2001

The Role of Kernel Epicuticular Wax in Zea Mays Resistance to Aspergillus Flavus and Aflatoxin Production.

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THE ROLE OF KERNEL EPICUTICULAR WAX IN *ZEA MAYS* RESISTANCE TO *ASPERGILLUS FLAVUS* AND AFLATOXIN PRODUCTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and
Crop Physiology

by
You-Keng Goh
B.S., L.S.U., 1995
December, 2001

To
Mom and Dad

ACKNOWLEDGEMENTS

My sincerest gratitude goes to my advisor, Dr. K. E. Damann, who despite my performance in his class, agreed to take me under his wing. His guidance, understanding, care, and most of all, patience allowed me to work at my own pace and made it possible for me to accomplish this work. I would like to thank my former advisor, Dr. J. S. Russin, for giving me an opportunity to pursue my graduate studies here at Louisiana State University and Agricultural and Mechanical College. His guidance, encouragement, and friendship mean so much to me. To my minor professor, Dr. R. A. Laine, I offer my gratitude for his guidance, constructive comments, and interest in my work. Dr. R. L. Brown and Dr. T. E. Cleveland have been most helpful in providing comments and suggestions. Their interest in my work and my well-being and their efforts to encourage me to do my very best are very much appreciated. I thank Dr. M. C. Rush, whose example taught me professionalism in the research community. His passion for teaching and research have inspired me in many different ways. I must thank my entire committee and Dr. W. Todd, the Graduate School Dean's representative, collectively for their confidence in me. Their insistence for me to acquire the knowledge to survive in ever expanding field of science made it possible for me to make the proper attitude and professional adjustments. I am proud to say I have the best team of committee members any graduate student could ever hope for!

I thank Dr. J. P. Snow, the Plant Pathology and Crop Physiology department head, for his help in many different ways. His impartiality in dealing with various issues has been an example for me. Mrs. P. Hives has been an angel in looking over all the graduate students in the department, despite the office duties she has. Her encouragement and help

in making sure all the paper work was done and all the procedures were followed has made life a lot easier. Miss C. Warr and the entire office team have been the "silent" angels in helping me in many different ways.

Special thanks to Dr. C. C. Grimm of USDA-SRRC for his help in GC-MS analysis. My project could not have been completed without his help. I would also like to thank Dr. M. Sasser of MIDI Inc. for his help in analyzing fatty acids from kernel epicuticular wax and providing the cost of my trip to visit his company. I thank the many student helpers who were involved in my work. One that deserves special acknowledgement is Mr. Hoe Hin Ong. His patience in helping me to process literally hundreds of samples and spotting each one of them onto TLC plates made it possible for me to complete my work. I thank my former/present collegues for their help, cooperation, and encouragement. They are Dr. P. Lotrakul, Dr. P. Kuruppu, Dr. S. Stetina, Dr. K. Tubajika, Dr. B.-Z. Guo, Dr. Z.-Y, Chen, Mrs. C. Wientjest-Carter, Mrs. C. Giles, Ms. G.-P. Su, and Mr. K. Stetina. It had been said, "it takes a community to raise a child." Likewise, it took the entire department, university, and research community to make me who I am today. It would be impossible to thank every single one of them.

I would especially like to thank Pastor K. L. Priest and Pastor D. Williams and the entire LSU Chi Alpha Christian Fellowship and Hosanna First Assembly for their prayers and encouragement. To the Malaysian friends I've come to know here at LSU, I express my gratitude for their friendship and provisions for a home away from home.

I express my sincerest gratitude to my mom, dad, brothers and sisters, and the rest back home in Malaysia for the sacrifices they have made to send me here. I could never

have made it this far without their prayers, support, and encouragement. They mean the whole world to me.

I thank the USDA-SRRC for providing the funding for this work. Most importantly, I thank God for His provisions and blessings. All glory and honor belong to Him!

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ABSTRACT

The physical and chemical roles of *Zea mays* (corn) kernel epicuticular wax in conferring resistance to aflatoxin production were examined. There was no evidence of a correlation between the amount of epicuticular wax and aflatoxin production in a kernel development experiment. Aflatoxin levels decreased as kernels developed. Epicuticular wax removal reduced aflatoxin at the earlier stages of development but increased aflatoxin in some genotypes at the later stages, indicating the importance of epicuticular wax during late-stage development and hinting of different defense mechanisms between the early- and late-stage kernels. The amount of epicuticular wax from mature kernels of 23 commercial hybrids and the aflatoxin resistant GT-MAS:gk genotype was compared with aflatoxin production in wax-extracted and non-wax-extracted kernels. Unextracted GT-MAS:gk kernels produced the lowest aflatoxin in a kernel screening assay but ranked fourth in weight of extracted wax per seed. Data from all 24 genotypes in the regression test resulted in an r-square value of 0.042 (positive correlation) and of 0.0325 (negative correlation) between amount of wax per seed and aflatoxin in wax extracted and non-wax-extracted kernels, respectively. Regression analysis for a selected group of 16 genotypes produced r-square values of 0.1528 and 0.5659 for wax-extracted and non-wax-extracted kernels, respectively, indicating a decrease in aflatoxin with increasing extractable wax. Epicuticular wax analysis indicated alkanes of odd numbered chain lengths C_{27}, C_{29}, and C_{31}, predominated in all genotypes at all stages of development. However, sterol analysis indicated quantitative differences between resistant and susceptible genotypes. Epicuticular wax profiles for resistant genotypes showed differences in epicuticular wax for each genotype. Four chemical components were

detectable exclusively in the resistant genotypes. A thin layer chromatography plate bioassay showed antifungal spots in waxes from Pioneer 3154 and T115. Fatty acid analysis of GT-MAS:gk and those of 13 commercial hybrids showed that epicuticular wax of GT-MAS:gk was the most different. This study represents the first report of epicuticular wax accumulation and aflatoxin production during kernel development.

CHAPTER 1. INTRODUCTION

1.1 Aflatoxin and Mycotoxicology

Colonization of corn kernels by *Aspergillus flavus* Link:Fr and *Aspergillus parasiticus* Speare results in aflatoxin contamination, which causes economic losses at all levels in the production, marketing, and utilization of farm animals (Nichols, 1983) and was also shown to cause liver cancer in humans (Goldblatt, 1969). Aflatoxin was first discovered in 1960 as a result of the turkey-X disease in England caused by contaminated Brazilian peanut meal. Subsequent aflatoxin research marked the beginning of modern mycotoxicology as a field of study (Lillehoj, 1987). Aflatoxicosis, a pathological condition in which animals are affected by aflatoxin, causes severe clinical disease and death. Subacute aflatoxicosis causes poor feed conversion, lowered productivity, and decreased resistance to diseases.

1.2 Aflatoxin in Corn and Possible Resistance Mechanisms

Efforts to breed *Zea mays* (corn) hybrids which are resistant to aflatoxin contamination have been ongoing since the early 1970's, when it was first discovered that aflatoxin contamination also was a preharvest problem (Anderson *et al.*, 1975). Some corn hybrids have shown variable resistance (Gardner and Wallin, 1980; Gardner *et al.*, 1987; Guo *et al.*, 1995; King and Scott, 1982), but the corn population GT-MAS:gk showed consistent resistance to aflatoxin contamination (Guo *et al.*, 1994; Guo *et al.*, 1995; Widstrom *et al.*, 1987). Some studies (Brown *et al.*, 1995; Guo *et al.*, 1995; Russin *et al.*, 1997) suggested that this resistance may be due in part to kernel surface wax acting as a physical barrier against fungal infection. After testing 13 commercial hybrids and the corn population GT-MAS:gk, Guo *et al.* (1995) found that non-wounded kernels of GT-

MAS:gk and the four commercial hybrids DK 689, G 4666-21, G 4666-51, and ORO 200W inoculated with *A. flavus* produced lower aflatoxin levels when compared to inoculated wounded kernels. Russin *et al.* (1997) reported that the GT-MAS:gk corn population contained more kernel epicuticular wax than the susceptible Asgrow RX 947, Deltapine G-4666, and Pioneer 3154 hybrids both in weight and as observed by scanning electron microscopy. They further showed that epicuticular wax extracted from GT-MAS:gk reduced *A. flavus* colony radial growth by about 35%. Thus, kernel pericarp level resistance to *A. flavus* may either be physical, i.e. the thickness of wax prevents the fungus from invading the kernels, or chemical, i.e. certain chemical compound(s) in kernel epicuticular wax surpress the growth of the fungus and perhaps aflatoxin accumulation, or both.

Resistance to aflatoxin production in corn is not limited to kernel surface factor(s). Brown *et al.* (1993) reported that living embryo was required to express resistance to aflatoxin production. Other studies reported low levels of aflatoxin in GT-MAS:gk even when kernels were either wounded (Guo *et al.*, 1995) or treated (Guo *et al.*, 1996) to breach kernel cuticular layers. Although non-wounded, endoperm wounded, and embryo wounded kernels of resistant inbred MI82 produced increasing levels of aflatoxin in that order, they were still significantly lower than susceptible corn inbred 33-16 (Brown *et al.*, 1997). The effect of wounding corn kernels on aflatoxin production is, however, not consistant among all genotypes. Wounding the kernels produced significantly higher levels of aflatoxin in inbred CI2 but not in inbreds T115 and M182 or in the GT-MAS:gk population (Brown *et al.*, 1995). Thus, it appears that corn resistance

to aflatoxin production involves multiple mechanisms. In some genotypes, pericarp factor(s) may play a significant role; in others, internal factor(s) may be more important.

Indeed, reports on the existance of antifungal compounds in corn kernels have been published. In susceptible and resistant corn genotypes, both germinating and dry kernels contained antifungal proteins that reacted with zeamatin and ribosome-inactivating protein (RIP) antisera in Western blot analyses (Guo *et al.*, 1997). When protein extracts from dry kernels were examined using polyacrylamide gel electrophoresis, several proteins were reported to exist either exclusively in the resistant corn genotypes GT-MAS:gk and Mp420 or at higher concentrations than in the susceptible genotypes Pioneer 3154 and Deltapine G-4666 (Guo *et. al.*, 1998). Protein extracts from resistant genotypes suppressed hyphal growth of *A. flavus* more than extracts from the susceptible ones. Chen *et al.* (1998) identified a 14-kDa protein from corn associated with *A. flavus* infection and inversely correlated with aflatoxin accumulation in 11 corn genotypes.

A. flavus is known to secrete enzymes with abundant lipase activities when invading oil seed hosts. It was hypothesized that volatilized aldehydes that have antifungal activities are produced via the "lipoxygenase pathway" when an invading fungus secretes extracellular lipases and other enzymes in soybean (Doehlert *et al.*, 1993). Fungal lipases lyse soybean cotyledon cells and hydrolyze triglyceraldehydes to fatty acids, which become the substrate for the pathway and result in the production of hexanal, which is antifungal. Such a reaction could inhibit further fungal growth thus contributing to the increased resistance of soybeans to *A. flavus* when compared to other oil seeds (Gardner *et al.*, 1987). The lipoxygenase pathway in soybeans was suspected to

have protected soybeans against *A. flavus* invasion (Doehlert *et al.*, 1993). This pathway, believed to be triggered by secretion of lipase from *A. flavus*, resulted in the production of hexanal, which in turn inhibited further fungal growth and invasion. Although maize possesses the lipoxygenase pathway, 9-hydroperoxide is produced instead of hexanal (Gardner and Weisleder, 1971).

1.3 Plant Cuticle and Fungal Invasion

Infection of a corn kernel by *A. flavus* can be halted at various levels (Martin, 1964). It may be stopped at the surface of the plant or in the cuticular layer, epidermis, or intracellularly. Martin (1964) listed several aspects of the relationship between host cuticle and the invading pathogen: 1) Cuticle may act as the physical barrier that obstructs invasion of the pathogen or it may contain substances detrimental to the growth of the pathogen. 2) The pathogen may penetrate the cuticle via naturally occurring channels. 3) The pathogen may produce enzymes that soften and degrade the material in its path. 4) Cuticular components of the host may provide nutrients or growth promoters for the growth of the pathogen. Removal of cuticular wax from isolated fruit (*Lycopersicon, Capsicum, Solanum*) and leaf (*Citrus, Olea, Pyrus, Ficus*) cuticles resulted in an increase of permeability of 2,4-dichlorophenoxy acetic acid by several orders of magnitude (Riederer and Schönherr, 1985).

Some fungi are able to penetrate plant cuticles directly (Dickinson, 1960; Wood, 1960). Prior to 1960, there was a strong belief among researchers that fungal invasion was done primarily via mechanical pressure (Baker and Bateman, 1978; Kolattukudy *et al.*, 1981). Purdy and Kolattukudy (1975) demonstrated the ability of several phytopathogenic fungi to produce cutinolytic enzymes to aid fungal invasion of host

tissues. Baker and Bateman (1978) showed degradation of apple cutin by a wide range of phytopathogenic fungi. In *A. flavus* invasion of corn, Guo *et al.* (1996) demonstrated the production of cutinase by *A. flavus* in an *in vitro* plate assay where cutin was the sole carbon source. Both susceptible (Pioneer 3154) and resistant (GT-MAS:gk) corn kernels treated with a fraction of *A. flavus* culture filtrate, having the putative cutinase activity, resulted in levels of aflatoxin comparable to wounded kernels and to kernels treated with bacterial cutinase. In contrast, intact, untreated kernels produced lower levels of aflatoxin.

1.4 Biochemistry of the Plant Cuticle

Cuticular membrane, the extracellular layer of plants, can be defined both chemically and anatomically (Holloway, 1982a). Chemically, cuticular membrane is comprised of two specific groups of lipid substances, namely insoluble polymeric cutins and soluble epicuticular and cuticular waxes. The former makes up the framework of the membrane and are polymers of substituted aliphatic acids predominantly C_{16} and C_{18} in chain length (Holloway, 1982b) whereas epicuticular waxes are deposited on the surface and cuticular waxes are embedded within the cutin matrix (Baker, 1982). Cuticular membrane of plants is neither structurally nor chemically uniform but may vary in the number of layers, thickness, demarcation, and chemical constitution (Holloway, 1982a). These characteristics may vary according to species, part of the plant (Bianchi and Avato. 1984), and the stage of plant development (Baker *et al.*, 1982; Viougeas *et al.*, 1995).

The outermost layer of the plant is composed of epicuticular wax, which may be definite or indefinite in form. In the former, this layer may be comprised of definite semi-crystalline or crystalline structures (Baker, 1982). Wax morphology is genetically

controlled (Bianchi and Marchesi, 1960) but the configuration, size, and distribution of the crystalline waxes can be modified by environmental conditions (Baker, 1982). Embedded (intracuticular) waxes are made up of short-chain fatty acids of C16 and C18 (Baker, 1982). Beneath the epicuticular wax lies the cuticle proper sensu von Mohl (Holloway, 1982a), which has no cellulose or cell wall materials and can be completely dissolved by treatment with alkali (von Mohl, 1847). The cuticle proper is formed by the secretion and subsequent apposition of cutin and cuticular wax onto the outside of the outer epidermal wall (Holloway, 1982a). The cutinized layer, which represents incrustations of epidermal cell wall formed by the "intussusception of cutin and cuticular wax beneath the cuticle proper" (Holloway, 1982a), bridges the cuticle proper and the pectin layer. The interface between the cuticular membrane and the epidermal cell wall is marked by the pectin layer, also known as the pectin lamella (Holloway, 1982a; Martin, 1964).

The major classes of plant epicuticular wax are long chain aliphatics (hydrocarbons, alkyl esters, aldehydes, and primary alcohols), pentacyclic triterpenoids, sterols, and flavonoids (Baker, 1982; Eigenbrode and Espelie, 1995; Kolattukudy, 1980; Kolattukudy, 1996). The range of carbon chains and major components in each class are listed in Table 1.1 (Baker, 1982; Kolattukudy, 1996). Even-carbon-numbered homologues of C_{22}-C_{34} predominate in wax classes primary alcohols, acetates, aldehydes, and fatty acids (Baker, 1982; Eigenbrode and Espelie, 1995; Kolattukudy, 1996); even-carbon-numbered homologues of C_{36}-C_{72} predominate alkyl esters and C_{52}-C_{60} predominate diol esters. Wax classes containing predominantly odd-numbered carbon atoms are hydrocarbons (C_{17}-C_{35}), secondary alcohols (C_{21}-C_{35}), ketones (C_{23}-C_{33}),

Table 1.1 Common chain lengths of major plant epicuticular wax components based on Baker (1982) and Kolattukudy (1996).

Class	Chain length			
	Range		Major	
	Kolattukudy	Baker	Kolattukudy	Baker
Hydrocarbons				
n-Alkanes	C_{21}-C_{35}	C_{17}-C_{35}	C_{27}, C_{29}, C_{31}	C_{29}, C_{31}, C_{33}
Branched				
Iso	C_{21}-C_{35}		C_{27}, C_{29}, C_{31}	
Anteiso	C_{22}-C_{36}		C_{26}, C_{28}, C_{30}, C_{32}	
Primary alcohols	C_{22}-C_{34}	C_{22}-C_{32}	C_{26}, C_{28}	C_{26}, C_{28}, C_{30}
Secondary alcohols	C_{21}-C_{35}	C_{21}-C_{33}	C_{27}, C_{29}, C_{31}	C_{29}, C_{31}
Ketones	C_{21}-C_{35}	C_{23}-C_{33}	C_{27}, C_{29}, C_{31}	C_{29}, C_{31}
β-diketones		C_{29}-C_{33}		C_{31}, C_{33}
Hydroxy β-diketones		C_{29}-C_{33}		C_{31} derivatives
Fatty acids	C_{16}-C_{34}			
Short series		C_{12}-C_{18}		C_{16}, C_{18}
Long series		C_{20}-C_{32}		C_{26}, C_{28}
Aldehydes	C_{23}-C_{35}	C_{22}-C_{32}	C_{26}, C_{28}, C_{30}, C_{32}	C_{26}, C_{28}, C_{30}
Alkyl esters		C_{36}-C_{72}		$C_{16,18}$-OH + $C_{15,17,19,21}$-COOH
Cyclic constituents				
Triterpenoid acids				ursolic acid, oleanolic acid
Triterpenols				β-amyrin, α-amyrin, lupeol
Triterpenoid esters				β-amyrenyl acetate, taraxeryl acetate, lupeyl acetate
Triterpenoid ketones				taraxerone, lupene-3-one

ketols, β-diketones, and their hydroxy- and oxo-derivatives (Baker, 1982; Eigenbrode and Espelie, 1995; Kolattukudy, 1980; Kolattukudy, 1996). Other minor and unusual wax components are unsaturated hydrocarbons, branched-chain hydrocarbons, branched-chain primary alcohols, branched-chain acids, branched-chain esters, hydroxy ketones, α,ω-diols, α,ω-diol esters, γ-diols, oxo-β-diketones, acetates, polyestolides, sterols, steryl esters, uvaol, erythrodiol, dihydroxy triterpenoid acids, flavanoids, and flavanoid glycosides (Baker, 1982).

1.5 Corn Epicuticular Wax

Bianchi *et al.* (1984) examined the epicuticular waxes of corn husks and leaves (15 d after silk extrusion), seedlings, and mature kernels of corn inbred WF9. Leaf epicuticular wax contained 17% alkanes, 9% aldehydes, 14% alcohols, 14% acids, 42% esters, and 4% sterols. Husk epicuticular wax contained 4% alkanes, 3% aldehydes, trace alcohols, 8% acids, 64% esters, and 21% sterols. Kernel epicuticular wax contained 6% alkanes, trace aldehydes, 2% alcohols, 11% acids, 76% esters, and 5% sterols. Seedling epicuticular wax contained 1% alkanes, 20% aldehydes, 63% alcohols, trace acids, 16% esters, and trace sterols.

