RESISTANCE OF MAIZE SILK TO *FUSARIUM GRAMINEARUM*

by

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of the requirements for the degree of

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To my father and mother, Fred and Rose,

FOREWORD

This thesis is submitted in the form of original papers suitable for journal publications. Chapter 4 is a literature review presenting the theory and previous knowledge on the thesis topic. The next eight chapters are presented in manuscript form. Chapter 13 is a general discussion of the results obtained in all of the manuscripts. This is followed by three chapters which summarize the conclusions of the research, the originality of the work, and suggestions for future research.

The thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines Concerning Thesis Preparation, section B.2 "Manuscripts and Authorship", which are as follows:

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- Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (eg. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported.
- The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

It is acceptable for thesis to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and

supplementary explanatory material is always necessary.
- Photographs or other materials which do not duplicate well must be included in their original form.

- While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear.

All the work reported here was the responsibility of and conducted by the candidate (unless otherwise stated as in Chapter 6). The project was supervised by Dr. D.E. Mather, Department of Plant Science of McGill University. For consistency and convenience, all manuscripts follow the same format. The copies that will be sent to the respective journals, however, will follow the requirements of each journal. All manuscripts are co-authored by L.M. Reid, D.E. Mather, R.I. Hamilton, and A.T. Bolton.

The manuscripts of Chapters 5 and 6 will be submitted to the Canadian Journal of Plant Pathology. When submitted for publication, the Chapter 5 manuscript will include data on *in vitro* tests and field evaluations of an additional 12 inbreds and 12 hybrids (data not part of thesis) and the Chapter 6 manuscript will include an additional field season of data on the effect of time of inoculation on hybrids.

The Chapter 7 manuscript is also co-authored by J.T. Arnason and has been submitted to the Canadian Journal of Botany.

The Chapter 8 manuscript will be co-authored by D. Spaner and will be

submitted to Canadian Journal of Plant Pathology. When submitted, it will include results of similar isolate studies carried out on hybrids in 1990 (Bolton and Hamilton), and 1991 (Reid and Hamilton) and on high-lysine corn germplasm (Spaner and Mather) in 1990 and 1991.

The Chapter 9 manuscript will be submitted to the Canadian Journal of Plant Science. When submitted for publication, data obtained by Drs. A.T. Bolton and R.I. Hamilton which initially indicated that the inheritance of silk infection was simple and dominant will be added to the manuscript as well as an additional analysis of 'CO272' crossed to another highly susceptible line being carried out in 1991 by Dr. R.I. Hamilton and L. Reid (not part of thesis).

The manuscript of Chapter 10 has been submitted to the Canadian J. of Plant Science.

The Chapter 11 manuscript is being submitted to Maize Genetics Cooperation Newsletter as a note.

The Chapter 12 manuscript will begin submitted to the Canadian J. of Microbiology. When submitted for publication it will include data obtained by Dr. A.T. Bolton on the correlation between visual ratings and toxin content of 20 Ontario hybrids.

GENERAL ABSTRACT

The characteristics and inheritance of maize silk resistance to *Fusarium graminearum* ear rot were investigated. In an *in vitro* test, genotypic differences in the degradation of detached silk tissue by F. graminearum were correlated to field evaluations of resistance. Susceptibility to infection decreased with silk age. Total phenolics of silk channel silk tissue increased in response to infection in resistant inbreds but decreased in susceptible inbreds. The flavones iso-orientin, iso-vitexin, maysin, luteolin, and apigenin were identified in the silk. No significant genotype by isolate interaction effects were found when 13 inbred lines were inoculated with three *F. graminearum* isolates. Simple models of quantitative and qualitative inheritance were not adequate to explain the inheritance of resistance. Disease severity ratings were bimodally distributed in the F_1, F_2, and backcross generations. In a complete diallel cross among 12 inbred lines, general and specific combining ability effects were significant for both disease incidence and disease severity. A screening of 12 accessions of exotic maize germplasm with resistance to either *Aspergillus flavus* or *Heliothis zeae*, identified several possible new sources of resistance to *F. graminearum*. Visual evaluations of resistance were correlated to deoxynivalenol levels of the ear.

RESUME

Les caractéristiques et les propriétés héréditaire de résistance des soies du mais à la fusariose de l'épi, *Fusarium graminearum*, ont été étudiées. Une étude, in vitro, de tissu isolées des soies de l'épi a permis de déterminer que les soies constitue un substrat approprié pour la croissance de *F. graminearum*. Les différences génotypiques observées en laboratoire, de l'assimilation du tissu des soies isolées, ont été corrélées à des évaluations de la résistance dans le champ. Ainsi, la sensibilité à l'infection diminu avec l'âge des soies. De plus, la quantité totale de composés phénoliques dans les cannaux du tissu des soies augmente suite au développement de la fusariose chez les sonsanguins génotypes résistants et diminue chez les lignées susceptibles. Les flavones iso-orientine, iso-vitexine, maysine, lutéoline et apigénine ont été identifiées dans les soies. Aucun effet significatif de l'interaction génotype de mais - isolat de champignon n'a été trouvé lors de l'inoculation de 13 lignées consanguines avec 3 isolats de *F. graminearum*. Les modèles simples connues de l'hérédité quantitative et qualitative n'étaient pas appropriés pour expliquer l'hérédité de la résistance. Ainsi, les évaluations de la sévérité de la maladie ont montré une distribution bimodale pour les générations F_1, F_2, et leur rétrocroisement. Lors de croisements en diallèle de 12 lignées consanguines, il fut démontré que les affinités générales et spécifiques des croisements avaient un effet significatif sur l'incidence et la sévérité de la maladie. Un examen de 12 variétés exotiques de mais avec divers degrés de résistance à *Aspergillus flavus* ou *Heliothis zeae*, a permis d'identifier plusieurs nouvelles sources de résistance à *F. graminearum*. Les évaluations visuelles de la résistance ont été corrélées au niveau de déoxynivalenol dans l'épi.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my research supervisor, Dr. Diane E. Mather, for her guidance, encouragement and unlimited input throughout this project. Her enthusiasm in the many discussions of my research and conscientious reviews of each version of every manuscript was deeply appreciated and very helpful.

I am indebted to Drs. R.I. Hamilton and A.T. Bolton of the Plant Research Centre of Agriculture Canada in Ottawa for their guidance, enthusiasm and endless help. The development of this research could not have happened without their preliminary studies. Dr. Hamilton generously provided the necessary facilities to carry out the research and often technical staff from his own very busy crew. I greatly appreciate the help of all of Dr. Hamilton's crew especially that of Ross MacDonald, Don Born, Clayton Barr, Trevor Henry, and Peter Tytor for always providing answers to my endless requests.

Special thanks to Tsegaye Woldemariam for preparing the inoculum for this study and technical assistance whenever needed.

Sincere thanks are extended to the members of my research advisory committee, Drs. Hamilton and Bolton, Dr. J.T. Arnason of the University of Ottawa, and Dr. B.E. Coulman of McGill University, for their valuable criticisms and suggestions at my graduate committee meetings.

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CHAPTER 1

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

Chapters 5-12 correspond to manuscripts or parts of manuscripts by Reid et al. All manuscripts are co-authored by myself, L.M. Reid, my supervisor at McGill University, Dr. D.E. Mather, and by Drs. R.I. Hamilton and A.T. Bolton of the Plant Research Centre of Agriculture Canada, in Ottawa.

Dr. Mather provided normal supervisory guidance from the outset of the research to the reviewing of the manuscripts. Dr. Mather also arranged for technical assistance during field seasons. Dr. Mather provided advice on the analysis of the genetic data and after I carried out the analyses she discussed the results with me and offered suggestions for organizing it in manuscripts.

Dr. Hamilton provided supervisory guidance throughout the research and arranged for me to obtain the use of field sites, appropriate field equipment, greenhouse space, storage space, computers and technical assistance at Agriculture Canada, in Ottawa. Dr. Hamilton and his technical staff made all of the crosses necessary for the genetic studies as well as arrangements for crossing in the winter nursery as part of the Plant Research Centre corn breeding program. They also planted and fertilized all field plots.

Dr. Bolton provided technical advice throughout the research and with his technician T. Woldemariam, provided me with *Fusarium graminearum* inoculum. The manuscript corresponding to Chapter 6 is also co-authored by

T. Woldemariam who carried out the hybrid study included in this manuscript.

The manuscript corresponding to Chapter 7 is also co-authored by Dr. J.T. Arnason of the University of Ottawa who provided advice on the phytochemical analysis of silk tissue and laboratory facilities.

I performed all tasks and techniques of the research but technicians and field hands assisted during compressed time periods of inoculations and harvesting of plant material. I also received assistance to control weeds. I conducted all laboratory analyses.

CHAPTER 2

INTRODUCTION

Maize or corn is the most important grain crop in Quebec and Ontario (Canada Grains Council, 1990). In some years, diseases significantly reduce yields. The fungus *Fusarium graminearum* causes ear rot of maize, which can result in yield loss and the presence of mycotoxins in the grain. These compounds include deoxynivalenol, a tricothecene fungal metabolite, and zearalenone, a compound with estrogen-like activity. Presence of these toxins destroys the market value of the grain. This disease has occurred infrequently but can be very severe under specific climatic conditions. When this has happened it was a serious threat to food and feed safety and to the value of the Canadian corn crop.

Although infection can occur through insect wounds, *F. graminearum* primarily enters the maize ear by invasion through the silk channel. In lines exhibiting almost complete resistance the fungus does not reach the grain or the rachis, suggesting that resistance mechanisms may be operating in the silk or the silk channel. No studies have investigated the mechanism of resistance in the silk to *F. graminearum* infection.

Perhaps the only feasible solution to *F. graminearum* control is the development of resistant maize hybrids through plant breeding. Techniques for screening germplasm have been developed and a range of susceptible to

moderately resistant genotypes have been reported. Recently the inbred line 'CO272' has been found to exhibit very high resistance under artificial inoculation conditions. Initial observations suggested that this resistance may be simply inherited, and that controlling gene(s) expressed complete dominance. 'CO272' and other lines exhibiting high resistance may serve as important sources of resistant germplasm for future plant improvement programs.

CHAPTER 3

MAJOR GOALS

The two major goals of this research were:

(1) To determine the role of silk in resistance to *F. graminearum* ear rot.

(2) To investigate the mode(s) of inheritance of resistance to *F. graminearum* infection via the silk, with emphasis on the highly resistant inbred line 'CO272'.

CHAPTER 4

GENERAL LITERATURE REVIEW

4.1 The Host - Maize

Maize or corn, *Zea mays* (L.), is a member of the grass family, Gramineae (Poaceae), and shares several features in common with other members of this family: conspicuous nodes on the stem; a single leaf at each node; parallel-veined leaves; and leaves composed of a sheath surrounding the stem and a blade connected to the sheath by a blade joint. Most of the maize plant body is composed of long narrow leaves spaced alternately in two rows on strong erect stalks. Tillers may originate from subterranean nodes and develop into structures identical to the main stem although somewhat smaller. The presence of tillers varies among maize varieties. It is generally considered an undesirable trait since few tillers produce grain. Unlike most of its relatives, maize is an annual monoecious plant with staminate flowers in tassels at the top of the stem and pistillate flowers in ears in the axils of the leaves. Most varieties have one or two ears per plant.

Many divergent types of maize are grown over a wide range of climatic conditions. Maize can be grown throughout temperate, subtropical, and tropical zones wherever rainfall or irrigation is adequate. Various soil conditions are tolerated, but the best is a deep, well drained, fertile loam soil with a slightly acidic pH of approximately 6.0 (Bockholt, 1979). Maize is a warm-weather crop requiring

mean summer day temperatures higher than 19°C and night temperatures higher than 13°C (Hartman et al., 1981). It is classified as a short-day plant; longer days tend to increase the duration of the vegetative stage and the size and number of leaves (Bockholt, 1979).

Maize is the world's third largest cereal crop after rice and wheat. The major producer is the U.S. followed by China, Brazil, the U.S.S.R., Romania, Yugoslavia, Mexico, South Africa, Argentina, and India (Janick et al., 1981). More than 100 million hectares of land are planted each year and produce more than 250 million metric tons (Shurtleff, 1984). More than 100 million people in the world rely on maize as a major food crop (Chiang, 1978). Maize is also the most important feed grain in the world, and is an important source of industrial products.

Diseases and insects are major limiting factors to increasing maize yields (Jugenheimer, 1985). All parts of the maize plant are susceptible to diseases that reduce both yield and quality. Some of the more serious diseases are those that affect the ear.

4.1.1 The Development and Structure of the Female Inflorescence of Maize

The female inflorescence of maize, usually referred to as the ear, has been extensively studied since it is the grain-bearing structure. According to the scale of Ritchie et al. (1986), ear shoots (potential ears) first become apparent in the V6

(vegetative) stage of growth as protrusions from leaf axils. At first, ear shoots develop from every above-ground node except the last four to five nodes below the tassel. However, growth of most lower stalk ear shoots eventually slows, and only the upper one or two ear shoots ever develop into a harvestable ear.

By the V12 stage the number of rows of kernels per ear has been established. Silks have commenced growth from the upper ear by the V15 stage, however the whole ear is still not visible since it is surrounded by the leaf sheath.

By the V18 stage silk elongation has been initiated. Silks from the basal ear ovules are the first to elongate and those from the tip ovules are the last to elongate. At this time, silks are still under the husk and are not yet visible.

Approximately one week later, the first reproductive stage (R1-silking) begins with the first silks emerged and visible.

Pollination takes place when pollen grains liberated from the tassel land on the silk and grow down the silk to the ovule, and fertilize the ovule which develops into a kernel. Generally 2-3 days are required for all silks on a single ear to emerge and be pollinated. Silks continue to elongate and grow 2.5-3.8 cm per day until fertilized. Embryos have not yet developed and the kernels are covered by glumes, lemmas, and paleas.

The R2-blister stage occurs 10-14 days after silking. The silks, after serving a function in fertilization, begin to senesce by dehydrating and darkening in colour. Silk senescence is highly influenced by the environment. Kernels become white, blister shaped, approximately 85% moisture, and have a clear inner fluid. Small

embryos are developed and the cob is close to or at full size.

Approximately 18-22 days after silking the ear reaches R3-milk stage. Most silks are brown and dry. Kernels are approximately 80% moisture and have an inner fluid which is milky white in appearance due to accumulation of starch. Actual colour of the kernel may be starting to change at this time, usually to yellow. Embryos are rapidly developing and are quite visible by this stage.

In the next stage, the R4-dough, ears are 24-28 days post-silking and silks are completely brown and dry. The inner fluid of the kernels has now thickened to a doughy consistency due to continued accumulation of starch in the endosperm. Kernels are approximately 70% moisture and may begin to dent in dent varieties.

Denting may continue in the next stage, R5-dent, which is approximately 35-42 days after silking. By now kernels are approximately 55% moisture.

The final stage is R6 or physiological maturity. All kernels have reached their maximum dry weight or dry matter accumulation, are 30-35% moisture, and the black or brown abscission layer has formed. Husks and leaves of the plant are also dry and no longer green.

4.1.2 The Structure and Function of Maize Silk

The silks or styles of maize vary in abundance, length, and colour according to genotype and environmental conditions. The silk channel is the portion of the ear between the tip of the rachis and the place where the silks emerge from the

husk. The length of the silk channel decreases as the ear matures.

The tip of each silk is forked and it is this portion that is the most receptive to pollen and is referred to as the stigma (Miller, 1919; Kiesselbach, 1949). Silks may contain 2-3 vascular strands which terminate in the branched tip. A cross-section of silk shows that it is grooved on both upper and lower surfaces and that vascular bundles are located near the edge. The surface of silk is covered with numerous, often branched, epidermal hairs or trichomes. The trichomes and the stigma create a rough surface upon which pollen grains lodge. Trichomes are more numerous on the edges of the silk and near the tip, but pollination can take place along the entire length of the silk. At the base of the silk is a meristematic zone where new cells develop, causing elongation of the silk until pollination. Pollen grain germination is highly dependent on the water potentials of both the silk and the pollen grain and therefore under a great deal of environmental influence (Westgate and Boyer, 1986; Schoper and Martin, 1989). The pollen tube may follow the surface of the hair but usually enters between the cells of the hair and then into the main body of the silk. It then follows the sheath cells surrounding the vascular tissue to the base of the silk and passes into the cavity of the ovary where fertilization takes place . As the pollen tube passes between the bundle sheath cells it causes very little disturbance in the position of these cells which quickly return to their normal form and position. About 24 hours are required for the pollen tube to reach the embryo sac (Miller, 1919).

4.2 The Causal Organism - *Fusarium graminearum*

The major ear rotting pathogen of maize in Quebec and Ontario is *Gibberella zeae* (Schw.) Petch. This is the sexual stage of *Fusarium roseum* f. sp. *cerealis* "Graminearum", which is more commonly referred to as *F. graminearum* Schwabe. *Fusarium graminearum* is classified in the section Discolor according to the taxonomic system of Nelson et al., (1983). This pathogen is also the cause of stalk rots and seedling blights of maize (Shurtleff, 1984; Mesterhazy and Kovacs, 1986).

It should be noted that other fungal pathogens can cause similar ear rot diseases in maize. The major ones include: *F. moniliforme*, *Aspergillus* spp., *Diplodia* spp., and *Rhizopus* spp..

4.2.1 Pathogenicity and Disease Distribution

Fusarium graminearum attacks a wide range of plant species in addition to cereals (Martin and Johnston, 1982), but the major hosts are wheat and maize. There exist two populations of *F. graminearum*: Group 1 and Group 2. The groups are mainly distinguished by the fact that perithecia have not been observed in Group 1, but are readily formed on both hosts and in culture in Group 2 (Cook, 1981; Burgess, 1981). Group 1 pathogens are more common in drier areas and are usually associated with crown and root rots. Members of this group are classified as soil-borne pathogens, while members of Group 2 are classified as air-borne pathogens. The latter classification is based upon the fact that Group 2

pathogens usually infect aerial plant parts such as heads of wheat, barley and oats; stalks and ears of maize; and, stalks of pearl millet (Burgess, 1981). These air-borne pathogens are common under warm, humid conditions which favour formation of perithecia and ascospore discharge.

The incidence of *F. graminearum* diseases in maize is worldwide. It has been reported in maize-producing regions in Canada (Gordon, 1959; Sutton et al., 1980a), the United States (Hesseltine and Bothast, 1977; Koehler, 1959; Ullstrup, 1977), southern and eastern Europe (Bottalico, 1977; Milic et al., 1969), central and southern Africa (Marasas et al., 1979), the USSR (Manannikova, 1979), and China (Tanaka et al., 1988).

4.2.2 Disease Cycle

4.2.2.1 Inoculum Dispersal

The major source of inoculum for *F. graminearum* is believed to be infested host debris such as old stalks and ears of maize (Sutton, 1982). Such refuse may give rise directly to infectious mycelium, or may serve as a food base for sporulation and dissemination. The use of infected grain as seed may result in diseased seedlings. Actual inoculum propagules may be ascospores, macroconidia, chlamydospores, or hyphal fragments. In epidemics, the major means of inoculum dispersal is aerial; ascospores and macroconidia are therefore the most important inoculum types. Birds and insects can also be vectors of *F. graminearum* and wounds created by feeding may predispose the ear to further

fungal invasion (Sutton et al, 1980b; Enerson and Hunter, 1980; Attwater and Busch, 1983). Damage of ears by hail has also been found to increase the incidence of infection (Abbas et al., 1988).

4.2.2.2 Infection of Maize Ears

Susceptibility of maize ears to infection has been reported to be highest shortly after silks emerge and then decreases thereafter (Ullstrup, 1970; Enerson and Hunter, 1980). Normally, the disease invades the ear from the ear tip (Koehler, 1942; Hesseltine and Bothast, 1977), however butt and shank infection can occur especially if ears are wounded by birds or insects (Sutton et al.,1980b). It is a common belief that *F. graminearum* primarily enters the ear via the silk channel and that the silk may serve as a potential substrate for fungal establishment (Sutton, 1982; Hesseltine and Bothhast, 1977). However, research has mostly concentrated on the infection process in the kernels rather than on the path of infection through the silk channel. The earliest work was carried out by Koehler (1942) who reported that *F. graminearum* colonies were first formed on the crown half of the kernel. Koehler plated surface-sterilized kernels on potato dextrose agar and found that most infection originated through the tip of the ear. Entry into very young kernels occurred through the pericarp and testa, but as kernels developed, the testa offered increased resistance and limited fungal entrance to the tip cap. No infection was found in pedicels, the vascular cylinder, or the butt of the cob. Hesseltine and Bothast (1977) examined developing maize

ears for the presence of various pathogens including some *Fusarium* species. They found that silks were the first tissue of the ear to become infected and that after pollination when the silk dies, it may provide a good fungal substrate and pathway to the kernels. Smart (1987) found that when ears were inoculated with *F. graminearum*, hyphae grew into the pedicel of wounded kernels and onto adjacent kernels. Penetration of the pericarp and the testa was followed by endosperm liquefaction. Initial colonization of kernel tips was also confirmed by Sutton and Baliko (1981).

Studies have been reported on the colonization of silk tissue by *Aspergillus flavus* Link ex Fries, an ear-rotting pathogen that produces the mycotoxin aflatoxin. It has been found that silk condition greatly influenced how well the fungus developed on external silks and moved down the silks to infect the kernels. Payne (1987), using scanning electron microscopy, found that the greatest amount of fungal spore germination occurred on silks that were yellow-brown in colour, i.e. just starting to senesce. Silks were penetrated through cracks as well as intercellular gaps. Hyphae were observed only in the parenchymatous tissue and growth was oriented parallel to the silk axis. Before fungal growth reached the kernel an abscission layer formed and detached the silk from the kernel. This is a normal process after pollination is completed and may cut off the pathway of fungal growth. In addition, as with *F. graminearum, A. flavus* penetration of the kernel seems to be primarily via the tip region (Marsh and Payne, 1984). This suggests that the mycelium arrives at the silk attachment site then grows onto the

adjacent pericarp and colonizes the kernel surface rather than penetrating the abscission layer into the kernel (Marsh and Payne, 1984).

Pollen seems to play a critical role in the establishment of *A. flavus* growth on external silks since germination of fungal spores is greatest nearest pollen grains (Payne, 1987). The pollination process may decrease plant defenses and increase nutrient supply to the fungus. As the pollen tube grows down the silk, vacuoles contract, cytoplasmic shrinkage occurs, membrane permeability is increased, and there is a general necrosis of stigma cells. Furthermore, pollen itself is a rich source of carbohydrates, amino acids, and minerals. Naik and Busch (1978) have reported that maize pollen stimulated conidial germination, germ tube growth and colonization of detached silks for *F. graminearum*. They also reported that although sugars from pollen were not important in this activity, other compounds, such as choline, may play a role.

4.2.2.3 Influence of Environment and Ecological Requirements

Growth of *F. graminearum* requires periods of warm weather with persistent wetness. Almost all stages of infection require temperatures of 15-35°C with an optimum of about 25-32°C (Sutton, 1982). Low intensity ultraviolet light (< 390 nm) and the presence of the hormone zearalenone (which is a mycotoxin) are also required for perithecial formation (Tschanz et al., 1976; Wolf et al., 1972). Production of ascospores and macroconidia requires temperature conditions similar to those needed for production of perithecia, as well as moist conditions

and, possibly, a specific light requirement.

