A Study of the Nature of Lipid and Sterol Fractions of Healthy and Rust-Infected Primary Leaves of Wheat

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A STUDY OF THE NATURE OF LIPID AND STEROL FRACTIONS OF HEALTHY AND RUST-INFECTED PRIMARY LEAVES OF WHEAT

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THESIS FORMAT

A major criticism of graduate programs is that the young professional scientists graduating from these programs have not received sufficient training to present scientific papers in an acceptable form. For this reason, the results of these studies are presented in the following sections in the same form as that required for submission to a scientific journal. In addition, a speculative discussion of the type encouraged in a thesis is presented in the general discussion.

ABSTRACT

Lipids and sterols were extracted from two near-isogenic lines of healthy and rust-infected wheat (Triticum aestivum L.), and analyzed by thin-layer and gas-liquid chromatography. The lines of wheat differ in that one contained the temperature-sensitive gene Sr6 from Red Egyptian Spring wheat which provided resistance to race 56 of stem rust at mean temperatures below 20°C.

Rust infection had no effect on the level of the total lipids, polar and non-polar lipids, and unsaponifiable lipids in leaves of Chinese Spring wheat grown at 20°C. In leaves maintained at 25°C, where both resistant and susceptible lines displayed a susceptible phenotype, and in four other wheat varieties that were susceptible to race 56 and maintained at 20°C, infection did not affect appreciably the lipid content. Wheat leaves contained at least 11 fatty acids. Six of these were present in appreciable amounts. Infection was not accompanied by qualitative changes in the lipid and fatty acid spectrum and no significant changes were observed in constituents of either non-polar or polar fractions.

Wheat leaves of susceptible and resistant lines contained cholesterol, campesterol, stigmasterol, β-sitosterol, and an unknown sterol that accounted for 75% to 80% of total sterol content. A further sterol, stigmast-7-enol, occurred in all rust-infected leaves and in one of eleven samples of healthy leaves. The level of stigmast-7-enol in infected

leaves appeared to be correlated with the amount of fungal mass in the host.

Various lipid fractions from healthy and rust-infected wheat leaves were not phytotoxic. They did not inhibit rust development in susceptible leaves nor did they promote it in resistant leaves.

The results suggested that the lipid and sterol fractions are not related to susceptibility or resistance.

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INTRODUCTION

Much is known about the metabolic disturbances that occur in host plants after infection with parasites (5,10,16,46,58). Some of these changes may be related directly to altered membrane integrity and permeability of host cells as a consequence of pathogenic attack. However, the nature of the changes in the affected cell membranes have not been elucidated.

While much work has yet to be done on the nature of structural components in host cell membranes, it is generally accepted that all cellular membranes are composed of two principal classes of compounds: lipids and proteins (51). The principal structural lipids in plant membranes are the phospholipids, glycolipids, sulfolipids, and isoprenoids (37,51).

The amphipathic nature of the first three classes of lipids suggests their primary importance to be in the maintenance of membrane structure and integrity. However, it is not unlikely that they may also serve other important metabolic functions such as in the transport of metabolites. The last group of lipids, plant isoprenoids, may function in cell membrane structure, permeability, and transport. Kemp *et al* (21) suggested that unesterified plant sterols have a structural role in membranes while plant sterol esters may be involved in membrane transport. Grunwald (14) proposed that phytosterols stabilize plant membranes by controlling

their permeability.

Involvements of this kind by lipid and sterol derivatives in the regulation of cellular membrane activity could greatly influence the nature of the response by a host plant to an invading pathogen and/or its toxins. Phospholipids have been the subject of studies on wheat leaves infected with Erysiphe graminis tritici D.C. (26) and Puccinia graminis Pers. f. sp. tritici (Eriks. & E. Henn.) (36). However, no studies on the role of sterols in rust-infected cereal leaves have been reported.

At the present time our knowledge of plant membrane chemistry is based almost entirely upon studies of the distribution and general structure of constituents in lipid extracts, primarily from seeds and leaves. Most of these lipid constituents are probably specific to cellular membranes (51). In the present study, the lipid and sterol contents from two near-isogenic lines of healthy and rust-infected wheat leaves were investigated, with the hope that some insight into the mechanisms of resistance and susceptibility might be gained.

LITERATURE REVIEW

It is generally accepted that the breakdown of membrane structure during the course of the host-parasite interaction leads to increased permeability of host membranes resulting in disorganization of cellular components. Consequently, intracellular changes involving increased catabolic processes and eventual senescence may ensue (5,10,16,46,58). Changes in the properties of membranous components of host cells could be expected to be reflected in alterations in the nature of those lipids associated with lipo-protein membrane structures. However there are few detailed studies on the nature of alterations in the biochemical and biophysical properties of cellular membranes of the host in response to an invading pathogen and/or its toxins.

I. Alterations to membrane permeability

The literature contains numerous reports which imply disease symptom production as a consequence of phytotoxin interaction with plant cell membranes (c.f. 10, 39,58).

Gottlieb (12) reported that a fluid obtained from tomato stems infected with Fusarium oxysporium f. lycopersici increased the permeability of tomato pith cells.

Gäumann and co-workers (8) carried out extensive studies on changes in permeability induced in a variety of plant tissues by low molecular weight toxins produced by Fusarium oxysporium f. lycopersici. Damage to cell membranes

appeared to be an early effect.

Gnanam (9) observed changes in conductivity of cotton plant tissues infected with Fusarium vasinfectum. He observed that conductivities were not affected until fifteen days after infection. Later on the conductivity of the leaf sap from infected plants was higher than that from healthy plants.

Kern and Naef-Roth have isolated four phytotoxic pigments from various cultures of Fusarium solani and from diseased pea (22,23,24). The toxins presumably caused wilting by affecting the water permeability of the cell membranes.

Glycopeptide toxins have been isolated from species of Corynebacterium (41,49). The available evidence suggests that they play a role in production of disease symptoms by their interactions with cellular membranes, and not simply by a plugging action of the vascular elements.

Colletotin is a toxin produced by the phytopathogenic fungus Colletotrichum fuscum. The toxin appears to act by causing disintegration of leaf mesophyll and palisade tissue (29).