Although alkanes with C_{22}-C_{35} were identified, odd numbered alkanes of C_{27}-C_{33} were dominant in all the corn organs examined. Aldehydes of C_{23}-C_{34} were found in the leaf, with even numbered C_{26}-C_{32} being the major components. In corn husks, only odd numbered aldehydes of C_{25}-C_{29} were found. Seedlings had aldehydes of C_{28}-C_{32}, with C_{32} constituting 96% of total aldehydes detected. No aldehyde was detected in kernels. Likewise, no alcohol was detected in the corn husk. Alcohols of C_{18}-C_{34} were detected in other organs examined, with even numbered C_{20}-C_{32} chains dominating. Acids of C_{16}-C_{35}

were detected in the organs examined, but even numbered C_{16}-C_{34} were dominant. Esters of C_{38}-C_{60} were detected in all the organs, with even numbered C_{40}-C_{56} being dominant, depending on the organ examined. Campesterol, stigmasterol, and β-sitosterol together constituted 100, 71, and 18% of total sterols in husk, leaf, and kernels, respectively.

In general, the range and dominant species of wax classes in corn are consistent with the generalized observation in plants described in the previous section (see also Table 1) (Avato *et al.*, 1987; 1990; Bianchi *et al.*, 1975; 1977; 1979; 1982;1984; 1985; 1989; Bianchi and Salamini, 1975; Yang *et al.*, 1992; 1993). Guo *et al.* (1995) identified 61 sterols (m.w. 384- 442) in corn seed, pollen, cultured hypocotyl cells, roots, coleoptiles (sheaths), and blades. Avenasterol, campesterol, cholesterol, fucosterol, sitosterol, and stigmasterol were among those found.

1.6 Examples of Antifungal Compounds in Plant Wax

Chemical substances associated with the leaf surface layer of several plants were shown to prevent the germination and growth of fungal spores. Wax from the chrysanthemum leaf surface was shown to contain components that would either stimulate or suppress the germination of *Botrytis cinerea* spores (Blakeman and Atkinson, 1976). Beet (*Beta vulgaris*) leaf surface wax contains components that inhibit the germination of *Botrytis cinerea* (Blakeman and Sztejnberg, 1973). Wax components of *Ginkgo biloba* were shown to inhibit germination and growth of germ tubes of *Monilinia fructicola* and *Stemphylium sarcinaeforme* (Johnston and Sporston, 1965). Hargreaves *et al.* (1982) showed that the surface layer of white lupin (*Lupinus albus* L.) leaves contained two isoflavones, luteone (Harborne *et al.*, 1976) and wighteone, which showed antifungal activities against *Cladosporium herbarum*.

Wax from corn genotype GT-MAS:gk showed antifungal activity against *A. flavus* in plate assays (Russin *et al.*, 1997). Thin-layer chromatography (TLC) showed that this wax contained unique components at Rf 0.30, 0.81, and 0.40 in benzene:chloroform (7:3), chloroform:ethyl acetate (1:1), and methylene chloride, respectively. Gas chromatography (GC) showed two unique peaks at retention times greater than 20 min (Guo *et al.*, 1995). The antifungal activity of this compound, however, was not tested.

1.7 Perspective

Infection of a corn kernel by *A. flavus* can be halted at various levels (Martin, 1964). Corn epicuticular wax appears to provide frontline protection against the invading pathogen. The mechanism of this so-called pericarp resistance may be physical, chemical, or both. Comparing susceptible and resistant corn genotypes for the amounts of epicuticular wax they produce will provide information on pericarp resistance mechanism, particularly, if higher amounts of epicuticular wax provide an effective physical defense barrier. For this to hold true, resistant corn genotypes must consistantly produce higher amounts of epicuticular waxes thn susceptible genotypes. It was previously shown that the epicuticular wax of GT-MAS:gk contained antifungal components (Russin *et al.*, 1997).

Epicuticular wax composition varies with development of plants (Baker *et al.*, 1982; Holloway, 1982a; Viougeas *et al.*, 1995). Therefore, evaluating aflatoxin production in kernels of resistant and susceptible genotypes at different stages of development and determining wax components and their amounts at those stages of kernel development will allow comparisons to see if correlations exist between aflatoxin

production and epicuticular wax components and their amounts. The same can be done to compare GT-MAS:gk with other genotypes. The objective of this research was to examine the physical and chemical roles of kernel epicuticular wax in corn resistance to afltoxin production.

CHAPTER 2. AFLATOXIN PRODUCTION IN RELATION TO EPICUTICULAR WAX ACCUMULATION IN DEVELOPING CORN AND MATURE KERNELS

2.1 Introduction

Since the early 1970's, when it was first discovered that aflatoxin contamination was a preharvest problem, researchers increased efforts to breed corn hybrids resistant to aflatoxin contamination (Anderson *et al.*, 1975). In earlier studies the corn population GT-MAS:gk showed consistent resistance to aflatoxin contamination (Guo *et al.*, 1994; Guo *et al.*, 1995; Widstrom *et al.*, 1987). Some studies (Brown *et al.*, 1995; Guo *et al.*, 1995; Russin *et al.*, 1997) suggested that this resistance may be due in part to kernel surface wax acting as a physical barrier against fungal infection.

Russin *et al.* (1997) reported that the GT-MAS:gk corn population contained more kernel epicuticular wax than susceptible Asgrow RX 947, Deltapine G-4666, and Pioneer 3154 hybrids both in weight and in scanning electron microscopy visualizaton. Thus, kernel pericarp level resistance to *A. flavus* may either be physical, i.e. the thickness of wax prevents the fungus from invading the kernels, or chemical, i.e. certain chemical compound(s) in kernel epicuticular wax surpress the growth of the fungus and perhaps aflatoxin accumulation, or both.

The purpose of this study was to examine epicuticular wax amounts and effects on aflatoxin production in aflatoxin resistant and susceptible corn genotypes during kernel development and to determine if the amount of wax correlated with aflatoxin production in mature kernels of 24 corn genotypes.

2.2 Materials and Methods

2.2.1 Maize Genotypes and Kinetics of Wax Production

Resistant GT-MAS:gk and T115 and susceptible Asgrow RX 938 and Pioneer 3136 were grown at the Ben Hur Research Station, Baton Rouge, LA, and selfed in 1998. Developing corn ears were harvested at 1, 2, 3, 4, 5, 6 weeks (wks) post selfing. Susceptible Deltapine G4666 and Pioneer 3154 were added to the susceptible list in 1999 and corn ears were harvested at 2, 3, 4, 5, 6, 7, 8, 9 wks post selfing. Experiments also explored the relationship between epicuticular wax amounts and aflatoxin production among mature kernels of 23 commercial hybrids (Dekalb hybrids DK 687, DK 679, DK 668, DK 689, Cargill hybrids 6888, 7731 IMI, 8412, 7821 BT, Mycogen hybrid 2888 IMI, Garst hybrids 8300, 8513 IT, 8325, Pioneer hybrids 3223, 33V08, 3395IR, 31B13, 3260, 33K81, 32Z18, 3394, 33R87, 32K61, and Deltapine hybrid G-4666) and corn population GT-MAS:gk.

2.2.2 Wax Extraction

Epicuticular wax was extracted by immersing developing corn ears in 500 mL chloroform for 1 min at room temperature. In the mature kernel analysis, kernels (100 g) were immersed in 200 mL chloroform for 1 min at room temperature. The chloroform wax extract was allowed to evaporate to dryness. Wax was transferred into preweighed 10 mL vials by washing the beakers containing the dried wax 3 times with 2 mL of chloroform, again evaporated, and weighed to determine the wax weight of each sample collected. Wax weights were expressed as grams per ear of corn in the developing ear experiment. In the mature kernel experiment, wax weight was expressed as grams per

kernel, determined by dividing total wax weight by the estimated number of kernels in 100 g.

2.2.3 Fungus Culture

A. flavus (strain AF13) was obtained from P. J. Cotty (SRRC/ARS/USDA, New Orleans, LA). The strain produces large quantities of aflatoxin in culture and in developing cottonseeds and maize kernels. (Brown *et al.*, 1991; Cotty, 1989).

2.2.4 Kernel Screening Assay

Extracted and unextracted developing corn ears were dried at 60°C for 7 d to allow removal of kernels. The kernels were stored at -20°C before being used in a kernel screening assay. Kernels were surface sterilized in 5% NaOCl solution for 1 min and rinsed 3 times with sterile distilled water. Twelve replicates containing 4 kernels per replicate from each wax extracted and unextracted ear at each harvest period were placed in culture dishes (Costar®, 6 well cell culture cluster, Corning Incorporated, Corning, NY 14831) and individually inoculated with 3 μL of *A. flavus* spore suspension (10^6/mL). The kernels were incubated for 7 d in a moist chamber made up of plastic containers lined with paper towels containing 500 mL distilled water. Kernels were dried at 60°C for 72 hours (hrs) to stop fungal growth and aflatoxin production. The official method of the American Oil Chemists Society (Anonymous, 1988) with modifications (Brown *et al.*, 1993 and Guo *et al.*, 1994) was used to assess aflatoxin levels in each sample. Kernels (4) were powdered, weighed, and mixed with 30 mL methylene chloride in 50 mL flasks. The flasks were shaken for 30 min, contents filtered into 50 mL beakers (Whatman® #1, Whatman International Ltd., Maidstone, England), and the filtrates were allowed to evaporate. Beakers were rinsed with methylene chloride, transferred to 8 mL vials and

evaporated to dryness. Residue was dissolved in 2 mL benzene:acetonitrile (98:2). Ten microliters of the extract was spotted onto thin layer chromatography (TLC) plates (Silica Gel 60 F_{254}, 20 X 20, EM Science, 480 Democrat Road, Gibbstown, NJ 08027), and developed in ether:methanol:water (96:3:1). Aflatoxin B1 levels were quantified by comparing them to a commercial aflatoxin B and G mixture standard (Sigma Chemical Co., 3500 Dekalb St., St. Louis, MO 63118) using a scanning densitometer with fluorometry attachment (Model CS-930; Shimadzu Scientific Instruments, Inc., Tokyo, Japan).

2.2.5 Germination Test

Ten corn kernels were lined up in a row on three sheets of germination paper (Anchor regular weight seed germination paper, Anchor, 480 Broadway St., St. Paul, MN 55101) in plastic containers (10 cm x 10 cm x 1 cm). Twenty mL of distilled water was added to the chamber and the chambers were incubated at 25°C for 7 d in the dark. The primary root length was measured at day 7 and expressed as an average of the ten seeds.

2.2.6 Data Analysis

Aflatoxin levels were transformed using log (aflatoxin + 1) before being subjected to Bonferroni analysis at α=0.05.

2.3 Results

2.3.1 Developing Ear Wax Weight

Wax yield per ear of corn in 1998 followed a general trend of increase from weeks 1-6 for Asgrow RX 938, GT-MAS:gk, T115, and Pioneer 3136 (Table 2.1). In 1999, however, wax yield per ear of corn was highly variable over time. Asgrow RX 938, T115, Pioneer 3136 and 3154 showed an increase in wax yield per ear for the first 3

weeks (Table 2.1). Wax yield per ear fluctuated during the later weeks. In GT-MAS:gk, wax yield per ear increased over weeks 2 through 8. Wax yield per ear for Deltapine G4666 increased between weeks 2 and 3, but subsequent yields were lower than the initial yields. In contrast, for all other genotypes, wax yields during the later weeks never fell below the initial week. Interestingly, wax yield per ear at week 9 decreased when compared to week 8 for all genotypes.

2.3.2 Mature Kernel Wax Weight and Aflatoxin

Non-wax-extracted kernels of GT-MAS:gk produced the lowest level of aflatoxin (Table 2.2). Extracting epicuticular wax from GT-MAS:gk, Dekalb hybrids DK 687 and DK 679, Cargill hybrids 8412 and 7821 BT, Pioneer hybrids 3223, 3395IR, 3260, 33K81, 32Z18, 33R87, and 32K61, and Deltapine G4666 resulted in higher levels of aflatoxin production when compared to their non-wax-extracted kernels. Extracting epicuticular wax from GT-MAS:gk kernels rendered it's aflatoxin level not statistically different from many of the other genotypes' wax-extracted kernels. In the wax-extracted kernels, Cargill 6888 produced the lowest amount of aflatoxin, followed by DK 668, Deltapine G4666, DK 689, and so forth. Cargill 6888 and 7731 IMI, Garst 8325, 8513 IT, and 8300, Mycogen 2888 IMI, DK 668 and 689, and Pioneer 33V08, 3394, and 31B13 produced lower levels of aflatoxin when their kernels were wax extracted. Deltapine G4666 had the highest amount of wax per seed, followed by Cargill 8412, Mycogen 2888 IMI, GT-MAS:gk, and so forth. A regression test was done to determine if there was any correlation between the amount of wax per seed and aflatoxin production in wax extracted and untreated kernels. Data from all 24 genotypes in the regression test resulted in an r-square value of 0.042 (positive correlation) and of 0.0325 (negative correlation)

Table 2.1. Aflatoxin production (ppb) in wax-extracted and non-wax-extracted kernels and wax weight (g) per ear of corn in 1998 and 1999. [a] Boxes highlighted in yellow indicate a higher level of actual, non-transformed aflatoxin production. [b] Based on Bonferroni grouping at α=0.05, after log (aflatoxin + 1) transformation.

		1998				1999			
GENOTYPE	WEEK	UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR	UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR
RX938	1				1.07E-03				
RX938	2				1.80E-03				5.02E-03
RX938	3	2061	988		2.02E-03	1917	1196		6.47E-03
RX938	4	838	1484		3.95E-03	1402	183		9.06E-03
RX938	5	1602	23		5.77E-03	960	280		7.30E-03
RX938	6	532	2502		5.72E-03	153	383		9.50E-03
RX938	7					6	593		7.85E-03
RX938	8					89	1663		9.15E-03
RX938	9					5	106		8.80E-03
GT-MAS-gk	1				8.10E-04				
GT-MAS-gk	2				2.06E-03				5.77E-03
GT-MAS-gk	3	2496	3464		1.76E-03	1351	42		6.10E-03
GT-MAS-gk	4	1002	40		3.56E-03	3030	288		7.65E-03
GT-MAS-gk	5	325	804		3.02E-03	1620	225		7.80E-03
GT-MAS-gk	6	921	530		4.05E-03	347	23		8.10E-03
GT-MAS-gk	7					3	20		8.05E-03
GT-MAS-gk	8					36	443		9.70E-03
GT-MAS-gk	9					2	14		7.85E-03

Table 2.1. (continued)

		1998				1999			
GENOTYPE	WEEK	UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR	UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR
T115	1				1.26E-03				
T115	2				1.15E-03				6.15E-03
T115	3	2100	2548		1.62E-03	855	823		9.75E-03
T115	4	1324	1849		2.96E-03	552	1279		9.95E-03
T115	5	743	678		3.90E-03	172	773		9.75E-03
T115	6	311	2955		3.75E-03	3	722		8.95E-03
T115	7					1	119		6.65E-03
T115	8					37	417		7.90E-03
T115	9					276	61		7.55E-03
PIONEER 3136	1				1.33E-03				
PIONEER 3136	2				1.76E-03				5.40E-03
PIONEER 3136	3	2757	355		1.40E-03	50	561		5.43E-03
PIONEER 3136	4	867	73		2.94E-03	93	355		8.30E-03
PIONEER 3136	5	1673	799		3.44E-03	351	306		8.00E-03
PIONEER 3136	6	3	1063		4.67E-03	4	320		8.00E-03
PIONEER 3136	7					8	406		7.40E-03
PIONEER 3136	8					2	580		7.35E-03
PIONEER 3136	9					4	537		6.20E-03

Table 2.1 (continued)

GENOTYPE	WEEK	1998				1999			
		UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR	UNEXTRACTED[a]	EXTRACTED[a]	SIGNIFICANCE[b]	WAX/EAR
D. G4666	1								
D. G4666	2								9.65E-03
D. G4666	3					2067	614		1.14E-02
D. G4666	4					1099	314		8.55E-03
D. G4666	5					245	232		8.35E-03
D. G4666	6					23	24		9.45E-03
D. G4666	7					3	69		8.20E-03
D. G4666	8					16	96		9.35E-03
D. G4666	9					11	50		7.10E-03
PIONEER 3154	1								
PIONEER 3154	2								5.60E-03
PIONEER 3154	3					757	25		6.37E-03
PIONEER 3154	4					1289	8		8.35E-03
PIONEER 3154	5					1296	191		6.90E-03
PIONEER 3154	6					212	7		6.70E-03
PIONEER 3154	7					79	262		7.10E-03
PIONEER 3154	8					185	873		6.25E-03
PIONEER 3154	9					262	738		6.10E-03

Table 2.2. Aflatoxin production in wax-extracted (Ex) and non-wax-extracted (Unex) kernels (100 g), total wax, and wax per seed of the GT-MAS:gk genotype and 23 commercial hybrids. * Numbers are based on means obtained from 12 samples after log (aflatoxin + 1) transformation. Numbers with the same letter are not significantly different, based on Bonferroni (Dunn) test at $\alpha = 0.05$.

GENOTYPE	AFLATOXIN Unex*	AFLATOXIN Ex*	TOTAL WAX (g)	WAX/SEED (g)
DK 687	6.5886 a b	7.6628 a b c d	0.0098	1.62E-05
DK 679	8.6640 a	8.9591 a	0.0126	2.52E-05
DK 668	5.9617 a b	4.0038 f g	0.0088	1.76E-05
DK 689	5.8061 a b	4.4412 e f g	0.0093	1.53E-05
CARGILL 6888	6.1689 a b	2.5798 g	0.0074	1.50E-05
CARGILL 7731 IMI	4.9737 b c	4.7015 d e f g	0.0119	1.90E-05
CARGILL 8412	6.7254 a b	7.9719 a b	0.0102	2.75E-05
CARGILL 7821 BT	6.5218 a b	7.0043 a b c d e f	0.0092	1.61E-05
MYCOGEN 2888	8.2788 a	5.6451 b c d e f	0.0098	2.72E-05
GARST 8300	6.3263 a b	5.4785 b c d e f g	0.0091	2.23E-05
GARST 8513 IT	7.0274 a b	5.1072 b c d e f g	0.0094	2.12E-05
GARST 8325	8.4963 a	5.7576 b c d e f	0.0093	2.51E-05
PIONEER 3223	6.0588 a b	6.6389 a b c d e f	0.0078	1.83E-05
PIONEER 33V08	6.2793 a b	4.4680 e f g	0.0086	2.00E-05
PIONEER 3395IR	6.2714 a b	7.3373 a b c d e	0.0077	2.02E-05
PIONEER 31B13	6.4662 a b	6.4268 a b c d e f	0.0082	2.11E-05
PIONEER 3260	5.6576 a b	7.6122 a b c d	0.0095	2.16E-05
PIONEER 33K81	6.8966 a b	7.4672 a b c d e	0.0086	1.87E-05
PIONEER 32Z18	6.1040 a b	6.5818 a b c d e f	0.0097	2.59E-05
PIONEER 3394	6.3711 a b	6.2969 a b c d e f	0.0088	2.13E-05
PIONEER 33R87	5.7493 a b	6.7357 a b c d e f	0.0102	2.24E-05
PIONEER 32K61	7.5405 a b	7.8369 a b c	0.0083	2.22E-05
DELTAPINE DG4666	2.3132 c d	4.2424 f g	0.0106	3.15E-05
GT-MAS:gk	1.7448 d	4.8528 c d e f g	0.0084	2.65E-05

between amount of wax per seed and aflatoxin in wax extracted and non-wax-extracted kernels, respectively. Using Bonferroni grouping, group "f" in the treated kernels produced the most desirable r-square for untreated kernels. The group consisted of Cargill 7731 IMI and 7821 BT, Deltapine G4666, DK 668, 689, Garst 8300, 8325, and 8513 IT, GT-MAS:gk, Mycogen 2888 IMI, and Pioneer 31B13, 3223, 32Z18, 3394, 33R87, and 33V08. The r-square value for the group was 0.1528 (positive correlation) and 0.5659 (negative correlation) for treated and untreated kernels, respectively.