Rainfall, especially in August and September (post-pollination), is a key factor in epidemics of *F. graminearum* ear rot (Koehler, 1959; Sutton, 1982). It has been speculated that high rainfall favours infection by prolonging wetness duration in maize ears (Sutton, 1982). Colonization of maize kernels is highest on kernels with moisture contents greater than 35% (Eugenio et al., 1970; Sutton and Baliko, 1981).

These environmental influences may explain the sporadic nature of this disease, as well as its increased incidence in cooler, wetter short-season growing areas.

4.2.3 Disease Symptoms

The major symptom of *F. graminearum* infection on maize ears is a characteristic pink to reddish mold on kernels and between husks and kernels. Husks may adhere tightly to the kernels in severely infected ears. Infection is usually found near the tip of the ear although disease symptoms also have been found around tunnels made by insect feeding such as that of the European corn borer, *Ostrinia nubilalis* Hbn., and the corn earworm, *Heliothis zeae* Boddie (Christensen and Schneider, 1950; Koehler, 1959). If the season is long and wet, perithecia of *F. graminearum* may develop on infected husks, kernels, and stalks.

4.3 Mycotoxins of *Fusarium graminearum*

Grain with the characteristic mycelial growth poses a major problem and threat to livestock production since this contaminated grain may harbour mycotoxins. Smith and Moss (1985) define mycotoxins as "low molecular weight, nonantigenic fungal metabolites which may elicit toxic responses in man and animals." *F. graminearum* produces a number of mycotoxins including the estrogenic metabolite zearalenone, and the trichothecenes deoxynivalenol (DON) and nivalenol (Mirocha, 1984). This results in a combination of toxic poisoning symptoms in animals which consume the infected plants or seeds. The two main mycotoxins, zearalenone and DON, may occur together in cereal grains.

4.3.1 Zearalenone

Zearalenone is a natural metabolite produced by *F. graminearum* possibly for the induction of perithecia (Mirocha and Christensen, 1974). Zearalenone is the trivial name for 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-resorcyclic acid lactone (Urry et al., 1966). This compound was first isolated from maize by Stob et al. (1962). It is usually produced in storage rather than in the field.

Corn is the major feed of swine and zearalenone is commonly associated with swine poisoning (Mirocha, 1984). Zearalenone causes swine estrogenic syndrome by binding to specific oestrogen receptor proteins of the animals and by competing for binding sites with oestradiol (Mirocha et al., 1989). Estrogenism in swine is characterized by such symptoms as vulvovaginitis which can lead to

the abortion of fetuses. Other symptoms of zearalenone poisoning include infertility in male swine, reduced litter size, diarrhea, refusal of feed, loss of weight gain, and haemorrhagia (Mirocha and Christensen, 1974). Concentrations of 1 to 5 ppm of zearalenone are sufficient to cause estrogenism in swine (Mirocha and Christensen, 1974).

4.3.2 Deoxynivalenol

Deoxynivalenol is a trichothecene toxin and as such is characterized by the presence of a tetracyclic 12,13-epoxy-trichothec-9-ene skeleton (Ciegler, 1979). Deoxynivalenol is the trivial name for 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one. Acetylated derivatives of DON, 15- and 13-acetyldeoxynivalenol (15- and 13-ADON) are also produced by *F. graminearum* and are found in contaminated grain along with DON (Miller et al., 1983a; Munoz et al., 1989). Deoxynivalenol was first detected in barley in Japan (Morooka et al., 1972). Since then it has been reported and confirmed in naturally moldy field maize in the U.S. (Vesonder et al., 1981), mixed feeds, wheat, triticale and rye (Mirocha and Pathre, 1979; Hart et al., 1984; Neish et al., 1983; Scott et al., 1984).

Concentrations of DON greater than 2 ppm will make cereal grains unfit for either food or feed (Scott et al., 1983). Deoxynivalenol gained the generic name vomitoxin due to its ability to induce emesis in swine which is characterized by vomiting, feed refusal, and decreased weight gain (Vesonder et al, 1981). When administered orally, DON is acutely toxic even at low concentrations and often

results in death due to haemorrhaging of the digestive system from severe gastroenteritis (Mirocha and Christensen, 1974). Pigs have been found to be able to tolerate only up to 2 mg/kg of feed before effects occur, while poultry are less sensitive, tolerating at least 5 mg/kg (Trenholm et al., 1984). The LD_{50} for DON in mice is 78 mg/kg oral and 49 mg/kg intraperitoneal (Forsell et al., 1987). Deoxynivalenol is also a skin irritant causing inflammation, desquamation, subepidermal haemorrhaging, and general necrosis (Mirocha and Christensen, 1974). Vesonder et al. (1981) have shown that DON possesses antibiotic activity to various molds.

4.3.3 The Biosynthesis and Accumulation of Mycotoxins

Biosynthesis of mycotoxins occurs in senescing maize plants in the field and in harvested grain. Information on physical and biological factors that regulate mycotoxin biosynthesis has been obtained, by necessity, with *in vitro* cultures due to the many interacting and complicated variables affecting biosynthesis in nature. Mycotoxins can be produced in the laboratory in solid-substrate fermentation using rice, maize, wheat and barley, or in liquid cultures.

In liquid cultures, mycotoxin production can be stimulated by reduced oxygen levels, depletion of carbohydrate in the medium, changes in pH, and possibly by the presence of a low concentration of an organic nitrogen source (Miller et al., 1983a). In solid-substrate cultures, temperature, initial moisture content, pH, oxygen and carbon dioxide can influence biosynthesis (Greenhalgh

et al., 1983).

Mycotoxins of *F. graminearum* represent at least two biochemical origins. Zearalenone biosynthesis has been found to be by head-to-tail condensation of acetate units via the acetate-malonyl-coenzyme A enzyme system (Steele et al., 1974; Mirocha and Pathre, 1979). Deoxynivalenol and other trichothecenes are derived from the condensation of three mevalonate units to give trans-farnesyl pyrophosphate, which cyclizes via 1,2-methyl and 1,5-hydrogen shifts to give the characteristic tetracyclic sesquiterpene structure (Ciegler, 1979).

It has been reported that some strains of *F. graminearum* which are able to produce zearalenone in culture, fail to do so in plant tissue (Neish et al., 1983; Miller et al., 1983a; Bennett et al., 1988). This may be because the enzymes responsible for zearalenone production are induced or activated at low temperatures, i.e. 12-18^{o}C (Eugenio et al., 1970; Mirocha and Christensen, 1974). Such temperatures are not optimal for fungal growth, but rather ones of physiological stress. It has been hypothesized that at low temperatures there is a decrease in the fungal growth rate and a subsequent switching of carbon metabolism to other biosynthetic pathways including those leading to zearalenone production (Sherwood and Peberdy, 1972). This may account for the observation that the largest production of zearalenone occurs not in the field but when maize is stored (Mirocha and Christensen, 1974). In contrast, the production of DON is highest at about 30^{o}C (Vesonder et al., 1981; Greenhalgh et al., 1983). The production of DON and other trichothecenes has been found to be isolate-specific,

especially between isolates from various countries (Mirocha et al., 1989). It has been reported that translocation of mycotoxins in the plant can occur (Miller et al., 1983b). This raises serious questions as to the use of contaminated maize as silage and the introduction of toxins into the soil by litter. Sutton et al. (1976) found some evidence for the translocation of zearalenone from inoculated maize stems to uninoculated ears; however, inconsistency of results among plants indicated that a number of factors were involved. It was suggested that the observed translocation may be associated with metabolite migration from the stem to the ear. No translocation was found for zearalenone from the ear to the stem. However, Young and Miller (1985) reported that both the fungal metabolite ergosterol and DON were translocated from the ear to the husk, axial stem, and stalk after ears were inoculated with colonized toothpicks.

Movement of the infection down the tip of the ear or spreading from wounds, results in zones of differing kernel rot severity along the ear. It has been reported that most toxin production is in the severely infected and heavily damaged kernels and underlying cobs (Bennett et al., 1988; Hamilton et al., 1988).

4.4 Economic Impact

Although the occurrence and severity of *F. graminearum* ear rot can be somewhat sporadic and localized from year to year, losses may be appreciable. Primary losses are a result of infected kernels and decreased palatability to

livestock. Indirect losses occur from seedling blight when diseased kernels are used as seed. The extent of yield loss depends upon the maize genotype, the aggressiveness of the fungus, and the season.

Because grain corn is often processed or milled, it is important to know the fate of mycotoxins in food and feed processing. In wet-milling, where high-quality, clean maize is required, inadvertent contamination does sometimes occur especially with zearalenone (Bennett and Anderson, 1978). Studies with wheat have shown that with milling there are increases in DON and ergosterol levels in the bran and a decrease in levels in flour (Young et al., 1984). The combined effects of milling, baking, and dilution result in an overall reduction of approximately 66% DON (Young et al., 1984; Scott et al., 1983). The dry-milling of maize from zearalenone-contaminated lots has been reported to remove 3-10% of the zearalenone; however, all product fractions produced by dry-milling of cleaned maize can be contaminated with zearalenone (Bennett et al., 1976). Also, in the same study it was found that grit fractions contained the lowest levels of zearalenone while germ and feed fractions had the highest.

In most of Mexico and Latin America, maize is consumed by humans as a principle food in their daily diet. A study conducted by Abbas et al. (1988) found that in the process of making tortillas from maize, 59-100% of the zearalenone, 72-82 % of the DON, and 100% of the 15-ADON was either destroyed or degraded to other unknown compounds.

The Food and Agriculture Organization (FAO) estimates that 25% of the

world's food crops are affected by mycotoxins (Mannon and Johnson, 1985). The recommended Canadian limit of DON in feedstuff is currently 2 ppm. In a major epidemic of *F. graminearum* ear rot in Ontario in 1986, it was reported that 7% of over 1500 samples analyzed had zearalenone levels between 1 and 20 ppm with an average of 2.3 ppm, and of over 1000 samples analyzed, 80% had DON levels between 0.5 and 64 ppm with an average of 6 ppm (Hough and Jones, 1987). Multimillion dollar losses occur annually but it is difficult to estimate these accurately (Trenholm et al., 1988). It has been estimated that the economic impact on the hog industry in Ontario is $9 million for zearalenone and $12 million for DON, although these levels may be as high as $50 million annually (Hough and Jones, 1987).

4.5 Control Measures

Obviously, there is a tremendous need to control both the incidence and the severity of *F. graminearum* ear rot. No one measure has been found to give adequate control of both infection and mycotoxin development.

Various management strategies have been found to give some degree of control. Since crop residues are believed to be the principle source of inoculum, plowing under of such residues is encouraged as this will hasten decay of the residue and the pathogen. This suggests that control will be difficult to achieve in conservation tillage systems (Seaman, 1982). Effective weed control and crop rotation with nongraminaeous crops has been found to decrease the inoculum

potential (Martin and Johnston, 1982). Use of early-maturing hybrids and early harvesting may allow plants to escape infection. Over-wintering of maize before harvesting should be avoided since it allows further fungal infection and mycotoxin accumulation (Farnworth and Neisch, 1980). As with most diseases, maintaining balanced fertility is important (Shurtleff, 1984). Increased levels of nitrogen fertilizer have been found to increase disease incidence (Enerson and Hunter, 1980; Shurtleff, 1984). Seedling vigour should be maximized by using high quality and fungicide-treated seed (McGee, 1988). Foliar fungicides, such as maneb and benomyl sprays, decrease infection slightly but have no effect on the amounts of mycotoxins produced (Martin and Johnston, 1982; Seaman, 1982).

Post-harvest fungal growth and contamination can be prevented with proper grain drying, storage of grain at low moisture levels, and sanitation of feed preparation and delivery systems (Seaman, 1982; Enerson and Hunter, 1980; Shurtleff, 1984; Martin and Johnston, 1982).

Dilution of contaminated grain with clean grain has been used but it is not fully satisfactory and is not usually practical for growers who produce their own feed grains. Decontamination of grain by chemical treatment (e.g. ammonium nitrate) is not economically feasible (Seaman, 1982; Martin and Johnston, 1982). Because the pathogen utilizes the maize matrix, infected kernels are significantly lighter than uninfected kernels. Density segregation can therefore be used to remove damaged kernels at harvest (Bennett et al., 1988).

The most promising control measure is the development and use of

resistant hybrids and varieties (McGee, 1988; Shurtleff, 1984). This is also the most economical and efficient means of controlling maize diseases and is the control measure that is most readily accepted by growers.

4.6 The Concept of Disease Resistance

Disease resistance may be considered a character of the host plant causing it to have less disease or less overall loss than another plant, cultivar, or species subjected to the same disease stress. For the purposes of this study, disease resistance is defined as "the ability of a plant to prevent, restrict, or retard disease development" and may occur at high, moderate, or low levels (Bell, 1981).

Resistance has been classified in a number of different ways. Van der Plank (1968) divided resistance into two categories: vertical and horizontal. Vertical resistance is effective against some races of a pathogen but not others and therefore assumes an interaction between the host and the pathogen. Horizontal resistance, on the other hand, is equally effective against all races, i.e. across the entire pathogen population. These two classes of resistance also have been referred to as specific and general resistance, respectively (Thurston, 1971). Resistance can be conditioned by one to a few major genes (monogenic resistance) or by a number of minor genes (polygenic resistance). Schafer (1971) breaks resistance down into four classes: escape, exclusion, inhibitory host-parasite interaction, and tolerance. A tolerant cultivar is one that is less damaged than an equally diseased susceptible cultivar.

4.6.1 The Host-Pathogen Relationship or "How Plants Defend Themselves"

Throughout their life cycle plants are subjected to attempted infection by many different microorganisms yet few of these attempts are successful. Since plants are immobile, they must have developed efficient strategies which provide defence against microorganisms and herbivores.

There are three major defense strategies of plants: (1) mechanical protection such as trichomes and thorns; (2) growth strategies such as regeneration of diseased tissue; and (3) chemical protection. These strategies often operate cooperatively against pathogen or herbivore attack. Chemical protection is the most elaborate of the three strategies and can be further subdivided into four groups: (1) cuticular waxes which may contain antibiotic and repellent compounds and flavonoids; (2) carbohydrates and lignin in cell walls or induced production at the site of infection or wounding; (3) protein and enzyme inhibitors which degrade microbial cell walls or phenolases which could help to inactivate phytotoxins of microbial origin; and (4) secondary metabolites with repellent or toxic properties. In the case of fungal pathogens the above defense strategies can be further subdivided into those that act preinfectionally and those that act postinfectionally.

4.6.1.1 Preinfectional Defense Strategies

During the first contact between a fungal pathogen and a host plant the

fungus is often exposed to unfavourable climatic conditions and therefore is unlikely to be successful. However, if environmental conditions are favourable, fungal growth occurs and the initial interaction between the fungus and the plant takes place. For a fungal lesion to successfully develop a number of events must occur in sequence: (1) the fungal spore must arrive at a plant site where the environment is favourable for spore germination; (2) the fungal germ tube then must penetrate the plant surface and establish itself; and (3) further fungal growth and sometimes reproduction then must proceed. The result is establishment of disease symptoms.

Pre- and postinfectional barriers may block each or all of these fungal processes. Preformed or preinfectional defense mechanisms require no plant response to be effective and can be divided into physical defense mechanisms and physiological defense mechanisms (Creasy, 1985).

The physical defense mechanisms consist of preformed mechanical barriers normally present in healthy plants, such as cell walls with higher concentrations of cutin, suberin, lignin, and cellulose and pectic substances (Bell, 1981; Schigeyasu and Fukutomi, 1977). These barriers are usually more impermeable and insoluble to pathogen exudates than are normal cell walls.

Physiological preinfectional defense mechanisms come into play because successful invasion of a plant through wounds, stomata, or other natural openings is influenced by the host's physiological status. There is a precise interplay of nutritional factors of the host and requirements of the pathogen. In addition,

chemical factors, such as specific proteins on cell surfaces of both the host and the pathogen permit "recognition" without which the pathogen cannot bind to the host.

4.6.1.2 Postinfectional Defense Strategies

For plants to respond defensively to the presence of microorganisms they must be able to detect invasion by pathogens (Ayers et al., 1985). Postinfectional defence mechanisms are usually induced. These mechanisms play the major role in preventing pathogens from penetrating and establishing themselves in the host. Like preinfectional defense mechanisms, postinfectional defence mechanisms can be divided into physical and physiological mechanisms.

Physical postinfectional mechanisms include physical changes in plant structure. Some common examples of this type of defense are: vascular occlusion of xylem elements by formation of tyloses and gels; deposition of additional wall layers; growth and differentiation of cells to form protective tissue; and hardening of cells by calcium accumulation.

Physiological defense mechanisms consist of a number of different chemical barriers such as the production of various hydrolases like chitinase which attack pathogen cell walls and the production or release of fungal toxins. Plants may counteract a successful fungal attack with rapid *de novo* synthesis of a fungitoxin. The pathogen itself induces the production of an elicitor molecule within the host plant, which then catalyses production of the fungitoxin. This series of metabolic

events is known as a phytoalexin response (Creasy, 1985; Bell, 1981). The term phytoalexin encompasses those defense chemicals which are not present in healthy tissue but are synthesized and accumulated after exposure to microorganisms and to stress (Friend, 1977).

4.6.2 The Role of Secondary Metabolites in Disease Resistance

A characteristic of higher plants is their capability of synthesizing enormous numbers and varieties of organic molecules, the so-called secondary metabolites. These compounds were originally called secondary since they seemed to play no known role in primary metabolism of the plant. The role of these compounds in plants has been a controversial subject over the last 100 years. They were first believed to be waste products but are now generally regarded as defence compounds and in some cases they may have metabolic functions such as nitrogen transport and storage (Swain, 1977). They are also now known to play a role as attractants for pollination and seed dispersal.

Secondary metabolites have been known to play a major role in host resistance to fungi. These compounds may be present already in the plant, be activated by wounding, or be synthesized *de novo* in response to infection. Such compounds are usually synthesized or stored at strategically important sites, such as the cell wall, or in plant parts valuable for reproduction and survival, such as fruits, roots, seeds, and flowers. The secondary metabolites involved in interactions between plants and fungal pathogens are often phenolic compounds.

Phenolic compounds accumulate rapidly during host-parasite interactions and have been known to mediate disease suppression through inactivation of fungal enzymes or strengthening of plant structural components. Phenolic compounds are known to be toxic to a great number of microorganisms. In fact, many biocides used in pest control have phenolic groups in their structures. Phenolics are widely distributed in higher plants. The variety of structures provide a basis for a degree of specificity as antimicrobial agents.

Phenolic compounds are recognized by their hydrophilic nature and by their common origin from the precursor shikimic acid via the shikimic acid pathway. All phenolic compounds have an aromatic ring bearing one or more hydroxyl (-OH) substituents. Since most occur with sugar as glycosides, they are water-soluble and usually found in the cell vacuole. Of the more than 1000 naturally occurring phenolic compounds, the flavonoids are the largest group.

Approximately 2% of all carbon photosynthesized by plants is converted into flavonoids and related compounds (Markham, 1982). The basic structure of most flavonoids is a flavonoid glycoside consisting of an aglycone attached to a sugar moiety. The aglycone contains 15 carbon atoms arranged in a C6-C3-C6 configuration, i.e. two aromatic rings linked by a three carbon unit which may or may not form a third ring. The sugar moiety may be attached to hydroxyl groups of the aglycone by an acid-labile hemiacetal bond, and thus form a flavonoid *O*-glycoside. Less commonly, the sugar may be attached directly to the benzene nucleus, usually at the 6- or 8-C positions, by a carbon-carbon bond which is acid

resistant. This type of bonding is characteristic of flavonoid C-glycosides.

In maize, there are 25 different genes known to affect flavonoid biosynthesis with variation existing in terms of compounds, concentrations, tissue specificities, and patterns within a tissue (Styles and Ceska, 1975, 1977). Many of these genes are believed to be under the genetic control of the P locus (Styles and Ceska, 1977).

Flavonoid compounds and other phenolics have been shown to be possible resistance factors in other crops and with different species of *Fusarium*. In tomato, resistance to *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) is associated with incorporation of phenolic and lignin-like materials into cell wall appositions (papillae) and into modified cortical cell walls in roots. Application of the herbicide glyphosate (N-phosphonomethyl-glycine) inhibits the shikimic acid pathway and lowers resistance in normally resistant cultivars (Brammall and Higgins, 1988). This is believed to be due to induced inhibition of lignification and phenolic deposition in cell wall appositions. Phenolic compounds have been found to inhibit germination of fungal spores and to reduce hyphal growth of FORL (Kasenberg and Traquair, 1988). Ismail et al. (1987) tested the effect of 12 phenolic compounds on the germination and growth of FORL and *A. flavus* and reported that toxicity increased with the molecular weight of the phenolic compounds and that phenolics with hydroxyl groups arranged in the ortho-position to one another on the benzene nucleus were the most toxic.

Phenolic compounds, especially flavonoids, may not have any anti-fungal

or anti-microbial activity *per se*, but may become active when oxidized by phenol-oxidizing enzymes or phenolases. Such enzymes catalyze the reaction:

(o-diphenyl: O_2 oxidoreductases)

$$o\text{-diphenol} + 1/2O_2 \text{ ---> } o\text{-quinone} + H_2O \qquad (1)$$

The products of such a reaction, i.e. quinones, react with proteins and intracellular amino acids. Quinones inhibit enzymes by non-specific binding to the enzymes, by competing or reacting with the substrate or cofactor, by producing H_2O_2 during the oxidation, by oxidizing sulphydryl groups controlling tertiary enzyme structure, and by complexing metal ions which participate in the catalysis (McClure, 1975; Kosuge, 1969).

Since phenolases are usually localized in plastids and mitochondria of healthy tissue, they are released and form quinones with flavonoids and other polyphenols when tissues age or are wounded by pathogens. This may be an important resistance mechanism when invading fungi produce toxins and extracelluar enzymes that kill host tissue in advance of fungal growth. In addition, the resulting products could have antifungal action by polymerizing and forming a protective seal on cell walls. Once a phenolic substrate is oxidized, the quinone or radicals formed may mediate oxidation of other phenolics which are not a substrate for the given enzyme. Thus, indirectly many phenolics and other compounds which are not substrates can be oxidized (Leatham et al., 1980). In the host itself, there is a rapid reduction of *o*-quinones to prevent damage to the host (Kosuge, 1969).

Two of the major phenol-oxidizing enzymes are peroxidase (PO) and polyphenoloxidase (PPO). Peroxidase catalyses the oxidation of phenolic compounds in the presence of the oxidant, hydrogen peroxide, while PPO catalyses the oxidation of phenolics in the presence of oxygen. The levels and activity of these enzymes have been found to increase when plants are inoculated with a pathogen (Friend, 1977). Gentile et al. (1988) found that activity of PO and PPO increased in tomato roots, stems, and leaves following inoculation with both pathogenic and non-pathogenic *Fusarium* species or treatment with heat or chloroform. Such increases in phenolase activity may not result from *de novo* synthesis; they may result from increased solubilization of phenolase from particulate material or from activation of latent phenolase (Kosuge, 1969).

A characteristic of phenolase activity is the formation of brown pigmentation. Such browning is a common degradative phenomenon of injured or senescent tissue. It usually indicates that damage to cells has decompartmentalized enzymes and their phenolic substrates. The resulting polymerization of phenolics and proteins results in the formation of a brown pigmentation. In vascular wilt infections caused by various *Fusarium* pathogens vascular browning is indicative of cell damage and is more extensive in susceptible plants than in resistant plants (MacHardy and Beckman, 1981). However, in some plants, extensive vascular browning is indicative of a high content of phenolic substrates (Levings and Stuber, 1971) and therefore possibly of high resistance potential.