Leaf and stem blight in zinnia have been attributed to the fungus Alternaria zinniae. The symptoms appear to be caused by zinniol, a toxin isolated from culture filtrates of the fungus (57). The toxin caused rapid loss of pigment and

drastic shriveling of cut red beet stems. These observations tend to suggest that cell membrane damage is an early effect.

Ascochitine is a phytotoxic hemiquinone derivative which has been isolated from culture filtrates of Ascochyta fabae, the causal fungus of brown spot disease of broad bean (38). The toxin caused epidermal cells of Rhoeo discolor to leak pigment and to lose plasmolytic ability, suggesting alterations of the cell membranes.

In recent years more detailed studies have been directed toward studies on the nature of these phytotoxin-plant cell membrane interactions. Two such studies involve the toxins victorin and syringomycin.

Work with victorin, a polypeptide toxin of Helminthosporium victoriae suggests that its primary effect is an alteration in cell membrane permeability. Wheeler and Black (53,54) showed that toxin-treated or infected tissue from a susceptible oat variety rapidly begins to lose electrolytes into a bathing solution. This electrolyte loss began within five minutes after toxin treatment. Tissue from resistant oat varieties was not affected. The authors concluded that these are host specific and not general effects. Further evidence for this membrane-alteration theory was obtained by Samaddar and Scheffer (43) using plant cell protoplasts. Protoplasts from susceptible oat tissue quickly stopped protoplasmic streaming and ruptured within one hour after

toxin treatment. Protoplasts from resistant oats were unaffected. Luke and co-workers (30) have obtained electron microscopic evidence for selective toxicity toward cell membranes. The endoplasmic reticulum, nuclear envelope, and mitochondrial membranes were more resistant to toxin damage. These findings have been corroborated by studies showing that neither succinoxidase activity (13,27,44), respiratory control (55), nor permeability (53,54) of mitochondria isolated from susceptible oat tissue were affected by toxin treatment.

Backman and Devay (3) have studied the membrane action and biogenesis of the phytotoxin syringomycin, isolated from Pseudomonas syringae var. Hall. This toxin causes a rapid necrosis in two to five hours when injected into plant tissue. The contact angle of water to hydrophobic surfaces was found to be reduced, suggesting that it possessed detergent-like properties shown by many membrane-active polypeptide antibiotics. The authors suggested that the site of action for this toxin was most likely on cell membranes. Corroborating evidence came from findings that the toxin caused rapid leakage of haemoglobin from washed rabbit erythrocytes. Electron micrographs of toxin treated cells of Geotrichium candidum indicated that at lethal doses, massive disruption of membrane integrity occurred within ten minutes and that membrane abnormalities were caused at sub-

lethal doses. Application of ^{14}C-toxin to plant tissues showed that radioactive toxin was concentrated primarily over the plasmalemma and nuclear envelope. Studies with membrane components indicated that the site of syringomycin binding might be membrane lipids. They postulated that the mechanism of reduction of syringomycin toxicity in vitro caused by some lipids, for example α-lecithin, was similar to the binding of the toxin by cell membranes. From their findings the authors believe syringomycin to be a product of the cell wall of P. syringae and that its primary role is the rapid, detergent-like lysis of cellular membranes by its binding to membrane lipids.

There are very few reports on alterations to membrane permeability of host tissues following rust infection.

Thatcher (50) used plasmolytic techniques to measure changes in permeability which occur in a number of plant diseases. Infection by rusts was found to increase permeability of cells adjacent to invaded, killed cells.

Silverman (48) extracted a toxin from Marquis wheat infected with race 38 of the wheat stem rust fungus. The toxin was isolated from Marquis seedlings grown at high temperatures but not from those grown at low temperatures. Chlorosis appeared in seedlings of Marquis infiltrated with the toxin only when these test plants were grown at low temperatures; it did not appear on plants grown at the high tem-

peratures. Little Club wheat, which is susceptible to race 38 at all temperatures, did not become chlorotic following infiltration with the toxin regardless of the temperature preceeding or following infiltration. However, Little Club is T. campactrum not T. aestivum. The difference in reaction to the toxin may therefore have been species dependent. Microscopic examination of chlorotic areas produced by the toxin revealed that the chloroplasts were affected; however the cell walls of the host remained normal.

II. Lipid metabolism

Very few studies on the relationship between lipid metabolism and host-parasite interactions have been reported.

Maciejewska-Potapczyk (33) examined the phosphorus compounds, nuclease, and phosphatase activities in healthy and tumorous tissues of Datura stramonium L. The author reported an increase in the phospholipid fraction in the tumorous tissues.

Scott et al (45) investigated the phosphorus-containing fractions in normal and tumor tissues of Beta vulgaris. They reported that the residual-lipid phosphate per unit fresh weight was three times higher in tumors. However per microgram DNA-phosphate, it was about the same in both tissues.

Mukherjee and Shaw (36) studied the effect of stem rust on the phosphate fractions in wheat leaves and reported that rust infection caused striking increases in the lipid

phosphate fraction. They suggested that this increase was of considerable interest since an increased rate of synthesis of lipids could promote the operation of the hexose monophosphate pathway by promoting the oxidation of $NADPH_2$.

The incorporation of ^{32}P into the phospholipid fraction of wheat leaves infected with powdery mildew was investigated by Kljajic and Plesnicar (26). They observed that the incorporation was higher in the resistant than in the susceptible variety.

Epton and Deverall (7) have found that leaves of Phaseolus vulgaris resistant to halo blight disease have larger quantities of a lipid than susceptible leaves. On acid hydrolysis this lipid yielded linolenic and linoleic acids, and was thought to be a galactosyl diglyceride. Following homogenization and incubation, the amounts of free linolenic acid increased eight-fold in halo blight-resistant leaves; in susceptible leaves there was a four-fold increase.

Keen and Williams (20) studied the synthesis and degradation of starch and lipids following infection of cabbage by Plasmodiophora brassicae. They noted that lipids did not accumulate in the host cytoplasm during disease development, and no pronounced changes occurred in lipase, esterase, or fatty acid synthetase activities. Specific activities of fatty acid synthetase were forty to seventy times higher in extracts of isolated P. brassicae plasmodia than in noninfected or infected tissue extracts.