2.3.3 Aflatoxin Kinetics

No correlation was found between the amount of wax produced in each week and aflatoxin production. Aflatoxin levels were higher during earlier weeks of kernel development in the non-wax-extracted kernels of Asgrow RX 938 (weeks 3 and 5), GT-MAS:gk (week 4), T115 (week 4), and Pioneer 3136 (weeks 3 and 4) in 1998 (Table 2.1 and Figure 2.1). Wax-extracted kernels from the final week had significantly higher aflatoxin levels than non-wax-extracted kernels of Asgrow RX 938, T115, and Pioneer 3136 (Table 2.1 and Figure 2.1). Although non-wax-extracted kernels of GT-MAS:gk produced higher aflatoxin level than wax-extracted kernels at week 6, they were not statistically different. However, wax-extracted kernels at week 5 produced significantly higher level of aflatoxin compared to the non-wax-extracted kernels. Although not always statistically different, aflatoxin levels in the earlier weeks were higher in the non-wax-extracted kernels of Asgrow RX 938 (weeks 3-5), GT-MAS:gk (weeks 3-6), T115 (week 1), Deltapine G4666 (weeks 3-5), and Pioneer 3154 (weeks 3-6) in 1999 (Table 2.1 and Figure 2.2). The only exception to this observation was Pioneer 3136. As in 1998, aflatoxin levels in wax-extracted kernels were higher than non-wax-extracted kernels of

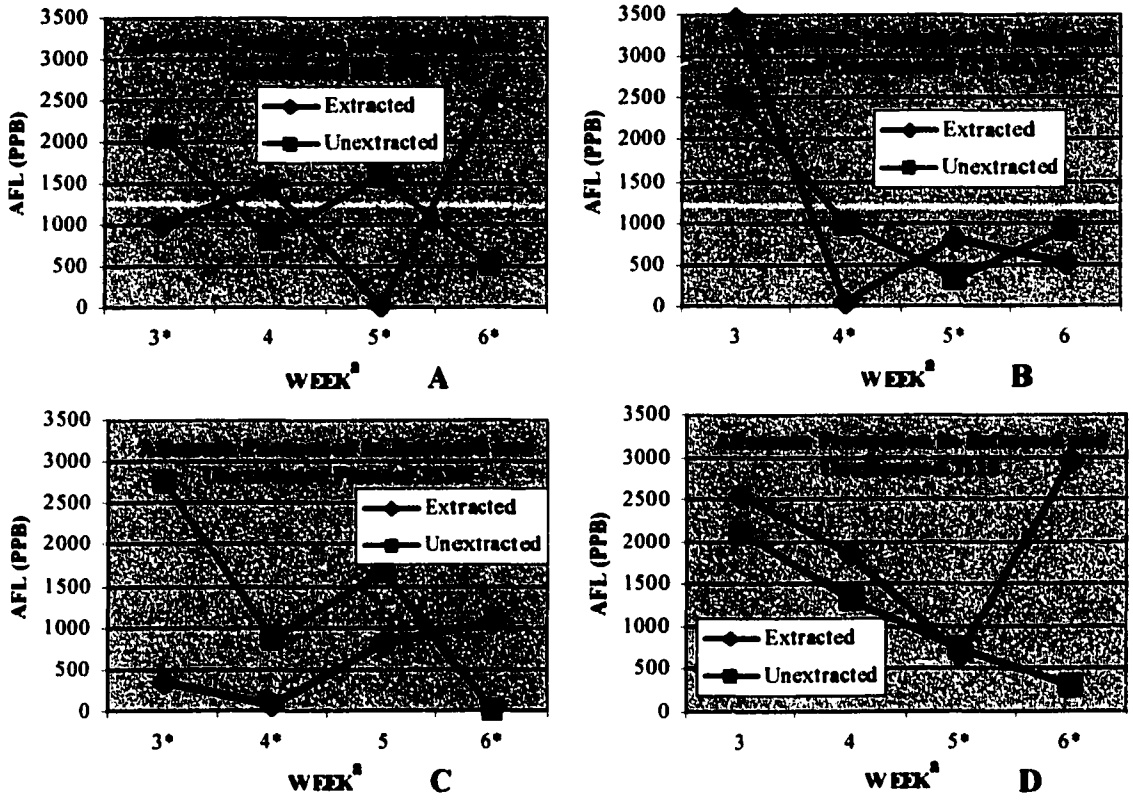


Figure 2.1. Aflatoxin production (weeks 3-6) in wax-extracted and non-wax-extracted kernels of Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), and T115 (D) in 1998. [a] In weeks marked with (*) aflatoxin production in wax-extracted and non-wax-extracted kernels are statistically different based on Bonferroni grouping at $\alpha = 0.05$ after log(aflatoxin + 1) transformation.

Asgrow RX 938 (weeks 6-9), GT-MAS:gk (weeks 7-9), T115 (weeks 4-8), Pioneer 3136 (weeks 6-9), Pioneer 3154 (weeks 7-9), and Deltapine G4666 (weeks 6-9) during the later stages of kernel development (Table 2.1 and Figure 2.2). Although non-wax-extracted kernels produced higher aflatoxin level than wax-extracted kernels at week 9 in T115, they were not statistically significant. When epicuticular wax was extracted from week 8 kernels of Asgrow RX 938, GT-MAS:gk, Pioneer 3136, and Pioneer 3154, the kernels produced the highest levels of aflatoxin. Although not the case with T115 and Deltapine G4666, their wax-extracted week 8 kernels were higher compared to weeks 7 and 9.

2.3.4 Germination Test

Germination tests were conducted to see whether kernels collected in 1999 were germinable. The tests indicated that non-wax-extracted kernels from wks 6-9 of GT-MAS:gk, T115, Deltapine G4666, and Pioneer 3154, and wks 7-9 of RX938 and Pioneer 3136 germinated (Figure 2.3). With the exception of week 9 in T115, aflatoxin levels measured when kernels were germinable were always lower than the non-germinable kernels (Figure 2.3). In the wax-extracted kernels, the only germinable seeds were those from week 9 of Asgrow RX 938, GT-MAS:gk, and Deltapine G4666, and weeks 8 and 9 of T115 (Figure 2.4). Germinable wax-extracted week 9 kernels produced the lowest levels aflatoxin in Asgrow RX 938, GT-MAS:gk, and T115, and the second lowest level in Deltapine G4666 (Figure 2.4).

2.4. Discussion

2.4.1 Wax Yield and Aflatoxin

Wax yield per ear of corn followed a general trend of increase for Asgrow RX 938, GT-MAS:gk, T115, and Pioneer 3136 in 1998 (Table 2.1). Coindentally, 1998 was a severe

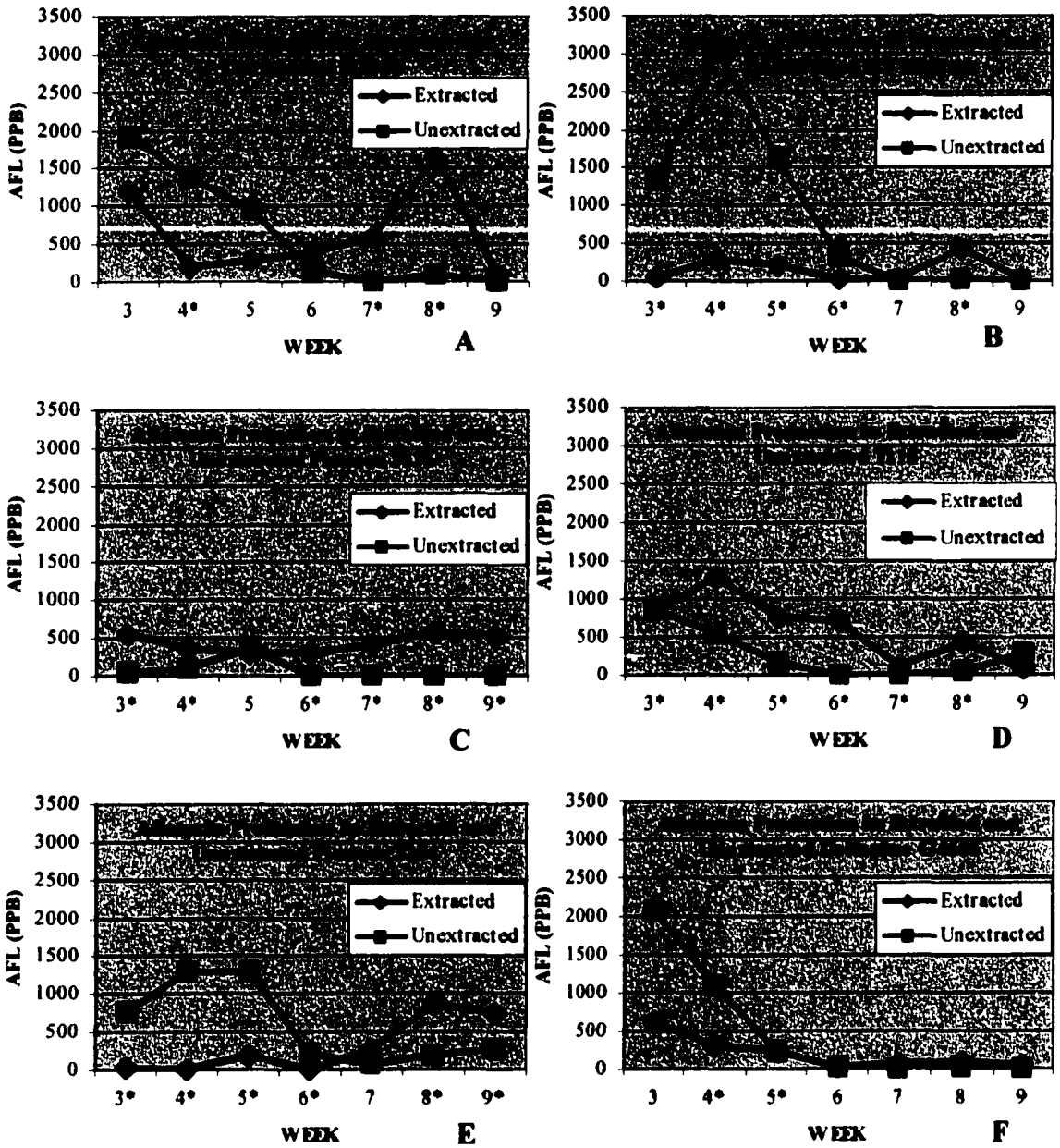


Figure 2.2. Aflatoxin production (weeks 3-9) in wax-extracted and non-wax-extracted kernels of Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), T115 (D), Pioneer 3154 (E), and Deltapine G4666 (F) in 1999.[a] In weeks marked with (*) aflatoxin production in wax-extracted and non-wax-extracted kernels are statistically different based on Bonferroni grouping at $\alpha = 0.05$ after log(aflatoxin + 1) transformation.

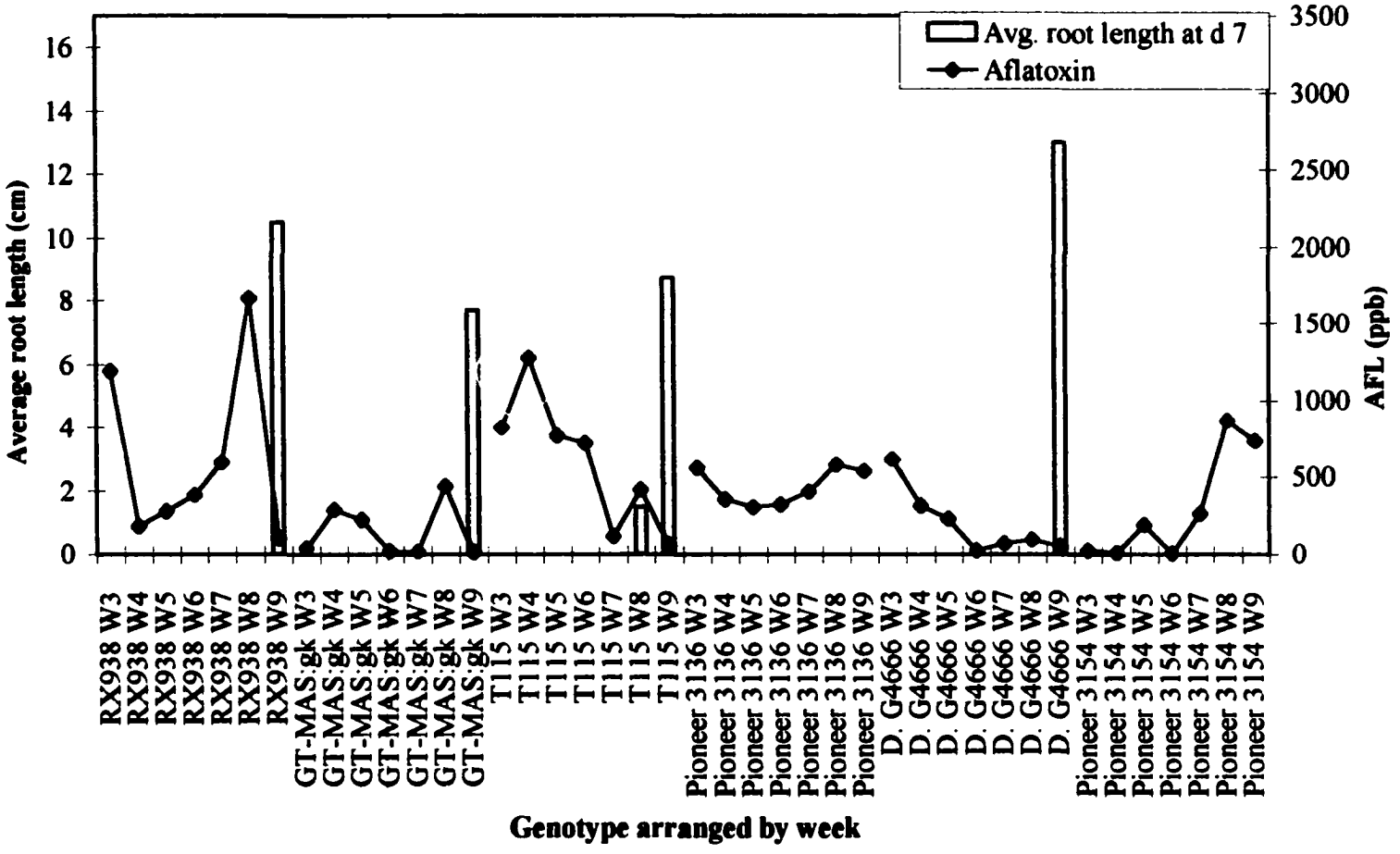


Figure2.3. Average root lengths and aflatoxin levels of wax-extracted kernels of Asgrow RX 938, GT-MAS:gk, T115, Pioneer 3136, Deltapine G4666, and Pioneer 3154 in 1999.

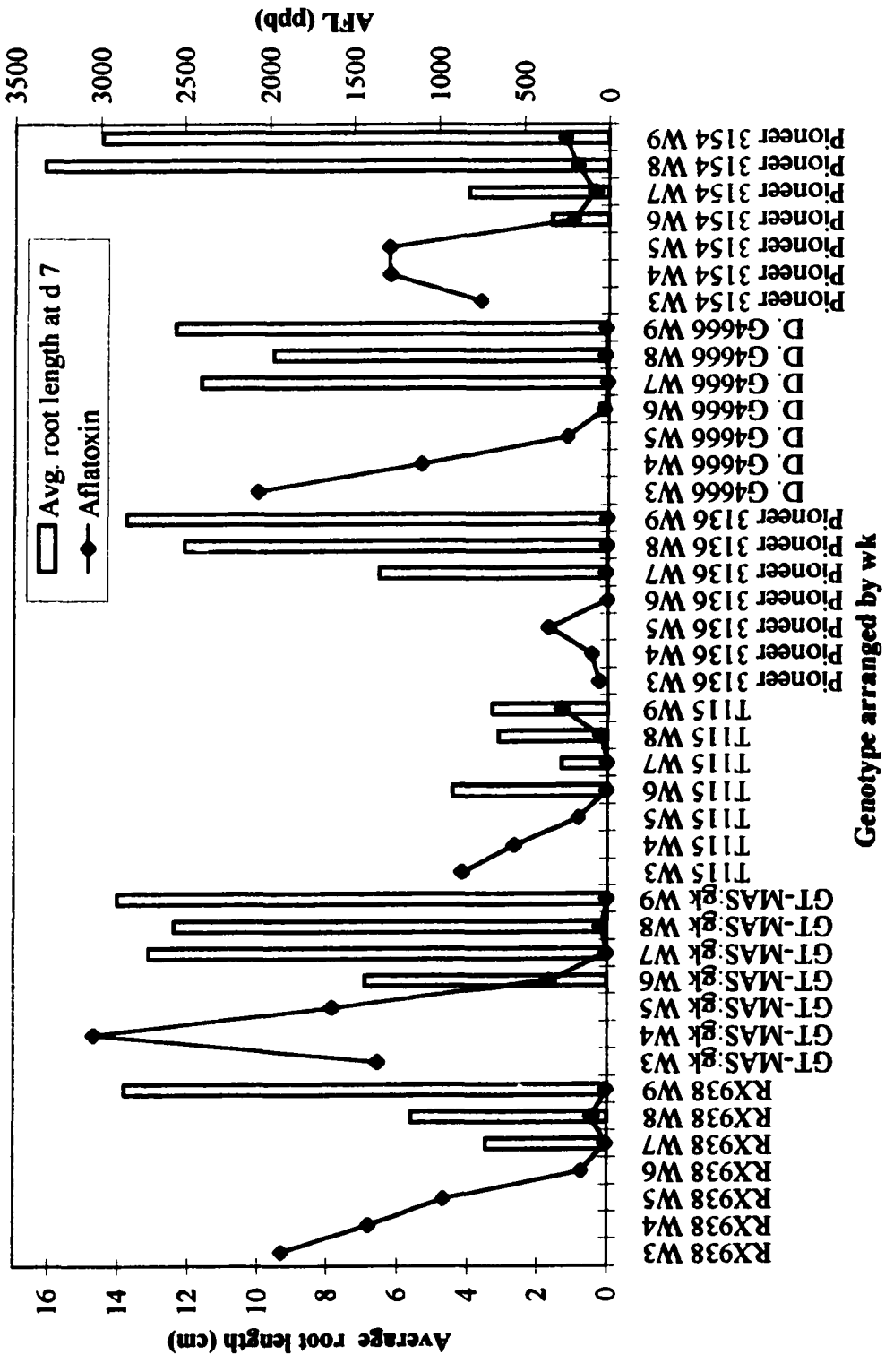


Figure 2.4. Average root lengths and aflatoxin levels of the non-wax-extracted kernels of Asgrow RX 938, GT-MAS:gk, T115, Pioneer 3136, Deltapine G4666, and Pioneer 3154 in 1999.

drought year. Perhaps the increase in wax yield was the result of the extreme drought conditions experienced in 1998. Hydrophobic characteristics of epicuticular wax are thought to play a significant role in water balance of the plant (Lemieux, 1996). Epicuticular wax may confer plant resistance to drought, frost, pathogens, UV radiation, as well as influencing the retention of chemicals applied to plant surfaces (reviewed by Martin and Juniper, 1970). Also, physical environmental conditions affect the amount of wax produced (Walton, 1990). Factors that increase the amount of wax produced include increases in energy flux or decreases in humidity and soil moisture.