4.7. Resistance of Maize to *Fusarium graminearum* Ear Rot

4.7.1 Types of Resistance

Schroeder and Christensen (1963) have summarized resistance to *F. graminearum* in wheat to fall into two categories: Type I resistance, or resistance to initial infection; and Type II resistance, or resistance to hyphal invasion. Host responses to *F. graminearum* infection of maize kernels have been found to include false plasmolysis ahead of the hyphae, tannin accumulation in the rachilla and rachis, and tylose accumulation in the xylem (Smart, 1987). In tomato, the cultivar 'CR6' possesses a single dominant gene for resistance to FORL. This resistance is believed to be in part due to modification of the cortical cell wall, suberization of the hypodermis and growth of papillae containing phenolics and lignin-like materials (Brammall and Higgins, 1988). Carnation varieties resistant to *F. oxysporum* f.sp. *dianthi*, have been found to respond to infection by rapid and extensive gelation of the xylem vessels in advance of fungal colonization resulting in physical containment of the pathogen (Harling and Taylor, 1985).

The role, if any, of *F. graminearum* mycotoxins in disease development is not clear. They may aid in overcoming host resistance or may play a role in symptom induction. *Fusarium* toxins are not host-specific and may affect a wide range of plants (Drysdale, 1984). They may have various effects on plant tissues, such as wilting, chlorosis, necrosis, inhibition of indoleacetic acid, and inhibition of growth of whole plants or organs (Brian et al., 1961). Zearalenone has been reported to kill cereal seedlings and inhibit germination (Brodnik, 1975) and to

interact with the plasma membrane and inhibit ATPase activity (Vianello and Macri, 1978). Deoxynivalenol, on the other hand, has been found to be a potent inhibitor of protein synthesis and to inhibit incorporation of leucine in wheat and maize (Hart et al., 1987; Casale and Hart, 1988). Hart et al. (1987) reported that DON inhibited protein synthesis in both maize leaf discs and cell-free systems, at concentrations less than that found in infected tissue. This indicates that DON may act directly on the protein synthetic mechanism. This has important implications since such a toxin could overcome or delay host defenses, including induced defenses which may be under transcriptional or translational control. However, sensitivity to DON was not found in all susceptible maize lines tested by Hart et al. (1987). The sensitivity to DON was not a factor in highly resistant lines but was more important in lines with intermediate resistance. Some other high-level resistance mechanism must exist.

The fungal metabolite ergosterol has been found to be a good indicator of *Fusarium* presence in maize (Seitz and Bechtel, 1985). It has been used for *F. graminearum* studies since a significant correlation ($r=0.89$) has been reported between ergosterol and DON contents in toothpick-inoculated maize ears (Miller et al., 1983b). Ergosterol and fungal propagule counts have been observed to increase rapidly until six weeks after inoculation and then to decline rapidly, possibly due to lack of moisture and nutrients or to end-product inhibition by DON (Miller et al., 1983b). In the same study, DON was found to increase to approximately 580 ppm in the first 6 weeks then slowly decline to 20 ppm. No

appreciable amount of zearalenone (10 ppm) was found until 9 weeks after inoculation.

The decrease in DON content as the fungal population in the ear declined has been suggested to be a result of the action of maize enzymes that continue to metabolize and degrade toxins (Miller et al., 1983b), and/or of degradation by bacteria and other microorganisms (Ueno et al., 1983). Similar decreases in DON also have been observed in wheat (Scott et al., 1984; Miller and Young, 1985; Miller and Arnison, 1986). An important observation is that in both maize and wheat, this decrease in DON concentration most often occurs in varieties with higher resistance. Miller et al. (1985) found that susceptible cultivars of wheat, rye, and triticale have higher levels of ergosterol and DON than resistant cultivars although appreciable amounts of ergosterol were found in resistant cultivars. They suggested that susceptible cultivars have lower resistance to fungal invasion (Type 2 resistance), but that resistant cultivars have a third type of resistance, the prevention of DON synthesis and the promotion of DON degradation.

4.7.2 *Fusarium graminearum* Ear Rot Resistance Factors in Maize

Various factors have been implicated in host resistance to ear rot of maize. Three primary factors are: ear declination, husk coverage and tightness, and inherent resistance including chemical and physiological resistance factors (Koehler, 1959). Enerson and Hunter (1980), after evaluating resistance of 13 maize hybrids, reported that upright ears averaged 108% more ear mold than

pendant ears and ears with tight husks averaged 241% more mold than ears with loose husks. However, they speculated that husk tightness may be due to fungal mycelium binding the husks to the ears, which would delay drying, producing an environment conducive to further fungal development.

4.7.3 The Potential of Silk Resistance Factors

If fungal infection of maize ears is mainly via the silk and silk channel, it is reasonable to assume that an initial barrier to fungal growth and establishment may exist in the silk of resistant genotypes.

Tight husks can favour silk balling; a condition resulting when silk at the apex of the ear, which is the last to elongate, can not or does not grow through the silk channel which is already filled with silk from the rest of the ear. Instead, the silk from the apex piles up in layers to form an N-shaped ball. These silks are often not pollinated and the tip of the ear does not get fertilized ("tip blanking"). It has been suggested that these silk balls may form a physical barrier to ear penetration by the earworm and by fungal pathogens (Luckmann et al., 1964).

The possibility of chemical resistance factors in the silk has not been explored. Silk tissue possesses a wide array of volatile compounds and flavonoids, some of which are active against insects.

4.7.3.1 Flavonoids of Maize Silk

Although no studies have examined the possible role of flavonoids in

resistance to *F. graminearum*, a number of studies have established that phenolic compounds including flavonoids are abundant both in the kernels and in the silk. Some of these have been correlated with resistance to various insects. As well, silk contains a number of volatile compounds which have been found to be attractants for insects that feed upon the silks, in particular adult rootworms, *Diabrotica* species (Prystupa et al., 1988). Some volatiles of the husks, kernels and silks have been found to inhibit the growth of *A. flavus* on maize and aflatoxin accumulation (Wilson, 1987a).

By far, the greatest amount of work has concentrated on a flavonoid compound in the silks which is believed to confer resistance to the earworm, *Heliothis zeae* Boddie. The Mexican landrace 'Zapalote Chico' (P.I. 217413) frequently has been reported to have a high level of resistance to the corn earworm. It was first believed that a "lethal" factor existed in silks of this dent field maize (Walter, 1957). However, when lyophilized silk powder was incorporated into the diet of earworm larvae it was found that a feeding deterrent or growth inhibitor was present in the silk (Wiseman et al., 1985). In 1979, Waiss et al. isolated the flavonoid believed to be responsible for this. It is a yellow flavonoid C-glycoside, rhamnosyl-6-C-(4-ketofucosyl)-5,7,3',4' tetrahydroxyflavone, or maysin. Incorporation of maysin into the diet of earworm larvae resulted in a decrease in larval growth, delayed pupation, and decreased weight gain but did not kill the larvae (Waiss et al., 1979; Reese et al., 1982). This suggested that maysin acts as a growth inhibitor or antifeedant rather than as an acute toxicant. Similar effects

have been observed when Zapalote Chico silks are incorporated into diets of fall armyworms, *Spodoptera frugiperda*, J.E. Smith (Wiseman and Widstrom, 1986). However, it has been shown that maysin is not the only factor responsible for resistance to these insects (Wiseman et al., 1985; Henson et al., 1984).

Environmental effects have been reported for maysin contents, with different environments apparently more conducive to the production of higher levels (Widstrom et al., 1982). Broad-sense heritability of maysin concentration has been estimated to be 79.8%, and in an S_1 selection procedure with a winter nursery and 10% selection intensity, the expected genetic response has been estimated to be 0.232 mg/ml or 25.7% per cycle (Henson et al., 1984). A single dominant gene may condition low maysin level in some inbred lines of maize (Widstrom et al., 1989).

4.7.3.2 Silk Browning

Silks of some genotypes undergo rapid browning (5-10 minutes) when ground in the presence of water or when cut in preparation for pollination. This indicates the presence of polyphenolic oxidation by phenolases. The silks of Zapalote Chico have been reported to undergo such browning, however it is not known if silk browning plays a role in resistance to the earworm (Byrne et al., 1989; Waiss et al., 1979).

Levings and Stuber (1971) found that both browning and non-browning genotypes contained the enzymes required for browning, but non-browning silks

lack phenol substrates for these enzymes. Further analysis indicated that the major enzyme responsible was polyphenoloxidase. Additional analysis of the phenol extracts revealed that browning genotypes had at least 12 more phenolic compounds than non-browning genotypes and that some of these compounds were C-glycoslfylavones. Non-browning silks also have been found to have higher pH than browning silks (Byrne et al., 1989).

The inheritance of silk browning has been studied. Crosses between browning and non-browning genotypes indicate that browning is conditioned by one completely dominant gene (Levings and Stuber, 1971). The dominant allele for browning has been given the symbol *Fv* while the recessive allele was given the symbol *fv*. It was suggested that flavonoid synthesis is blocked in non-browning *fvfv* genotypes. The block is not complete for all flavonoids; anthocyanins were present in the non-browning silks. The block is limited to silks since numerous flavonoids were found in leaves and tassels of non-browning genotypes. Silk browning has been found to be related to cob colour; almost all browning genotypes also have red cobs while non-browning genotypes have white cobs (Coe, 1985). These traits are controlled by two closely linked loci, both of which exhibit complete dominance (Coe and Han, 1986; Widstrom, 1987; Han and Coe, 1987).

4.8 Breeding for Increased Resistance to *Fusarium graminearum* Ear Rot

Genetic resistance is the first line of defense against plant pests. Disease resistance may be conditioned by one to many genes. This leads to a classification of the inheritance of resistance into three broad categories: oligogenic or Mendelian (qualitative traits); polygenic (quantitative traits); and extrachromosomal.

4.8.1 Screening for Resistant Germplasm

Due to the sporadic and unpredictable nature of *F. graminearum* epidemics, artificial inoculation techniques are needed to enhance incubation and infection and to overcome variability of infection during years when natural contamination is too low to identify genotype differences. Unfortunately, this has been a major limiting factor in breeding for resistance since methods of inoculation and screening have not been consistent. A diversity of results, many of which are conflicting, have been obtained with different inoculation and postinoculation treatments. Growth stage of the host, inoculum type, position of inoculum, wounding of the ear, and weather are among the variable factors (Ullstrup, 1970).

Two types of inoculum have been used: colonized substrates and spore suspensions. Colonized substrates include toothpicks or cereal kernels which are usually overgrown with mycelium. Both may be inserted into the silk channel near

the tip of the ear, however colonized toothpicks are usually inserted into the centre of the ear after artificial wounding (Ullstrup, 1970; Fajemisin et al., 1987; Sutton and Baliko, 1981; Enerson and Hunter, 1980; Koehler, 1959). Spore suspensions may be injected directly into the silk channel or sprayed onto exposed silks, or a pipecleaner impregnated with the spore suspension may be inserted into the centre of the ear after artificial wounding (Ullstrup, 1970; Enerson and Hunter, 1980; Fajemisin et al., 1987).

The use of colonized substrates does not simulate natural infection since the inoculum source is not spores. If a colonized toothpick or cereal kernel is placed in the silk channel it can be displaced away from the ear tip as silks elongate. This can result in a low level of infection (Sutton and Baliko, 1981). High levels of infection are obtained when a colonized toothpick is inserted into the ear. However, even normally resistant genotypes may become infected and it becomes difficult to distinguish them from susceptible genotypes (Sutton and Baliko, 1981). The introduction of colonized toothpicks or spore-impregnated pipecleaners directly into wounded kernels circumvents any physical barriers or resistance factors outside the kernel that could otherwise exclude the pathogen.

Spraying of a spore suspension on silks closely simulates natural infection because the inoculum source is spores and because the host is not wounded. This technique augments natural infection and differs only by providing for a greater volume, higher concentration, specific time of application, and more uniform distribution of inoculum than in natural infection. Spraying of a spore

suspension on silks is often followed by bagging of the ear to prevent desiccation. However, bagging often results in excess water on ear surfaces, encouraging bacterial growth and reducing the level of infection. Injection of spore suspensions into the silk channel can give better results than spraying and subsequent bagging. Infection has been found to be greatest when inoculation is made 0.5-1.0 cm above the cob tip (Sutton and Baliko, 1981). Ullstrup (1970) reported that the amount of infection was the same when the spore suspension was approximately 450,000 spores/ml as when it was 450 spores/ml.

Since some forms of host resistance function only against certain isolates or races of a pathogen it is important to consider which isolate(s) will be used in screening for resistance. Mesterhazy and Kovacs (1986) found a significant genotype X isolate interaction for lines with intermediate resistance but not for lines that had high resistance or susceptibility. They concluded that more than one isolate should be used if exact differences between genotypes are desired but this was not necessary for general screenings. Atlin et al. (1983) tested six different isolates on six different hybrids and also concluded that genotype X isolate interactions were not large enough to warrant the use of more than one isolate for resistance breeding.

Environmental effects and genotype X environment interactions also have been found to be significant factors and may affect studies of inheritance of resistance. Hunter et al (1986) reported significant genotype by environment interactions for 12 short-season hybrids grown in five different environments.

However, they suggested that some of this interaction may have been due to the use of different isolates of *F. graminearum* at some of the sites.

Various methods have been used to evaluate the severity of ear rot after inoculation, including whole-row ratings (Koehler, 1942), kernel sorting (Koehler, 1959), kernel plating (Koehler, 1959), and ear ratings using visual rating scales (Ullstrup, 1970; Sutton and Baliko, 1981; Enerson and Hunter, 1980). The latter method is the most widely used since it is the least time consuming, increments on the scale are easily discernable, data recording is simplified, and it allows for rapid screening of a large number of genotypes.

4.8.2 Inheritance of Resistance

Studies have suggested that resistance to *F. graminearum* ear rot is inherited in a quantitative manner, is associated with additive genetic effects, and is partially dominant. However, the lack of uniformity in inoculation and screening methods make comparisons among studies difficult. In addition, most studies have concentrated on resistance to infection via wounds rather than via the silk.

Initial studies on inheritance to ear rot were concentrated on *F. moniliforme*. Boling and Grogan (1965) assessed resistance to *F. moniliforme* in two inbred lines, one resistant and one susceptible. Maize ears were inoculated by shooting a spore covered BB pellet into the ear about 5 cm below the tip. By gene effects analysis, the average degree of dominance was estimated to be approximately 0.5, and the number of gene pairs involved was estimated to be 1.27 to 1.42. Additive

gene effects, and additive X dominance epistatic gene effects were reported to be important. King and Scott (1981) relied on natural infection of *F. moniliforme* to assess resistance in crosses among resistant and susceptible lines. They reported that the progeny of crosses between two resistant parents had 11% infection, progeny of crosses between two susceptible parents had 55% infection and progeny of crosses between susceptible and resistant parents had 33% infection.

Studies concentrating on inheritance of resistance to *F. graminearum* have revealed similar findings to those with *F. moniliforme*. Odiemah and Manninger (1982) carried out a 7 X 7 diallel and a generation means analysis on the six generations: P1, P2, F_1, F_2, BC1, and BC2. Two methods of artificial inoculation were used: injection of spore suspensions into the silk channel, and insertion of colonized toothpicks into the centre of the ear. Results indicated that additive gene action was predominant, and that there was some dominance and additive X dominance epistatic action. These results were confirmed by Gendolf et al. (1986) who also found additivity to be the major gene effect in a generation means analysis of two resistant and two susceptible lines. As well, all F_1's were rated as intermediate to the parents suggesting partial dominance. Gendolf et al. (1986) also reported a maternal influence in one set of reciprocal crosses. Odiemah and Manninger (1982) found resistance to be partially dominant with some lines having more dominant genes than others.

Estimations of combining ability indicated that genetic variation for resistance is associated with highly significant general combining ability effects but

not with specific combining ability effects (Hart et al., 1984; Odiemah and Manninger, 1982). This also has been reported for *opaque-2* maize lines which are on average much more susceptible to ear rot (Szel, 1984). Chiang et al. (1987) carried out an 8 X 8 diallel using a range of susceptible to resistant lines. They inoculated by placing an infected toothpick in the silk channel at the tip of the ear. Again, additive gene action was reported to be the most important source of genetic variation. In addition, at least three groups of genes showed some degree of dominance, narrow-sense heritability was estimated to be 68.78%, and susceptibility was found to be a recessive character.

4.8.3 Sources of Resistant Germplasm

It is possible to find genotypes with resistance to *F. graminearum* ear rot, but no lines that are entirely resistant have been found. Most germplasm is susceptible. In a recent screening of many inbred lines, Dr. A. Bolton and Dr. R.I. Hamilton of the Plant Research Center, Agriculture Canada, Ottawa have revealed an inbred line with high resistance (person. comm.). This line, 'CO272', exhibits almost complete resistance when artificially inoculated with a spore suspension injected into the silk channel. Crosses of 'CO272' with susceptible lines suggest that inheritance of resistance in this line may be qualitative, and dominant. This line could prove to be a unique source of resistant germplasm. The details of the inheritance of resistance in this line and others exhibiting high resistance are not known.

4.9 Hypotheses

The overall hypothesis of the research was that initial barriers to infection of maize ears by *F. graminearum* were present in the silks of maize and were heritable. More specific hypotheses were:

- differences in the amount of infection of maize ears after artificial inoculation of the silk channel can be explained by differences in silk resistance

- susceptibility to *F. graminearum* infection via the silk and/or silk channel changes as the silk senesces

- a chemical resistance mechanism exists in the silk of resistant genotypes

- phenolic compounds, such as the flavone C-glycoside maysin, are present in the silk of resistant genotypes, are involved in resistance, and exist in higher concentrations in resistant genotypes

- resistance to *F. graminearum* infection via the silk and/or silk channel is not isolate-specific

- resistance to *F. graminearum* infection via the silk and/or silk channel, such as that expressed by the highly resistant inbred 'CO272', is heritable and due to one

or a few dominant genes

- exotic genotypes of maize with resistance to other ear-rotting organisms or to insects which feed upon the silk also have resistance to *F. graminearum* silk infection and may be new sources of resistant germplasm

- deoxynivalenol contents of *F. graminearum* infected maize ears are correlated to visual evaluations of the amount of infection

4.10 Objectives

The objectives of this research were:

- to compare the resistance of several genotypes to silk infection by *F. graminearum* both in the field and *in vitro*

- to determine if differences in silk resistance measured *in vitro* were associated with resistance to infection in the field

- to compare the amount of infection that develops on maize ears inoculated at different stages of silk development or age to determine if susceptibility changes with silk age

- to determine the stage of silk development in which ears should be artificially inoculated to achieve maximum differentiation between genotypes

- to examine the major phenolic constituents of maize silks to determine if resistant genotypes have higher levels of some compounds or if levels of phenolic compounds change with infection

- to compare the resistance of different genotypes to infection by three different isolates of *F. graminearum* to determine if resistance was isolate specific

- to investigate the mode of inheritance of resistance to *F. graminearum* silk infection with emphasis on the highly resistant line 'CO272' by examining the resistance of F_1, F_2, and backcross generations of two resistant X susceptible crosses, and by conducting a complete 12 X 12 diallel cross

- to assess the potential of the inbred line 'CO272', and others exhibiting high resistance, in future breeding programs

- to assess the resistance of exotic maize lines with resistance to *A. flavus* and to the earworm *H. zeae*

- to determine if deoxynivalenol content of infected maize ears was correlated to

visual evaluations of resistance to silk infection

Several objectives are addressed in each of the following eight chapters and in the general discussion chapter.

Preface to Chapter 5

The experiments reported in Chapter 5 were designed to determine if there are genotypic differences in the resistance of silk to *F. graminearum*. Results of an *in vitro* test of detached silk tissue were compared to field evaluations of resistance. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table and figure is presented on the page following the first referral to it.

CHAPTER 5

GENOTYPIC DIFFERENCES IN THE RESISTANCE OF MAIZE SILK TO FUSARIUM GRAMINEARUM

5.1 Abstract

Detached maize silk tissue was used in a *in vitro* test for *Fusarium graminearum* resistance. The test consisted of incubating, in a petri dish, 1-g samples of silk tissue sprayed with a spore suspension of *F. graminearum*. Degradation of the tissue by *F. graminearum* was measured by the percentage reduction in dry weight relative to a control silk sample sprayed with sterile water. Silk tissue from five field grown inbreds was tested with incubation periods of 24, 72, and 168 hrs. Degradation increased with time and significant genotypic differences were found among inbreds by 168 hrs. Exposed silk tissue (from outside the husk) was less degraded than silk channel silk tissue. Silk channel silk tissue was tested from 12 inbreds grown in the field and in a greenhouse and incubated for the full 168 hrs. Greenhouse grown tissue was more readily degraded than field grown tissue, but percentage reduction of dry weight of field and greenhouse grown tissue was correlated ($r=0.94$). Field evaluations of resistance to silk infection were conducted on the same 12 inbreds. A spore suspension was injected into the silk channel of individual ears and, at harvest, the spread of infection from the ear tip was rated. Both field and greenhouse *in vitro* tests were correlated with field evaluations of resistance ($r= 0.85$ and 0.80,

respectively) and the most resistant lines had the lowest degradation of silk tissue. These results suggested that resistance mechanisms exist in the silk of some genotypes.

5.2 Introduction

Entry into maize or corn (*Zea mays* L.) ears via the silk and/or the silk channel is believed to be a major mode of entrance of various ear-rotting pathogens including some *Aspergillus* species and *Fusarium* species such as *F. graminearum* Schwabe [sexual state: *Gibberella zeae* (Schw.) Petch] (Koehler, 1942; Marsh and Payne, 1984). Both of these pathogens produce mycotoxins which may contaminate the grain. In Quebec and Ontario as well as parts of the northeastern U.S., Europe, Asia, and Africa, *F. graminearum* is a major problem to both maize and wheat crops (Sutton, 1982). In maize, *F. graminearum* causes both stalk rot and ear rot.

The major symptom of *F. graminearum* ear rot is a reddish mold on the kernels often causing the husks to adhere tightly. The disease cycle of *F. graminearum* is not well known due to the sporadic occurrence of epidemics. Infected host debris seems to be the major source of inoculum (Sutton, 1982). Aerial dispersion is the major means of inoculum dispersal although insects have been known to be vectors as well (Sutton, 1982). *F. graminearum* can enter the ear by spores germinating on the silks and then growing down the silks to the kernels and rachis, with the silks possibly serving as substrates for fungal growth

(Koehler, 1942; Hesseltine and Bothast, 1977). Infection then spreads down the ear from the tip with both the kernels and rachis being infected, thus resulting in a yield loss.

Since silk is usually the first tissue to come into contact with the pathogen, it is reasonable to hypothesize that in maize lines exhibiting no infection, initial resistance barriers to infection may exist in the silk or the silk channel. Silk varies in abundance, length, and colour according to genotype. Genotypic differences presumably exist for biochemical and physiological factors which may have an affect on any resistance characteristics.

The purposes of the present study were to: (1) determine if genotypic differences exist in the resistance of silk to *F. graminearum*; and (2), determine if these differences are correlated with field resistance to infection via the silk and/or silk channel.