The choline content of healthy and rust-infected primary leaves of wheat were examined by Kim (25). Rust infection was found to increase the content of free choline, but not the content of lipid choline in susceptible or resistant leaves. It was concluded that the postinfectional changes in choline concentrations were not related to susceptibility or resistance because rust infection increased the choline content equally in both lines of wheat.

Hoppe (18) investigated the phosphatid and glycolipid metabolism, and permeability behaviour of Phaseolus vulgaris after infection with Uromyces phaseoli var. typica. In infected halves of leaves, an increase in leaching of ions, amino acids, and sugars was observed from the fourth day after infection. Only in later stages of pathogenesis was it possible to observe increased leaching from non-infected halves of leaves compared to those of uninfected control leaves. Infection was not accompanied by qualitative changes in the lipid spectrum, and no significant changes were observed regarding the concentration of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine in infected halves of leaves. In relation to control leaves, a fifty percent decrease occurred in the concentration of phosphatidylglycerol, monogalactosyldiglyceride, and digalactosyldiglyceride, while phosphatidylserine and phosphatidic acid increased. Compared with uninfected con-

trols, uninfected halves of infected leaves contained marginally smaller amounts of all phosphatids and glycolipids. In infected halves of leaves, radioactive phosphorus was incorporated to a larger extent into phosphatidylserine and to a smaller extent into phosphatidylglycerol, compared to non-infected controls. In non-infected halves of infected leaves, phosphatidic acid contained a larger proportion and the other phosphatids a smaller proportion of the total incorporated activity. The specific activity of all phosphatids was increased in infected halves of leaves. Infection did not affect the qualitative fatty acid composition of any of the phosphatids and glycolipids, and neither did it change the proportion of individual fatty acids in galactolipids. The phosphatids, however, contained an increased proportion of unsaturated fatty acids in infected tissues. This was especially evident with phosphatidylethanolamine and with phosphatidylcholine, and to a smaller extent with phosphatidylglycerol. The affect of infection on the fatty acid composition of phosphatids in infected tissues occurred concomitantly with increased leaching, starting three to four days after infection. Phospholipase activity was found to increase between the fourth and sixth days after infection in inoculated leaves. In non-infected halves of infected leaves phosphatidase activity was not affected.

III. Steroid metabolism

The possible functions of steroids both in membrane structure and function (51) and in the sexual reproductive cycle of many species of fungi (17) suggests that they play a role in determining the extent to which a pathogen is able to establish itself within a host plant. However, very few studies on the relationship between steroid metabolism and host-parasite interactions have been reported.

Baur et al (4) and Richardson et al (42) reported that tobacco mosaic virus infection did not appear to have any effect on sterol biosynthesis or distribution in tobacco plants.

Jennings et al (19) studies sterol levels in maize leaves infected with Helminthosporium carbonum and observed changes in total sterol content and composition in inoculated leaves. They suggested that the toxin produced by H. carbonum might interact with the host sterols changing the host cell permeability thereby eventually leading to loss of sterols during disease development.

Locci and Kuc (30) studied the amount of steroid alkaloids in potato tubers and reported that the amount of these compounds present in fresh tissues was low, while an increase in their quantity and number occurred in aged tissues. Similar increases were also observed when tubers were inoculated with a non-pathogen of potato Helminthosporium carbonum. In the case of a compatible pathogen Phytophthora

infestans, there was a slight loss in these compounds. The authors suggested that the accumulation of steroid alkaloids in potato was a response to physiological stress.

Allen and Kuc (1) reported that extracts from potato tubers contained fungitoxic glycoalkaloids, l-solamine and l-chacomine that may be associated with resistance of this tissue to certain fungi.

McKee (34) reported that the rapid accumulation of steroid glycoalkaloids near wounds in potato tubers increased resistance to Fusarium coeruleum Lib. and concluded that the free bases of the steroid glycoalkaloids were toxic to the fungus.

Naturally occurring steryl glycosides have been reported to exhibit biological activity against some microorganisms, but their role in host-parasite interactions is not known. Dewey and Kidder (6) reported that the reversal of the inhibition of the growth of Tetrahymena pyriformis W. by the analog, 6-methylpurine, could be obtained with small amounts of a fraction from saponified lecithin of soybeans. The active material had properties which indicate its identification as a β-D-glycoside of β-sitosterol. Synthetic β-sitosterol-β-D-glycoside had growth-promoting properties approximately equal to those of the isolated material. Both materials were more active than free β-sitosterol. Ma and Schaffer (32) tested extracts of commercially dried grape-

fruit pulp for antibiotic activity and reported that methanol extracts exhibited some antifungal activity but little or no antibacterial activity. In searching for the material responsible for this antifungal activity, the authors isolated β-sitosteryl-D-glucoside and β-sitosterol, neither of which had been isolated previously from grapefruit. These two compounds accounted for most of the antifungal activity observed; however these compounds were designated as having a low order of antifungal activity. Goto et al (11) studied the antimicrobial glycosides of Euptelea polyandra and reported the inhibition of the antimicrobial activity of eupteleoside A.B. caused by β-sitosterol.

Ulrychova and Limberk (52) studied the effect of sterols on plant mycoplasma. They found that the application of two essential sterols, cholesterol and stigmasterol, to grafts from tomato plants infected with potato witches' broom, stimulated growth of the diseased grafts and delayed symptom manifestation. The stimulation was more evident with stigmasterol but the delay in symptom manifestation and intensity was more distinct with cholesterol. They suggested that the exogenous supply of sterols probably promoted additional multiplication of mycoplasma in already infected grafts, thereby allowing the plants these essential metabolites for the development of reproductive organs.

Mellano et al (35) studied the role of host sterols

in the development of tolerance in snapdragon seedlings to infection by Pythium ultimum Trow. The authors suggested that substances such as β-sitosterol, present in tolerant host tissues, may switch fungal development from vegetative proliferation to reproductive activity. The end-result was suggested to be a lessening of virulence.