Wax yield per ear of corn in 1999 was inconsistent with the exception of GT-MAS:gk, where wax yield per ear increased from weeks 1-8. Regression analysis of wax yield per ear and aflatoxin production did not show any correlation. Because kernels cannot be removed from the cob during the earlier stages of development, whole ears were dipped in chloroform to extract epicuticular wax. Most likely waxes from the cob were extracted along with kernel epicuticular wax, complicating the estimation of kernel epicuticular wax. Thus the estimated amount of wax yield may overestimate the amount of wax on kernel surface.

The mature kernel experiment was done to test if the amount of epicuticular wax provides a physical barrier against pathogen invasion to the 23 commercial hybrids and the GT-MAS:gk corn population. The latter produced the lowest level of aflatoxin when its epicuticular wax was intact. Extracting epicuticular wax from GT-MAS:gk increased its aflatoxin level. Likewise, 12 of the 23 commercial hybrids also increased levels of aflatoxin when kernels were wax-extracted. This shows the importance of epicuticular

wax in providing resistance to aflatoxin production in these genotypes, particularly, in GT-MAS:gk.

There was negative correlation (the higher the amount of wax per seed, the lower the aflatoxin produced, r-square value of 0.5659) between the amount of wax per seed and aflatoxin production in the untreated kernels in the Cargill 7731 IMI and 7821 BT, Deltapine DG4666, DK 668, 689, Garst 8300, 8325, and 8513 IT, GT-MAS:gk, Mycogen 2888 IMI, Pioneer 31B13, 3223, 32Z18, 3394, 33R87, and 33V08 group. Although the r-square value for the wax extracted kernels in this group was much lower (0.1528), it is interesting that the relationship was a positive one (the more epicuticular wax is extracted from the kernels, the higher the level of aflatoxin).

Surprisingly, epicuticular wax removal resulted in lower aflatoxin production in Cargill 6888 and 7731 IMI, Garst 8325, 8513 IT, and 8300, Mycogen 2888 IMI, DK 668 and 689, and Pioneer 33V08, 3394, and 31B13. Likewise, extracting epicuticular wax from kernels at earlier stages of development for all genotypes examined in 1998, and all but Pioneer 3136 in 1999, resulted in lower levels of aflatoxin production. Visual examination of the kernels showed denser *A. flavus* sporulation on the surface of untreated kernels. Perhaps, certain factors exist on the surface of untreated kernels that promote sporulation of *A. flavus*. Calvo *et al.* (1999) reported the promotion of *Aspergillus* spp. sporulation by linoleic acid. GC-MS analyses of the extracted epicuticular waxes did not reveal any detectable level of linoleic acid (data not shown). Other chemical components on the surface may have promoted sporulation or certain internal factors may have been removed or destroyed while extracting the epicuticular

waxes from the young kernels. This shows that young kernels are highly susceptible to aflatoxin contamination.

Extracting epicuticular wax from kernels at later stages of development resulted in higher levels of aflatoxin production at these stages, with the exception of GT-MAS:gk in 1998 and week-9 of T115 in 1999. However, in both cases, aflatoxin levels in the wax-extracted and non-wax-extracted kernels were not statistically different. The remarkable consistency seems to suggest that epicuticular wax may provide kernel resistance to aflatoxin production and/or *A. flavus* at later stages of development. Previous studies support the current observation. When mature kernels (GT-MAS:gk and the four commercial hybrids DK 689, G 4666-21, G 4666-51, and ORO 200W) were wounded and inoculated with *A. flavus,* higher aflatoxin levels were produced than in inoculated, non-wounded kernels (Guo *et al.,* 1995). Russin *et al.* (1997) reported that the GT-MAS:gk corn population contained more kernel epicuticular wax than the susceptible Asgrow RX 947, Deltapine G-4666, and Pioneer 3154 hybrids both in weight and as observed by scanning electron microscopy. The present mature kernel analysis, however, showed that Deltapine G4666 has more epicuticular wax than GT-MAS:gk.

Another interesting observation is that the highest levels of aflatoxin production was in wax-extracted week-8 kernels of Asgrow RX 938, GT-MAS:gk, Pioneer 3136, and Pioneer 3154 in 1999. Although aflatoxin production was not the the highest in T115 and Deltapine G4666, their wax-extracted week-8 kernels produced higher levels of aflatoxin compared to weeks 7 and 9. The unextracted week-8 kernels of all genotypes except for T115 produced much lower levels of aflatoxin.This may indicate that week-8

kernels are most vulnerable to *A. flavus* contamination without epicuticular wax protection.

2.4.2 Germinability and Aflatoxin

Germinable kernels, regardless of whether they were wax-extracted or not, produced among the lowest levels of aflatoxin (Figures 2.3 and 2.4), illustrating the importance of a living embryo in conferring kernel resistance to aflatoxin production. Brown *et al.* (1993) reported that living embryo was required for GT-MAS:gk to express resistance to aflatoxin production. In another study, although non-wounded, endosperm wounded, and embryo wounded kernels of resistant inbred MI82 produced increasing levels of aflatoxin in that order, they were still significantly lower than susceptible corn inbred 33-16 (Brown *et al.*, 1997).

Non-germinable kernels at stages immediately prior to attaining germinability [Asgrow RX 938 (week 6), T115 (week 5), Pioneer 3136 (week 6), and Deltapine G4666 (week 6)] produced low levels of aflatoxin comparable to the germinable stages (Figure 2.4). Aflatoxin production decreased as non-germinable kernels developed from weeks 3-5 in Asgrow RX 938, T115, and Pioneer 3136 (Figure 2.4). Thus, a living embryo is not the only factor conferring resistance to aflatoxin production. Other internal resistance factors may build up during kernel development in corn genotypes Asgrow RX 938, T115, and Deltapine G4666.

Although the low aflatoxin levels in wax-extracted kernels may be attributed to internal factors, such factors alone cannot explain the observation. Non-germinable, wax-extracted, week 6 kernels of Pioneer 3136 produced higher levels of aflatoxin than non-wax-extracted kernels (Figures 2.3 and 2.4). Also, extracting wax from germinable week-

8 kernels of T115 increased aflatoxin. It is unlikely that the internal resistance factor(s) were removed during the process of epicuticular wax extraction judging from the fact that non-germinable wax-extracted kernels of GT-MAS:gk (weeks 6 and 7), T115 (week 7), Deltapine G4666 (weeks 7 and 8), and Pioneer 3154 (week 6) produced equally low amounts of aflatoxin as did the germinable untreated kernels during the same weeks (Figures 2.3 and 2.4). These observations suggest epicuticular wax may contribute to resistance to aflatoxin production during the later stages of kernel development.

Kernel resistance to aflatoxin production is complicated and may involve many factors acting in concert. The present study provided evidence that both epicuticular wax and kernel internal factors may be involved. Removing epicuticular wax from mature kernels resulted in higher levels of aflatoxin production in 11 commercial hybrids and especially in the GT-MAS:gk population. The difference in aflatoxin production in kernels at the earlier and later stages of development suggests that they may have different defense mechanisms. During the later stages of development, epicuticular wax became more important in lowering aflatoxin production. Germination tests provided evidence of the role of internal factors that confer resistance. The fact that non-germinable wax-extracted kernels (week 6 in Pioneer 3136) produced higher levels of aflatoxin than non-wax-extracted kernels and germinable wax-extracted kernels in week 8 of T115 produced higher levels of aflatoxin than germinable non-wax-extracted kernels provided evidence that epicuticular wax may also confer resistance to aflatoxin production. Many questions remain to be answered. In particular, why wax-extracted kernels resulted in lower levels of aflatoxin production in the early stages of kernel development and why extracting epicuticular wax from week-8 kernels resulted in a dramatic increase in aflatoxin production.

CHAPTER 3. COMPOSITION OF EPICUTICULAR WAX IN DEVELOPING AND MATURE CORN KERNELS AND FATTY ACID ANALYSIS OF EPICUTICULAR WAX FROM SELECTED CORN GENOTYPES

3.1 Introduction

The cuticular membrane of plants may vary in the number of layers, thickness, demarcation, and chemical constitution (Holloway, 1982a). The major chemical classes of plant epicuticular wax components are long chain aliphatics (hydrocarbons, alkyl esters, aldehydes, and primary alcohols), pentacyclic triterpenoids, sterols, and flavonoids (Baker, 1982; Eigenbrode and Espelie, 1995; Kolattukudy, 1980; Kolattukudy, 1996). In general, the range and dominant species of wax classes in corn are consistent with those in many other plants (Avato *et al.*, 1987; 1990; Bianchi *et al.*, 1975; 1977; 1979; 1982;1984; 1985; 1989; Bianchi and Salamini, 1975; Yang *et al.*, 1992; 1993).

The objectives of this study were: 1) to examine long chain alkanes and selected sterols from epicuticular wax in corn genotypes during kernel development; 2) to compare epicuticular wax composition of mature, aflatoxin resistant and susceptible corn kernels and to perform bioassays for antifungal activity present in epicuticular wax extracts; 3) to compare fatty acid profiles of the 13 commercial genotypes with GT-MAS:gk using MIDI Sherlock System.

3.2 Materials and Methods

3.2.1 Maize Genotypes and Harvesting Dates

Aflatoxin resistant GT-MAS:gk and T115 and aflatoxin susceptible Asgrow RX 938 and Pioneer 3136 corn genotypes were grown at the Ben Hur Research Station, Baton Rouge and selfed in 1998. Corn ears were harvested at weeks 1-6 after selfing.

Susceptible corn genotypes Deltapine G4666 and Pioneer 3154 were added to the susceptible list in 1999 and corn ears were harvested at weeks 2-9 after selfing. In the mature kernel assay, susceptible corn genotypes Deltapine G-4666 and Pioneer 3154 were compared with resistant corn genotypes GT-MAS:gk and T115. In the fatty acid analysis, 13 commercial corn genotypes, Pioneer 3165, 3136, Deltapine G4666, Dekalb 689, Asgrow RX 789, 770, *770, Mycogen 2677, 2725, 2759, 7250, 7059, and GT-MAS:gk population were used.

3.2.2 Wax Extraction

Epicuticular wax was extracted by immersing corn ears in 500 mL chloroform for 1 min at room temperature. In the mature kernel assay and fatty acid analysis, corn kernels (100 g) were immersed in 200 mL chloroform for 1 min at room temperature. The chloroform wax extract was allowed to evaporate to dryness. Dried wax was transferred into pre-weighed 10 mL vials by washing the beakers containing the dried wax 3 times with 2 mL of chloroform. Contents in the vials were evaporated and weighed to determine the wax yield. The wax was reconstituted with 2 mL chloroform and kept at -4°C before being analyzed using GC-MS or MIS whole fatty acid analysis by gas chromatography.

3.2.3 GC-MS Analysis

The following parameters were used in GC-MS (HP 5970, Agilent, 395 Page Mill Rd., Pala Alto, CA 94303) analysis : Injector was in splitless mode for 1 min at 270°C. Column used was DB-5, 30m x 0.25 mm, with a 1 μm film thickness. Oven was at 100°C for 1 min, then ramped 5°C /min to 150°C, then ramped 10°C /min to 300°C and held for

7 min for a total run time of 33 minutes. The MS was operated in electron ionization mode and scanned from m/z 45 to 650.

3.2.4 TLC Plate Bioassay

Epicuticular waxes from each genotype were spotted onto TLC plates (Silica Gel 60 F_{254}, 20 X 20, EM Science, 480 Democrat Road, Gibbstown, NJ 08027) (Holloway and Challen, 1966). Plates were developed in the following solvent systems: benzene-chloroform (7:3 v/v) (Guo *et al.*, 1995), methylene chloride for the separation of alcohols (Zweig and Sherma, 1972), benzene-ethanol (9:1 v/v) for the separation of anthracene derived hydrocarbons (Zweig and Sherma, 1972), benzene-methanol-acetic acid (45:8:8) for the separation of carboxylic acids, benzene-chloroform (1:1 v/v) for the separation of essential oils, petroleum ether-ethyl acetate (2:1 v/v) for the separation of flavonoids, petroleum ether-diethyl ether-acetic acid (90:10:1 v/v) for the separation of lipids, petroleum ether-diethyl ether-acetic acid (70:30:1 v/v) for the separation of fatty acids, and chloroform:ethyl acetate (1:1 v/v) (Maloney *et al.*, 1988). Spores from 7-d-old culture of *A. flavus* (2 mL, 10^6 spores/mL) were suspended in autoclaved nutrient broth (15mL). The suspension was thinly applied onto the developed TLC plates using a chromatogram sprayer. Germination and growth of the fungus was examined periodically throughout a 7-day incubation in a moist chamber (Woodward and Pearce, 1985).

3.2.5 Fatty Acid Analysis Using MIDI Sherlock System:

Reagent 1 (Saponification reagent/NaOH in aqueous methanol)

NaOH (certified ACS)	45 g
Methanol (HPLC grade)	150 mL
Deionized distilled water	150 mL

Reagent 2 (Methylation reagent/HCl in aqueous methanol)	
6N HCl	325 mL
Methanol (HPLC grade)	275 mL
Reagent 3 (Extraction solvent/hexane in methyl-tert butyl ether)	
Hexane (HPLC grade)	200 mL
Methyl-tert butyl ether	200 mL
Reagent 4 (Base wash/dilute NaOH)	
NaOH (certified ACS)	10.8 g
Deionized distilled water	900 mL

Saponification: One milliliter Reagent 1 was added to vials containing 2 mL corn epicuticular wax in chloroform, vortexed for 10 s, incubated in a 100°C hot water bath for 5 min, vortexed again for 10 s, and incubated in a 100°C hot water bath for 25 min. Methylation: The cooled, saponified wax extract was vortexed for 10 s after the addition of 2.0 mL Reagent 2 and then incubated in an 80°C hot water bath for 10 min. Extraction: Reagent 3 (1.25 mL) was added to the cooled, saponified, and methylated wax extract and placed on a shaker (12 rpm) for 10 min. The bottom phase was removed. Base wash: The remaining top phase was mixed with 3 mL Reagent 4 and placed on a shaker (12 rpm) for 5 min. Two-thirds of the top phase was removed and placed into GC vials for analysis.

3.3 Results

3.3.1 Wax Composition During Kernel Development

The percent areas for the fifty tallest peaks in each GC chromatogram for each wax sample period were generated. In 1998, out of the total peak area, an average of

34.58% represented long chain alkanes (C_{21}-C_{35}), 35.16% represented the 14 sterols examined, and 30.27% represented other unidentified compounds (Table 3.1). In contrast, in 1999, an average of 21.31% represented long chain alkanes, 20.82% represented the 14 sterols examined, and 57.66% represented other unidentified compounds (Table 3.1). Overall, the trend was for long-chain alkanes to decrease and sterols to increase over the developmental period.

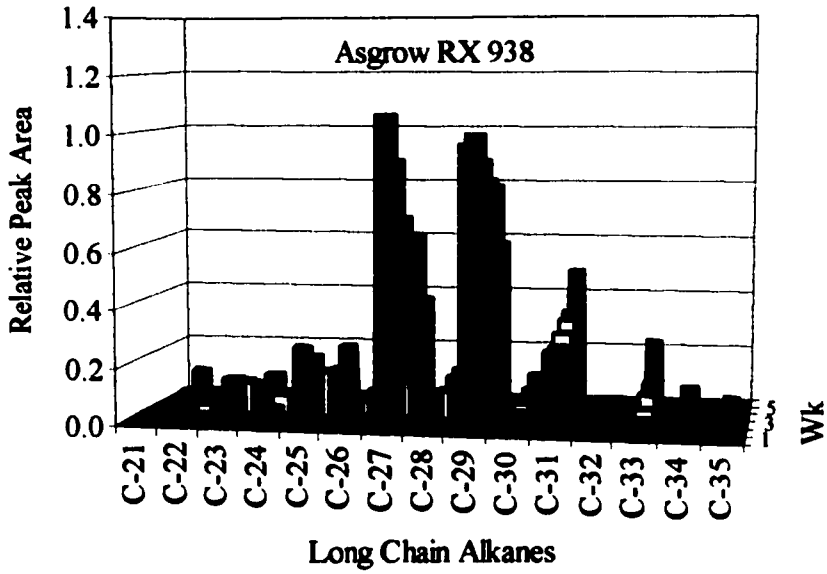
C_{27}, C_{29}, and C_{31} dominated the long-chain-alkane group in all genotypes examined 1998 and 1999 (Figures 3.1 and 3.2). In 1998, C_{27} showed a general trend of decrease during kernel development for Asgrow RX 938, GT-MAS:gk, T115, and Pioneer 3136 (Figure 3.1). C_{29} showed a similar trend, with the exception of Pioneer 3136. C_{31} increased with kernel development for all but Pioneer 3136. All four genotypes showed the same trend for C_{33}. In 1999, C_{27} decreased during kernel development for Asgrow RX 938, GT-MAS:gk, T115, Pioneer 3154, and Deltapine G4666 (Figure 3.2). C_{29} decreased only in GT-MAS:gk and T115. The levels of C_{29} in other genotypes tended to fluctuate, although they remained high. All six genotypes examined in 1999 increased in the levels of C_{31}. Likewise, all but T115 showed the same trend in the levels of C_{33}. The levels of C_{31} and C_{33} during the later stages of kernel development (weeks 8 & 9) in GT-MAS:gk were higher than those of other genotypes (Figure 3.2B). Although the levels of C_{31} and C_{33} also increased in Asgrow RX 938, Pioneer 3136 and 3154, and Deltapine G4666, the levels of increase were not as dramatic as GT-MAS:gk (Figures 3.2 A, C, E, and F, respectively). In contrast, levels of C_{31} and C_{33} in T115 were fairly stable (Figure 3.2D).

Table 3.1. Total long chain alkanes, sterols, and other undetermined compounds based on 50 tallest peak area percentage generated from gas chromatogram.