5.3 Materials and Methods

To test for resistance of silk to *F. graminearum* an *in vitro* test of detached silk tissue was conducted. Each genotype was grown in a 12 plant row (3.8 m long, 76 cm between rows) on a sandy loam soil at the Plant Research Centre, Agriculture Canada, Ottawa. When the silk was elongated and pollinated (5 to 7 days post-silk emergence), the primary ears from the center ten plants in the plot were harvested. From each ear, two 1-g samples of a cross-section of the silk mass were cut from the silk channel (the area between the cob tip and the point

where the silk emerges from the husk) or from the exposed silk (sild that had grown out of the husk). Each sample was placed in a petri dish containing two pieces of filter paper moistened with 3 ml of sterile water. One of the two samples was sprayed with 3 ml of a *F. graminearum* spore suspension (5×10^5 spores/ml) with an atomizer and the other was sprayed with 3 ml of sterile water. Ten replicates (10 ears) were used for each genotype. The petri dishes were placed in a randomized arrangement in a growth chamber and incubated at 25°C and 95% relative humidity. Following incubation, samples were dried in an oven at 80°C for 48 hrs and weighed. The percentage dry weight reduction of the inoculated silk with respect to the water control silk [[(control dry wt. - inoculated dry wt.)/control dry wt.] x 100] was measured and taken as an indicator of the amount of tissue degraded by the pathogen. This technique is similar to that used by Naik and Busch (1978) for testing the effects of maize pollen on *F. graminearum* growth.

Cultures of *F. graminearum* were maintained on potato dextrose agar (PDA). Inoculum for this study was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 pprn ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing

mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour at 4 hour intervals for eight days. Spore counts in these flasks were approximately 2 million/ml.

In 1990, to study silk channel silk vs. exposed silk assimilation, five inbreds ('CO272', 'CO266', 'F7', 'F2', and 'A641') were grown as previously in single-row plots in a completely randomized design. Thirty ears were harvested from each inbred and two 1-g samples were cut from both the silk channel silk and the exposed silk tissue and placed in petri dishes. Thirty petri dishes of each tissue type were sprayed with a spore suspension and the other 30 with sterile water as per the *in vitro* test described above. Each group of 30 petri dishes was then divided into three groups of ten petri dishes each and incubated for 24, 72, or 168 hrs.

In a separate experiment, also conducted in 1990, twelve inbreds including the five used above and 'CO265', 'CO282', 'CO267', 'CK44', 'Mo17', 'CO325', and 'CO317' were grown in a randomized complete block design with four blocks. Two-row plots of each genotype were randomized in each block. One row of each two-row plot was used for the *in vitro* test of detached silk tissue. A total of 10 primary ears per inbred were randomly sampled from the four blocks. The *in vitro* test was conducted as described above except that all samples were incubated for the full 168 hrs and only silk channel silk tissue was used.

The second row of each plot was used for field evaluations of resistance.

Primary ears of the center ten plants were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of a 2 X 10^6 spores/ml spore suspension into the silk channel of each ear (Appendix 1) when the silks were elongated, pollinated, and had some tip browning (approximately 6 days post-silk emergence). After inoculation, plots were irrigated for four weeks at a rate of 2-5 mm twice daily. Evaluations for resistance to spread of infection made in mid-October used a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected (Appendix 2). Ears were rated individually and a mean rating was calculated for each row.

Statistical analysis of disease ratings was carried out using Friedman's two-way analysis of variance by ranks and the corresponding multiple comparison test (Daniel, 1990), but analysis of *in vitro* results was made using parametric statistics (Steel and Torrie, 1980). Correlations between *in vitro* tests and field evaluations were examined using Spearman's rank correlation (Daniel, 1990).

In February and March of 1991, the *in vitro* method described above was applied to silk channel silk tissue collected from greenhouse grown plants of the same 12 inbreds. Plants were grown singly in large (23 cm) pots arranged in a completely randomized design. Five replications per genotype were used. Greenhouse conditions consisted of temperatures of 25°C (day) and 18°C (night) with the photoperiod supplemented to 16 hr with low pressure sodium lights.

5.4 Results and Discussion

Silk of the five inbreds differed significantly with respect to the amount of tissue degradation by *F. graminearum* measured as the percentage reduction in silk dry weight relative to the water control (Fig. 5.1). No significant difference between genotypes was apparent with silk from the silk channel after 24 hrs incubation. Little degradation had occurred by 72 hrs for 'CO272, 'F7', and 'A641', however 'F2' had been 15.6% degraded and 'CO266' 21%. Significant differences were apparent between all five inbreds after 168 hrs incubation with the least degraded 'CO272' followed by 'A641', 'F7', 'F2', and 'CO266'. Similar results were obtained when exposed silk tissue was used. Exposed silk tissue of 'CO272', 'F7', and 'A641' was more degraded by 72 hrs than silk channel tissue of the same genotypes, but by 168 hrs, exposed silk tissue from all inbreds was less degraded than silk channel silk tissue. This difference between silk-channel silk and exposed silk is probably due to the greater environmental influence, such as light, lower water content and advanced stage of senescence in exposed tissue.

Significant differences in the amount of silk tissue degraded after 168 hrs incubation were found among the 12 inbreds for both field-grown and greenhouse-grown silk tissue (Table 5.1). Although degradation of field-grown silk was highly correlated to greenhouse-grown silk ($r=0.94$, $p<0.01$, Pearson), the latter was almost three times greater for 'CO272' and approximately 1.5 times greater for the remaining inbreds. Greenhouse-grown tissue was more susceptible to *F. graminearum*. Lower light intensities decrease resistance of other plants to

FIGURE 5.1

Percentage reduction in silk dry weight after 24, 72 and 168 hrs. incubation with *F. graminearum*. SD= standard deviation.

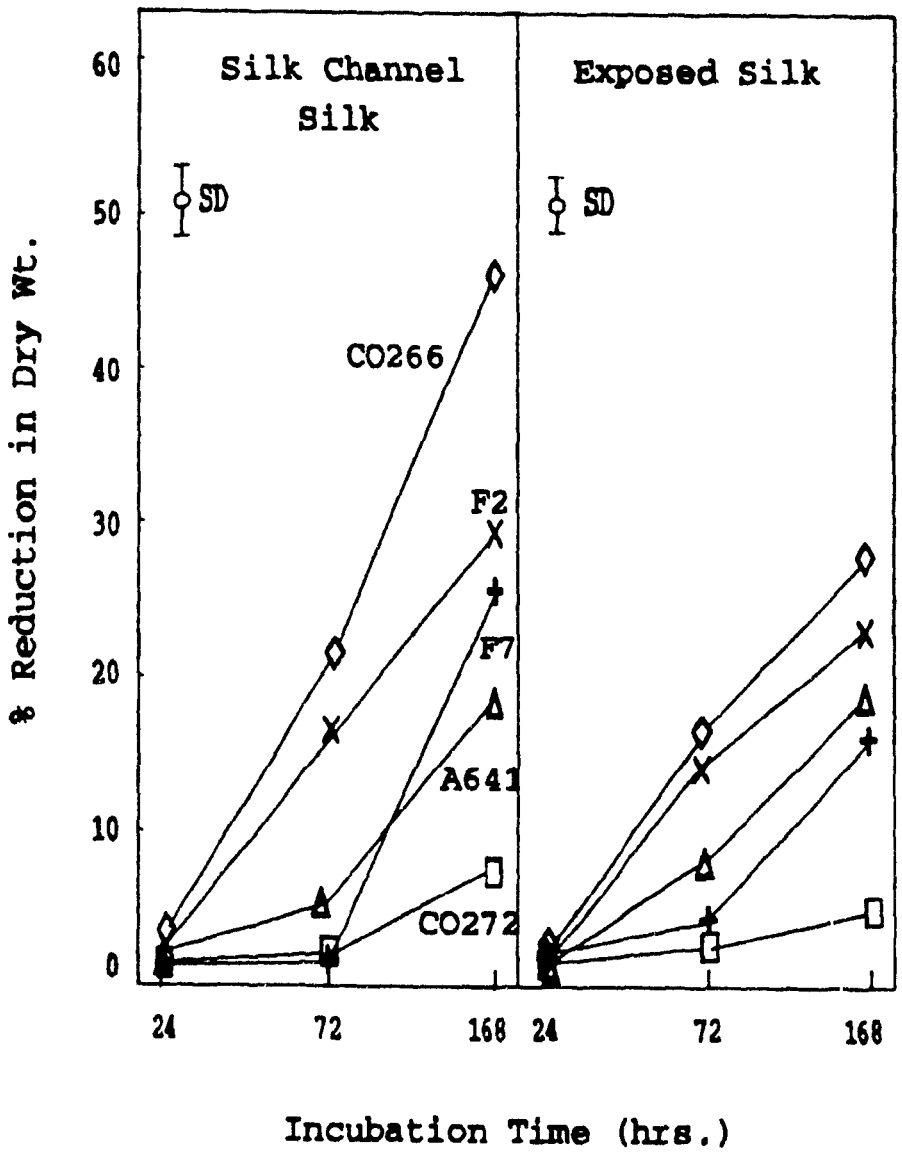

Silk Channel Silk
Exposed Silk
SD
SD
CO266
F2
F7
A641
CO272
60
50
40
30
20
10
0
% Reduction in Dry Wt.
24
72
168
24
72
168
Incubation Time (hrs.)

Table 5.1. Percentage of field grown and greenhouse grown silk tissue degrded by *F. graminearum* after 168 hrs incubation.

	Percentage Reduction in Dry Weight[a]	
Inbred	Field Tissue	Greenhouse Tissue
CO272	7.2 a	23.7 a
A641	18.5 b	32.1 b
F7	24.9 b	35.5 bc
CO325	26.4 bc	33.3 b
CO265	27.2 bc	41.8 c
F2	28.0 bc	43.2 cd
CO282	32.5 cd	49.2 de
CK44	33.5 cd	51.5 de
MO17	36.0 de	49.7 de
CO317	36.8 de	44.2 d
CO267	43.8 e	53.9 e
CO266	45.2 e	59.1 f

[a] Percentage reduction of a 1-g sample of silk tissue due to fungal assimilation during 168 hrs of incubation at 25°C, 95% RH. Mean of 10 replicates for field tissue and 5 replicates for greenhouse tissue. Means followed by the same letter for a given tissue are not significantly different at the 0.05 level by Duncan's multiple range test.

diseases (Bell, 1981) including maize resistance to anthracnose, *Colletotrichum graminicola* (Ces.) G.W. Wils. (Hammerschmidt and Nicholson, 1977). This suggested that resistance may be linked to the presence of biochemical factors, such as phenolic compounds, whose biosynthesis is light-activated (Bell, 1981).

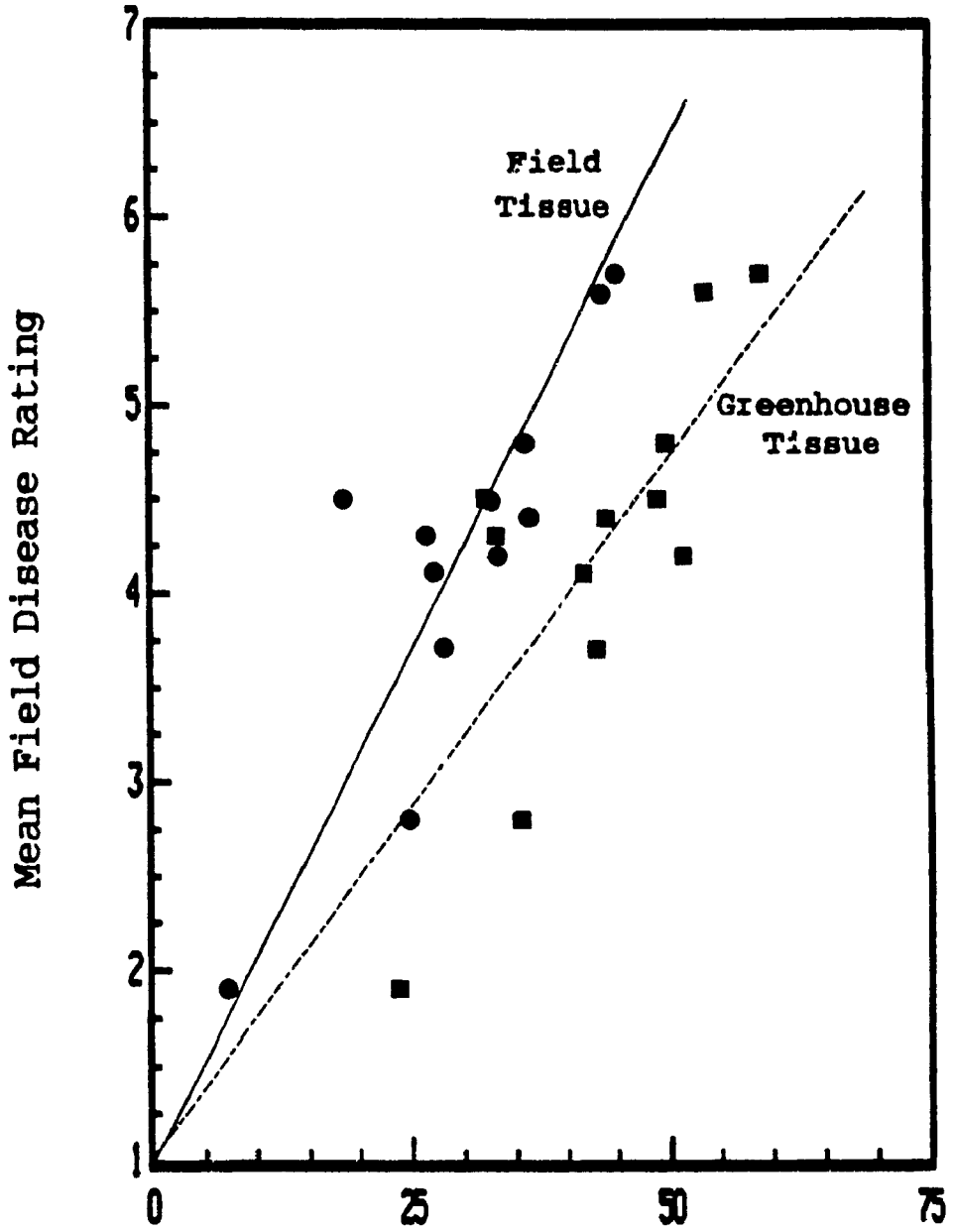
The amount of tissue degraded was correlated to field resistance ratings for both field silk tissue ($r=0.85$, $p<0.01$) and greenhouse silk tissue ($r=0.80$, $p<0.01$) (Fig. 5.2). This suggested that silk plays an important role in resistance to *F. graminearum* ear rot. This role still has to be determined. It may involve morphological, physiological, and/or biochemical resistance factors. It also may be associated with silk age.

The *in vitro* test of detached silk tissue could be an additional method of screening germplasm for resistance to *F. graminearum*. The method was rapid and allowed evaluation of silk resistance as soon as silk appeared and could be adapted for a non-destructive silk evaluation with the ear shoots still available for subsequent pollination and seed generation or production. Furthermore, it is possible to use this technique outside of the normal growing season.

In conclusion, genotypic differences existed in the resistance of maize silk to *F. graminearum*. These differences were correlated with field resistance to infection via the silk and/or silk channel. Some genotypes appeared to have resistance mechanisms in the silk. Selection for silk resistance in breeding programs should improve the probability of new lines with superior resistance.

FIGURE 5.2

Relationship between *in vitro* silk resistance to *F. graminearum* and field evaluations of resistance. Disease ratings are on a scale of 1-7, where lower values indicate less infection.


Mean Field Disease Rating
7
6
5
4
3
2
1
0
25
50
75
Field
Tissue
Greenhouse
Tissue
% Reduction in Dry Wt.

Preface to Chapter 6

In Chapter 5 it was shown that genotypic differences existed in the resistance of maize silk to *F. graminearum* and that these differences were correlated to field evaluations of resistance to infection via the silk and/or silk channel. Since the silk rapidly senesces after pollination its resistance may also change. The following chapter, Chapter 6, was designed to determine if resistance to silk infection changes with silk age. The hybrid experiment in the following chapter was conducted by A.T. Bolton and T. Woldemariam and was analyzed by L.M. Reid. This experiment was included in this chapter since it clearly indicated the effect of time of inoculation on resistance and added significantly to the overall purpose of the study. All other experiments were carried out by L.M. Reid. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table and figure is presented on the page following the first referral to it.

CHAPTER 6

EFFECT OF SILK AGE ON RESISTANCE OF MAIZE TO *FUSARIUM GRAMINEARUM*

6.1 Abstract

One of the major modes of entrance of *Fusarium graminearum* into maize ears is via the silk, a tissue which rapidly senesces after pollination. Experiments to investigate the effect of silk age on resistance of maize to fungal infection and its implications for screening programs were carried out. A moderately resistant hybrid ('Pride K127') and a more susceptible hybrid ('Pioneer 3953') were inoculated at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and to 18 days post-silk emergence by injecting a spore suspension into the silk channel. Five inbreds ('CO272', 'CO266', 'F7', 'F2', and 'A641') were inoculated at three stages of silk development (early, intermediate, and late) using two inoculation techniques, namely, silk channel injection and spraying exposed silks. Evaluation of resistance to infection at harvest u sed a seven-class disease severity rating scale. In addition, detached silk channel and exposed silk tissue from the inbreds at each stage of silk development was examined *in vitro* for resistance to *F. graminearum*. Resistance of both hybrids and inbreds was dependent on silk age. *In vitro* degradation of both exposed and silk channel silk tissue decreased as silk aged. Decreases in silk channel length and silk water content as silk aged were also recorded and may account for some of the change in resistance. It is recommended that

screening for resistance should be conducted using consistent times of inoculation, preferably at the intermediate stage of silk development when silk is elongated, pollinated, and has some tip browning. Inoculation later than 4-7 days after silking should be avoided because the level of infection obtained declined and failed to correctly assess resistance and to differentiate among genotypes.

6.2 Introduction

A major ear-rotting pathogen of maize or corn (*Zea mays* L.) in eastern Canada and the northeastern USA is *Fusarium graminearum* Schwabe, the asexual state of *Gibberella zeae* (Schw.) Petch. This ear rot occurs sporadically, but is significant because of mycotoxicoses that occur when contaminated grain is fed to swine (Sutton, 1982). Entry of *F. graminearum* into the ear occurs primarily via the silk and/or the silk channel, although entry also can occur through bird or insect wounds (Sutton, 1982). Infection spreads down the ear from the ear tip with the silks possibly providing a pathway to the kernels and rachis (Koehler, 1942; Hesseltine and Bothast, 1977).

The silks or styles of maize rapidly senesce after pollination (Heslop-Harrison, 1979). This physiological change has the potential to alter the suitability of silk as a substrate for the growth of ear-rotting organisms. There are only a few reports about the possible role of maize silks or the effect of silk age on infection of maize with *F. graminearum*. Some studies have indicated that susceptibility is highest shortly after silks emerge and decreases thereafter (Ullstrup, 1970;

Enerson and Hunter, 1980; Sutton and Baliko, 1981). In contrast, Hesseltine and Bothast (1977) reported that as silk tissue senesced it became a more suitable substrate for the growth of *F. graminearum* as well as a variety of other ear-rotting organisms. For otherr fungal pathogens, Marsh and Payne (1984) found less kernel infection when older, brown silks were inoculated with *Aspergillus flavus* Link ex. Fries than when young, fresh silks were inoculated. Recent studies on sweet maize inbreds by Headrick et al. (1990) and Headrick and Pataky (1991) reported that colonization of silks by *F. moniliforme* J. Sheld. did not occur until after the onset of silk senescence.

The purpose of this study was to determine the effect of silk age on infection of maize with *F. graminearum* and the implications for breeding programs evaluating genotypes for resistance.

6.3 Materials and Methods

F. graminearum cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These

flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour at 4 hour intervals for eight days. Spore counts reached 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Plants were grown in 12 plant rows (3.8 m long, 76 cm between rows) of which the primary ears of the ce er 10 plants were inoculated and evaluated for resistance to infection at the Plant Research Centre of Agriculture Canada, Ottawa. Ears were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear. After inoculation, plots were irrigated for four weeks at a rate of 2-5 mm twice daily. Evaluations for resistance to spread of infection were made in mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected. Ears were rated individually and a mean rating was calculated for each sub plot. Statistical analysis of results was carried out using Friedman's two-way analysis of variance by ranks and the corresponding multiple comparison test (Daniel, 1990).

Hybrid Study

In 1990, a 2 x 13 factorial experiment in a split-plot design with four replicates was conducted. Each main-plot unit consisted of 13 rows of one of two

hybrids, 'Pride K127' (a moderately resistant hybrid) or 'Pioneer 3953' (a susceptible hybrid), subdivided into 13 sub-plot units (single 12-plant rows) to which 13 times of inoculation were randomized. The inoculation times used were: 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 18 days after the first appearance of silk on that plant.

Inbred Study

In 1989 and 1990, 5x3x2 factorial experiments arranged in split-split plot designs were conducted with four replicates in one location in 1989 on a sandy loam soil, and with four replicates in each of two locations in 1990, one on a sandy loam soil and the other planted ten days later than the first on a clay soil. The main-plot units were five inbreds ('CO272', 'F7', 'F2', 'CO266', and 'A641'), the sub-plot treatments were three times of inoculation corresponding to stages of silk development, and the sub-sub-plot treatments were two methods of inoculation. Each sub-sub-plot consisted of a single 12-plant row. Each main plot unit of six rows was bordered on either side with a non-inoculated row of the same genotype.

The three stages of silk development used were: early, when silks were immature and just exposed; intermediate, when silks were elongated, pollinated, and had some tip browning; and late, when silks were brown and dry. Using the scale of Ritchie et al. (1986) these stages correspond roughly to: early R1 (reproductive) silking stage, early R2 blister stage, and late R3 milk stage.

One of the inoculation techniques was the silk channel injection technique described above while the other was a spray technique designed to simulate natural infection as much as possible. It consisted of spraying approximately 2 ml of a spore suspension (2×10^6 ml^{-1}) from an atomizer onto the exposed silks. Ears were then bagged with glassine ear shoot bags to maintain humidity, minimize contamination by other ear-rotting organisms, and to identify inoculated ears. Within each plot, randomly selected plants were inoculated with sterile water to serve as control treatments. Silk channel injections at the early and late stages were conducted only in 1990.

Final evaluation of resistance to spread of infection was made at harvest as above. However, ears were harvested at an equal number of days after inoculation, in order to counteract any effect due to differences in time available for fungal growth.

At each stage of silk development silk channel length was measured, and silk water content was determined for both silk within the channel and exposed silk. Ten randomly selected plants from each main-plot unit were sampled. Water content was measured by drying 4 replicates each of 1 g samples of silk tissue at 80^oC for 48 hrs then weighing the samples and calculating the percentage water content as the decrease in weight after drying.

In Vitro Tests of Detached Silk Tissue

In vitro tests of detached silk tissue of different ages from field grown plants

were conducted to assess differences in resistance to degradation by *F. graminearum*. From each ear, two 1-g samples of silk tissue were cut from the silk channel and two were cut from the exposed silk and placed in four petri dishes containing two pieces of filter paper moistened with 3 ml of sterile water. Three millilitres of spore suspension (5×10^5 spores/ml) were sprayed evenly on one of the silk channel samples and one of the exposed silk samples of each ear with an atomizer. The remaining samples were sprayed with 3 ml of sterile water to serve as controls. Samples were taken from each of the five inbreds used in the inbred study at each of the three stages of silk development. Ten replicates were carried out for each treatment combination. The petri dishes were placed in a randomized arrangement in a growth chamber at 25°C and 95% relative humidity, incubated for 7 days, dried in an oven at 80°C for 48 hrs and then weighed. The amount of tissue degraded by the pathogen was measured as the percentage dry weight reduction from the water control. This technique is similar to that described by Naik and Busch (1978) for testing the effect of maize pollen on *F. graminearum* growth. Analysis of results was made using parametric statistics (Steel and Torrie, 1980). Relationships among *in vitro* results, inbred characterizations and disease evaluations were examined using Spearman's rank correlation coefficient (Daniel, 1990; Steel and Torrie, 1980).