In a study on the physiological activity of the toxin syringomycin produced by Pseudomonas syringae isolates pathogenic to Prunus sp., Backman et al (3) reported that cell membrane disruption caused by the toxin could be greatly reduced by the addition of cholesterol, β-sitosterol, and viosterol. In contrast, ergosterol was less active, and squalene and cholestanol were inactive.

The disruption of host cell membranes caused by substances of pathogenic origin undoubtedly forms an integral part of the host-parasite interaction. Changes in the properties of membranous components of host cells can also be induced by physicochemical forms of environmental stress such as temperature, water, radiation, and chemicals (28). However, from a plant pathological standpoint, the findings of Backman et al (3) that biologically active lipid and steroid derivatives could reduce or arrest such processes raised the question as to how the metabolism of these compounds in wheat leaves is affected by rust-infection.

SECTION ONE

LIPIDS OF HEALTHY AND RUST-INFECTED PRIMARY LEAVES OF WHEAT

ABSTRACT

Lipids extracted from two near-isogenic lines of healthy and rust-infected wheat (Triticum aestivum L.) six days after inoculation were separated into non-polar and polar fractions by preparative thin-layer chromatography and analyzed by gas-liquid and thin-layer chromatography.

Rust infection had no effect on the level of the total lipids, polar and non-polar lipids, and unsaponifiable lipids in leaves of Chinese Spring wheat grown at 20°C. In leaves maintained at 25°C, where both resistant and susceptible lines displayed a susceptible phenotype, and in four other wheat varieties that were susceptible to race 56 and maintained at 20°C, infection did not affect appreciably the lipid content. Wheat leaves contained at least 11 fatty acids. Six of these were present in appreciable amounts. Infection was not accompanied by qualitative changes in the lipid and fatty acid spectrum and no significant changes were observed in constituents of either non-polar or polar fractions. Lipid levels were not correlated with susceptibility or resistance.

INTRODUCTION

Early cytological investigations by Thatcher (21, 22, 23) on the rust/wheat interaction demonstrated that membrane permeability of resistant reacting host cells differed from that of susceptible reacting cells. These findings and those of related work were reviewed extensively by Wheeler and Hanchey (24). Few cytological studies involving obligate parasites have been carried out in recent years, but use of the electron microscope has provided some insight into pathogenically induced structural changes occurring at the subcellular level. For example, recent work (20) on the host/parasite interaction between Trifolium pratense L. and Erysiphe polygoni DC. showed that plasmalemma and nuclear membranes were rapidly destroyed, and chloroplast membranes were broken down in the resistant reaction. Similar changes were observed in the resistant reaction of cowpeas (Vigna sinensis (Torner) Savi) to Uromyces phaseoli var. vignae (Barcl.) Arth. where depositions of phospholipid-like material were detected at the interface (5). No such changes were observed in susceptible interactions. It is not clear how any of the ultrastructural differences are related to changes in function, but they may interfere with the transfer of metabolites across the host/parasite interface.

Phospholipids and glycolipids are of interest to studies on host/parasite interactions because they are im-

portant and universal constituents of all biological membranes (18). Phospholipids have been the subject of studies on wheat leaves infected with Erysiphe graminis tritici DC. (10) and Puccinia graminis Pers. f. sp. tritici (Eriks. & E. Henn.) (13). In both cases, infection increased the level of these compounds. Infection with a virulent race of Uromyces phaseoli (Pers.) caused quantitative but no qualitative changes in the glycolipid and phospholipid content of bean leaves (7). The lipid metabolism of resistant reacting tissues was not investigated.

In the present study, we compared the lipid content of susceptible and resistant reacting wheat leaves infected with stem rust. The sterol content of these tissues was the subject of a previous report (14).

MATERIALS AND METHODS

Plant Material and Fungus

Near-isogenic lines of wheat (*Triticum aestivum* L.) and race 56 of stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.) were used. The lines of wheat differ in that one contains the gene *Sr6* from Red Egyptian Spring wheat which conditions resistance to race 56 of stem rust at mean temperatures below 20°C. Six days after seeding (i.e. 1 day before inoculation), the ambient temperature was increased from 20°C to 25°C. The plants were maintained at the higher temperature until 1 day after inoculation (17). They were then returned to 20°C to induce the expression of resistance in *Sr6*-containing plants. By using this procedure it was felt that any metabolites produced in the resistant reaction might be present in higher concentration due to the greater abundance of fungal material in the infected host tissue.

The plants were harvested 6 days after inoculation. In each experiment, a set of four differently treated plants was used: healthy and rust-infected plants of the susceptible lines, and healthy and rust-infected plants of the resistant line. Each of the four samples consisted of 3g of primary leaves cut in 4-in. lengths (approx. 10 cm). Experiments were repeated once with different plant material and the data was presented as the averages of duplicate determinations.

Extraction of Lipids

Total lipids were extracted in a VirTis homogenizer with isopropanol-chloroform-methanol, 3:2:1 (v/v/v). The homogenate was filtered and the remaining residue washed with additional solvent until it was colorless. The filtrate was concentrated to dryness *in vacuo*, taken up in chloroform-methanol, 2:1 (v/v), and washed according to the procedure of Folch *et al* (3). After phase separation, the lower phase was filtered through anhydrous sodium sulfate, dried *in vacuo*, and taken up in chloroform-methanol. Aliquots were taken to dryness under nitrogen, and weighed to give the weight of "total lipids". Variation between duplicate determinations did not exceed 5%.

Class Separation of Lipid Extracts

Lipids were separated into non-polar and polar components by thin-layer chromatography (TLC) on 0.5 mm thick layers of silica gel G (19) with chloroform-acetone-methanol-acetic acid-water, 65:20:10:10:3 (11). Fractions were eluted from gel scrapings with chloroform and chloroform-methanol, 2:1 (v/v), concentrated to dryness *in vacuo*, and redissolved in chloroform-methanol.

Weight of Unsaponifiable Lipids and Fatty Acid Content

Aliquots of the extracts containing "total lipids" were saponified with 1N KOH in absolute methanol (16). Unsaponifiable lipids were extracted from the mixture with diethyl ether. The ethereal phases were combined, evaporated to dryness *in vacuo* and weighed. The residual aqueous fractions

were acidified with 10N H_2SO_4 and extracted with diethyl ether (16). The ethereal phases were concentrated in vacuo and the fatty acids were esterified with 14% BF_3-methanol (12).

