. sativum infected plants the sub-epidermal cells

displayed a degradation pattern which became progressively worse beginning at the margin and extending towards the center of the lesion. However, in H. vagans infected plant tissue healthy cells were often found interspersed among the collapsed and pigmented cells within the lesion. Germination and penetration of H. vagans on Merion and common Kentucky bluegrass was also similar to that of H. sativum infected tissue.

Thus the resistance of Merion to H. vagans and H. sativum is believed to be due to the lower number of successful penetrations by the causal organisms when compared to common. Another facet of the resistant response is the lower number of penetrations at the juncture of the epidermal cells. An explanation for the differences between Merion and common Kentucky bluegrass was suggested to be attributed to differences in the chemical constituents of the cuticular layers of these two bluegrass varieties. A toxic component is believed to be present in the Merion cuticle resulting in the abnormal growth of germ tubes and a lower number of successful penetrations.

Enzymatic studies with H. sativum, and Merion and common Kentucky bluegrass indicates differences in the type of pectolytic and cellulolytic enzymes with regard to the host (68). In vitro studies have demonstrated that H. sativum is capable of producing two pectolytic enzymes, polymethylgalacturonase and a calcium stimulated methyl-trans-eliminase, when the carbon source was pectin or sodium polypectate; however no pectolytic enzymes were found when glucose was the carbon source. These results suggest that these enzymes may be inductive enzymes or that their synthesis may be repressed by glucose. When diseased tissue from Merion and common Kentucky bluegrass was analyzed for

enzyme activity four extracellular enzymes were identified. In the Merion extracts pectin methylesterase, methyl-trans-eliminase, and polymethylgalacturonase were the pectolytic enzymes while one cellulolytic enzyme, cellulose (Cx), was also isolated. In extracts from common the same cellulolytic enzyme was present while the pectolytic enzymes were the same except that polygalacturonase was produced instead of polymethylgalacturonase. The Merion-H. sativum enzyme extract had a higher enzyme activity level than did the common-H. sativum extract. It was noted earlier by Muse (68) that lesions on Merion caused by H. sativum were generally larger than on common. An explanation for the increased lesion size for Merion was suggested to be due to higher enzyme activity level in Merion and/or the presence of polymethylgalacturonase. The enzymes, pectin methylesterase and cellulose (Cx), were believed to be synthesized by the host and not the pathogen since these enzymes were not present in the in vitro study.

The implication that the nonstructural carbohydrate content of plant tissues is related to disease incidence was first shown for the tomato-Alternaria solani interaction (early blight of tomato) (51). When sugar levels within the tomato leaves decreased due to age and/or fruit development the incidence of early blight increased. Because of this early blight was classified as a low-sugar disease. Helminthosporium leaf spot of cereals was also classified as a low-sugar disease. The reverse situation may be the case in which the host attacked has a high sugar content. Examples of high-sugar diseases are the rust and powdery mildew diseases of cereals (51).

The carbohydrate level in bluegrasses is not only dependent on varietal differences but other factors such as environmental conditions and cultural practices may also influence the carbohydrate content (34, 35, 59, 96, 107). Investigations relating leaf spot and melting-out of Kentucky bluegrass to sugar level have been reported (34, 35, 59). Leaf sugar concentration as affected by varietal differences of the host, cutting height, and light intensity to disease incidence in the field were examined (59). The resistant varieties of bluegrass tested were Merion and Windsor, while the intermediate and susceptible varieties were Newport, Park and common. The correlation coefficient between the disease rating and the amount of leaf sugar was $-.96$ (59). As sugar content decreased disease incidence due to H. vagans increased. Varietal differences in sugar concentrations were observed with Merion having the highest levels and common the lowest. Also mowing height and light intensity affected carbohydrate content. When the clipping height and light intensity were lowered, the carbohydrate level decreased with subsequent increases in the severity of Helminthosporium disease (59).