6.4 Results and Discussion

Hybrid Study

Effects due to time of inoculation were significant for both hybrids [$p = P(X^2_3 \geq X^2_{rcal}) < 0.01$, where $X^2_{rcal} = 31.07$ and 37.28 for 'Pride K127' and 'Pioneer 3953' respectively]. For each hybrid, susceptibility increased initially, peaked at 4-6 days, then decreased rapidly and finally stabilized around 14 days (Figure 6.1). Statistically significant differences between the two hybrids were obtained with inoculations made between two and seven days after silking.

Inbred Study

In both years, lower disease ratings were found for all inbreds inoculated with the spray technique compared to the injection technique (Table 6.1). Differences between inoculation techniques were statistically significant [$p = P(X^2_3 \geq X^2_{rcal}) < 0.05$] for all inbreds at the early stage of inoculation and for all inbreds at the intermediate stage except 'CO272'. Only the most susceptible inbred, 'CO266', had significant differences in inoculation technique effects at the late inoculation stage. Ears of susceptible lines could become highly infected with both techniques. Wicklow and Caldwell (1990) have suggested that spray inoculation is ineffective, because they could not obtain infection when the silks of the hybrid Dekalb XL-12, grown in a controlled environment chamber, were sprayed with *F. graminearum* spores. We also have experienced difficulty in achieving infection when silks are spray-inoculated in greenhouse environments but not in the field.

FIGURE 6.1

Effect of time of inoculation on amount of infection by *F. graminearum*. Disease ratings are on a scale of 1-7, where lower values indicate less infection. SD= standard deviation.

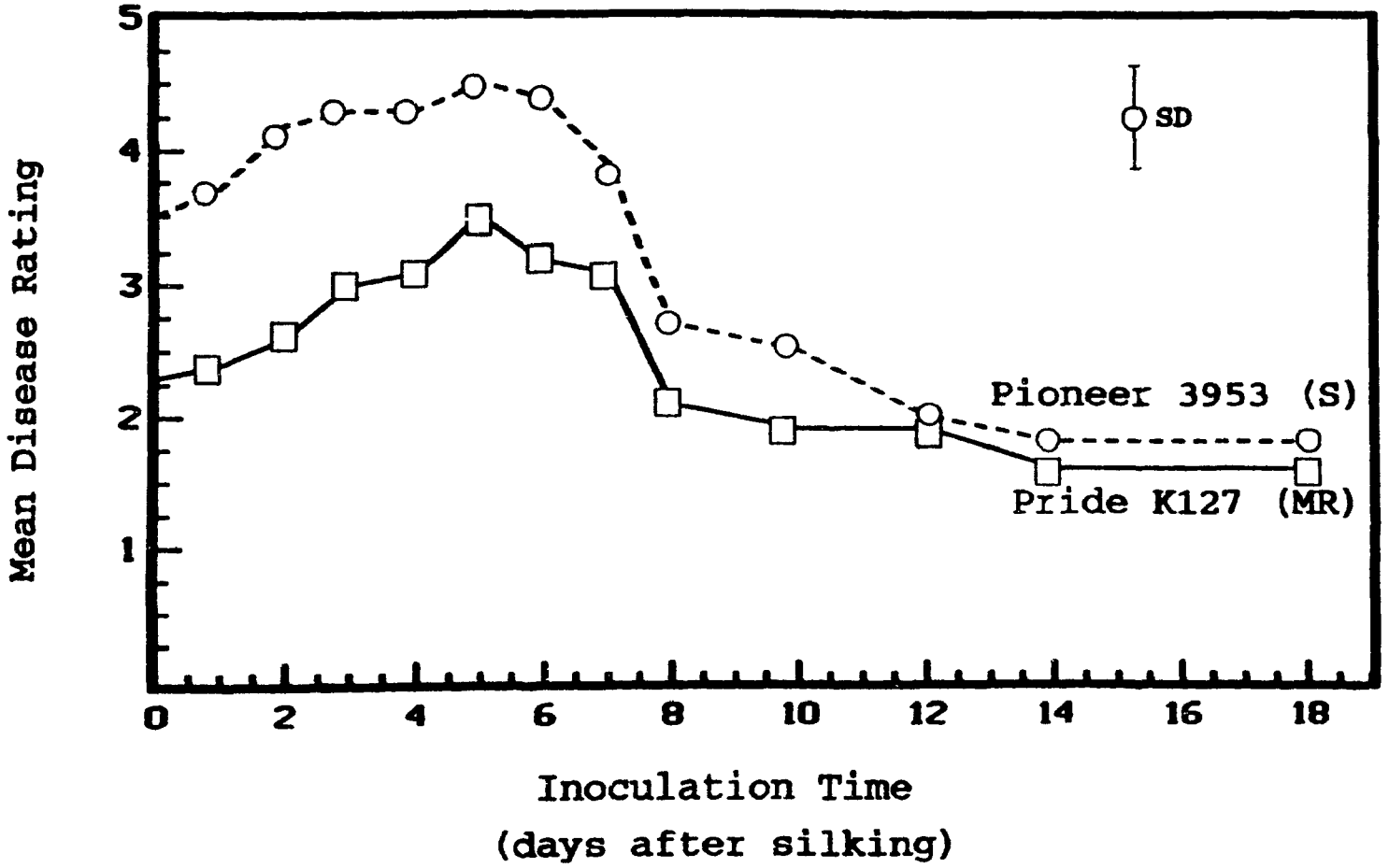

Mean Disease Rating
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
SD
Pioneer 3953 (S)
Pride K127 (MR)
Inoculation Time
(days after silking)

Table 6.1. Mean disease ratings in 1989 and 1990 for six inbreds inoculated with *F. graminearum* at three stages of silk development and with two methods of inoculation.

		Mean Disease Ratings[a]			
		1989		1990	
Inbred	Silk Stage[b]	Spray	Inject	Spray	Inject
CO272	Early	1.1 a	---	1.1 a	2.3 a
	Intermediate	1.2 a	1.6 a	1.2 a	1.9 a
	Late	1.0 a	---	1.2 a	1.5 a
CO266	Early	2.5 a	---	4.6 c	6.1 e
	Intermediate	2.9 b	5.1 c	3.6 b	5.7 e
	Late	2.2 a	---	3.0 b	5.0 d
F7	Early	1.0 a	---	1.0 a	3.8 bc
	Intermediate	1.0 a	2.9 b	1.0 a	2.8 b
	Late	1.0 a	---	1.0 a	1.0 a
F2	Early	1.1 a	---	1.1 a	4.6 cd
	Intermediate	1.4 a	3.3 b	1.1 a	3.7 bc
	Late	1.5 a	---	1.1 a	1.8 a
A641	Early	2.2 a	---	2.3 ab	4.8 d
	Intermediate	3.0 b	4.7 c	2.9 b	5.0 d
	Late	2.2 a	---	2.7 b	3.3 b

[a] Scale of 1-7, where lower values indicate less infection, averaged over all replicates for 1989 and replicates and locations for 1990. Inoculation techniques: spraying of spore suspension on external silks and injection of spore suspension into silk channel. Means followed by the same letter within a column are not significantly different at the 0.15 experimentwise error rate by the multiple comparison test (Daniel, 1990). ---= no data.

[b] Early= immature, just exposed silks; Intermediate= elongated, pollinated and some tip browning; Late= brown and dry.

The reason for this is not known.

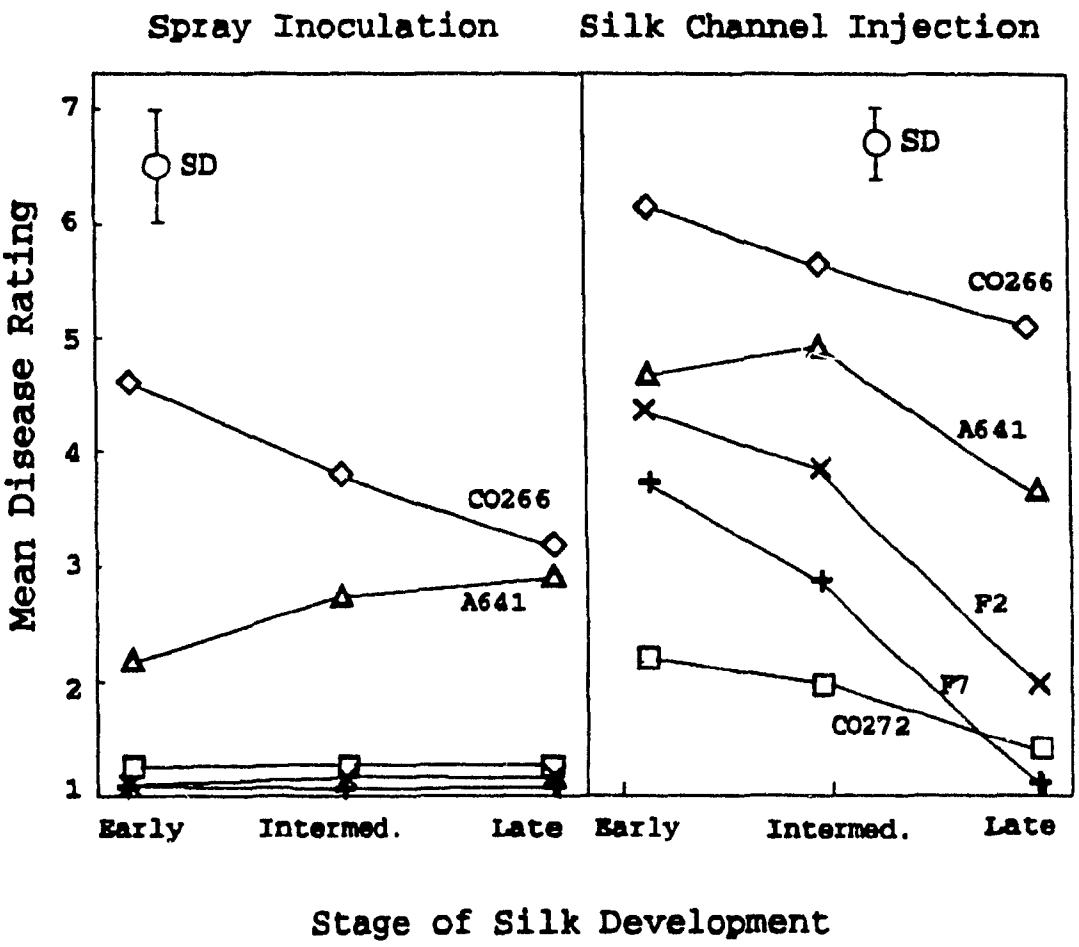
Effects due to time or stage of inoculation were tested for each inbred-inoculation technique combination. Significant effects were only found for 'A641' and 'CO266' with the spray technique, perhaps due to the overall low level of infection with this technique (Fig. 6.2). Effects due to stage of inoculation for the silk channel injection technique were significant at the 0.05 level for all inbreds except 'CO272' (Fig. 6.2). For 'F7', inoculations at the early and intermediate stages were significantly different from inoculation at the late stage. For the inbreds 'A641', 'F2', and 'CO266', only the early and the late stages of inoculation were significantly different from each other.

Significant genotype effects were found for all treatment combinations. Greater differentiation between inbreds was obtained with the silk channel method of inoculation and at the intermediate stage of silk development.

The early and intermediate stage of development occurred 1 and 5-7 days post-silking for all inbreds; however, the time required to reach the late stage of development varied considerably. The inbreds 'F2', 'F7', and 'A641' reached the late stage by 16-18 days, whereas the most resistant inbred 'CO272' and the most susceptible inbred 'CO266' took 21 days to reach the late stage. Thus those genotypes which took longer for the silk to senesce were not necessarily more resistant. This result was different from that of Headrick and Pataky (1991) who reported that in sweet maize, lines with delayed senescence were more resistant to *F. moniliforme*.

FIGURE 6.2

Effect of inoculations at different stages of silk development on disease ratings in 1990. Stages of silk development: Early= immature, Intermediate= elongated, pollinated, and some tip browning, and Late= brown and dry. SD= standard deviation.


Spray Inoculation
Silk Channel Injection
Mean Disease Rating
7
6
5
4
3
2
1
SD
SD
CO266
A641
CO266
A641
F2
F7
CO272
Early
Intermed.
Late
Early
Intermed.
Late
Stage of Silk Development

It was noted that silk colour varied between inbreds, with 'CO266' and 'F2' both having anthocyanin pigmentation. This was apparently not associated with resistance since 'CO266' was very susceptible but 'F2' was intermediate in resistance. The colour of exposed silk changed more with age than did the colour of the silk channel silk; senescence was apparently accelerated in the exposed silk.

As would be expected, in all inbreds, silk channel length decreased with silk age due to growth of the cob (Table 6.2). At a given inoculation time, very susceptible lines such as 'CO266' had much shorter silk channels than more resistant lines such as 'F7' and 'CO272'. Silk channel length may play a partial role in natural resistance. Longer silk channels may prolong the length of time the fungus must grow before it can reach the nutrient-rich reserves in the cob and kernels. If infection was too late the silk and ear tissue might dry out and become less suitable substrates by the time the fungus reached the cob. Furthermore, the greater abundance of silk in a longer silk channel may enhance any resistance barriers present in the silk or silk channel.

The water content of silk channel silks remained approximately 91%, whereas that of the exposed silk tissue decreased with age. No significant genotypic differences were found among exposed silk water content at the early or intermediate stages, when water content averaged 91% and 89% respectively. Significant genotypic differences were found in the late stage silk. The susceptible inbred 'A641' had the lowest water content of 65.9 % followed by the highly

Table 6.2. Silk channel length of inbreds at each stage of silk development[a].

Inbred	Silk Channel Length (cm) at each Stage of Development		
	Early	Intermediate	Late
C0272	10.1 ± 2.62	8.8 ± 2.28	7.3 ± 1.75
F7	11.7 ± 2.76	8.2 ± 2.71	7.5 ± 1.54
F2	10.8 ± 1.23	8.6 ± 2.98	6.1 ± 2.05
A641	6.8 ± 3.02	3.8 ± 2.07	3.2 ± 2.01
C0266	6.5 ± 2.14	6.4 ± 2.30	5.7 ± 2.49

[a] Early= immature, just exposed silks; Intermediate= elongated, pollinated, and with some tip browning; Late= brown and dry. Mean ± standard deviation of 20 ears, measured from the tip of the cob to the point where the silks protrude from the husk.

resistant inbred 'CO272' with 71.3 % and 'F7', 'F2', and 'CO266' with 81-90 %. Thus although changes in water content could partially account for changes in the amount of infection with age in a given inbred, silk water content does not account for differences among inbreds. The hybrid study showed that early inoculations developed less infection than the peak at the intermediate stage. Younger silk may be more resistant or a poorer substrate for the fungus since it is actively growing. Headrick et al. (1990) and Marsh and Payne (1984), working with *F. moniliforme* and *A. flavus* respectively, found more invasion of silks as senescence began. Slightly different results were obtained with the inbred study. The earliest inoculation stage was more infected than the intermediate stage. Susceptible inbreds may therefore be more open to earlier infection in the silk channel. These results agreed with those of Ullstrup (1970), and Sutton and Baliko (1981).

In Vitro Tests of Detached Silk Tissue

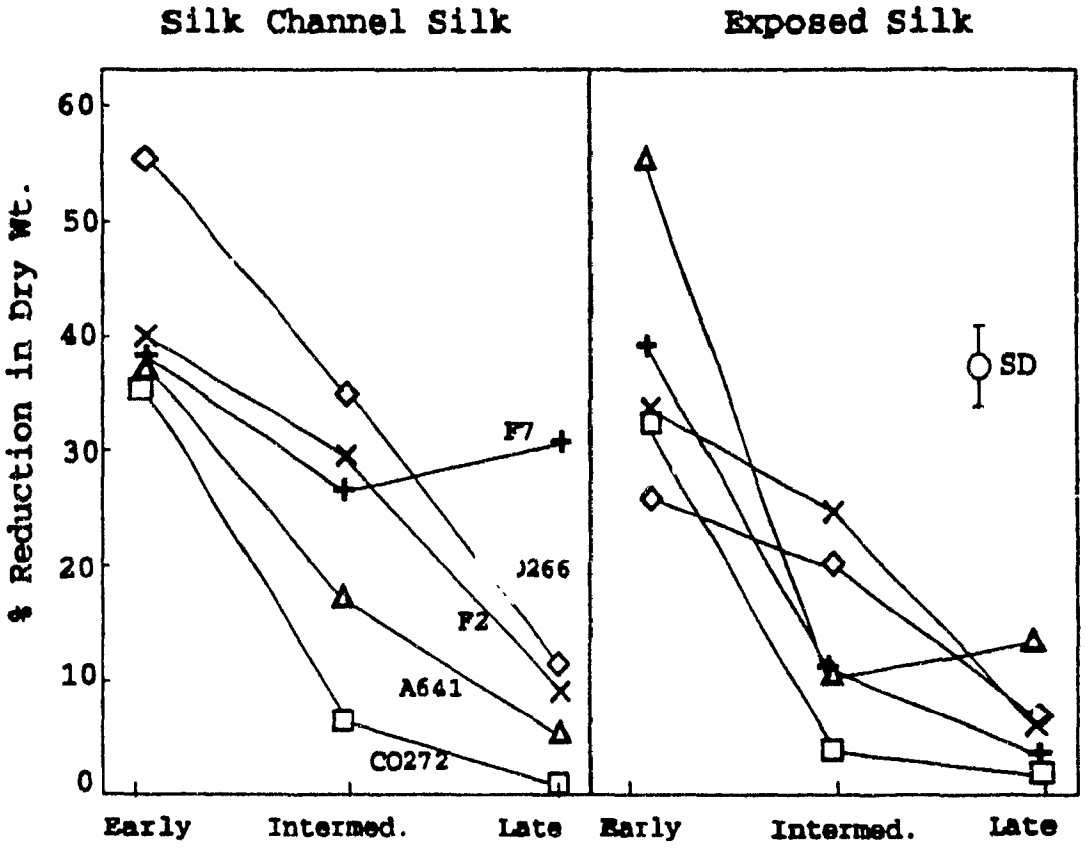
F. graminearum was able to digest detached silk tissue. Genotype, stage of silk development, tissue (silk channel silk vs exposed silk), and genotype-tissue interactions were all significant at the 0.05 level of probability.

Dry weight depletion decreased with silk age for all inbreds except for the 'F7' silk channel tissue and the 'A641' exposed silk tissue which both increased slightly by the late stage (Fig. 6.3). On average, exposed silk tissue was less degraded than silk channel tissue.

The greatest differentiation between inbreds occurred with intermediate-

FIGURE 6.3

Stage of silk development and percentage dry weight reduction of silk tissue by *F. graminearum*. Stages of silk development: Early= immature and just exposed, Intermediate= elongated, pollinated, and some tip browning, and Late= brown and dry. SD= standard deviation.


Silk Channel Silk
Exposed Silk
% Reduction in Dry Wt.
60
50
40
30
20
10
0
F7
0266
F2
A641
CO272
SD
Early
Intermed.
Late
Early
Intermed.
Late
Stage of Silk Development

stage silk channel silk tissue. Significant differences were not obtained among inbreds at the early and late inoculation times. Silk from genotypes with greater field infection had increased degradation of silk tissue by the fungus.

It was also apparent that differences existed in the silk resistance of the inbreds studied in both the field and *in vitro* studies. The nature of this resistance may be either physical, chemical, or a combination of both.

In conclusion, as silk aged, both maize hybrids and inbreds exhibited an increase in resistance to *F. graminearum* infection via the silk. This was also manifested in a decrease in the ability of *F. graminearum* to degrade detached silk tissue of later developmental stages. Thus evaluations of resistance carried out on ears inoculated at late silk development stages may result in low levels of infection and therefore do not provide accurate assessments of resistance. Earlier inoculations may result in too much infection and those lines exhibiting some resistance may be overlooked.

The results of this study suggested that in evaluating resistance to *F. graminearum* ear rot, it is important to inoculate all genotypes at the same stage of silk development. This could be more time-consuming, but is essential when genotypes differing in maturity are being evaluated. Optimum results were obtained by inoculating with the silk channel injection technique when silks were elongated and pollinated. This stage occurred at approximately 4-7 days after silk emergence.

Preface to Chapter 7

The results of Chapters 5 and 6 indicated that differences existed in the resistance of various genotypes of maize to *F. graminearum* infection via the silk and/or silk channel and in the degradation *in vitro*, of silk tissue by this pathogen, and that within a given genotype resistance changed with silk age. The mechanism(s) of this resistance is not known. The following chapter, Chapter 7, was designed to study the phytochemistry of corn silk and determine if any phenolic compounds in the silk are associated with resistance. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table and figure is presented on the page following the first referral to it.

CHAPTER 7

CHANGES IN HOST PHENOLICS OF MAIZE SILK INFECTED WITH FUSARIUM GRAMINEARUM

7.1 Abstract

Total phenolics in the silk of five maize inbred lines ('CO272', 'F7', 'F2', 'A641', and 'CO266') of varying susceptibility to *Fusarium graminearum* were determined for three stages of silk development (early, intermediate, and late). Total phenolics of non-inoculated silk tissue increased with silk age for two of the inbreds but this did not appear to be associated with resistance. A suspension of *F. graminearum* spores was sprayed on the exposed silk. Silk tissue was collected from the silk channel one to 14 days after spraying and analyzed for total phenolics. Total phenolics increased above that of a water control in the most resistant line 'CO272', and decreased below water controls in susceptible lines. High performance liquid chromatographic analysis of the phenolic extracts revealed that flavones were the major phenolic compounds present. Five flavones were identified: iso-orientin, iso-vitexin, maysin, luteolin, and apigenin.

7.2 Introduction

A major mode of entrance of *Fusarium graminearum* Schwabe (sexual state: *Gibberella zeae* (Schw.) Petch.) into maize or corn (*Zea mays* L.) ears is via the silk and/or silk channel (Sutton, 1982; Koehler, 1942). Infection then spreads down

the ear from the tip (Koehler, 1942; Hesseltine and Bothast, 1977). Althougn the incidence of this ear-rotting pathogen is sporadic, infections are serious due to the production of mycotoxins that can contaminate feed (Mirocha and Christensen, 1974; Vesonder et al., 1981).

The possibility of the presence of chemical resistance barriers to infection in the silk has not been explored. Silk tissue possesses a wide array of volatile compounds as well as phenolics, some of which are active against insects. One such compound is the flavonoid C-glycoside maysin which has been seen to reduce growth and development of the corn earworm *Heliothis zeae* Boddie (Wiseman et al., 1985; Waiss et al., 1979; Reese et al., 1982).