Gas Chromatography

Fatty acid methyl esters were analyzed with a Packard gas chromatograph Model 7500, equipped with hydrogen flame detectors. A column of 6 ft. x 4 mm inner diameter containing 5% butanediol succinate on Anakrom ABS, 90/100 mesh, was used with a flow rate of 50 ml N_2/min. Operating conditions were: inlet, 215°C; column, 180°C; outlet, 205°C; detectors, 195°C. Peaks of unknowns were assigned identities if the retention times were within 4% of those for corresponding authentic fatty acid methyl esters. Methyl heptadecanoate was used as internal standard to determine the concentrations of endogenous fatty acids in each sample (15). Variation between duplicate determinations did not exceed 1%.

Thin-Layer Chromatography

Lipid components were separated from each other on pre-coated sheets of silica gel F 254, 0.25 mm thickness (E. Merck, Darmstadt) with the following solvent systems: for non-polar lipids, diethyl ether-benzene-ethanol-acetic acid, 40:50:2:0.2, followed by air-drying and development in the same direction with diethyl ether-hexane, 6:94 (4); for polar lipids, chloroform-methanol-acetic acid-water, 85:15:10:3.5 (1). The air-dried plates were charred with 10% H_2SO_4 at 120°C for 10 min. (19) and then scanned at 365 nm with a Zeiss chromatogram spectrophotometer.

RESULTS AND DISCUSSION

The lipid content of healthy and rust-infected wheat leaves harvested 6 days after inoculation is shown in Table 1. There were differences in lipid content between infected and non-infected leaves, but most of these differences were small. The close agreement between the values from infected and non-infected leaves of Chinese Spring wheat grown at 20°C indicates that infection with stem rust has no effect on the level of the total lipids, polar and non-polar lipids, and unsaponifiable lipids. Increased levels of polar lipids had been reported in wheat leaves following infection with mildew (10) and stem rust (13). In leaves maintained at 25°C, where both resistant and susceptible lines displayed a susceptible phenotype, and in four other wheat varieties that were susceptible to race 56 and maintained at 20°C, infection did not affect appreciably the lipid content.

Gas chromatographic analysis showed that wheat leaf lipids contained 6 major fatty acids: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), and behenic acid (22:0). Additional fatty acids were detected in small concentrations. These included: n-tridecaneic acid (13:0), n-pentadecanoic acid (15:0), methyl 14-pentadecanoic acid (iso 16:0), palmitoleic acid (16:1), and methyl 16-heptadecanoic acid

Table 1

Lipid content of healthy and rust-infected wheat leaves harvested 6 days after inoculation

Plant material	Ambient temperature C°	Total lipid	Lipid fractions		
			Non-polar	Polar	Unsaponifiable
		mg/g fresh weight#			
Chinese Spring					
Susceptible, healthy	20	11.4	4.7	6.2	8.6
infected	20	12.4	5.5	6.2	7.3
Resistant, healthy	20	10.8	4.8	5.1	7.8
infected	20	13.1	5.4	5.3	5.8
Susceptible, healthy	25	11.9	6.3	4.2	9.9
infected	25	11.2	6.5	3.1	10.7
Resistant, healthy	25	13.7	6.3	4.5	9.5
infected	25	11.7	7.0	1.8	11.1
Little Club+, healthy	20	7.7*	5.3	2.4	5.7
infected	20	9.4*	6.3	3.1	8.4
Prelude+, healthy	20	10.6*	4.7	5.9	5.9
infected	20	13.1*	5.8	7.3	7.6
Red Bobs+, healthy	20	8.0*	5.0	3.0	6.4
infected	20	9.6*	5.5	4.1	8.6
Reliance+, healthy	20	6.1*	3.6	2.5	4.6
infected	20	8.1*	4.9	3.2	6.8

\# variation between duplicate determinations did not exceed 5%

\+ susceptible to race 56 wheat stem rust

* values calculated from the weights of the non-polar and polar lipids

(iso 18:0). In none of the lipid extracts did these minor components exceed 2% of the total fatty acid content.

The effect of rust infection on the level of 6 major fatty acids of wheat leaf lipids is shown in Table 2. Some of the changes in fatty acid content after infection were very considerable, but did not follow a readily recognizable pattern. Infection tended to increase the contents of palmitic and stearic acids in Chinese Spring grown at 20°C and 25°C, but this pattern was not observed in the four other varieties. Previous reports had shown that the fatty acid spectrum of susceptible reacting plants was not accompanied by qualitative changes (7,8).

Two unusual changes were observed in the concentrations of linolenic and behenic acids in Chinese Spring wheat. Firstly, the level of linolenic acid showed an apparent five-fold increase upon infection in resistant plants grown at 20°C. Epton and Deverall (2) reported that this acid increased eight-fold in the resistant reaction of Phaseolus vulgaris L. leaves to halo-blight disease. Since linolenic acid is very susceptible to oxidation, we believe that the low level reported for healthy leaves of the resistant line may be in error.

Secondly, we observed a three-fold increase of behenic acid levels in susceptible reacting plants of Chinese Spring wheat grown at 25°C. This acid is a major constituent

Table 2

Fatty acid composition of the total lipids from healthy and rust-infected wheat leaves harvested 6 days after inoculation

Plant material	Ambient temperature C°	Fatty acid composition, µg/g fresh weight* Palmitic (C16)	Stearic (C18)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)	Behenic (C22)
Chinese Spring							
Susceptible, healthy	20	79	28	t**	26	389	78
infected	20	90	51	t	30	361	86
Resistant, healthy	20	37	14	t	3	57	70
infected	20	93	27	t	21	278	63
Susceptible, healthy	25	87	12	19	63	367	102
infected	25	94	25	15	46	283	305
Resistant, healthy	25	35	7	3	16	125	70
infected	25	51	20	9	18	93	81
Little Club†, healthy	20	29	9	10	5	21	188
infected	20	20	9	11	4	21	203
Prelude†, healthy	20	41	15	30	4	62	162
infected	20	40	15	18	3	31	180
Red Bobs†, healthy	20	23	10	11	n.d.#	24	198
infected	20	25	13	10	n.d.	20	210
Reliance†, healthy	20	54	23	31	13	27	153
infected	20	39	18	26	7	21	150

* variation between duplicate determinations did not exceed 1%

** t = trace amounts less than 1 µg

\# n.d. = not detectable

\+ susceptible to race 56 wheat stem rust

of the surface lipids of wheat leaves and of Puccinia striiformis West. (6). It is likely that stem rust also contains this substance, and the increased amount in infected susceptible leaves grown at 25°C could well reflect the more luxurient growth of the fungus at the higher temperature.