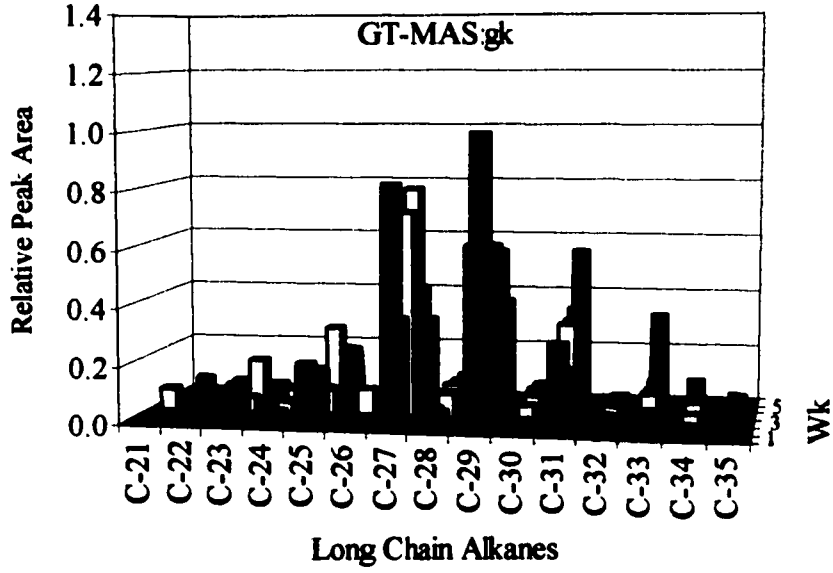
	1998			1999		
Genotype (wk)	Total LCA	Total sterols	Total others	Total LCA	Total sterols	Total others
RX 938 1	41.23	11.30	47.49			
RX 938 2	46.90	22.45	30.68	31.73	6.10	62.15
RX 938 3	44.95	32.96	22.11	33.99	4.40	60.35
RX 938 4	26.44	53.88	19.69	25.42	8.58	64.79
RX 938 5	24.62	47.11	28.24	28.00	27.59	44.01
RX 938 6	14.30	45.19	40.49	17.29	21.54	60.69
RX 938 7				34.86	13.93	50.95
RX 938 8				16.47	28.76	54.77
RX 938 9				17.77	36.02	45.96
GT-MAS:gk 1	42.59	10.90	46.52			
GT-MAS:gk 2	33.02	37.97	29.00	28.18	6.44	64.80
GT-MAS:gk 3	30.24	43.66	26.08	21.36	29.54	48.32
GT-MAS:gk 4	52.60	20.93	26.47	22.36	11.18	65.56
GT-MAS:gk 5	42.94	41.55	15.53	34.91	12.70	52.00
GT-MAS:gk 6	13.27	51.30	35.41	16.90	11.21	71.39
GT-MAS:gk 7				18.67	27.49	53.60
GT-MAS:gk 8				14.77	36.13	49.04
GT-MAS:gk 9				11.39	40.98	47.63
T115 1	45.43	12.49	42.07			
T115 2	33.66	39.29	27.07	41.81	18.54	39.20
T115 3	27.71	43.79	28.50	44.19	10.76	45.08
T115 4	32.06	44.09	23.87	30.28	11.60	58.17
T115 5	35.92	31.36	32.76	31.66	12.25	56.09
T115 6	12.88	43.91	43.23	15.91	17.00	67.10
T115 7				19.49	14.60	65.93
T115 8				29.34	8.49	62.14
T115 9				24.20	10.65	65.14
P 3136 1	54.80	6.48	38.70			
P 3136 2	42.33	34.46	23.23	11.48	22.65	65.92
P 3136 3	44.81	37.12	18.07	14.93	26.87	57.82
P 3136 4	41.05	33.59	25.36	9.54	16.96	73.51
P 3136 5	20.84	49.99	29.20	9.19	28.39	62.44
P 3136 6	25.26	48.01	26.72	6.39	22.04	71.27
P 3136 7				12.31	36.70	50.72
P 3136 8				9.27	32.96	57.52
P 3136 9				10.14	42.68	47.18

Table 3.1 (continued)

Genotype (wk)	1998			1999		
	Total LCA	Total sterols	Total others	Total LCA	Total sterols	Total others
G4666 1						
G4666 2				23.73	6.97	68.84
G4666 3				30.17	19.29	49.75
G4666 4				30.85	11.73	56.71
G4666 5				21.74	21.68	56.18
G4666 6				23.36	24.60	51.58
G4666 7				16.10	30.95	52.51
G4666 8				15.63	42.52	41.80
G4666 9				14.87	52.79	32.09
P 3154 1						
P 3154 2				21.58	10.26	67.79
P 3154 3				26.39	14.16	58.69
P 3154 4				15.63	12.20	72.17
P 3154 5				20.44	17.12	62.43
P 3154 6				14.51	20.41	64.67
P 3154 7				11.50	10.34	77.72
P 3154 8				27.61	20.79	51.20
P 3154 9				14.37	27.74	57.52
Max	54.80	53.88	47.49	44.19	52.79	77.72
Min	12.88	6.48	15.53	6.39	4.40	32.09
Average	34.58	35.16	30.27	21.31	20.82	57.66

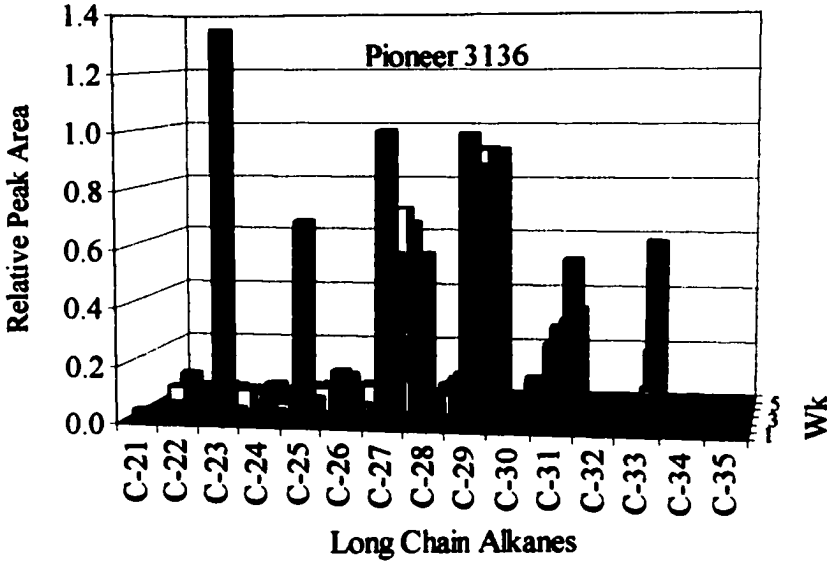


A



B

Figure 3.1. Long chain alkane development for Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), and T115 (D) in 1998. Figures were generated after normalizing target response of each component relative to C_{29}. To illustrate the development of C_{29}, its target response was normalized relative to C_{31}.



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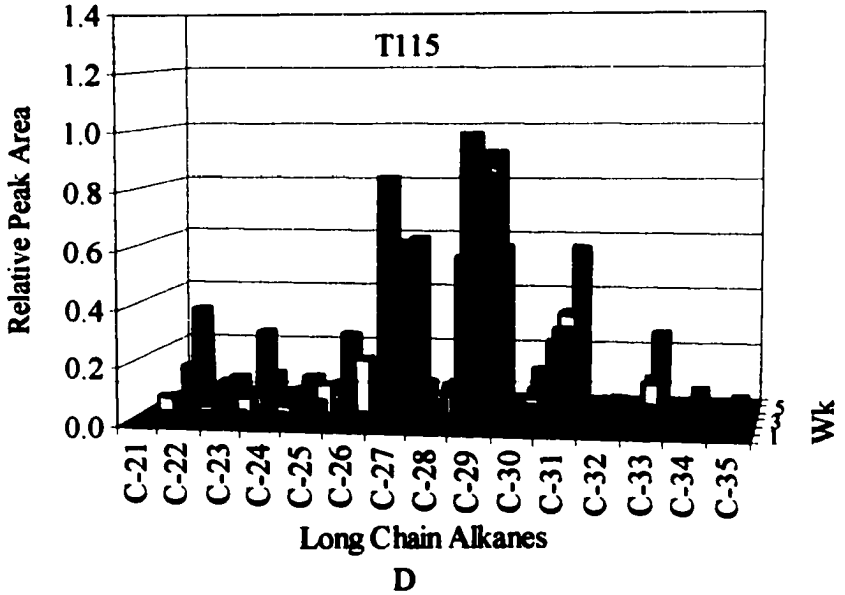


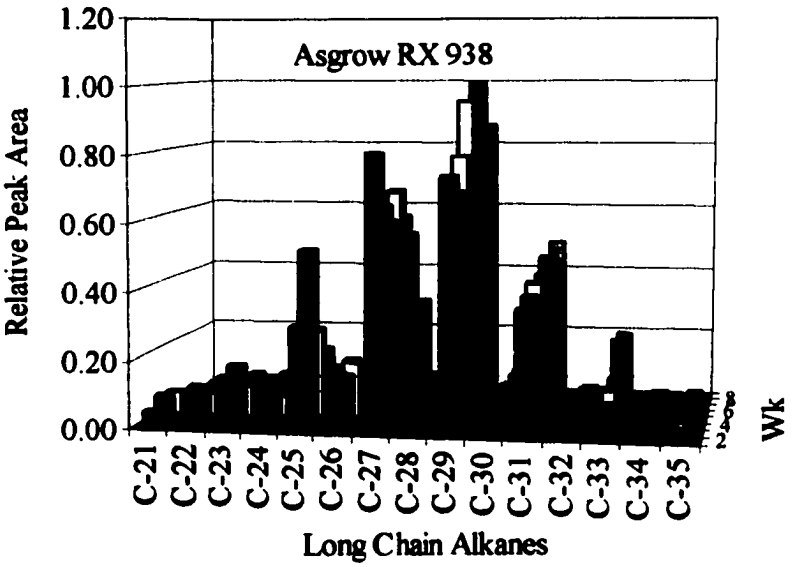
D

Figure 3.1 (continued)

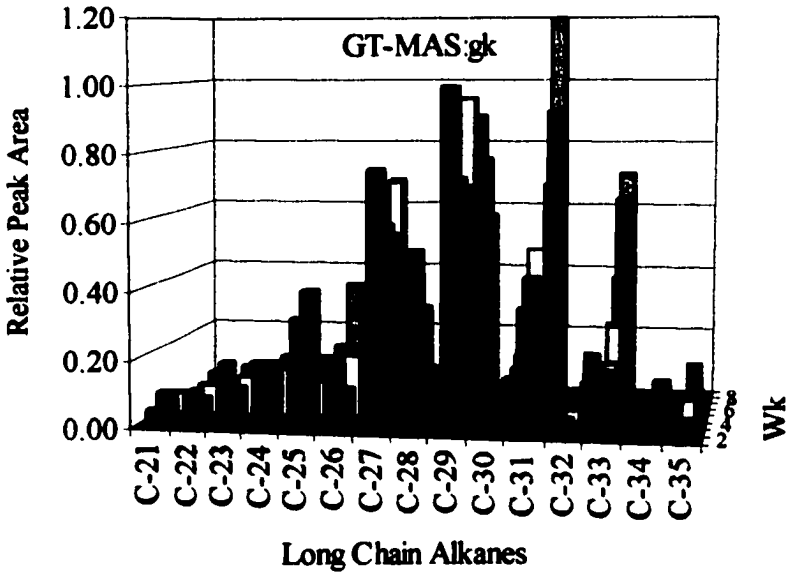
Fourteen sterols (stigmasterol, campesterol, sitosterol, "ergosterol-like sterol," β-tocopherol, cholesterol, vitamin E, "steroid 442-A," "steroid 424-A," β-amyrin, "steroid 424-B," α-amyrin, "steroid 426-A," and stigmast-4-en-3-one) were examined using GC-MS. Sterols that occurred in exceptionally high levels in at least one of the genotypes (3 times the levels of C_{29}) in 1998 were sitosterol (in Asgrow RX 938 and T115) and β-tocopherol (in Asgrow RX 938 and Pioneer 3136) (Figure 3.3). In 1999, only sitosterol occurred in exceptionally high levels (in GT-MAS:gk and Pioneer 3136) whereas the levels of β-tocopherol were among the lowest (Figure 3.4). Interestingly, "steroid 424-A," "steroid 424-B," and "steroid 426-A," increased in notable levels in GT-MAS:gk and T115 in 1999 and but the increase was not as dramatic in 1998. Alpha-amyrin in T115 and stigmast-4-en-3-one in GT-MAS:gk showed the same levels of increase. Tables 3.2 and 3.3 summarize sterol development in the genotypes examined in 1998 and 1999, respectively. Asgrow RX 938, GT-MAS:gk, and T115 increased in stigmasterol, campesterol, sitosterol, in both years.

3.3.2 Mature Kernel Assay

The fifty tallest peaks of each genotype were generated from the GC data. Based on chemical entries detectable only in the genotype concerned, Pioneer 3154 appereared to be the most different compared to other genotypes. Of the 50 tallest peaks, Pioneer 3154 had 12 chemical compounds that were undetectable in other genotypes, whereas GT-MAS:gk, T115, and Deltapine G4666 had 6, 7, and 8, respectively (Table 3.4, cells shaded gray). Four of the chemicals that were detectable in all other genotypes were undetectable in Pioneer 3154; GT-MAS:gk, T115, and Deltapine G4666 had 1, 5, and 1, respectively (Table 3.4, cells shaded yellow). Pioneer 3154, GT-MAS:gk, and T115 each

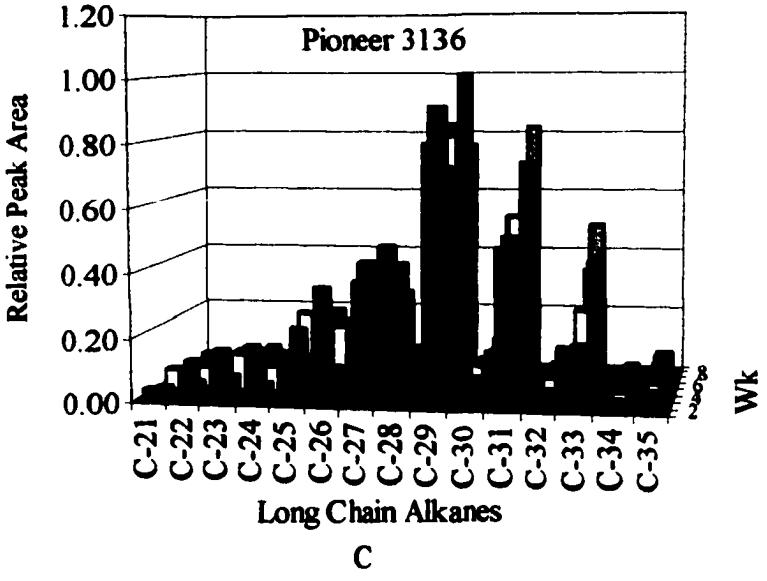


A



B

Figure 3.2. Long chain alkane development for Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), T115 (D), Pioneer 3154 (E), and Deltapine G4666 (F) in 1999. Figures were generated after normalizing target response of each component relative to C_{29}. To illustrate the development of C_{29}, its target response was normalized relative to C_{31}.



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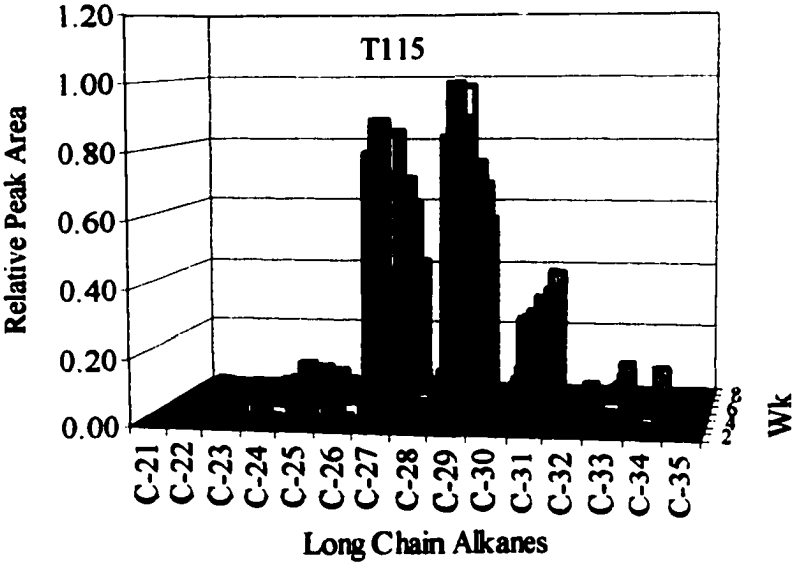
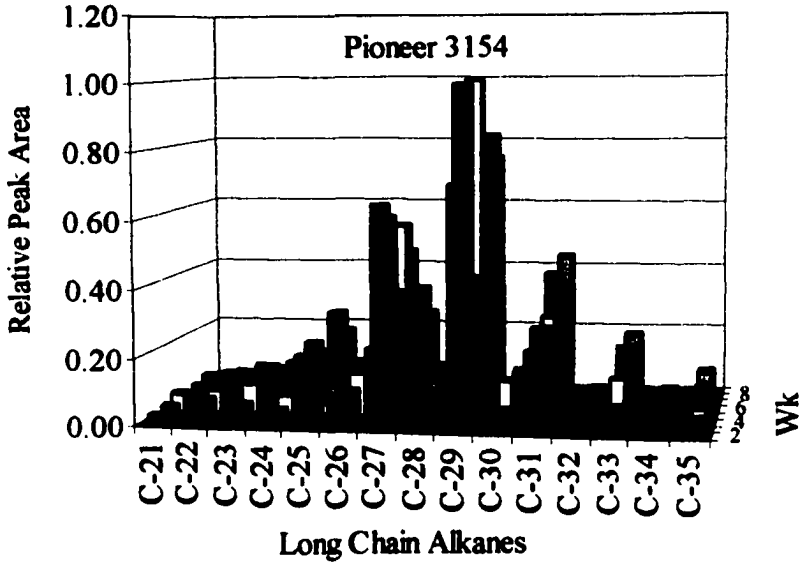


D

Figure 3.2 (continued).



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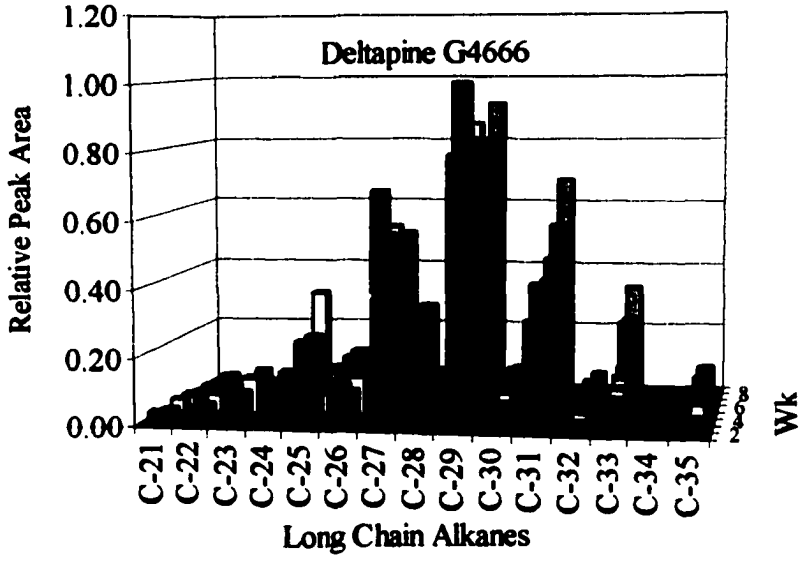


F

Figure 3.2 (continued).