Phenolic compounds, especially flavonoids, are widely distributed in higher plants and many have been implicated in mechanisms of disease resistance (Friend, 1977). Many studies have shown that phenolics are growth inhibitors or are toxic towards various pathogens. Phenolics accumulate rapidly during host-parasite interactions and can mediate disease suppression through inactivation of fungal enzymes or strengthening of plant structural compor ents (Bell, 1981). Upon infection, active phenolics and flavonoids may be produced in a single step by hydrolysis of their glycosides (Creasy, 1985; Bell, 1981). Hammerschmidt and Nicholson (1977) found flavonoids accumulated more rapidly in resistant than in susceptible maize lines in response to infection by anthracnose, *Colletotrichum graminicola* (Ces.) B.W. Wils.

Phenolic compounds have been reported to be involved in the resistance

of plants to infection by various *Fusarium* pathogens. The low molecular weight phenolics caffeic acid, ferulic acid, and catechol have been seen to inhibit the growth of *F. oxysporum* f.sp. *radicis-lycopersici* (Kasenberg and Traquair, 1988). Ismail et al. (1987) tested the effect of 12 phenolic compounds on the percentage germination and germ-tube growth of spores of *F. oxysporum* f.sp. *lycopersici* (Sacc.) Snyder and Hansen as well as *Aspergillus fumigatus* Fresenius and found that inhibitory action varied with dosage, chemical structure and organism tested. Ghosal et al. (1978) found reduced infection of sweet corn ears by *F. moniliforme* Sheld. if either before or after infection the ears were sprayed with three polyphenolic compounds, mangiferin, 1,3,6,7-tetrahydroxyxanthone, and 2,2',4-trihydroxybenzophenone. These three compounds caused lysis of hyphal cells and reduced fungal growth *in vitro*.

The purpose of the present study was to determine: (1) if preinfection or initial phenolic levels are correlated with resistance to *F. graminearum*; (2) if phenolic levels change with silk age; (3) if phenolic levels change with infection by the pathogen; and (4) the nature of the major phenolic compounds in the silk and whether these compounds are associated with resistance.

7.3 Materials and Methods

Inoculations and Tissue Collection

In 1990, five inbred lines, 'CO272', 'F7', 'F2', 'A641', and 'CO266' (in order of increasing susceptibility to *F. graminearum* silk infection), were each planted in

10-row plots randomized to each of four blocks in a randomized complete block design at the Plant Research Centre of Agriculture Canada, Ottawa. Each row (3.8 m long, 76 cm between rows) consisted of 12 plants of which the primary ears of the middle 8 plants were used for analysis. To simulate natural infection as much as possible and to avoid damaging the silks, *F. graminearum* inoculations were made by spraying 2 ml of a spore suspension (2×10^6 spores per ml) of *F. graminearum* from an atomizer onto the exposed silks. Ears were then bagged with glassine ear shoot bags to maintain humidity, minimize contamination by other ear-rotting organisms, and to identify inoculated ears. Uninoculated plants were sprayed with sterile water and bagged to serve as controls.

Cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour at 4 hour intervals for eight days. Spore counts in these flasks reached 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Inoculations were made at three different stages of silk development or age: early, when silks were immature and just emerged; intermediate, when silks were elongated, pollinated, and may have some tip browning; and late, when silks were brown and dry. For each treatment combination (inbred-inoculation time) four plants were inoculated with a spore suspension and four plants were inoculated with sterile wa ter. A sample of sild-channel silk tissue (1.5 g fresh weight) was collected from each plant in the early morning by removing ears from the plant, cutting ears in half crosswise, cutting off and discarding external silk, carefully unravelling the husk and cutting off and discarding silk overlying the cob. Samples were placed in plastic bags, frozen in liquid nitrogen, and stored at -70°C.

Initial Total Phenolics

Four replicates of uninoculated silk tissue each treatment combination (inbred-inoculation time) to be harvested. Silks were cut into 0.5 cm lengths and steeped in 20 ml of 70% methanol at room temperature for 24 h. Extracts were then filtered through four layers of cheesecloth and the residue was discarded. The filtrate (crude extract) was used for analyses.

Total phenolics were determined from crude extracts using the method of Swain and Hillis (1959). Results were expressed as mg/g fr. wt. gallic acid equivalent. Corrections for differences in water content of silk of different maturities were made by drying an additional two samples of each treatment in an oven at 80°C for 48 hrs to obtain moisture contents.

Changes in Total Phenolics with Infection

Silk was collected and total phenolics were analyzed from each of the five inbreds at each of nine collection times: 0, 1, 2, 4, 6, 8, 10, 12, and 14 days following inoculation at the intermediate stage of silk development with either a *Fusarium* spore suspension or sterile-water. In addition, for both 'CO272' and 'CO266' only, silk was collected at each of these times after inoculation at the early and late stages of development to determine if response to infection was different as the silk aged. The percentage change (increase or decrease) in total phenolics in the 14 day period post-inoculation was calculated for each treatment.

Flavonoid Analyses

Preliminary separation of extracts was done by polyamide thin-layer chromatography (TLC) (Harborne, 1989). Further analyses were carried out using high performance liquid chromatography (HPLC). Extracts were evaporated to dryness at room temperature, redissolved in 10 ml methanol, and centrifuged with 1.0 g of polyvinyl polypyrrolidone (PVPP) (Sigma) for 10 min. at 3,000 rpm to remove condensed tannins. The supernatant was dried under vacuum at 30°C and resuspended in 1 ml methanol (HPLC grade) and filtered through a 0.5 micron nylon millipore filter with a 1.5 ml Luer lock syringe and a Millipore filter holder prior to HPLC analysis.

The HPLC system consisted of a Perkin-Elmer LC 480 Diode Array Scan Detector, an LC 250 Binary Pump, a Nelson 900 Interface, a LC 600 Autosampler

(20 µl injection volume), and a 4.6 mm Ultrasphere ODS C-18 reverse-phase column. A gradient elution system was used consisting of the solvents 100% methanol (solvent A) and 5% formic acid (solvent B) with peak detection at 280 nm and 350 nm (Vande Casteele et al., 1982). An online UV scan from 250 to 430 nm of eluted peaks also was carried out. Identification of flavones was made by comparison of retention times, UV spectra, and by peak enrichment with authentic standards.

Selected extracts were analyzed to determine if inbreds differ in the number and quantity of flavones present in silk and if any changes occurred upon inoculation. HPLC analysis was carried out on both inoculated and water control extracts of the following treatments: 2, 8, and 14 days for 'CO272' and 'CO266' inoculated at the intermediate stage of silk development; 8 days for 'F7', 'F2', and 'A641' inoculated at the intermediate stage of silk development; and 8 days for 'CO272' and 'CO266' inoculated at the early and late stages of silk development.

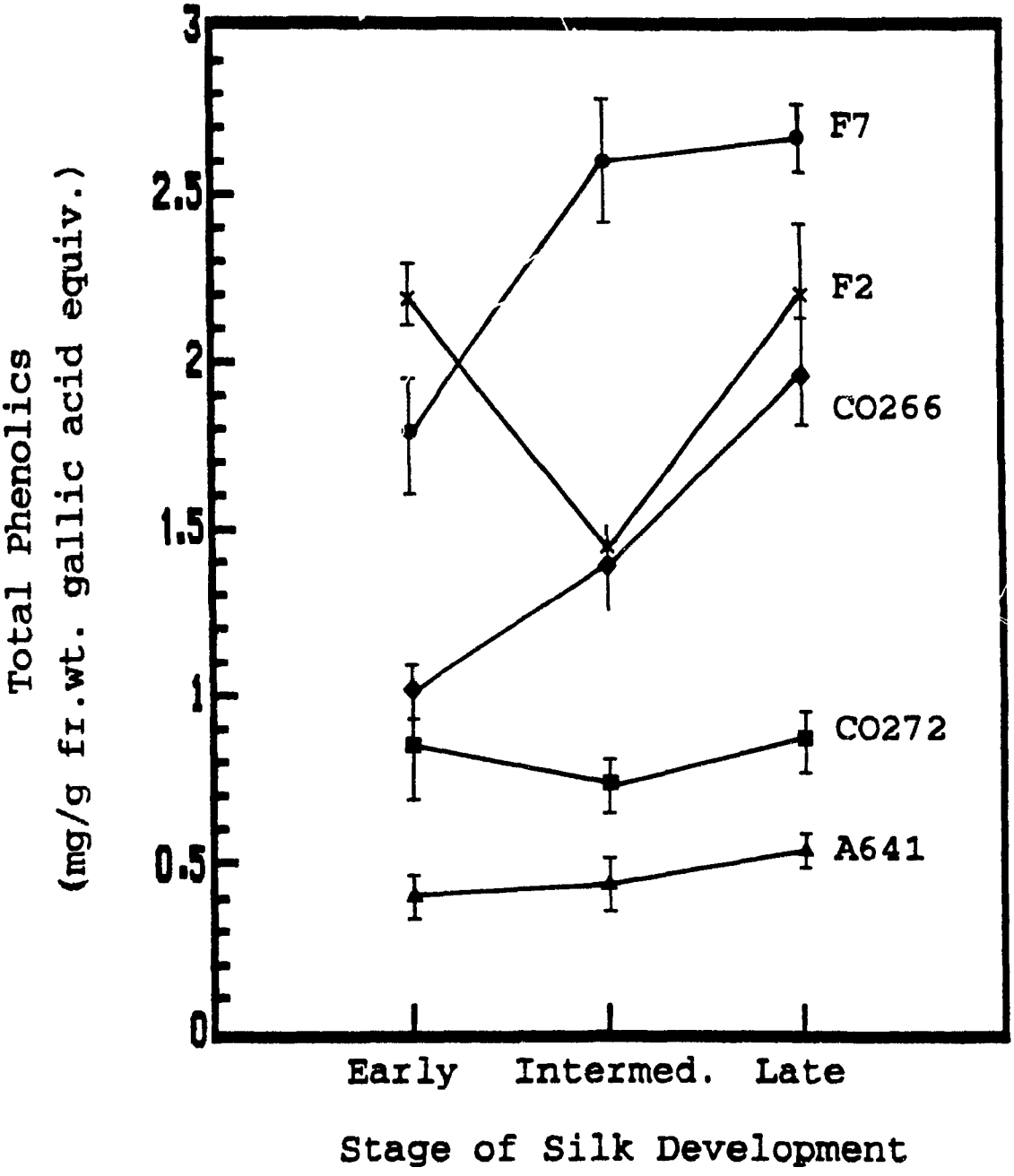
7.4 Results

Initial Total Phenolics and Silk Age

Significant differences ($P<0.01$) in total phenolics were found among inbreds (Fig. 7.1). The highly resistant line 'CO272' had lower total phenolics than the more susceptible lines 'F7', 'F2', and 'CO266' but was higher than the susceptible line 'A641'. Significant overall increases in total phenolics with age were found for both the susceptible line 'CO266' and the moderately resistant line

FIGURE 7.1

Effect of silk age on total phenolic levels of five inbred lines. Stages of silk development: Early= immature, Intermediate= elongated, pollinated, some tip browning; and Late= brown and dry.


Total Phenolics
(mg/g fr.wt. gallic acid equiv.)
3
2.5
2
1.5
1
0.5
0
F7
F2
CO266
CO272
A641
Early
Intermed.
Late
Stage of Silk Development

'F7'. Total phenolics in the inbred 'F2' decreased between the early and intermediate stages, but increased between the intermediate and late stages.

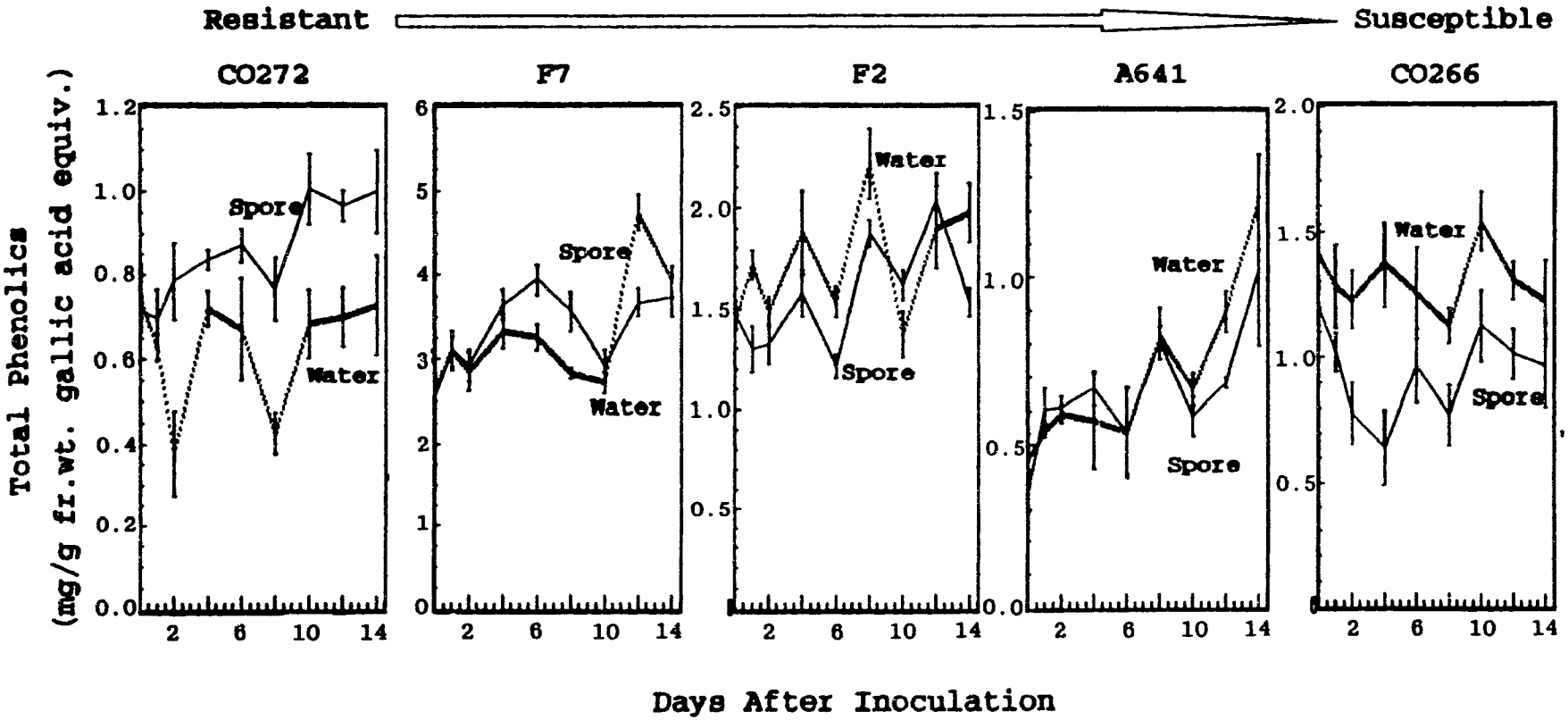
Changes in Total Phenolics with Infection

Significant differences in total phenolics during the 14-day period following inoculation were found between spore-inoculated and sterile water-inoculated control plants (Fig. 7.2). The five inbreds responded differently to infection when inoculated at the intermediate stage of silk development. The levels of phenolics increased significantly above that of the water control in the highly resistant line 'CO272'. Some increase in phenolics occurred in the moderately resistant line 'F7'; but levels decreased below the water control after 10 days. Phenolics decreased significantly below those of the water control for the more susceptible lines 'F2', 'A641', and especially for the very susceptible line 'CO266'.

The levels of total phenolics in inoculated tissue of all inbreds fluctuated with time but were parallel to those of the water controls. Such fluctuations may reflect the differing emergence times of silk of a given ear. Not all silk emerges at the same time. The silk at the butt of the cob emerges first while the silk at the tip is last (Ritchie et al., 1986). Thus, at a given time the silk channel contained silk at different stages of development and differing in response to infection. If phenolics were increased with infection, as was the case for 'CO272', the emergence of new silk at 8 days post-inoculation would lower the overall total phenolics in both the inoculated and water control tissue.

FIGURE 7.2

Total phenolic levels of *F. graminearum* spore-inoculated or water-inoculated control silks after inoculation at the intermediate stage of silk development.


Resistant
Susceptible
CO272
F7
F2
A641
CO266
Total Phenolics
(mg/g fr.wt. gallic acid equiv.)
Spore
Water
Days After Inoculation

Both time of inoculation and/or stage of silk development had a significant effect on the response of 'CO272' and 'CO266' to infection (Fig. 7.3). Immature silk of the resistant line 'CO272' responded with an increase in total phenolics above the water control. Phenolic content of both spore-inoculated and control silk increased in 'CO272' at the late stage of silk development. Immature silk of the susceptible line 'CO266' responded to infection with an initial increase in total phenolics but levels rapidly decreased below the water control by four days post-inoculation (Fig. 7.3) and were similar to the response at the intermediate stage (Fig. 7.2). Less response was seen in 'CO266' inoculated at the late stage of silk development.

Flavonoid Analysis

Polyamide TLC revealed that the major phenolic compounds present in the crude extracts were flavone or flavonol in nature. Many different flavone/flavonol compounds appeared in the extracts. Five flavone compounds were identified in HPLC chromatograms of the silk extracts of the different inbreds: the aglycones apigenin (5,7,4'-trihydroxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone), and the C-glycosides iso-orientin (6-C-∝-glucosyluteolin), maysin [6-C-(4-ketofucosyl)-5,7,3',4'-tetrahydroxyflavone], and iso-vitexin (6-C-∝-glucosylapigenin) (Appendix 3). Inbreds differed in both the number and distribution of flavones present and with respect to which compounds increased or decreased with inoculation (Table 7.1 and 7.2).

FIGURE 7.3

Total phenolic levels of *F. graminearum* spore-inoculated or water-inoculated control silks after inoculation at the early and late stages of silk development.

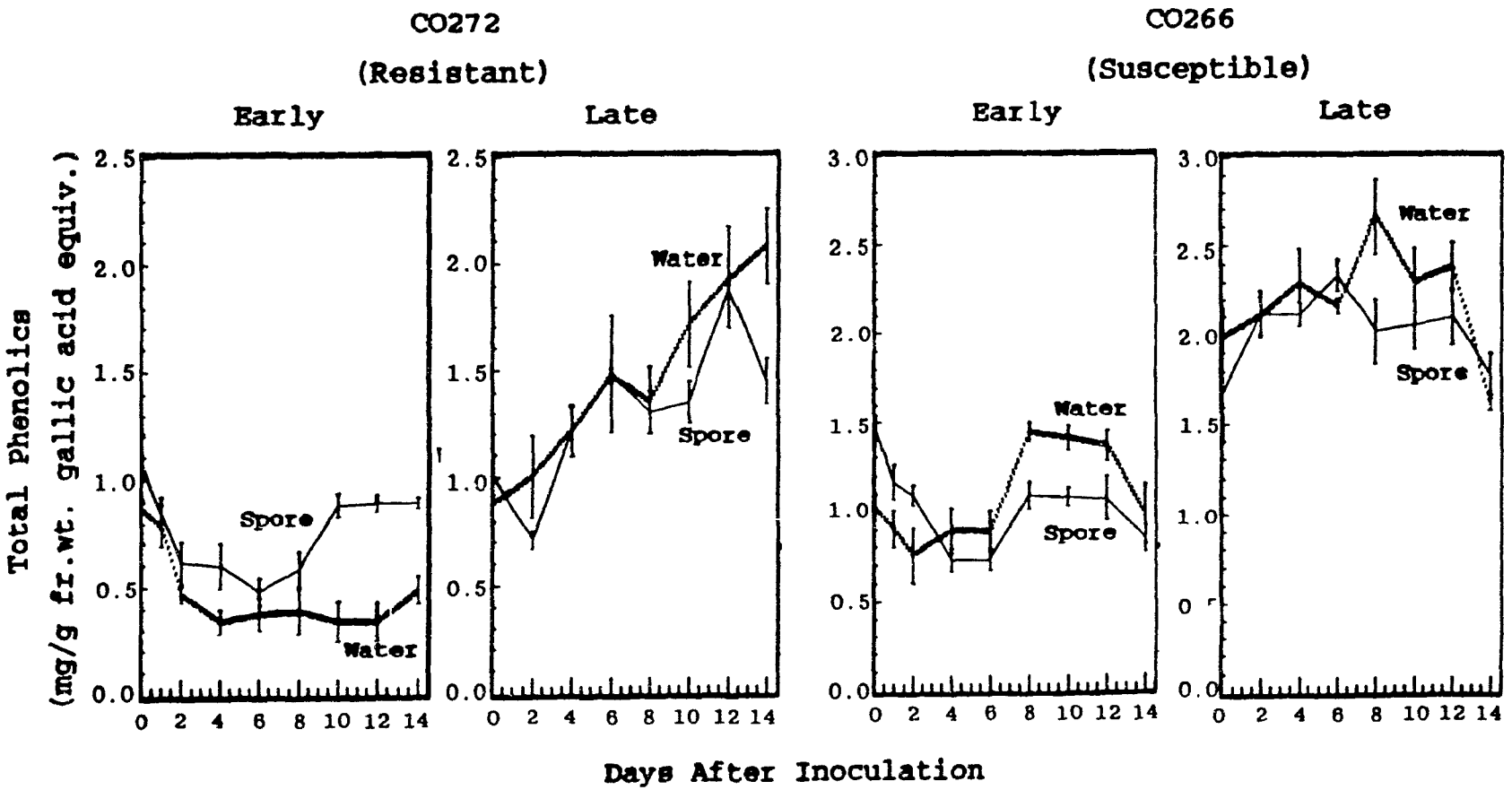

CO272
(Resistant)
CO266
(Susceptible)
Early
Late
Early
Late
Total Phenolics
(mg/g fr.wt. gallic acid equiv.)
Spore
Water
Water
Spore
Water
Spore
Water
Spore
Days After Inoculation

Table 7.1. Concentrations of the flavone glycosides iso-orientin, iso-vitexin, and maysin found in the silk of five maize inbreds before (water control) and after inoculation with *F. graminearum*.

			Flavone Concentration (mg/g fr.wt.)[c]								
	Inoculation	Harvest	Iso-orientin			Iso-vitexin			Maysin		
Inbred	Time[a]	Time[b]	Water	Inoculated	%	Water	Inoculated	%	Water	Inoculated	%
CO272	Early	8	0	0	0	0	0.327	--	0	0	0
	Intermed.	2	0	0	0	0	0.201	--	0	0	0
		8	0.767	0.819	6.7	0	0	0	0	0	0
		14	0	0	0	0.773	0	--	0	0	0
	Late	8	0	0	0	0.083	0	--	0	0	0
CO266	Early	8	0.937	0	--	1.791	1.108	-38.1	0	0	0
	Intermed.	2	1.059	0.809	-23.6	2.320	2.152	-7.3	0.602	0	--
		8	0.378	0.047	87.6	1.736	0.696	-59.9	0.138	0.036	73.9
		14	0.231	0	--	3.794	2.312	-39.1	0.668	0	--
	Late	8	0.284	0.212	-25.3	4.296	3.950	-8.0	1.266	0.778	-38.6
F7	Intermed.	8	0.027	0.029	7.4	0	1.485	--	0.221	0.227	2.7
F2	Intermed.	8	1.962	2.018	2.9	0	2.642	--	0.225	0.221	-1.8
A641	Intermed.	8	0.627	0	--	0	0	0	0	0	0

[a] Corresponds to stage of silk development (early, intermediate, and late).
[b] Days after inoculation until silks were harvested.
[c] Concentration in water-"inoculated" (control) and spore-inoculated silks; percentage change from water control.

Table 7.2. Concentrations of the flavone aglycones luteolin and apigenin found in the silk of five maize inbreds before and after inoculation with *F. graminearum*.