Thin-layer chromatography of polar and non-polar fractions revealed no major qualitative differences between resistant and susceptible reacting plants at either of the two temperatures. This is in agreement with work involving other host/parasite interactions (7,8) and it is consistent with earlier results on choline levels in rust-infected wheat (9).

However, important metabolic events may occur in the lipid metabolism and these need not lead to qualitative or quantitative changes that could be observed by analyzing crude extracts. Rather, they may involve rearrangements and replacements of certain phospholipid, glycolipid, or sulfolipid membrane constituents. These in turn could alter membrane structure or selectivity. The approach taken by us and other workers in this field would not reveal changes of this nature.

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SECTION TWO

STEROLS OF HEALTHY AND RUST-INFECTED PRIMARY LEAVES OF WHEAT*

* Taken from: Sterols of healthy and rust-infected primary leaves of wheat and of non-germinated and germinated uredospores of wheat stem rust.

R. NOWAK, W.K. KIM, and R. ROHRINGER. 1972. Can. J. Botany 50: 185-190.

Contribution No. 487, Research Station, Winnipeg, Manitoba.

ABSTRACT

Sterols extracted from healthy and rust-infected primary leaves of wheat were analyzed by thin-layer, column, and gas-liquid chromatography.

Wheat leaves of susceptible and resistant lines contained cholesterol, campesterol, stigmasterol, β-sitosterol, and an unknown sterol that accounted for 75% to 80% of total sterol content. A further sterol, stigmast-7-enol, occurred in all rust-infected leaves and in one of eleven samples of healthy leaves. Sterol levels were not related to susceptibility or resistance. The level of stigmast-7-enol in infected leaves appeared to be correlated with the amount of fungal mass in the host.

Sterol fractions from healthy and rust-infected wheat leaves were not phytotoxic. They did not inhibit rust development in susceptible leaves nor did they promote it in resistant leaves.

INTRODUCTION

Although sterols are known to be required for the growth and sexual reproduction of many species of fungi (8), little is known about their role in host-parasite interactions between fungi and higher plants. Extracts from potato tubers were reported to contain fungitoxic glycoalkaloids (1) that may be associated with resistance of this tissue to certain fungi. Naturally occurring sterylglycosides were also reported to have antifungal activity (4,6,13) but their role in host-parasite interactions is not known.

Successful colonization of a host by obligate parasites, such as the rust, depends on the integrity of membranes at the host-parasite interface. Sterols are important components of all membranes (19) and it was therefore of interest to determine their identity in the wheat host and to follow changes in sterol composition of the host-parasite complex during disease development. In addition it was of interest to test sterol fractions from healthy and rust-infected wheat leaf extracts for their effect on rust development in the host.

MATERIALS AND METHODS

Plant Material

Five varieties of wheat (Table 3) and two near-isogenic lines of wheat (*Triticum aestivum* L.) were used and grown under conditions previously described (17). The wheat lines differ in that one contains the temperature sensitive gene *Sr6* from Red Egyptian spring wheat, which provides resistance to race 56 of stem rust.

Extractions

Three grams of primary leaves of wheat, 3, 6, and 9 days after inoculation, were cut to 4-in. lengths and homogenized at 0°C with 50 ml of an isopropanol-chloroform-methanol mixture (3:2:1 v/v/v). The slurry was filtered and the residue washed with solvent until colorless. The combined filtrates were dried *in vacuo*.

The lipids thus obtained were dissolved in 10 ml chloroform-methanol (2:1 v/v) and partitioned with chloroform-methanol-0.02 M aqueous $CaCl_2$ (10:5:3 v/v/v) according to Folch *et al* (5). Both phases were dried *in vacuo*. The lipids were saponified according to Jackson and Frear (10).

The residue from the aqueous phase was extracted first with methanol and then with diethyl ether. The combined extracts were dried *in vacuo*, taken up with methanol, and monitored by thin-layer chromatography (TLC) for the presence of sterylglycosides (2). A portion of this material

was acid hydrolyzed and extracted with diethyl ether for the sterol moiety according to Ma and Schafter (13).

Column Chromatography

Total lipids from wheat leaves were fractionated on Florisil (60-100 mesh) using the stepwise elution apparatus of Hirsh and Ahrens (9). Columns were packed in petroleum ether with 15 g of Florisil. The elution sequence was petroleum ether, 50 ml; 5% diethyl ether in petroleum ether, 120 ml; 15% diethyl ether in petroleum ether, 150 ml; 25% diethyl ether in petroleum ether, 100 ml; 50% diethyl ether in petroleum ether, 100 ml; and methanol, 50 ml. Elution was carried out under nitrogen at the rate of 1 ml/min. Fractions were collected in bulk and evaporated to dryness *in vacuo*. Each fraction was assayed by TLC and those containing a single class of lipids were combined for further examination by TLC or gas-liquid chromatography.

Thin-Layer Chromatography

The sterols were separated on non-activated 0.25 mm silica gel G layers with benzene-ethyl acetate, 4:1 v/v (2). After air-drying, the plates were sprayed with 10% H_2SO_4 in ethanol and heated at 120°C for 10 min. (18).

Gas-Liquid Chromatography

All sterol analyses were performed with a Packard gas chromatograph Model 7500 equipped with hydrogen flame detectors. Free sterols, their trimethylsilyls ether (TMS)

and acetate derivatives were chromatographed using silanized glass columns containing 1% OV-1 on Anakrom ABS, 90/100 mesh (inlet, 270°C; column, 230°C; outlet, 250°C; and detectors, 240°C); an 8 ft. x 4 mm i.d. column was used with a flow rate of 85 ml N_2/min.