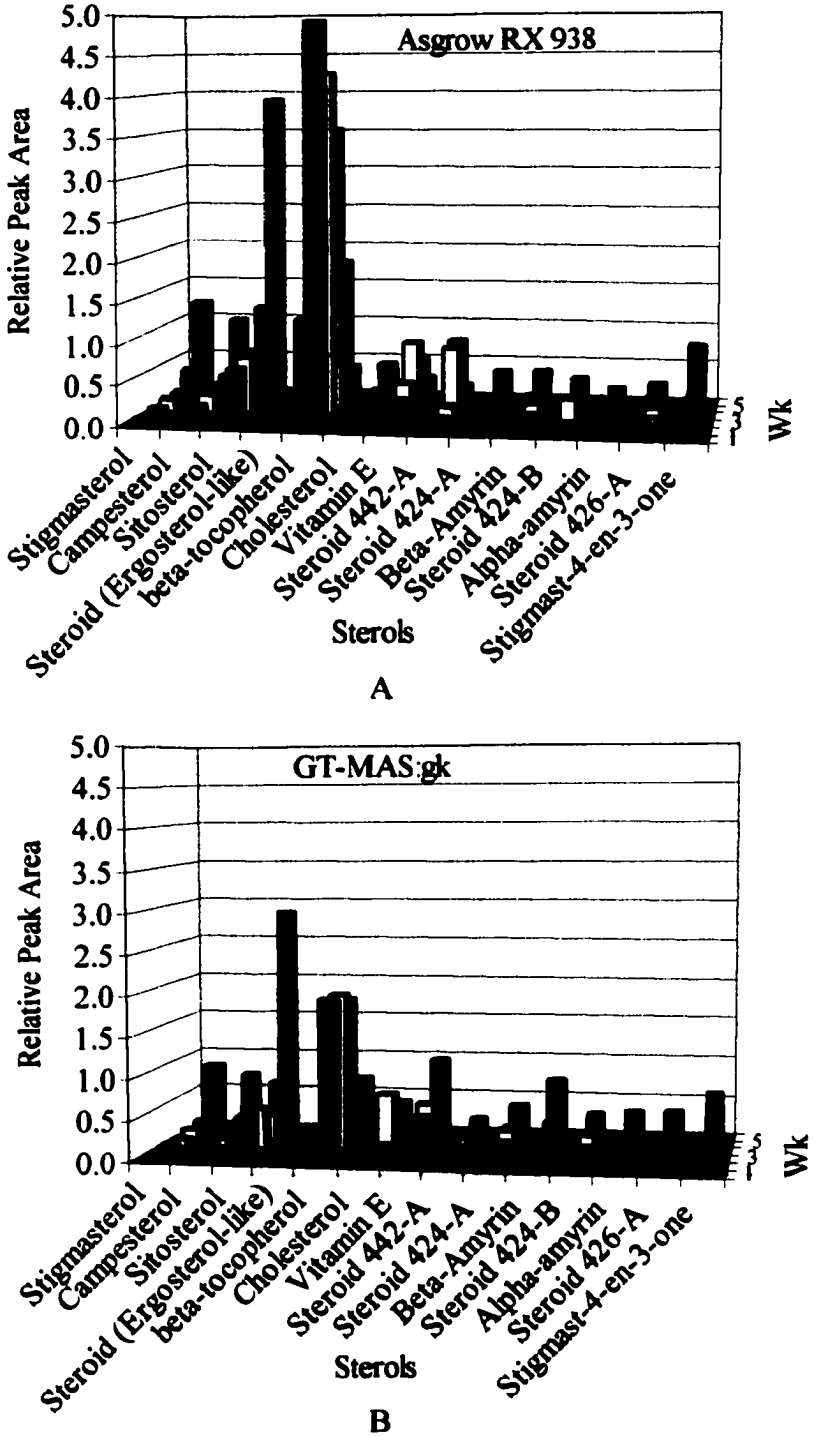
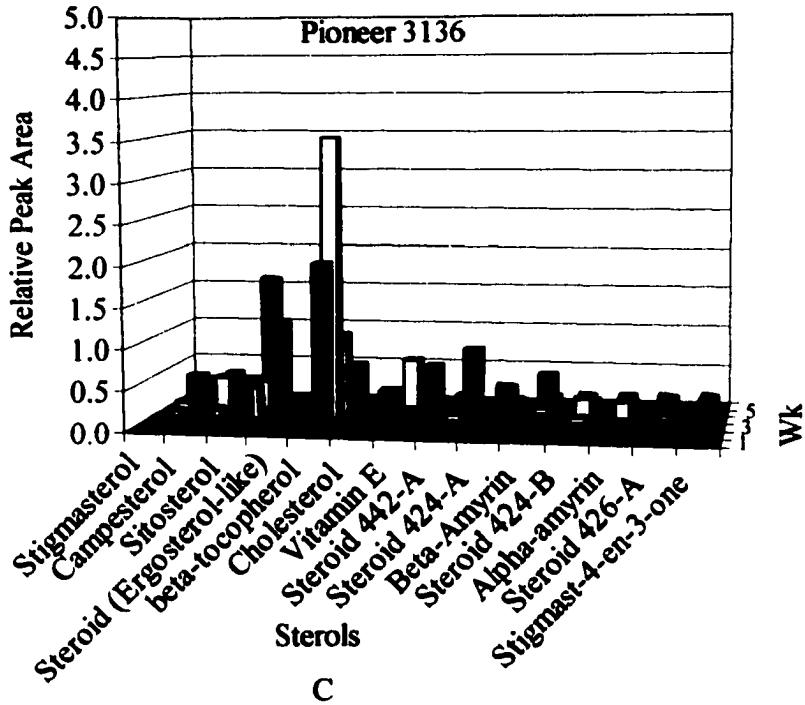


Figure 3.3. Sterol development for Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), and T115 (D) in 1998. Figures were generated after normalizing target response of each component relative to C_{29}.



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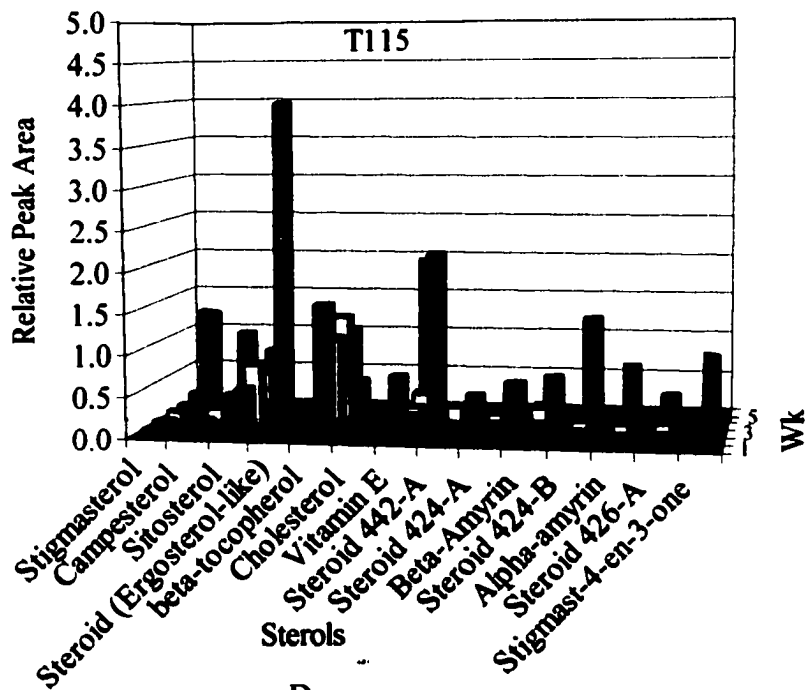
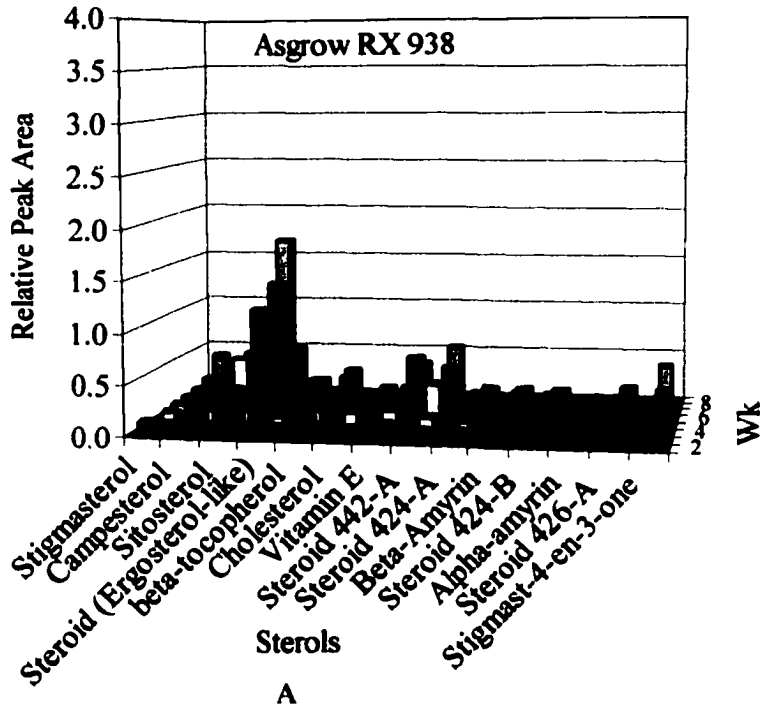


D

Figure 3.3 (continued).



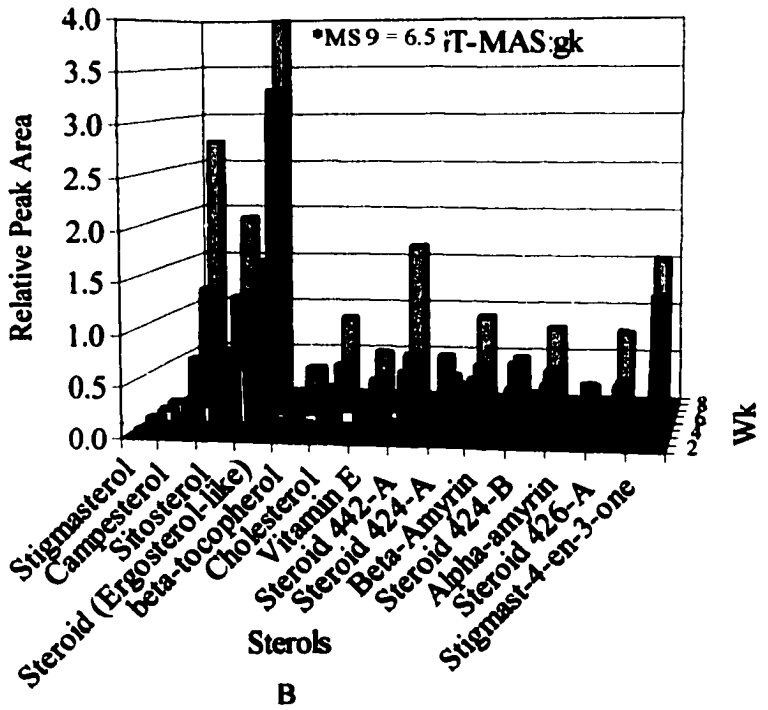
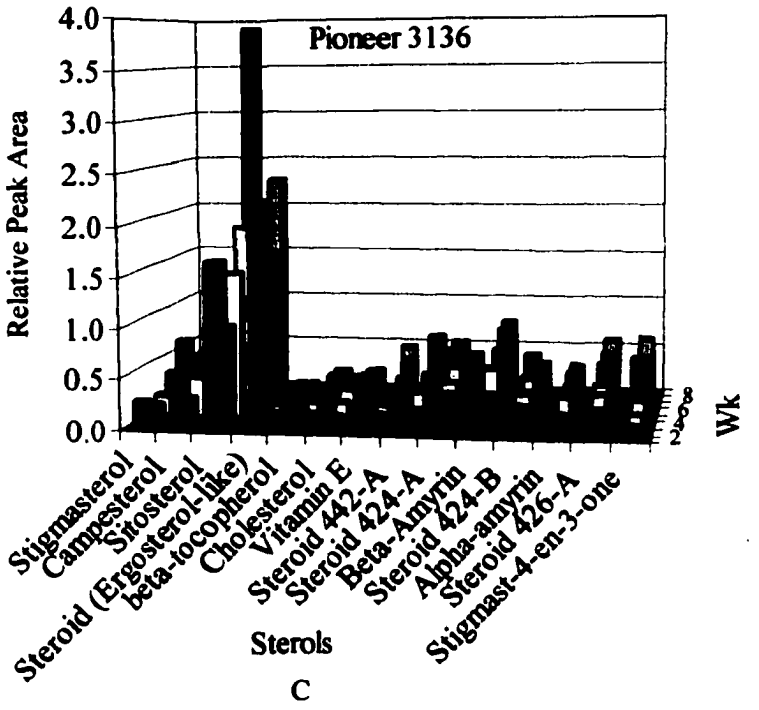


Figure 3.4. Sterol development for Asgrow RX 938 (A), GT-MAS:gk (B), Pioneer 3136 (C), T115 (D), Pioneer 3154 (E), and Deltapine G4666 (F) in 1999. Figures were generated after normalizing target response of each component relative to C_{29}.



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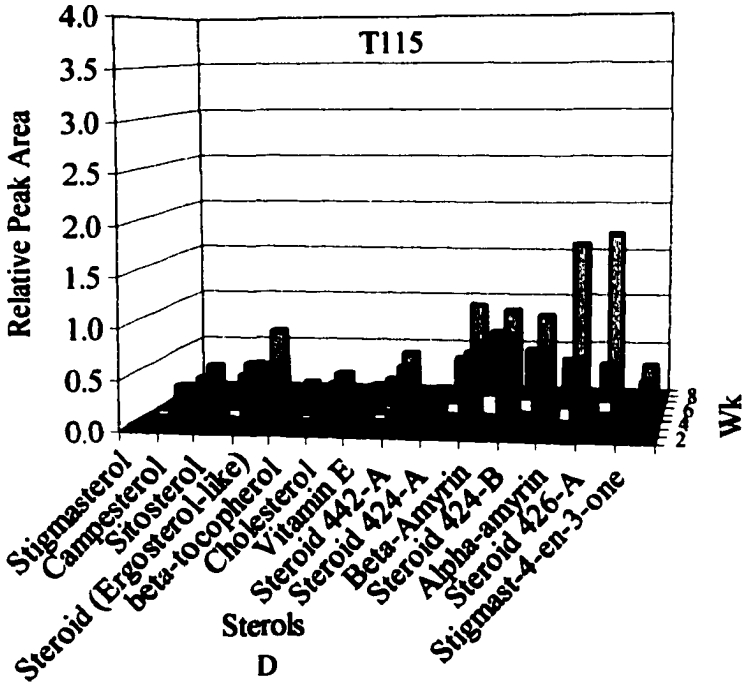

D

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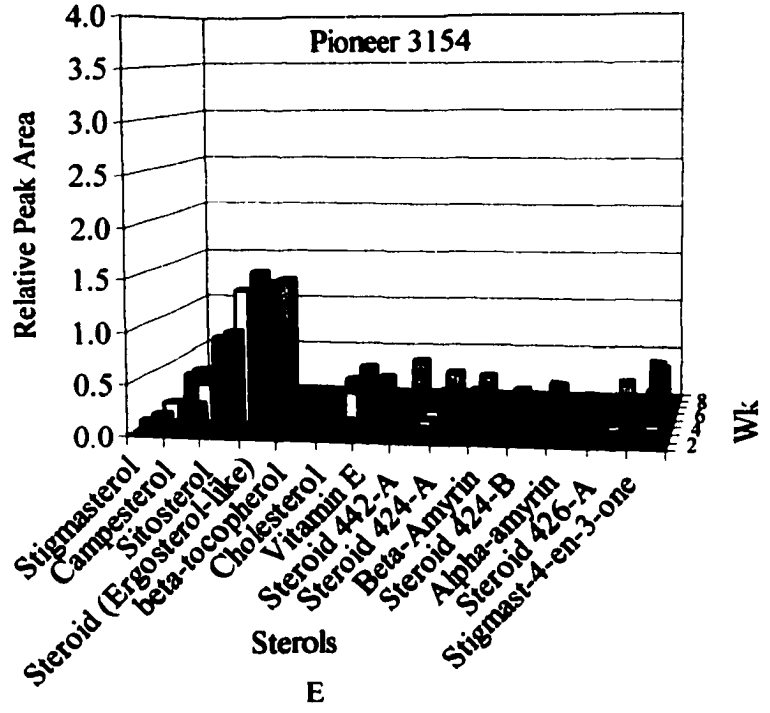

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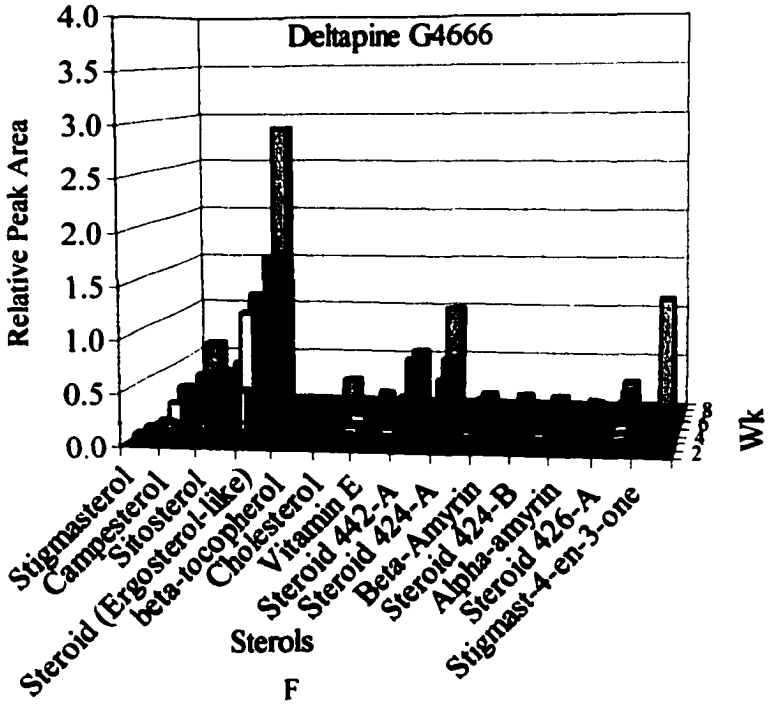

F

Figure 3.4 (continued).

Table 3.2. Sterol developmental trend and peak for RX 938, GT-MAS:gk, T115, and Pioneer 3136 in 1998.