			Flavone Concentration (mg/g fr.wt.)[c]					
	Inoculation	Harvest	Luteolin			Apigenin		
Inbred	Time[a]	Time[b]	Water	Inoculated	%	Water	Inoculated	%
CO272	Early	8	0.260	0.315	21.1	0	0	0
	Intermed.	2	0.198	0.466	135.3	0	0	0
		8	0.747	1.348	80.4	0	0.135	--
		14	0.656	0.749	14.2	0	0	0
	Late	8	0.683	1.202	76.0	0	0	0
CO266	Early	8	1.085	0.243	-77.6	0	0	0
	Intermed.	2	1.397	1.239	-11.3	0.186	0	--
		8	1.991	1.114	-44.0	0	0	0
		14	2.229	0	--	0	0	0
	Late	8	3.022	1.064	-64.8	0	0	0
F7	Intermed.	8	0.800	1.110	38.8	0	0	0
F2	Intermed.	8	0.651	0.522	-19.8	0	0.086	--
A641	Intermed.	8	0.161	0.155	-3.7	0	0	0

[a] Corresponds to stage of silk development (early, intermediate, and late).
[b] Days after inoculation until silks were harvested.
[c] Concentration in water-"inoculated" (control) and spore-inoculated silks; percentage change from water control.

7.5 Discussion

Initial total phenolics were not associated with differences in resistance of the inbreds used in this study. Furthermore, the changes in total phenolics with silk age can not explain changes in silk resistance over time, because the pattern of this response was not the same for all inbreds. Elliger et al. (1980) noted that for some maize varieties silk flavone content declined with age but remained constant in others. However, Wiseman et al. (1985) found no significant effect of silk age on maysin content.

Of more significance than initial phenolics or their response to silk aging was the change in phenolics in response to infection, and the differential response of the inbreds. Increases in total phenolics in response to infection, such as that found in the highly resistant inbred 'CO272', often have been found in disease-resistant plants (Bell, 1981; Hammerschmidt and Nicholson, 1977). The phenolic response observed in the silk presumably occurred in advance of actual infection in 'CO272', i.e. in the silk channel silk of ears in which only the exposed silk was inoculated. Phenolic compounds may not themselves have anti-fungal activity, but once oxidized by phenol oxidizing enzymes or phenolases, they may yield quinones, which are more toxic than phenolics. Decreases in total phenolic levels with infection, such as that found in the susceptible inbred 'CO266', are rarely observed. The observed decrease may be due to assimilation of the plant tissue by the pathogen and(or) to inhibition of plant metabolism by the pathogen. Such inhibition could be effected by protein-inhibiting toxins produced by the pathogen.

Fusarium graminearum produces mycotoxins, in particular deoxynivalenol, that are known to be protein inhibitors. It has been speculated that these toxins play a role in overcoming host plant defenses during infection (Wang and Miller, 1988; Hart et al., 1987; Casale and Hart, 1988). Resistant genotypes such as 'CO272' may be resistant to the toxins, or may be able to detoxify them. The increased level of phenolics induced in 'CO272' may play a role in toxin resistance, or may be a normal induced response that is allowed to proceed because of resistance to the fungal toxins.

Silk age had a significant effect on the response of silk tissue to infection. Differences in response time between silk of early and intermediate age accounted for some of the observed increase in resistance with age. However, the lack of response in older silk indicated that phenolic levels were probably not important at this stage of silk development. Water content may be more critical in determining susceptibility at this time. The advanced stage of senescence of older tissue would indicate it is less metabolically active.

Phenolics may be either stimulatory or inhibitory to pathogens, and in fact the same compound may be stimulatory to one pathogen but inhibitory to another (Boonyakiat et al., 1988). Analysis of the major phenolics in the present study revealed large differences among inbreds with respect to numbers and kinds of flavone compounds, and their responses to infection. Of the five flavones identified, only luteolin and iso-orientin were present in all inbreds. Luteolin is very common in plant extracts and has been reported previously in maize silk (Levings

and Stuber, 1971). Luteolin may play a role in resistance since levels of this compound did increase above the water control in the two most resistant inbreds, 'CO272' and 'F7', but decreased in the more susceptible inbreds. After inoculation at the intermediate stage of silk development, the levels of luteolin in 'CO272' more than doubled by 2 days post-inoculation then gradually declined yet always remained above that of the water control. However, in the most susceptible line 'CO266', by 2 days post-inoculation luteolin levels were lower in inoculated tissue and continued to decline until this compound was not present at 14 days. The largest increase in luteolin was found in 'CO272' silk inoculated at the intermediate stage of silk development. This was also the stage at which the largest decrease in 'CO266' luteolin levels occurred. The authors also have observed that artificial inoculation of genotypes at this stage of silk development rather than the early or late stages results in the greatest differentiation between genotypes on the basis of the severity of disease.

Maysin also has been reported previously in maize silk, and has been shown to have antifeedant activity against the earworm *H. zeae* (Wiseman et al., 1985; Waiss et al., 1979; Reese et al., 1982) but the results presented here suggest that it is not associated with the resistance of 'CO272' against *F. graminearum*.

Ultimately, bioassays of identified phenolics will be needed to confirm whether there is a causative connection between increased phenolic levels and resistance to infection by *F. graminearum*.

In conclusion, although initial levels of total phenolics and associated flavones were not correlated with resistance to silk infection by *F. graminearum*, changes in the levels of these compounds after inoculation were associated with resistance. Genotypic differences existed in the degree of the response to infection and with respect to which specific flavone compound(s) increased or decreased upon infection. It is possible that these compounds possess anti-fungal activity or are part of a phenol-oxidizing enzyme system.

Preface to Chapter 8

The results of Chapters 5 and 6 indicated that genotypic differences existed for resistance to *F. graminearum* silk infection. The following chapter, Chapter 8, prersents a study designed to determine if this resistance is isolate-specific in a set of selected inbred lines. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table is presented on the page following the first referral to it.

CHAPTER 8

LACK OF ISOLATE EFFECTS IN RESISTANCE OF MAIZE TO SILK INFECTION BY *FUSARIUM GRAMINEARUM*

8.1 Abstract

Genotype-isolate interactions in resistance of maize to silk infection by *Fusarium graminearum* were tested by inoculating 13 inbred lines with three *F. graminearum* isolates (ARC2, ORONO, and DAOM) and a sterile water control. Spore suspensions were injected into the upper silk channel to approximate natural infection. No significant isolate effects were found and no major rank reversals occurred for most inbreds. However, one inbred's, that of 'MO17' ranking was significantly changed from susceptible to moderately resistant when infected with the ORONO isolate. Though isolate effects in resistance to silk infection by *F. graminearum* were not sufficient to warrant the use of two or more isolates in screening, it would seem prudent to use a mix of isolates to avoid the possibility of intermediate symptoms caused by a less aggressive isolate.

8.2 Introduction

The development of ear rot resistant maize or corn (*Zea mays* L.) hybrids is an important plant breeding goal. An ear-rotting pathogen of great concern in Canada, some parts of the U.S. and other countries is *Fusarium graminearum* Schw. (sexual state: *Gibberella zeae* (Schw.) Petch) (Sutton, 1982). Infected

grains may be contaminated by mycotoxins produced by this pathogen.

A major mode of entrance of *F. graminearum* into maize ears is via the silk and/or silk channel with infection then spreading down the ear from the tip (Koehler, 1942; Hesseltine and Bothast, 1977). In most years, natural infection is too low to identify genotypic differences in resistance. Thus screening for resistance involves artificial inoculation of the silk or silk channel usually by injection of a spore suspension into the latter.

Host resistance to pathogens is sometimes isolate or race-specific as opposed to race-general resistance which is expressed against all isolates of a pathogen (Bell, 1981). Host-isolate interactions could complicate the identification of resistant genotypes and reduce the effectiveness of screening and selection programs.

Mesterhazy and Kovacs (1986), when testing for resistance to *F. graminearum* infection through wounds by inoculating maize ears by inserting a colonized toothpick through the husk into the centre of the ear, found a significant genotype X isolate interaction for lines with intermediate resistance but not for lines that had either high resistance or susceptibility. They concluded that more than one isolate should be used if measurement of exact differences between genotypes is desired but not for general screenings. Atlin et al. (1983) tested six different isolates on six different hybrids by inserting a colonized toothpick into the silk channel and also concluded that genotype X isolate interactions were not large enough to warrant the use of more than one isolate for resistance breeding.

However, neither of these inoculation methods reflected natural infection through the silk or silk channel.

The purpose of this study was to use three isolates of *F. graminearum* to determine if resistance to silk infection is isolate-specific in a sample of 13 maize inbreds, in order to establish whether it is necessary to use more than one isolate in screening programs.

8.3 Materials and Methods

A 13 x 4 factorial experiment arranged in a split-plot design with four replicates was conducted in two field seasons, 1989 and 1990, at the Plant Research Centre, Agriculture Canada, Ottawa. Each main plot unit consisted of four single-row sub-plot units (3.8 m long, 76 cm between rows) of 12 plants each. Thirteen inbreds ['CO272', 'CO267', 'F7', 'CK44', 'A641', 'Mo17', 'CO266', 'CO282', 'CO317', 'CO265', 'F2', 'CO325', and '8808' (the latter obtained from Drs. A.T. Bolton and R.I. Hamilton)] were randomized to the main plot units . The sub-plot units consisted of one control row inoculated with sterile water and three rows inoculated with one of three *F. graminearum* isolates: ARC2, isolated from diseased maize near Ottawa, Ont.; DAOM180378, obtained from the National Agriculture Canada culture collection; and ORONO 8894, isolated from infected maize near Orono, Ont. Slight morphological differences were found to exist between the three isolates but sporulation on low sugar medium (0.2% dextrose) was similar. Inoculum was prepared using a modified Bilay's liquid medium

consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of potato dextrose agar containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour periods at 4 hour intervals for eight days. Spore counts in these flasks reached 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Primary ears of the center 10 plants of each row or sub-plot were artificially inoculated by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear when the silks were elongated, pollinated, and had some tip browning (approximately 6 days post-silk emergence). To maintain a humid environment, inoculation was followed by overhead sprinkler irrigation at a rate of 2-5 mm twice daily. Evaluations for resistance to infection were made in mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected.

Independence of isolate effects was tested using Kendall's coefficient of concordance (Daniel, 1990). Friedman's two-way analysis of variance by ranks

was used to test the significance of effects for disease ratings (Daniel, 1990).

Kernels exhibiting symptoms of infection and symptomless kernels from randomly selected ears of each treatment were used to re-isolate the pathogen to verify that *F. graminearum* was the causal agent of the infection. Kernels were surface sterilized in 10% sodium hypochlorite (Javex) for 1.0 min., and plated on PDA with 2.0 % dextrose. Visual identification of *F. graminearum* was made 7 days later based on colony colour and spore formation according to Nelson et al. (1983).

8.4 Results

Significant year effects ($P<0.05$) were found for three of the 13 inbreds: 'CO272', 'CK44', and 'Mo17'. All three of these inbreds were more infected in 1990 than in 1989 (Table 8.1). Genotype effects within a given isolate in each year were all significant ($P<0.01$). Based on mean disease ratings over both years, 'CO272' and 'CO325' would be classified as highly resistant (rating <3.0), 'F2', 'CK44', and 'F7' as moderately resistant (rating 3-4), and 'CO265', 'A641', 'Mo17', '8808', 'CO267', 'CO266', 'CO282', and 'CO317' as susceptible (rating >4.0).

The probability of Kendall's coefficient of concordance ($X^2= 34.72$, 1989; $X^2= 31.74$, 1990) at 12 df was less than 0.005 in both years indicating that there was some association among the three sets of isolate rankings. Significant differences were found between *F. graminearum*-inoculated plants and sterile-water-inoculated plants for all inbreds except the most resistant line 'CO272'

Table 8.1. Results of the 1989 and 1990 inoculation tests for the 13 inbred lines inoculated with three *F. graminearum* isolates and a sterile water control.

Isolate	Mean Disease Rating[a]													
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2	8808	Mean
							1989							
ARC2	1.8a	4.9a	4.1a	2.2a	4.9a	5.0a	5.4a	3.0a	5.5a	4.9a	4.2a	4.2a	5.0a	4.2a
DAOM	1.6a	5.5a	4.0a	2.5a	4.4a	4.9a	5.8a	2.7a	5.8a	6.0a	4.9a	3.5a	5.7a	4.4a
ORONO	1.3a	4.6a	3.7a	3.2a	3.5ab	3.3b	4.2ab	1.6ab	4.1b	5.5a	4.0a	2.3ab	3.7b	3.4a
Water	1.0a	1.0b	1.0b	1.0b	1.8c	1.0c	2.0b	1.0b	1.1c	2.5b	1.0b	1.0b	1.7c	1.3b
Mean[b]	1.6	5.0	3.9	2.6	4.3	4.4	5.1	2.4	5.1	5.5	4.4	3.3	4.8	
							1990							
ARC2	1.8a	5.7a	4.7a	4.2a	5.2a	6.1a	5.6a	2.6a	6.0a	5.5a	4.4a	4.1a	5.1a	4.7a
DAOM	1.9a	5.4a	4.0a	3.9a	5.0a	5.6a	4.2a	2.1ab	5.1ab	5.1a	4.7a	3.5a	4.6a	4.2a
ORONO	1.9a	4.4a	3.1a	3.4ab	3.9a	3.4b	5.1a	2.3a	4.6ab	5.2a	2.7ab	2.2ab	4.2a	3.6a
WATER	1.2a	1.3b	1.1b	2.8b	1.0b	1.6c	1.5b	1.1b	1.4c	2.0b	1.8b	1.1b	1.8b	1.5b
Mean[b]	1.9	5.2	3.9	3.8	4.7	5.0	5.0	2.3	5.2	5.3	3.9	3.3	4.6	
Mean[c]	1.8	5.0	3.8	3.2	4.5	4.7	5.0	2.3	5.1	5.4	4.1	3.3	4.7	

[a] Disease ratings are on a scale of 1-7, where 1= no infection and 7= >76% of the kernels infected. Means followed by the same letter for a given inbred within a given year are not significantly different at the 0.025 pairwise error rate by the multiple comparison test to follow Friedman's two-way analysis of variance by ranks.

[b] Mean of isolate ratings (excluding water inoculations).

[c] Mean of 1989 and 1990 results.

(Table 8.1). 'CO272' was consistently ranked first for all isolates in both years (Table 8.2). Isolate effects were not significant in either 1989 or 1990 except in four of the more susceptible inbreds: 'Mo17', and 'CO282' in 1989 and 1990, and '8808' in 1989. In all of these cases, the rating with the ORONO isolate gave significantly lower ratings than the ARC2 and DAOM isolates. This resulted in a change in the classification of 'Mo17' as susceptible with ARC2 and DAOM to moderately resistant with ORONO in both years, and the same for '8808' in 1989. However, 'CO282' remained classified as susceptible in both years for all isolates. These differences resulted in rank changes only for 'MO17' in both years and for '8808' for 1989 (Table 8.2).

Although isolate effects were not significant for most of the inbreds, a trend was noted that the ORONO isolate was the least aggressive as evidenced by the lower disease ratings obtained with this isolate in both 1989 and 1990. Also in 1989, the DAOM isolate was more aggressive than the ARC2 isolate while in 1990 the reverse occurred with ARC2 being the more aggressive isolate. All infections, including water controls, were slightly higher in 1990 than in 1989, possibly due to the higher rainfall in the latter.

F. graminearum was re-isolated from all kernels exhibiting symptoms of infection. Both *F. graminearum* and some *F. moniliforme* were isolated from infected kernels of the control plants, especially of the more susceptible lines 'CO266', 'Mo17', 'CO317', 'CO265', and '8808', indicating that some natural infection had occurred. Almost all symptomless kernels from both inoculated and

Table 8.2. Rankings of inbreds within each isolate for 1989 and 1990.

Isolate	Inbred Rankings[a]												
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2	8808
	1989												
ARC2	1	8	4	2	8	10.5	12	3	13	8	5.5	5.5	10.5
DAOM	1	9	5	2	6	7.5	11.5	3	11.5	13	7.5	4	10
ORONO	1	12	7.5	4	6	5	11	2	10	13	9	3	7.5
Mean	1	9	5	3	6	7.5	11.5	2	11.5	13	7.5	4	10
	1990												
ARC2	1	11	6	4	8	13	10	2	12	9	5	3	7
DAOM	1	12	5	4	9	13	6	2	10.5	10.5	8	3	7
ORONO	1	10	5	6.5	8	6.5	12	3	11	13	4	2	9
Mean	1	11.5	5.5	4	8	9.5	9.5	2	11.5	13	5.5	3	7
Inbred Mean[b]	1	10.5	5	3	7	8.5	10.5	2	12	13	6	4	8.5

[a] Average rankings were assigned to ties.
[b] Mean of 1989 and 1990 results.

control plants were free of infection.

8.5 Discussion

In this study, differences in resistance to *F. graminearum* infection via the silk or silk channel were not isolate-specific. Thus resistance in this group of inbreds (except for possibly 'Mo17') may be race-general resistance, i.e. expressed against all isolates, as opposed to race-specific resistance which would be expressed against some but not other isolates of a pathogen (Bell, 1981). This agreed with the results of Atlin et al. (1983).

Differences in resistance classifications and rankings with the different isolates were only observed consistently for one inbred, 'Mo17'. This difference was due to the seemingly less aggressive isolate ORONO; however, this isolate was capable of differentiating between all other inbreds in agreement with the other two isolates. Thus, providing a sufficiently aggressive isolate is used, a breeder will be able to identify inbreds with useful resistance and discard inbreds with severe susceptibility. Also, the use of the most aggressive isolate available would allow for a greater range in disease ratings and more differentiation between genotypes.

Although these results indicate that one isolate should be sufficient to screen for resistance, in nature a plant would be exposed to many different isolates. A mixture of isolates of various levels of aggressiveness would closely model natural conditions.

Preface to Chapter 9

In the experiments reported in Chapters 5, 6, and 8, both inbreds 'CO272' and 'F7' had very high to moderately high resistance to silk infection by *F. graminearum*. In the following study, these lines were each crossed to a very susceptible line, 'CO266', and F_2 and backcross generations were derived to study the inheritance of silk resistance. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table and figure is presented on the page following the first referral to it.

CHAPTER 9

RESISTANCE OF MAIZE TO FUSARIUM GRAMINEARUM SILK INFECTION IN TWO RESISTANT X SUSCEPTIBLE PARENTAL COMBINATIONS

9.1 Abstract

The inheritance of resistance to silk infection of maize ears by *Fusarium graminearum* in two resistant X susceptible parental combinations was examined. The parental generations (P1=resistant and P2=susceptible) were used to derive F_1, F_2, BC1 (F_1 X P1), and BC2 (F_1 X P2) generations. All six generations were artificially inoculated with a spore suspension of *F. graminearum* injected into the silk channel of individual ears. Plants were rated for the spread of infection. Mean disease severity rating and percent disease incidence were calculated for each generation. Both were lowest in the resistant parents and intermediate in the F_1, F_2, and backcross generations. Histograms were plotted of the number of ears in each disease rating class (1 to 7) for each generation. Disease severity ratings were bimodally rather than normally distributed in the F_1, F_2, and backcross generations. The observed ratios of infected:uninfected plants were not consistent with simple one- or two-gene Mendelian models of qualitative inheritance. A simple additive-dominance model was inadequate to explain the variation among generations. It appears that genetic factors affect the success of the pathogen growing down the silk channel, but that environmental factors affect the spread of disease on the kernels. For exploitation of the sources of resistance studied here,

disease incidence may be a more useful selection criterion than disease severity.

9.2 Introduction

Fusarium graminearum Schw. [sexual state: *Gibberella zeae* (Schw.) Petch], is a major ear-rotting pathogen of maize or corn (*Zea mays* L.). During epidemics, this pathogen enters the maize ear primarily via the silk and/or silk channel (Koehler, 1942; Hesseltine and Bothast, 1977; Sutton, 1982). Infection then spreads down the ear from the tip resulting in a characteristic pinkish mold covering the kernels. When infected grain is fed to livestock, especially swine, mycotoxicoses may result due to two major mycotoxins, deoxynivalenol and zearalenone, which are produced by *F. graminearum* (Vesonder et al., 1981; Mirocha and Christensen, 1974).

Previous studies on the inheritance of resistance to *F. graminearum* have tested for resistance to infection after wounding of the ear or inoculation with a colonized substrate. The inheritance of resistance to these forms of infection appeared to be quantitative, and associated largely with additive gene effects but with some partial dominance (Odiemah and Manninger, 1982; Chiang et al., 1987; Hart et al., 1984; Boling and Grogan, 1965; Odiemah and Kovacs, 1990).

The inbred line 'CO272' appears to be highly resistant to infection via the silk. Preliminary studies conducted at the Plant Research Centre of Agriculture Canada, Ottawa, suggested that the inheritance of this resistance may be qualitative rather than quantitative, with possibly only one dominant gene involved

(Drs. A.T. Bolton and R.I. Hamilton, personal communication). The inbred 'F7' has also been found to exhibit moderate resistance, however, nothing is known about the inheritance of this characteristic.

The purpose of this experiment was to study the inheritance of resistance to *F. graminearum* infection via the silk in six generations derived from each of two resistant X susceptible crosses.

9.3 Materials and Methods

The parental inbreds used in this study were: 'CO272', which is highly resistant to *F. graminearum* silk infection; 'F7', which is moderately resistant; and 'CO266', which is highly susceptible. In 1988, crosses (including reciprocals) were made between 'CO272' (P1) and 'CO266' (P2) and between 'F7' (P1) and 'CO266' (P2) to produce F_1 seed of each cross. In the 1988-89 winter nursery in Chile, F_1 plants were selfed to produce F_2 seed. In addition, F_1 plants were backcrossed to each of their parents to produce BC1 (F_1 X P1) and BC2 (F_1 X P2) seed and additional F_1 seed was produced. Parental lines also were selfed to produce additional seed.

Field evaluations of resistance were carried out in 1989 and 1990 at the Plant Research Centre of Agriculture Canada, Ottawa. In 1989, a randomized complete block design with four blocks was conducted for the 'CO272' X 'CO266' cross. Within each block, each genotype plot consisted of a single row of 12 plants (3.8 m long, 76 cm between rows) of which only the primary ears of the

centre 10 plants were used for evaluations of resistance. Thus a total of 40 ears per generation were evaluated for resistance to silk infection. In 1990, two randomized complete block design experiments with four blocks each were carried out in two locations, both at the Plant Research Centre: one on a clay soil and one sown 10 days later on a sandy loam soil. Plot size was increased to allow for analysis of phenotypic ratios in the segregating generations. Each block consisted of one 5-row plot for each parental generation, one 5-row plot for each F_1 generation, eight 5-row plots for each F_2 generation, and three 5-row plots for each backcross generation. The center three rows of each 5-row plot were used for resistance evaluations, with the two outer rows acting as border rows.

F. graminearum cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour periods at 4 hour intervals for eight days. Spore counts were approximately 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as

inoculum.

Plants were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear when the silks were elongated, pollinated, and had some tip browning (approximately 6 days post-silk emergence). To maintain a humid environment, inoculation was followed by overhead sprinkler irrigation at a rate of 2-5 mm twice daily for four weeks. Evaluations for resistance to spread of infection were made in mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected. The incidence of disease was determined as the percentage of plants in a population with infection on the kernels, i.e. ratings of 2 and above.