Sterol TMS and acetate derivatives were prepared according to Van Den Heuvel and Courts (20) and Knights (12) respectively. 5α-Cholestane was used as an internal standard for the calculation of the content (15) and retention time of each sterol in the sample (3,16). Analyses were repeated once with different plant material and the data was presented as the averages of duplicate determinations. The variation between duplicate determinations did not exceed 2%.

Biological Activity

Extracts or column eluates were dried with a stream of nitrogen and the residues were taken up with double distilled water. Aliquots of these samples at concentrations from 0.1 g to 1.0 g fresh weight of leaves were injected either as suspensions or clear solutions using the method of Hagborg (7) into healthy or rust-infected leaves 2 days after inoculation. The test plants, which had been grown under the same conditions (17) as used for the analytical studies, were examined 6 to 8 days later for phytotoxicity and effect on rust development.

RESULTS AND DISCUSSION

During preliminary studies attempts were made to locate free sterols and their ester and/or glycoside derivatives in isopropanol-chloroform-methanol extracts from wheat leaves. However, both column and thin-layer chromatographic techniques failed to reveal the presence of either sterol esters or steryl glycosides. Consequently, subsequent studies were directed towards determining the nature of the free sterols in wheat.

Primary leaves of wheat contained five sterols (Table 1) of which four were identified as cholesterol (peak 1), campesterol (peak 3), stigmasterol (peak 4), and β-sitosterol (peak 5). Peak 3 was identified as campesterol rather than ergosterol despite the closeness in the relative retention times for their TMS derivatives. Campesterol has been found to be present in most plants including wheat (12), whereas ergosterol is usually considered to be the principal sterol in fungi (8). The sterol emerging in peak 2 was not identified but represented 75 to 80% of the total free sterols. One additional sterol, stigmast-7-enol, was present in all rust-infected wheat leaves and in only one of six samples of non-infected wheat leaves used for the time study (Table 2). Repetition of this experiment with new plant material confirmed the presence of stigmast-7-enol in 13-day old non-infected leaves of the susceptible line and its absence in all other samples of non-infected leaves. Additional

Table 1

Relative retention times of free sterols and their derivatives from wheat leaves

	Compounds	Free	Acetates	TMS	Assignment
1.	Reference standard				
	5α-cholestane	1.00	-	-	
	Cholesterol	2.05	2.89	2.30	
	7-dehydrocholesterol	-	3.02	-	
	Brassicasterol	-	3.21	-	
	Campesterol	2.53	3.60	-	
	Ergosterol	2.70	3.83	3.15	
	Ergost-7-enol	2.92	4.32	-	
	Stigmasterol	3.00	4.32	3.42	
	β-Sitosterol	3.40	5.30	3.99	
2.	Wheat leaves				
	Peak 1	2.05	2.86	2.40	Cholesterol
	Peak 2	-	3.31	2.89	?
	Peak 3	2.70	3.77	3.14	Campesterol
	Peak 4	3.00	4.14	3.43	Stigmasterol
	Peak 5	3.40	4.77	4.00	β-Sitosterol
	Peak 6*	3.73	5.21	4.47	Stigmast-7-enol?

* Occurred in rust-infected wheat leaves

Table 2

Effect of rust-infection on sterol content in primary leaves of wheat

Plant material	Days after inoculation	Total free sterols, µg/g fresh weight	Sterol composition, µg/g fresh weight*					
			Chole-sterol	Unknown A	Campe-sterol	Stigma-sterol	Sito-sterol	Stigmast-7-enol
Susceptible healthy	3	491	6	358	38	16	73	n.d.#
	6	561	Trace+	420	36	10	75	20
	9	535	Trace	398	39	20	78	n.d.
Susceptible rust-infected	3	570	7	424	42	18	79	n.d.
	6	620	Trace	441	37	23	71	48
	9	468	Trace	290	34	25	71	48
Resistant healthy	3	677	Trace	532	44	22	79	n.d.
	6	886	Trace	703	52	28	103	n.d.
	9	669	Trace	525	42	19	83	n.d.
Resistant rust-infected	3	680	Trace	525	44	25	86	n.d.
	6	821	Trace	628	52	29	91	21
	9	728	Trace	551	42	25	80	30

* 5α-cholestane equivalents; variation between duplicate determinations did not exceed 2%
\# n.d. = not detectable
\+ 0.01 to 0.05 µg

Table 3

Sterol content of healthy and rust-infected susceptible wheat leaves harvested 6 days after inoculation

Plant material	Total free sterols, µg/g fresh weight	Sterol composition, µg/g fresh weight*					
		Chole-sterol	Unknown A	Campe-sterol	Stigma-sterol	Sito-sterol	Stigmast-7-enol
Little Club, healthy	626	Trace+	493	36	20	77	n.d.#
Little Club, infected	637	Trace	405	35	28	73	96
Reliance, healthy	593	Trace	442	38	27	86	n.d.
Reliance, infected	637	Trace	371	33	27	65	141
Red Bobs, healthy	825	Trace	630	55	32	108	n.d.
Red Bobs, infected	882	Trace	565	44	29	103	141
Prelude, healthy	719	1.06	472	76	35	135	n.d.
Prelude, infected	1007	Trace	588	67	42	119	191
Kubanka, healthy	611	Trace	482	40	17	72	n.d.
Kubanka, infected	523	Trace	363	33	16	52	59

* 5α-cholestane equivalents; variation between duplicate determinations did not exceed 2%
\# n.d. = not detectable
\+ 0.01 to 0.05 µg

information on the occurrence of stigmast-7-enol was sought by analyzing healthy and rust-infected leaves of five other varieties. In all cases, infected leaves contained this sterol, but non-infected leaves did not (Table 3). It is not known why stigmast-7-enol was detected in 1 of the 11 samples of healthy leaves analyzed, but it should be pointed out that its occurrence there coincided with maximum sterol content reached in all leaves 13 days after seeding (Table 2).