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Stigmasterol	RX 938	General increase	6	
	GT-MAS:gk	General increase	6	
	T115	General increase	6	
	P 3136	General increase weeks 1-5, decrease week 6	5	Lowest levels among all genotypes
Campesterol	RX 938	General increase	6	
	GT-MAS:gk	General increase	6	
	T115	General increase	6	
	P 3136	Higher levels weeks 4-6	4	
Sitosterol	RX 938	General increase	6	
	GT-MAS:gk	General increase except lower levels weeks 2 & 4	6	
	T115	General increase	6	
	P 3136	Higher levels weeks 5 & 6	5	Lowest levels among all genotypes
Steroid (Ergosterol-like)	RX 938	Very low levels throughout		
	GT-MAS:gk	Very low levels throughout		
	T115	Very low levels throughout		
	P 3136	Very low levels throughout		
beta-tocopherol	RX 938	Increase then decrease	2	Week 2 highest levels among all genotypes
	GT-MAS:gk	Increase then decrease	3	
	T115	Increase then decrease	2	
	P 3136	Increase then decrease	3	

Table 3.2. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Cholesterol	RX 938	General increase	6	
	GT-MAS:gk	Higher levels weeks 4 & 6	4	
	T115	Low levels weeks 1-5, peak at week 6	6	
	P 3136	Peak at week 5, low levels all other weeks	5	Lowest levels among all genotypes
Vitamin E	RX 938	Increase then decrease	4	
	GT-MAS:gk	Higher levels weeks 3-6	6	
	T115	General increase	6	Week 6 highest levels among all genotypes
	P 3136	Higher levels weeks 2-6	2	
Steroid 442-A	RX 938	Increase then decrease	5	
	GT-MAS:gk	Higher levels weeks 3-6	3	Lower levels compared to R and 6
	T115	Increase then decrease weeks 1-5, increase week 6	3	
	P 3136	General increase	6	
Steroid 424-A	RX 938	General increase	6	
	GT-MAS:gk	Higher levels weeks 1 and 3-6	6	
	T115	Increase then decrease weeks 1-5, increase week 6	6	
	P 3136	General increase weeks 1-5, decrease week 6	5	Low level week 6
Beta-Amyrin	RX 938	General increase	6	
	GT-MAS:gk	General increase, except week 4	6	
	T115	Increase then decrease weeks 1-5, increase week 6	6	
	P 3136	Higher levels weeks 2-6	5	

Table 3.2. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Steroid 424-B	RX 938	Higher levels weeks 3 & 6	6	
	GT-MAS:gk	Higher levels weeks 3-6	6	
	T115	Increase then decrease weeks 1-5, increase week 6	6	Week 6 highest level among all genotypes
	P 3136	Increase then decrease	4	
Alpha-amyrin	RX 938	Geneal increase	6	
	GT-MAS:gk	Higher levels weeks 3 & 6	6	
	T115	Increase then decrease weeks 1-5, increase week 6	6	Week 6 highest level among all genotypes
	P 3136	Higher levels weeks 4 & 5	4	
Steroid 426-A	RX 938	General increase	6	
	GT-MAS:gk	Higher levels weeks 3-6	6	
	T115	Increase then decrease weeks 1-5, increase week 6	6	
	P 3136	General increase weeks 1-5, decrease week 6	5	
Stigmast-4-en-3-one	RX 938	General increase	6	Abrupt increase at week 6
	GT-MAS:gk	General increase	6	Abrupt increase at week 6
	T115	General increase	6	Abrupt increase at week 6
	P 3136	General increase weeks 1-5, decrease week 6	5	

Table 3.3. Sterol developmental trend and peak for RX 938, GT-MAS:gk, T115, Pioneer 3136, Deltapine G4666, and Pioneer 3154 in 1999.

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Stigmasterol	RX 938	General increase	9	
	GT-MAS:gk	General increase	9	Weeks 8 & 9 higher levels than any other genotype
	T115	Slight increase weeks 4-9	9	
	P 3136	Increase then decrease weeks 2-8, increase week 9	6	
	D G4666	General increase	9	
	P 3154	Roughly stable, slight increase weeks 6, 7, & 9	6	
Campesterol	RX 938	General increase	9	
	GT-MAS:gk	General increase	9	Weeks 8 & 9 higher levels than any other genotype
	T115	General increase	6	Very low levels across all weeks
	P 3136	Roughly stable, slight increase week 6	6	
	D G4666	General increase	9	
	P 3154	Roughly stable, slight increase weeks 4 & 6	6	
Sitosterol	RX 938	General increase	9	
	GT-MAS:gk	General increase	9	Week 9 higher level than any other genotype
	T115	Higher levels weeks 6-9	9	Lower levels than any other genotype
	P 3136	High across all weeks	6	
	D G4666	General increase	9	
	P 3154	Moderately high across all weeks	6	

Table 3.3. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Steroid (Ergosterol-like)	RX 938	General increase except very low levels weeks 3 & 5	9	
	GT-MAS:gk	General increase	9	Week 9 higher level than any other genotype
	T115	Slightly higher levels weeks 6-9	9	Lower levels than any other genotype
	P 3136	Increase weeks 2-6, irregular weeks 7-9	6	
	D G4666	Very low levels throughout	6	
	P 3154	Very low levels throughout	6	
beta-tocopherol	RX 938	Decrease weeks 2-5, increase weeks 6-9	2	Week 2 higher than any other genotype
	GT-MAS:gk	General increase	9	
	T115	General increase	9	Lower levels than R and M
	P 3136	Decrease weeks 2-4, increase weeks 5-9	8	Lower levels than R and M
	D G4666	Stably low weeks 2-5, slightly higher weeks 6-9	9	Lower levels than R and M
	P 3154	Stably low weeks 2-5, slightly higher weeks 7 & 9	8	Lower levels than R and M
Cholesterol	RX 938	Relatively low levels throughout	4	Lower levels than other genotypes
	GT-MAS:gk	Increase weeks 2-4, decrease weeks 5-7, increase weeks 8-9	9	
	T115	Relatively low levels throughout	6	Lower levels than other genotypes
	P 3136	Relatively hihg levels throughout	6	
	D G4666	Increase then decrease weeks 2-8, increase week 9	6	
	P 3154	Irregular	6	

Table 3.3. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Vitamin E	RX 938	General increase weeks 2-8, slight decrease week 9	8	
	GT-MAS:gk	Increase weeks 2-3, drastic decrease week 4, general increase weeks 5-9	9	Week 9 Highest among all genenotypes
	T115	General increase	9	
	P 3136	General increase	9	
	D G4666	General increase	9	
	P 3154	Very low levels weeks 2-5, increase weeks 6-9	9	
Steroid 442-A	RX 938	Irregular, very low levels weeks 2, 3, & 7	9	
	GT-MAS:gk	Very low levels weeks 2-6, higher weeks 7-9	8	
	T115	Very low levels throughout		
	P 3136	Irregular, relatively high thoughout	8	
	D G4666	Irregular weeks 2-4, increase weeks 5-9	9	Week 9 highest among all genotypes
	P 3154	Increase weeks 2-6, drastic drop week 7, increase week 8-9	9	
Steroid 424-A	RX 938	Relatively low levels throughout	7	Lower levels than other genotypes
	GT-MAS:gk	Increase weeks 2-3, decrease weeks 4-7, increase weeks 8-9	9	
	T115	Moderately low weeks 2-5, higher weeks 6-9	9	Week 9 highest among all genotypes
	P 3136	High levels throughout, roughly stable	6	
	D G4666	General increase	9	Lower levels than other genotypes
	P 3154	Moderately low levels throughout	9	

Table 3.3. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Beta-Amyrin	RX 938	Very low levels weeks 2-5, slightly higher weeks 6-9	8	Lower levels than other genotypes
	GT-MAS:gk	Low levels weeks 2-6, increase weeks 7-9	9	
	T115	Moderately low weeks 2-5, high weeks 6-9	9	
	P 3136	Moderately low weeks 2, 3, 4, & 6, high weeks 5, 7, 8 & 9	9	
	D G4666	Very low levels weeks 2-5, slightly higher weeks 6-9	9	Lower levels than other genotypes
	P 3154	Very low levels weeks 2-9, slightly higher week 9	9	Lower levels than other genotypes
Steroid 424-B	RX 938	Relatively low levels throughout	7	
	GT-MAS:gk	Increase weeks 2-3, decrease weeks 4-6 increase weeks 7-9	9	
	T115	Lower levels weeks 2-5, higher weeks 6-9	9	Week 9 highest among all genotypes
	P 3136	Moderately high levels throughout, roughly stable	6	
	D G4666	Very low levels throughout	9	
	P 3154	Relatively low levels throughout	3	
Alpha-amyrin	RX 938	Very low levels throughout	8	
	GT-MAS:gk	Very low levels weeks 2-6, slightly higher weeks 7-9	8	
	T115	Lower levels weeks 2-5, higher weeks 6-9	9	Weeks 6-9 higher than any other genotypes
	P 3136	Moderately high across all weeks	9	
	D G4666	Very low levels throughout	9	
	P 3154	Very low levels throughout		

Table 3.3. (continued)

COMPOUND	GENOTYPE	TREND	PEAK	COMMENT
Steroid 426-A	RX 938	Irregular, moderately low	6	
	GT-MAS:gk	Irregular	9	
	T115	Lower levels weeks 2-4 higher weeks 5-9	9	Week 9 highest level among all genotypes
	P 3136	General increase, moderately high throughout	9	
	D G4666	General increase, low levels	9	
	P 3154	Irregular, low levels throughout	9	
Stigmast-4-en-3-one	RX 938	Decrease weeks 2-4, increase weeks 5-9	9	
	GT-MAS:gk	Low levels weeks 2-5, abrupt increase weeks 6-9	9	Week 9 highest level among all genotypes
	T115	Low levels weeks 2-5, higher weeks 6-9	9	
	P 3136	Low levels weeks 2-4, higher weeks 5-9	9	
	D G4666	Very low levels weeks 2-8, abrupt increase week 9	9	
	P 3154	Low levels weeks 2-5, higher weeks 6-9	7	

had 1 compound that was at least twice the amount of others (chemicals that occurred exclusively in the susceptible or resistant genotypes were not considered) (Table 3.4, entries underlined). GT-MAS:gk and Pioneer 3154 each had 1 compound that occurred at least twice less than those any other genotypes (chemicals that occurred exclusively inthe susceptible or resistant genotypes were not considered), while T115 had 2 (Table 3.4, entries italicized). Four chemicals that were detectable exclusively in the resistant genotypes (GT-MAS:Gk and T115) eluted at the retention times 29.40, 30.18, 30.88, and 31.76.

Antifungal activity occurred at the origin in all solvents used for extracts from all 4 hybrids and R_f 0.75 for TLC plates developed in petroleum ether-ethyl acetate (2:1) (Figure 3.5). The antifungal spots appeared in waxes from Pioneer 3154 (40 μL) and T115 (20 μL). Antifungal activity observed at the origin indicated some antifungal components had not been separated by the mobile phase.

3.3.3 Fatty Acid Analysis

Based on fatty acid composition, epicuticular wax from GT-MAS:gk was the most distinct among the 14 genotypes tested (Figure 3.6). The dendogram (Figure 3.6) was divided into subclusters A, B, C, and diluted and undiluted GT-MAS:gk. Subcluster A consisted of Mycogen 2677, 2725, 2759, and 7059 (diluted and undiluted); subcluster B consisted Deltapine G4666, Asgrow RX 770, 789, 770*, Mycogen 7250, and Pionner 3165 and 3136; subcluster C consisted Mycogen 2674 (diluted and undiluted) and Dekalb 689.

The undiluted GT-MAS:gk epicuticular wax sample was the least similar to the next immidiate cluster, having a Euclidian distance of 26.46 (Figure 3.6). Because its

Table 3.4. Epicuticular wax profiles based on the 50 tallest peaks in GC-MS for GT-MAS:gk, T115, Deltapine G4666, and Pioneer 3154.[a] Entries in bold were confirmed by examining MS data for each peak; other entries were based on MS built-in library search. [b] Entries italicized had peak areas at least twice lower than other genotypes; those underlined had peak areas at least double those of other genotypes. Cells shaded yellow indicate the compound was undetected only in that genotype; cells shaded gray indicate the compound was detected only in that genotype; cells shaded blue indicate the compound was detected either in the two resistant or susceptible genotypes.

COMPOUND	RETENTION TIME	PEAK AREA			
		GT-MAS:gk	T115	D. G4666	P. 3154
Nonanoic acid	6.80	85,028		68,915	38,262
	7.82				
	9.30				
	13.13				
Diethyl phthlate	13.57	826,663	699,906	716,143	798,531
Hexadecane	13.78				
	16.04				
	17.02				
1,2-Benzenedicarboxylic acid, bis(	17.23		101,257	81,223	
	17.68				
	17.90				
Hexadecanoic acid, methyl ester (C	17.95	246,923	337,508	157,480	147,732
1,2-Benzenedicarboxylic acid, dibu	18.29	68,946	295,049	223,097	43,267
Hexadecanoic acid, ethyl ester	18.66	116,741		82,348	66,898
Hexadecanoic Acid	18.95				
Linoleic Acid	19.34				
Heneicosane	19.73	79,746	105,250	78,290	78,405
Octadecanoic acid, methyl ester	19.97	1,840,016	2,495,801	1,215,046	*115,183*
Heptadecanoic acid, 15-methyl-, et	20.59	199,721	167,002	183,966	138,671
Docosane	20.67				
Octadecanoic acid, isopropylester	20.85	120,478	192,207	86,344	
	21.44				
Tricosane	21.54		86,477	118,360	146,106
	21.61				
Epifriedelinol	21.71				
9-tert-butyl-7-oxo-6,10-diazatricy	21.77	192,802	193,497	169,540	289,942
14-.Beta.-H-Pregna	21.83		134,165		41,927
9,17-Octadecadienal	21.97	114,989	156,657	77,208	100,999
	22.07				
	22.27				
Tetracosane	22.38	77,308	192,344	287,016	77,966
Tetracontane-1,40-diol	22.63	203,687	112,274	156,886	74,930
9,12-Octadecadienoic acid	22.86	156,129	102,100	170,931	153,773

Table 3.4. (continued)

COMPOUND	RETENTION TIME	PEAK AREA			
		GT-MAS:gk	T115	D. G4666	P. 3154
Pentacosane	23.18	126,518	462,701	570,282	120,475
Di-n-octyl phthalate	23.43	761,640	404,537	488,224	932,782
	23.54				[illegible]
	23.87	166,146	119,306	244,145	281,942
Hexacosane	23.95	89,124	468,358	700,313	56,322
Oxirane, hexadecyl-	24.23	141,887		313,909	114,410
Heptacosane	24.70	195,936	2,719,938	1,059,100	369,178
	24.83	107,562	118,925	217,425	188,838
	24.93				[illegible]
	25.04	90,974		117,837	
	25.18				[illegible]
	25.39	96,836			86,776
Octacosane	25.42	100,407	730,840	773,828	141,091
2,6,10,14,18,22-Tetracosahexaene,	25.53	93,020	127,040	97,134	332,183
9,12-Octadecadienoic acid	25.70			[illegible]	
	25.73	112,902	152,346		226,063
Nonacosane	26.12	*601,786*	3,884,452	2,028,865	1,550,626
	26.48		[illegible]		
2,4-Cyclohexadien-1-one, 3,5-bis(1	26.60	67,463	100,511	143,490	127,604
Propanedioic acid, propyl-, dimeth	26.65	310,767	251,697	147,512	
Triacontane	26.86	157,752	436,232	469,854	154,741
	27.22			[illegible]	
beta-tocopherol	27.37		110,260	86,006	
1-Nonadecene	27.53		[illegible]		
	27.60	95,719	199,992	204,879	129,016
Hentriacontane	27.72	523,756	1,278,311	982,972	655,791
Vitamin E	28.13		353,700		57,648
	28.17	[illegible]			
	28.42				[illegible]
	28.56	439,983	364,567	229,606	
Dotriacontane	28.70		129,825	180,900	
	28.80			[illegible]	

Table 3.4. (continued)

COMPOUND	RETENTION TIME	PEAK AREA			
		GT-MAS:gk	T115	D. G4666	P. 3154
Campesterol	29.23	68,861		248,389	113,751
	29.40				
Stigmasterol	29.53	598,918	999,508	340,170	
	29.77				
Tritriacontane	29.86	1,326,179	368,493	728,577	617,082
Steroid 442-A	30.18				
(23S)-ethylcholest-5-en-3.beta.-ol	30.45	1,443,296	3,396,499	*256,017*	587,952
Stigmast-5-en-3-ol	30.55				
	30.62				
	30.77				
Beta-Amyrin	30.88				
Steroid 424-B	31.09	1,097,674	4,993,654	*214,685*	435,456
Alpha-amyrin	31.64	937,438	1,761,528	158,553	177,591
Steroid 426-A	31.76				
1,4-bis(diethylamino)-2,3-dimethyl	31.93				
Stigmast-4-en-3-one	32.07	5,561,807		1,311,734	723,788
	32.42				
Pentatriacontane	32.73				

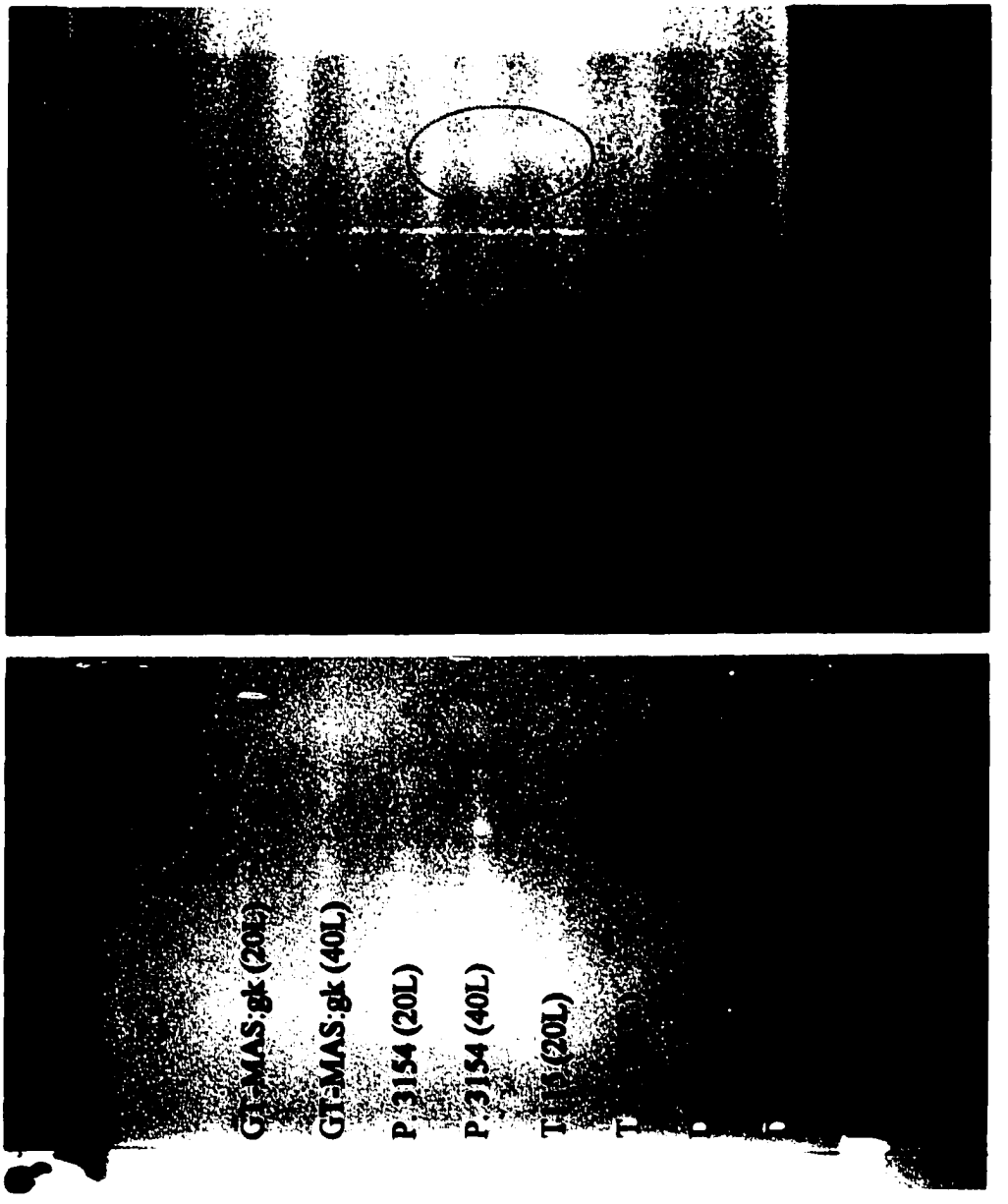

Figure 3.5. TLC plate bioassay showing antifungal spot. A, Experiment 1; B, Experiment 2. Spotted TLC plates were developed in petroleum ether-ethyl acetate (2:1) solvent system. Spores from 7-d-old culture of *A. flavus* (2 mL, 10^6 spores/mL) was suspended in autoclaved nutrient agar (15mL). Using a chromatogram sprayer, the suspension was thinly applied onto the developed TLC plates.

undiluted sample had peaks that were beyond the measurable threshold, the sample was diluted. The diluted sample had a Euclidian distance of over 16.54 compared to the combined B and C clusters. Cluster A had a Euclidian distance of over 19.84 compared to the combined diluted GT-MAS:gk, cluster B, and cluster C. Cluster B and C had the least Euclidian distance, measuring at about 13.23.