Resistance conferred to Kentucky bluegrass varieties possessing high carbohydrate levels is believed to occur as a result of one of two methods: (1) High levels of nonstructural carbohydrates may inhibit the synthesis of pectolytic enzymes or (2) Nonstructural carbohydrates may be an immediate source for the synthesis of fungitoxic phenols (59).

It has been shown that the activity of pectolytic enzymes is very slight when glucose is present in the medium for H. sativum and H. vagans (68). It is therefore suggested that resistant Kentucky bluegrass cultivars possess relatively high sugar concentrations which inhibit the synthesis of pectolytic enzymes of Helminthosporium spp. resulting in

the failure of the causal organism to grow intercellularly after penetration.

Fungitoxic phenols have been shown to be a factor in several host-parasite interactions (23,55). Since nonstructural carbohydrates are required for phenolic synthesis (86,90), Kentucky bluegrass varieties having relatively low carbohydrate levels may be unable to synthesize an adequate concentration of fungitoxic phenolic compounds.

In a subsequent investigation performed in the greenhouse with 13 varieties of Kentucky bluegrass, there was no correlation between a susceptible response and the concentration of either total and reducing carbohydrates or fructose (16). Another greenhouse study which compared eight varieties of bluegrass, including Merion, demonstrated that there was no correlation between sugar content and disease incidence (34).

Controversy over the influence of carbohydrate concentration in disease incidence exists since the term high versus low sugar levels is relative (34). It has been substantiated that factors such as light, temperature, season of the year, time of day, age of the plant and even extraction and analysis procedures will affect the reported carbohydrate level in bluegrasses (34,38,50,96,107). Therefore, factors such as these must be accounted for when comparisons between carbohydrate levels and disease incidence are reported.

The major cultural practices influencing the P. pratensis-Helminthosporium spp. interaction are nitrogen fertilization and mowing height (5,35,43,59). These two factors have been examined both individually and collectively. The effect of nitrogen fertilization on leaf spot disease was first investigated in field plots in Minnesota. Results obtained from four varieties of bluegrass under three fertilization treatments indicated no significant influence of fertility level on

leaf spot incidence (5). However, the negative effect of excessive nitrogen was reported at test sites in New Jersey (43). The melting-out phase due to H. vagans which was measured as the percentage of the total turf area destroyed or thinned out was greater on high fertility plots than on the low fertility plots (43). When disease ratings were taken in June 1961, the high fertility plots of Merion differed by only one percentage point from that of the low fertility treatment. However susceptibility of two common bluegrasses varied by as many as 15 to 17 percentage points between the two fertility treatments (43). The deleterious effects of excessive nitrogen fertilization appears to be more pronounced on the susceptible varieties than on resistant varieties. In this study it was also observed that in March and April less leaf spot damage occurred on the high fertility treatment but in May and June the melting-out phase was most destructive under the high fertility treatment. A greenhouse investigation, involving 13 varieties of bluegrass, also revealed that susceptibility to H. sativum increases with higher rates of nitrogen fertilization (16).

A two year study (1969-1970) conducted in Minnesota reported that in the first year leaf spot damage was significantly greater under the high fertility treatment than in the low fertility treatment for nine of the twenty lines of bluegrass examined (35). However, in the second year there was no significant difference between fertility treatments for any of the bluegrass cultivars.

The influence of Kentucky bluegrass mowing height on melting-out was demonstrated for several bluegrass varieties in New Jersey (43). Under a high fertility treatment Merion clipped at 3.7 cm had a lower disease index than the 1.8 cm mowing height. There was no difference

between mowing heights under the low fertility treatment for Merion. However, for a common cultivar the lower mowing height had a higher disease rating under both fertility treatments (43). Increased disease severity at lower mowing heights is believed to be due to the lowering of the carbohydrate level in the Kentucky bluegrass plants.

Opposite findings over the effects of mowing and fertilization practices have been reported in Minnesota (35). It was found that there was significantly less disease under the low mowing and low fertilization treatments than under the high mowing and high fertilization treatments. It has been suggested that the effects of mowing and fertilization are insignificant in disease development when compared to environmental factors such as moisture and temperature (35).