Total sterol content and content of unknown A differed widely between samples harvested at specified times after inoculation (Table 2). These differences appear smaller if the levels of individual sterols are expressed as percentages of total sterol content as reported by Jennings et al (11) for sterol levels in maize leaves infected with Helminthosporium carbonum (Ullstrup). However, it is felt that such a comparison is of little value when the sterol content differs widely. It was concluded that the changes of sterol levels which were observed in leaves after infection were not related to susceptibility or resistance. The level of stigmast-7-enol in infected leaves appeared to be correlated with the amount of fungal tissue in the host since infected susceptible tissues contain much more fungal material than infected resistant tissues especially at later stages of infection.

Since healthy wheat leaves do not contain stigmast-7-enol it could be assumed, that it is synthesized by the

host after infection with rust. However, it is simpler to assume that it is synthesized by the parasite and that the stigmast-7-enol detected in infected plants is of fungal origin. Nowak et al (14) have demonstrated that stigmast-7-enol is the major sterol in uredospores of both flax rust and wheat stem rust. In contrast Jackson and Frear (10) suggested that flax rust uredospores were unable to synthesize sterols and that stigmast-7-enol was derived from the host.

Sterol fractions from healthy and rust-infected leaves were tested for possible biological activity. These fractions were not phytotoxic or inhibitory to fungal development in the host, nor did they promote rust development in resistant reacting leaves.

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GENERAL DISCUSSION

Changes in the properties of membranous components of plant cells in response to the stresses of their environment might be expected to be reflected in alterations in the nature of those lipids and steroids associated with lipoprotein structures. The responses of plants to physicochemical types of environmental stresses have been reviewed by Levitt (28). Physicochemical forms of stress such as temperature, water, radiation, and chemicals are known to modify the lipid composition of plants. For example, total lipids and in particular, phospholipids, generally increase with decreasing temperature during the phenomenon called hardening. This is also accompanied by an increase in cell permeability which is suggestive of a change in the plasma membrane.

However, there is another form of environmental stress to which plants are normally subject, that being the biotic type. The latter belongs to the field of plant pathology and was not considered by Levitt (28). Very little is known about the effects of fungi on the lipid and sterol composition of plants. Since successful colonization of host plants by obligate parasites, such as the rusts, depends on the integrity of membranes at the host-parasite interface, it was hoped that, by investigating the nature of the lipid and sterol fractions of healthy and rust-infected wheat, some insight into the mechanisms of resistance and susceptibility

might be gained.

In the present investigation, minor increases were observed in the total lipid content of infected hosts in comparison to healthy hosts in both resistant and susceptible reacting plants at 20°C. At 25°C, this trend was reversed. Earlier reports had described the metabolism of phosphate-compounds to be enhanced upon infection by obligate parasites (26,33,36,45). Also infection was not accompanied by qualitative changes in lipid and fatty acid profiles, and no significant changes were observed regarding the constituents of either the non-polar or polar lipid fractions. These findings were in complete agreement with those of earlier works (18,20,25).

In view of the known effects of temperature (28) on the lipid composition of plants, it may be that the minor differences observed in the lipid composition in healthy Chinese Spring wheats grown at the two different temperatures were simply due to a temperature stress effect.

Some changes were observed regarding sterol levels in rust-infected leaves, but these differences were correlated with the amount of fungal mass in the host indicating that fungal constituents accounted for the increase. It was concluded that sterol levels were not related to resistance or susceptibility. Similar findings have been reported for tobacco infected with tobacco mosaic virus (4,42). In contrast, Jennings *et al* (19) reported changes in sterol content and

composition in maize leaves infected with Helminthosporium carbonum. The authors felt that an interaction, such as binding, between the toxin of the pathogen and sterol component of the host cell membranes was not sufficient to explain the observed changes in sterol content and composition. They suggested that the toxin produced by H. carbonum might interact with sterols of the host, changing the host cell permeability and leading to loss of sterols during later stages of the disease. The results of the present investigation do not appear to lend thenselves to this suggestion.

While it was not possible to demonstrate a correlation between resistance of wheat to stem rust and lipid content of the host/parasite complex, negative results do not preclude that a correlation exits. The techniques applied may simply have been inadequate to detect it. It is therefore desirable to restate the problem and to redefine objectives that may be helpful to future work in this field.

The breakdown of host cell membrane structure during the course of host-parasite interaction often leads to increased permeability of host membranes resulting in loss of compartmentation. The stability of these membrane components during the early attempts at establishment by the pathogen within the host may ultimately determine the degree to which susceptibility or resistance are expressed. Successful colonization of the host plant by obligate parasites such as the rusts and powdery mildews may depend upon maintenance

of the integrity of the cellular membranes at the host-parasite interface. By contrast, incompatible host-parasite interactions might be envisioned as resulting in the rapid loss of maintenance of membrane integrity. Cellular necrosis could then occur in such places of interaction and the overall effect would be the expression of the hypersensitive reaction.

One might postulate that following infection there is a disruption of membrane activity, accompanied by alterations in the lipoidal and steroidal components in the lipoprotein membrane complex. The well documented intracellular changes involving increased catabolic processes and eventual sensecence might be promoted by these alterations (5,10,16,46, 58). These changes may be associated with rearrangements or replacements of certain phospholipid, glycolipid, sulfolipid, and/or steroid constituents in the native membrane. In addition, alterations in the fatty acid profiles in these constituents may also act to alter membrane structure or selectivity.

The time after infection during which these suggested changes may occur would also be of major importance in determining their role in the expression of resistance or susceptibility. Two possibilities can be envisioned as occurring. First, the initial interaction between host and parasite might result in the alteration of host membrane integrity such that a cascading series of intracellular reactions

characteristic of events leading to cellular collapse ensues. This would suggest that membrane alterations and/or collapse would be a primary factor in determining the expression of the hypersensitive reaction. By contrast, the second possibility is that membrane collapse is a late expression of intracellular changes which were initiated following the passage of some biologically active product from the pathogen across the host-parasite interface. Substances capable of a high biological specificity, such as ribonucleic acids or peptides, might initiate the characteristic catabolic processes within the host and this in turn might lead to alterations to membrane structure and permeability.

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