3.4 Discussion

3.4.1 Kernel Development Assay

There were differences in chemical composition of epicuticular wax between 1998 and 1999. The 50 tallest GC peaks in 1998 averaged 34.58% long chain alkanes, 35.16% sterols examined, and 30.27% other unidentified compounds (Table 3.1). In contrast, 21.31% represented long chain alkanes, 20.82% represented the 14 sterols examined, and 57.66% represented unidentified compounds in 1999 (Table 3.1). The only difference between the years was the severe drought conditions in 1998. This data illustrates the role of environmental factors in wax composition as well as the amount of wax produced.

Bianchi *et al.* (1984) examined epicuticular wax of mature kernels of corn inbred WF9. It contained 6% alkanes and 5% sterols. The 50 tallest GC peaks in this study showed in epicuticular waxes extracted from the last week, alkanes represented a range of 12.88- to 25.26% in 1998 and sterols a range of 43.91- to 51.30%. In 1999, alkanes and sterols represented a range of 10.14- to 24.20% and 10.65- to 52.79%, respectively. The difference could be due to the different genotype used, environmental conditions, and/or the method of analysis. Bianchi *et al.* first separated different chemical groups using TLC before analyzing them by GC. This study analyzed total wax directly using GC-MS.

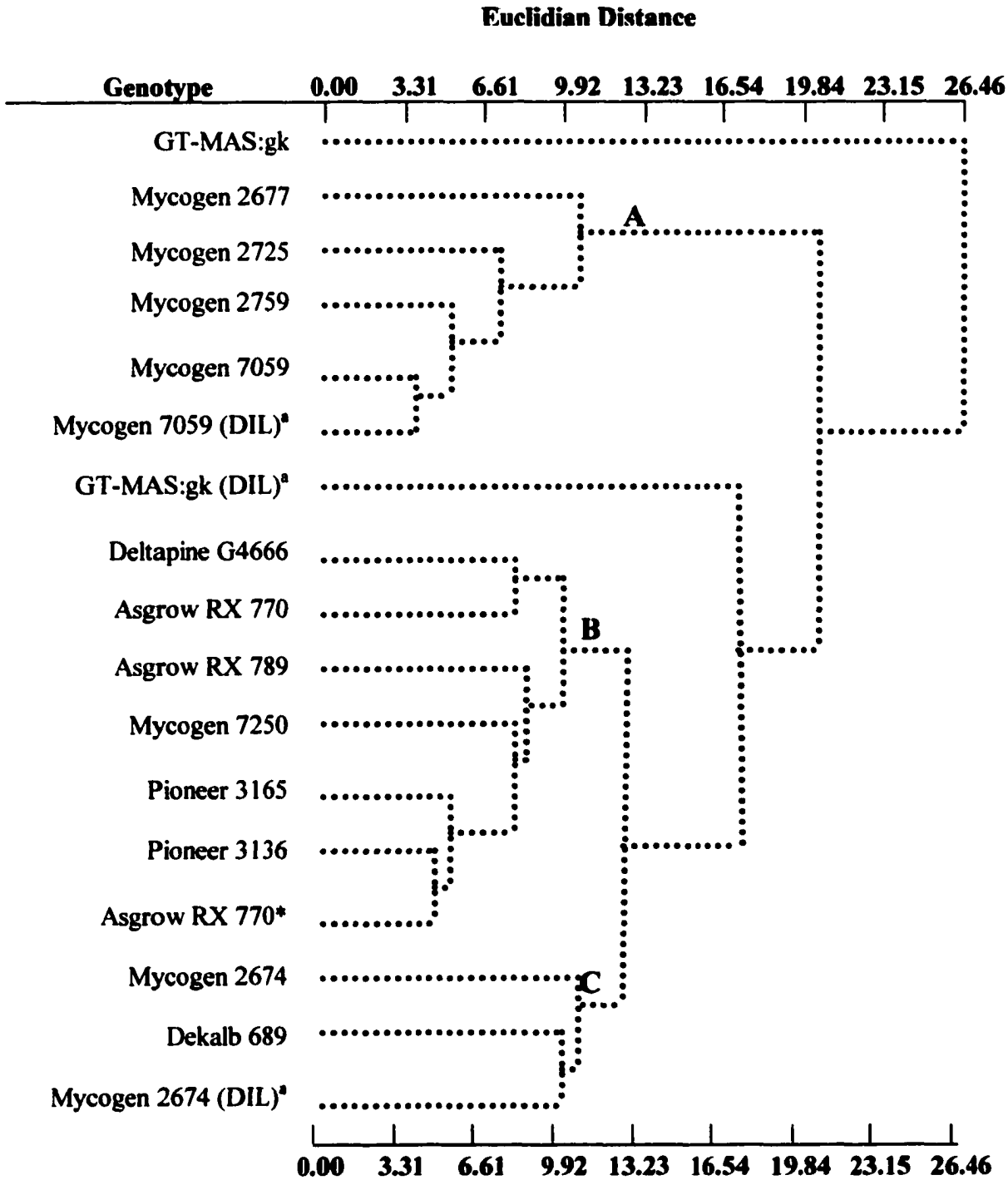

Figure 3.6. Dendogram of fatty acid compositions in epicuticular waxes from GT-MAS:gk, Pioneer 3165, 3136, Deltapine G4666, Dekalb 689, Asgrow RX 789, 770, *770, and Mycogen 2677, 2725, 2759, 7250, 7059 based on Euclidian distance. [a] Sample was diluted.

As expected, odd-numbered C_{27}, C_{29}, and C_{31} dominated the long-chain alkane group in all genotypes examined in both years (Figures 3.1 and 3.2). The data is consistent with other studies of corn epicuticular wax (Avato *et al.*, 1987; 1990; Bianchi *et al.*, 1975; 1977; 1979; 1982;1984; 1985; 1989; Bianchi and Salamini, 1975; Yang *et al.*, 1992; 1993).

In general, C_{27} and C_{29} tended to decrease as kernels developed while C_{31} and C_{33} showed the opposite trend. There appeared to be a difference in long chain alkane development between different corn genotypes. This difference is particularly evident in the 1999 data, where the levels of C_{31} and C_{33} during the later stages of kernel development (weeks 8 & 9) in GT-MAS:gk were higher than those of other genotypes (Figure 3.2B). Although the levels of C_{31} and C_{33} also increased in Asgrow RX 938, Pioneer 3136 and 3154, and Deltapine G4666, the levels of increase were not as dramatic as those of GT-MAS:gk (Figures 3.2 A, C, E, and F, respectively).

Just as long chain alkanes showed differences among genotypes, the 14 sterols (stigmasterol, campesterol, sitosterol, "ergosterol-like sterol," β-tocopherol, cholesterol, vitamin E, "steroid 442-A," "steroid 424-A," β-amyrin, "steroid 424-B," α-amyrin, "steroid 426-A," and stigmast-4-en-3-one) also showed differences among genotypes during kernel development (Figures 3.3 and 3.4). Interestingly, "steroid 424-A," "steroid 424-B," and "steroid 426-A," increased to notable levels in the resistant genotypes GT-MAS:gk and T115 in both years. Alpha-amyrin in T115 and stigmast-4-en-3-one in GT-MAS:gk showed the same levels of increase.

Differences in accumulation of chemical components of epicuticular wax between the resistant and the susceptible genotypes are of particular interest because wax from the

resistant genotype GT-MAS:gk has been shown to reduce *A. flavus* colony size by as much as 35% (Russin *et al.*, 1997). Regression analysis did not show a correlation between any of the components of epicuticular wax and aflatoxin production. However, many of the unidentified components have not been examined to see if they correlate with aflatoxin production.

3.4.2 Mature Kernel Assay

The data presented indicated differences in epicuticular wax compositions between the resistant (GT-MAS:gk and T115) and susceptible (Deltapine G4666 and Pioneer 3154) genotypes. There were a number of chemical components that were detected exclusively in epicuticular wax extract from each genotype. Four chemicals were detected exclusively in the resistant genotypes eluted at the retention times 29.40, 30.18, 30.88, and 31.76. Besides these differences, the overall epicuticular wax makeup for each genotype was also markedly different. Pioneer 3154, for example, not only had 12 chemical compounds that were undetectable in other genotypes but 4 of the compounds that were detected in other genotypes were undetectable in its epicuticular wax.

The TLC plate bioassay detected antifungal spots on plates developed in petroleum ether-ethyl acetate (2:1) solvent system (Figure 3.4). The spots appeared in waxes from Pioneer 3154 (40 μL) and T115 (20 μL). The absense of antifungal activity in the T115 (40 μL) spot could not be explained.

Some chemicals may exist on the surface of kernels that promote the growth of the fungus. Calvo *et al.* (1999) reported the promotion of *Aspergillus* spp. sporulation by linoleic acid. Interestingly, linoleic acid was detected only in epicuticular wax from

Pioneer 3154 in this study. When wax-extracted and untreated kernels from different genotypes at different stages of development were challenged with *A. flavus*, the untreated kernels at the earlier stages of development produced higher levels of aflatoxin (Chapter 2). Also, when wax-extracted and untreated kernels from 24 genotypes, including 23 commercial hybrids, were challenged with *A. flavus*, the untreated kernels from some of the commercial hybrids produced higher levels of aflatoxin (Chapter 2). Thus, the presence of chemical compounds in the pericarp appear to be important in stimulating aflatoxin production.

3.4.3 Fatty Acid Analysis

Fatty acid analysis of the epicuticular wax from the 13 genotypes confirmed the uniqueness of the aflatoxin resistant GT-MAS:gk epicuticular wax. For instance, the undiluted GT-MAS:gk epicuticular wax had a Euclidian distance of 26.46 compared to its next immediate cluster. This difference in GT-MAS:gk epicuticular wax is remarkable, considering many of the genotypes analyzed were susceptible to aflatoxin production whereas GT-MAS:gk was consistently resistant (Brown *et al.*, 1995;Guo *et al.*, 1994; 1995; Russin *et al.*, 1997; Widstrom *et al.*, 1987).

Fatty acid analysis has been widely used in differentiating bacterial strains (Sasser, 1997). The MIDI Sherlock System used in the study analyzes fatty acid composition using computerized high-resolution gas chromatography. Fatty acids identification and abundance were used to generate fatty acid profiles of each sample. The dendogram generated (Figure 3.6) was based on Euclidian distances of the collective fatty acids. Euclidian distance is the distance in two dimensional space between two genotypes or groups of genotypes when their fatty acid compositions are compared.

According to the technical notes provided by MIDI, when analyzing microbiological samples, samples linked at a Euclidean distance of 10 or less are of the same species; 6 or less are of the same subspecies or biotype; 2.5 or less are two different runs of the same strain.

Not only did this study confirm the uniqueness of GT-MAS:gk epicuticular wax, it also demonstrated the usefulness of using epicuticular wax to characterize the relatedness of corn genotypes. For example, 4 out of 6 Mycogen hybrids were grouped in subgroup A (Figure 6.1); both Pioneer hybrids were shown to be closely related as were the same with Asgrow hybrids. Because wax morphology is genetically controlled and the cuticle of corn is maternally inherited (Bianchi and Marchesi, 1960), it may be possible to identify corn lineages using fatty acid analysis.

3.4.4. Conclusion

The data presented confirmed the findings other studies (Avato *et al.*, 1987; 1990; Bianchi *et al.*, 1975; 1977; 1979; 1982;1984; 1985; 1989; Bianchi and Salamini, 1975; Yang *et al.*, 1992; 1993) that the major components of long chain alkanes in corn are C_{27}, C_{29}, and C_{31}. The data further showed that these odd numbered carbon long chain alkanes were dominant throughout kernel development. There was clearly a difference in sterol composition of epicuticular wax during kernel development between the resistant and the susceptible genotypes.

In the mature kernel experiment, epicuticular waxes from the genotypes examined were markedly different. The identification of components that were detectable only in the resistant genotypes suggests these chemicals may have a role in suppressing fungal growth and aflatoxin production. Antifungal spots were detected in TLC bioassay of

waxes of Pioneer 3154 and T115. Also, little or no fungal growth occurred at the origins on TLC plates, indicating that antifungal compounds existed in each of the epicuticular waxes.

Besides the differences in epicuticular wax composition accumulation during kernel development and differences in epicuticular wax chemical profiles in the mature kernel extract, fatty acid analysis on the 13 genotypes which included the resistant population GT-MAS:gk showed how different its epicuticular wax is, compared to other genotypes. The differences in epicuticular wax accumulation during kernel development, epicuticular wax chemical profiles in the mature kernels, fatty acid analysis of epicuticular wax, between the resistant and the susceptible genotypes all suggest that epicuticular wax may be involved in providing chemical resistance to aflatoxin production.

CHAPTER 4. SUMMARY AND CONCLUSION

4.1 Summary

The role of kernel epicuticular wax in conferring corn resistance to aflatoxin production was examined in this study. Chapter 2 examined the effect of the amount of epicuticular wax on aflatoxin production while Chapter 3 identified the chemical components in kernel epicuticular wax.

The amount of epicuticular wax and the individual epicuticular wax components were compared with aflatoxin production in the wax-extracted and non-wax-extracted kernels during kernel development. No evidence of a correlation between the amount of epicuticular wax or the selected individual epicuticular wax components and aflatoxin production was found. Removing epicuticular wax from kernels reduced aflatoxin in kernels at the earlier stages of development but increased the levels of aflatoxin in some, genotypes at the later stages. This observation suggests the vulnerability of less mature kernels to aflatoxin production and *A. flavus* invasion and may suggest a different resistance mechanism between the less mature kernels and the mature ones.

The amount of epicuticular wax from 23 commercial hybrids (Dekalb hybrids DK 687, DK 679, DK 668, DK 689, Cargill hybrids 6888, 7731 IMI, 8412, 7821 BT, Mycogen hybrid 2888 IMI, Garst hybrids 8300, 8513 IT, 8325, Pioneer hybrids 3223, 33V08, 3395IR, 31B13, 3260, 33K81, 32Z18, 3394, 33R87, 32K61, Deltapine hybrid G-4666) and GT-MAS:gk was compared with aflatoxin production in the wax-extracted and non-wax-extracted kernels. Cargill 6888 and 7731 IMI, Garst 8325, 8513 IT, and 8300, Mycogen 2888 IMI, DK 668 and 689, and Pioneer 33V08, 3394, and 31B13 produced lower levels of aflatoxin when their kernels were wax extracted. Data from all 24

genotypes in the regression test resulted in an r-square value of 0.042 (positive correlation) and of 0.0325 (negative correlation) between amount of wax per seed and aflatoxin in wax extracted and non-wax-extracted kernels, respectively.Regression analysis comparing the amount of wax and aflatoxin production for the Cargill 7731 IMI and 7821 BT, Deltapine G4666, DK 668, 689, Garst 8300, 8325, and 8513 IT, GT-MAS:gk, Mycogen 2888 IMI, and Pioneer 31B13, 3223, 32Z18, 3394, 33R87, and 33V08 group produced r-square values of 0.1528 (positive correlation) and 0.5659 (negative correlation) for treated and untreated kernels, respectively. Therefore, there was a correlation between increasing epicuticular wax and decreasing aflatoxin production in this group of corn genotypes.

Selected chemical components (long chain alkanes of C_{21}-C_{35} and 14 sterols: stigmasterol, campesterol, sitosterol, "ergosterol-like sterol," β-tocopherol, cholesterol, vitamin E, "steroid 442-A," "steroid 424-A," β-amyrin, "steroid 424-B," α-amyrin, "steroid 426-A," and stigmast-4-en-3-one) of the epicuticular wax were examined during the kernel development of aflatoxin resistant GT-MAS:gk, T115, and susceptible Asgrow RX938, Deltapine G4666 and Pioneer 3136 and 3154. In general, long chain alkanes decreased but sterols increased as kernels matured. Results showed that alkanes of odd numbered chain lengths C_{27}, C_{29}, and C_{31}, dominated in all genotypes at all stages of development. Development of sterols were not as consistant between genotypes and years, possibly due to differences in environmental conditions. Some of the sterols accumulated to much higher levels in the resistant genotypes.

Epicuticular wax profiles for resistant genotypes GT-MAS:gk and T115 and susceptible Deltapine G4666 and Pioneer 3154 were examined. Results indicated

differences in epicuticular wax profiles for each genotype. Pioneer 3154, for example, had 12 chemical components detectable exclusively in its epicuticular wax while 4 of the chemical components detectable in other genotypes were undetected. Four chemical components that were detectable exclusively in the resistant genotypes eluted at the retention times 29.40, 30.18, 30.88, and 31.76. Thin layer chromatography plates spotted with epicuticular waxes from the genotypes and developed in petroleum ether-ethyl acetate (2:1) solvent system showed antifungal spots in waxes from Pioneer 3154 and T115.

To further show how different GT-MAS:gk epicuticular wax was, in comparison to other genotypes, its fatty acids profiles and those from 13 commercial hybrids (Pioneer 3165, 3136, Deltapine G4666, Dekalb 689, Asgrow RX 789, 770, *770, and Mycogen 2677, 2725, 2759, 7250, 7059) were compared using MIDI Sherlock System. Results showed epicuticular wax of GT-MAS:gk was the most different compared to the rest.

4.2 Conclusion

This study demonstrates the importance of epicuticular wax in corn kernel resistance to aflatoxin production. Extracting epicuticular wax from the resistant GT-MAS:gk corn population consistently resulted in higher levels of aflatoxin production. While the regression tests in the developing kernels provided no evidence of a correlation between the amount of epicuticular wax and aflatoxin accumulation in the developing kernels, a correlation did occur when epicuticular waxes from selected commercial hybrids and GT-MAS:gk were compared with aflatoxin production in their non-wax-extracted kernels.

GT-MAS:gk kernel epicuticular wax was clearly distinct from that of other genotypes. The marked increase of certain epicuticular wax components (sterols) and components that were detected exclusively in the resistant genotypes suggest a possible role in providing chemically based resistance to aflatoxin accumulation.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Plant Health

Title of Dissertation: The Role of Kernel Epicuticular Wax in Zea Mays Resistance to Asperguillus flavus and Aflatoxin Production

Approved:

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