Friedman's two-way analysis of variance by ranks was used to test the significance of effects for disease ratings (Daniel, 1990). Parametric statistical methods were used for analysis of disease incidence data (Steel and Torrie, 1980). Normality of the distribution in each generation was tested using the univariate procedure of Statistical Analysis System (SAS/STAT User's Guide, 1988).

The observed numbers of non-infected (rating of 1) and infected plants (rating of 2-7) in the F_2 and backcross generations were compared to the expected numbers for two simple Mendelian models [(1) a monohybrid model with dominance, and (2) a dihybrid model with epistasis (i.e. resistance requires

dominant alleles at both loci)] using a Chi-square test for goodness-of-fit (Steel and Torrie, 1980). The models assumed dominance for resistance, but allowed for differences in gene expression between homozygotes and heterozygotes. Expected numbers in each class were therefore adjusted in accordance with the percentage expression observed in the parental (homozygous dominant and homozygous recessive) and F_1 (heterozygous) generations.

The individual scaling tests A, B, and C of Mather (1949) and Mather and Jinks (1977) and the joint scaling test of Cavalli (1952) as outlined by Mather and Jinks (1972) were carried out on both the mean disease ratings and the disease incidence data for the six generations.

9.4 Results

Infection in 1990 was on average higher than in 1989, but this difference was not statistically significant. No significant reciprocal differences were detected in the F_1 generations (Table 9.1). Location X genotype interactions were not significant in 1990. Data were therefore combined across reciprocals and across the two locations used in 1990. There were significant differences among maize genotypes in both 1989 (X^2= 16.07, $P<0.05$, 6 df) and 1990 [X^2=158.31 (clay soil), X^2= 141.68 (sandy loam soil), $P<0.01$, 12 df each]. For the 'CO272' x 'CO266' cross, the mean disease ratings of the parent inbreds were significantly different from each other and from those of the F_1, F_2, and most backcross generations (Table 9.1). However, the mean disease rating of 'Fy' was not

Table 9.1. Mean disease ratings and disease incidence data for the six generations in 1989 and 1990.

Generation	CO272 X CO266				F7 X CO266	
	1989		1990		1990	
	Rating[a]	Incid.[b]	Rating	Incid.	Rating	Incid.
Parental[c]						
P1	1.2a	30.5a	2.0a	52.9a	2.6a	48.7a
P2	5.6d	100.0d	5.5d	96.4d	---	---
F_1						
P1 X P2	2.7b	62.2b	3.1b	66.7bc	2.4a	69.5b
P2 X P1	3.1b	70.1bc	3.0b	76.1c	2.3a	69.5b
F_1 Mean	2.9b	66.1b	3.0b	71.4b	2.3a	69.5b
F_2	3.5bc	72.2bc	3.3b	77.8c	3.2bc	81.7c
BC1	3.1b	59.4b	3.3b	75.3c	2.7ab*	72.8b
BC2	4.0c	81.2c	4.0c	87.5d	3.9c*	86.8cd

[a] Disease ratings are on a scale of 1-7, where 1= no infection and 7= >76% of the ear infected. Means followed by the same letter for a given column are not significantly different at the 0.025 pairwise error rate.

[b] Disease incidence is the percentage of infected ears in a population, i.e. those with ratings of 2 or more. Means followed by the same letter for a given column are not significantly different at the 0.05 level by Duncan's multiple range test.

[c] In the designation of the cross, the first inbred was considered as P1 and the second inbred as P2.

* For the F7 X CO266 cross the backcross generations were reciprocals to that of the CO272 X CO266 cross, i.e. P1(P1 X P2) and P2(P1 X P2).

significantly different from the F_1 or BC1 generations. The F_1 generation means were not always significantly different from those of the F_2 or BC1 generations.

The resistant parents, 'CO272' and 'F7', were often non-infected (Fig. 9.1). When infection did occur in these lines, it was low to moderate in severity. The susceptible parent, 'CO266', was usually severely infected. The disease ratingdata for the F_1, F_2, and backcross generations are not normally distributed (Appendix 4). The distributions tended to be bimodal, with one peak at the rating class of '1' (no infection) and a second peak at the rating class of '4' (infection of 11-25% of the ear). The position and shape of the second peak gave information about the severity of infection in infected plants. In the F_1, F_2, and backcross generations of 'CO272' X 'CO266', the disease severity of infected plants was greater than in 'CO272' but less than in 'CO266'. In 'F7' X 'CO266', the disease severity of infected plants in the F_1, F_2, and backcross generations was similar to that of 'CO272' x 'CO266'. Among infected F_1 plants, there was considerable variability in the severity of infection, particularly in 'CO272' X 'CO266'. In that cross, the variance of disease severity ratings among infected plants was significantly greater in the F_1 than in the F_2 generation. In 'F7' X 'CO266', there was greater variability in the F_2 than in the F_1 generation.

Neither of the simple Mendelian models adequately explained the inheritance of resistance in these crosses. Chi-squared values were very large (Table 9.2). The smallest X^2 values were obtained for a dihybrid model with epistasis, especially for the 'F7' x 'CO266' parental combination, which had small

FIGURE 9.1

Histograms of the P1, P2, F_1, F_2, and backcross populations of two resistant X susceptible parental combinations. Disease ratings: 1= no infection, 2= 1-3 %, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= 76-100% of the ear infected. 1990.

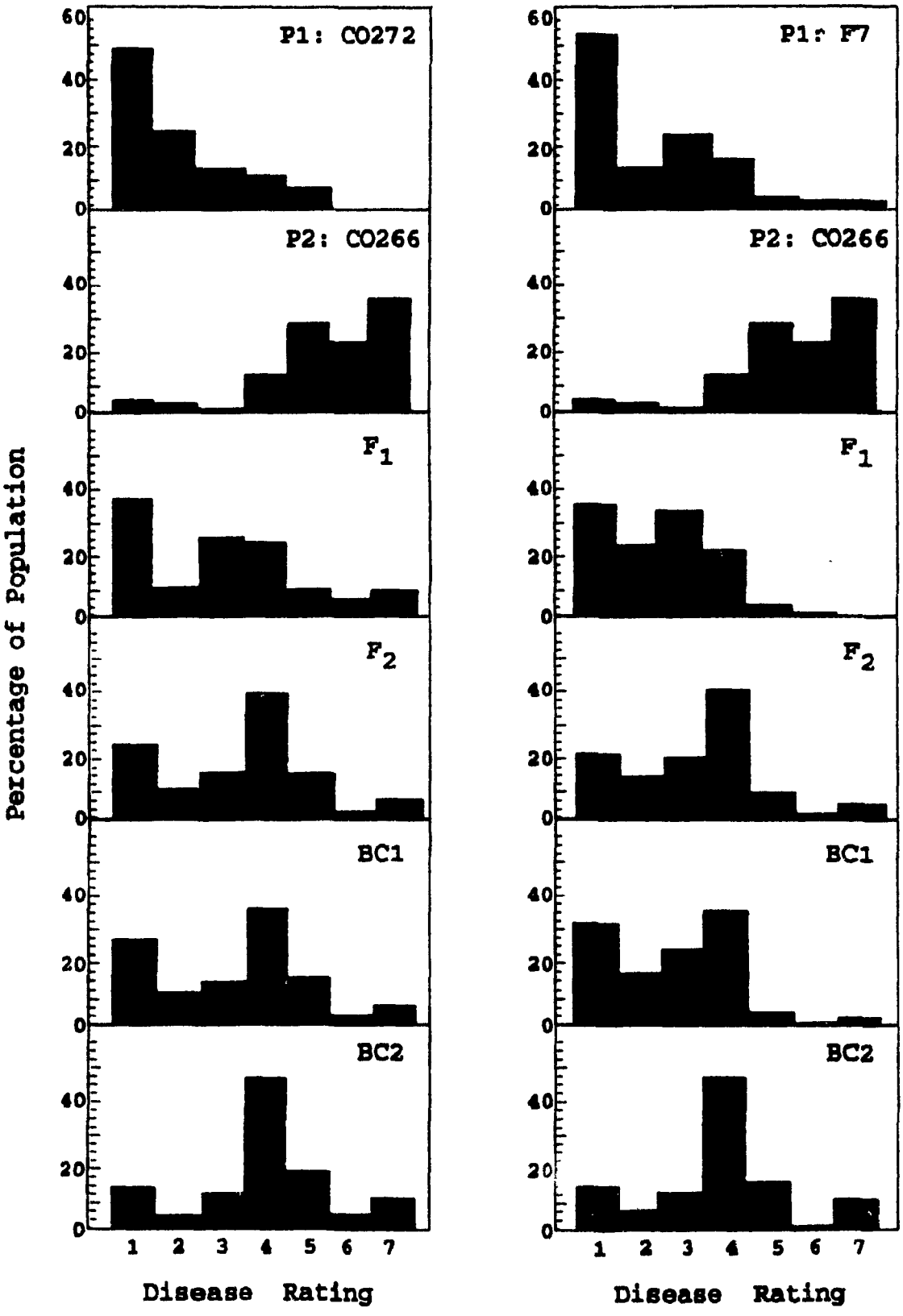

P1: CO272
P2: CO266
F1
F2
BC1
BC2
P1: F7
P2: CO266
F1
F2
BC1
BC2
Percentage of Population
Disease Rating
Disease Rating

Table 9.2. Mendelian models of inheritance tested with percentage of infected and uninfected plants and corresponding Chi-square values.

Model		Generation					
		F_2		BC1		BC2	
		Unfect.[a]	Infect.	Unfect.	Infect.	Unfect.	Infect.
				C0272 X C0266			
	Obs.[b]	372	1303	109	333	63	443
Monohybrid	Exp.	446	1229	166	276	80	426
	X^2	16.73**		31.34**		4.29	
Dihybrid (epistasis)	Exp.	303	1366	144	298	49	457
	X^2	15.75**		12.62**		4.43	
				F7 X C0266			
	Obs.	248	1106	140	375	28	184
Monohybrid	Exp.	392	962	211	304	36	176
	X^2	74.45**		40.47**		2.14	
Dihybrid (epistasis)	Exp.	271	1083	183	332	22	190
	X^2	2.44		15.76**		1.83	

[a] Unfect.= number of uninfected plants (rating of 1); Infect.= number of infected plants (ratings of 2-7).

[b] Obs.= observed number of plants; Exp.= expected number of plants based on the percentage of uninfected plants in the P1, P2, and F_2 generations.

** Chi-square value is significant at the 0.01 level and thus the observed is significantly different from expected and the model does not fit the data.

X^2 values for both the F_2 and BC2 generations (Table 9.2).

One of the individual scaling tests (B) showed significant departures from zero for incidence of infection and for mean disease ratings. Similarly, the joint scaling test detected significant deviations from expectations for anadditive-dominance genetic model ['CO272' X 'CO266', X^2= 29.28 (mean disease rating) and X^2= 23.18 (disease incidence), P<0.01, 3 df; 'F7' X 'CO266', X^2= 18.81 (mean disease rating) and X^2= 15.35 (disease incidence), P<0.01, 3 df). No further quantitative analyses were carried out.

9.5 Discussion

From the data collected in this study, it was possible to classify plants as either infected or uninfected to obtain a measure of disease incidence within each generation, and also to use disease ratings as a measure of disease severity for individual plants. The latter were averaged to obtain mean disease ratings for each generation.

Disease incidence was lowest in the resistant inbreds 'CO272' and 'F7', highest in the susceptible inbred 'CO266', and intermediate in the F_1, F_2, and backcross generations of resistant X susceptible crosses. There appeared to be some dominance in favour of resistance, in that disease incidences in the F_1 generations were somewhat closer to those of the resistant parent than to those of the susceptible parent. Disease incidences in the F_2 generations were higher than those of the F_1 generations.

The observed ratios of infected:uninfected plants within the F_2 and backcross generations are not consistent with those expected for simple qualitative models involving one or two dominant genes. The F_2 and BC2 generations of the 'F7' X 'CO266' cross fit a two gene model but the BC1 generation did not.

Dominance and/or gene expression may be incomplete.

Disease incidence and disease severity may be treated quantitatively so that methods of quantitative genetic analysis may be applied. Our intention was to apply mean effects analysis (Gamble, 1962) to these characters for the six generations evaluated. This analysis was not carried out because the data failed to satisfy certain assumptions of the analysis. For both disease incidence and disease severity, scaling tests indicated that a simple additive-dominance model was inadequate to explain the variation among generations. With only six generations, it is not possible to test the adequacy of more complex models involving digenic or higher order interactions. Furthermore, the distributions of disease severity within the various generations were not normal. The mean effects analysis is not particularly sensitive to minor deviations from normality, but its validity would be questionable for some of the obviously bimodal distributions observed. Finally, variation in disease severity among plants within the genetically heterogeneous F_2 generation of 'CO272' X 'CO266' was similar to that observed in the genetically homogeneous F_1 generation, suggesting that among infected plants, much of the plant-to-plant variation in the extent of infection on the ear was

not due to genetic differences. However, 'F7' may have some genetic variability for resistance to the spread of infection since variation in disease severity of the F_1 generation was less than that of the F_2 generation.

It appears t hat disease incidence is reduced in resistant inibreds such as 'CO272' and 'F7'; they can apparently impede the progress of the pathogen down the silk, and thus frequently prevent infection of the kernels. Nevertheless, the pathogen may sometimes succeed in reaching the kernels and moderate or even severe infection may result. Factors allowing this may include a high spore concentration of the inoculum, as was used in this study, and environmental conditions favouring fungal growth, such as the humid conditions experienced in 1990. A number of environmental factors, especially temperature and moisture, previously have been reported to affect the reaction of maize to *Fusarium* ear rot (Enerson and Hunter, 1980; Koehler, 1959; Sutton, 1982). Gendolf et al. (19856), when testing for resistance to infection via wounds, also found large variability in the F_1 generation and concluded that it was due to environmental factors. It is possible that heavy rainfall following inoculation may increase infection by forcing inoculum down the silk channel to the cob, thereby circumventing any resistance mechanisms residing in the silk tissue itself. Such factors, in combination with minor plant-to-plant differences is morphology and flowering time, could influence whether or not a particular plant would become infected. In a h ighly susceptible inbred, such as 'CO266', the pathogen is usually able to progress down the silk and infect the ear.

Variation in non-genetic factors apparently also affects the extent to which the pathogen colonizes infected ears, and thus influences the average disease rating of population. Despite the fact that disease ratings of infected plants of the more resistant parents tended to be lower than those of the susceptible parent, the data from F_1, F_2, and backcross generations of resistant X susceptible crosses shows no clear evidence that disease severity in infected ears is genetically controlled.

Although this experiment did not succeed in clearly elucidating the genetic control of the *F. graminearum* resistance carried by 'CO272' and 'F7', it did confirm that these inbreds carry heritable resistance which could be used in maize breeding. While the degree of resistance which could be used in maize breeding. While the degree of resistance possessed by these inbreds was not sufficient to completely prevent infection in this experiment, it should be noted that the conditions of this experiment were designed to deliberately favour the pathogen. Under non-inoculated, non-irrigated conditions used in commercial maize production, resistance derived from such inbreds might provide adequate protection from *F. graminearum* infection via the silk.

The patterns of variation observed in the generations studied here provide some guidance on appropriate selection methods for use in maize breeding. One selection strategy would be to eliminate each entry with one or more infected ears, on the assumption that the family or line is susceptible, and that non-infected individuals are 'escapes'. Such an approach wouwld have been unwise for the

germplasm and conditions used in this study, as evidenced by the fact that the ears of some plants of the inbreds 'CO272' and 'F7' became infected, even though these inbreds clearly carry useful heritable resistance. Desirable lines or families would be lost if all entries with one or more infected ears were discarded. To i dentify useful lines or families within a breeding program, it is important to evaluate several to many individuals, and to retain those entries with the lowest percentages of infected ears. With the high concentration of inoculum used, it was possible to obtain infection on almost all plants of the susceptible line 'CO266'. Thus, in a breeding program, it should be possible to eliminate such lines with confidence on the basis of of high disease incidence. Less attention should be paid to the severity of the infection where it occurs, because this may be more environmentally influenced.

Preface to Chapter 10

The results of Chapter 8 revealed that large differences existed in the susceptibility of corn inbreds to *F. graminearum* infection via the silk and/or silk channel. Only a few inbreds, such as 'CO272' and 'F7', were resistant. Chapter 9 revealed that this resistance is heritable. The following study, Chapter 10, was designed to determine the range of resistance in a larger group of inbreds, the resistance of hybrids of these inbreds with 'CO272', and to conduct a 12 X 12 diallel cross involving 12 of these inbreds. From the latter it was possible to select inbreds and hybrid combinations, if any, which would have the greatest potential in breeding for increased resistance. As well, further insights into the inheritance of resistance could be obtained. All literature cited in this chapter is listed in Chapter 17 of the thesis. Each table is presented on the page following the first referral to it.

CHAPTER 10

DIALLEL ANALYSIS OF RESISTANCE OF MAIZE TO *FUSARIUM GRAMINEARUM* INFECTION VIA THE SILK

10.1 Abstract

A set of 37 inbreds and a 12 X 12 complete diallel cross were evaluated at each of two locations for resistance to *Fusarium graminearum* ear rot infection via the silk and/or silk channel. Thirty-seven inbreds were artificially inoculated with a *F. graminearum* spore suspension injected into the silk channel of individual ears. Mean disease ratings, made on a scale of 1-7, revealed that most inbreds were susceptible. Most hybrids between each of these inbreds and the resistant inbred 'CO272' were moderately to highly resistant. Twelve of these inbreds, chosen to represent a range of susceptibility, were intercrossed in a complete diallel arrangement. Disease severity and incidence in the parents, crosses and reciprocals were evaluated at two locations. Genotype, general combining ability (GCA), and specific combining ability (SCA) effects were significant for both mean disease rating and disease incidence data. The GCA effects of the parents were correlated both to mean disease ratings and to disease incidence, with the most resistant parent 'CO272' having the largest negative GCA. The performance of hybrids could not be predicted simply on the basis of the performance or GCA of the parents. Reciprocal differences were significant only for mean disease ratings of one location. Four inbreds were identified as having significant GCA for

resistance to *F. graminearum* infection via the silk.

10.2 Introduction

Fusarium graminearum Schw., the asexual stage of *Gibberella zeae* (Schw.) Petch, is an important ear-rotting pathogen of maize (*Zea mays* L.) in Ontario, Quebec, and many parts of the US and other countries (Sutton, 1982). A major mode of entrance of this pathogen into the ear is via the silk and/or silk channel, especially during epidemics. Infection then proceeds down the silk channel to the kernels and rachis (Koehler, 1942; Hesseltine and Bothast, 1977). The major symptom of *F. graminearum* infection is a characteristic pink to reddish mold on kernels and between husks and cobs (Sutton, 1982). Husks may adhere tightly to the kernels in severely infected ears. Contaminated grain poses a major threat to livestock production, especially swine, since such grain may harbour mycotoxins (Vesonder et al., 1981).

Breeding for more resistant hybrids is one of the more feasible control methods for this pathogen. Studies have suggested that inheritance of resistance to ear rot is quantitative, associated with additive gene effects, and partially dominant (Odiemah and Manninger, 1982, Chiang et al., 1987; Hart et al., 1984; Boling and Grogan, 1965; Odiemah and Kovacs, 1990). In most of these studies ears were artificially inoculated by inserting a colonized toothpick directly through the husk into the center of each rachis. Such inoculation methods may simulate infection via bird or insect wounds but do not test for infection via the silk and/or

silk channel. Little is known about the inheritance of resistance to silk infection or the range of susceptibility that exists.

There is a need to identify inbreds with resistance and to determine which will have the greatest potential in breeding programs designed to increase resistance to *F. graminearum* infection.

The purposes of this study were: (1) to assess the susceptibility to infection via the silk of a group of inbred lines of varying genetic backgrounds and of the F_1 single crosses between these inbreds and the highly resistant line 'CO272'; and (2) to evaluate disease incidence and severity in a diallel cross involving 12 inbreds, including 'CO272', selected from the preliminary screening.

10.3 Materials and Methods

In 1989, the maize genotypes evaluated included 37 inbred lines (Table 10.1), and 37 F_1 hybrids between these inbreds and a highly resistant inbred 'CO272'. The experimental design was a randomized complete block with two replicates planted on a sandy loam soil at the Plant Research Centre of Agriculture Canada, Ottawa.

Based on the results obtained in 1989, 11 inbreds ('CO267', 'F7', 'CK44', 'A641', 'Mo17', 'CO266', 'CO325', 'CO282', 'CO317', 'CO265', and 'F2') were chosen to represent a range of susceptibility and were crossed, along with 'CO272', in a complete 12 x 12 diallel in the 1989-90 winter nursery. In 1990, all possible F_1 single crosses, including reciprocals, and parents were evaluated for

resistance in a randomized complete block design with four replicates at each of two locations at the Plant Research Centre in Ottawa: one on a clay soil and the other sown 10 days later on a sandy loam soil.

Each plot consisted of a row of 12 plants (3.8 m long, 76 cm between rows) of which the primary ears of the middle 10 plants were inoculated and later evaluated for disease symptoms. *F. graminearum* cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour periods at 4 hour intervals for eight days. Spore counts were approximately 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Plants were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear when the silks were elongated,

pollinated, and had some tip browning (approximately 6 days post-silk emergence). After inoculation, plots were irrigated for four weeks at a rate of 2-5 mm twice daily. Evaluations for resistance to spread of infection were made in mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected. In addition, for the diallel study, the incidence of disease was determined as the percentage of plants in a population with infection on the kernels, i.e. ratings of 2 or more.

Friedman's two-way analysis of variance by ranks was used to test the significance of effects for disease ratings (Daniel, 1990). Parametric statistics were used for analysis of disease incidence data (Steel and Torrie, 1980).

The number of days from planting to the time when 50% of the plants in a row had silk protruding at least 1 cm from the husk were recorded for each genotype. Spearman's rank correlation coefficient (Daniel, 1990; Steel and Torrie, 1980) was used to test the relationship between days to silking and resistance evaluations.

Diallel analysis was based on Griffing's (1956) model I, method 1 for a non-random (fixed) population and all p^2 combinations, where p is the number of parents. Analysis was carried out on both mean disease ratings and on disease incidence.

10.4 Results

In 1989, there were significant genotypic differences for both disease ratings ($p<0.01$, $F=5.30$, $df=74$) and relative days to silking ($p<0.01$, $F=4.19$, $df=74$). No significant correlation was found between resistance evaluations and days to silking.

The genotypes evaluated ranged from highly resistant to highly susceptible (Table 10.1). Based upon mean disease ratings genotypes were classified as: highly resistant (HR), rating less than 3.0; moderately resistant (MR), rating 3.0-4.0; or susceptible (S), rating greater than 4.0.

Of the 38 inbreds, only 'CO272' and 'F7', were classified as resistant (Table 10.1). Four inbreds ('CO289', 'CK44', '80P14', and 'A634') were moderately resistant, and all others were classified as susceptible. Hybrid combinations of these susceptible lines with 'CO272' exhibited more resistance; there were 23 resistant hybrids, four moderately resistant hybrids, and only four susceptible hybrids. None of the hybrids had mean disease ratings lower than that of 'CO272'. The hybrid combination of 'CO272' X 'F7' was only intermediate in resistance.

In the diallel analysis of mean disease ratings, the error mean squares of the two locations were heterogeneous ($p<0.01$, $F=2.0306$, $df=429$), with greater variability on the sandy loam soil. Genotype effects were significant in both locations [$F=2.0171$ (clay soil), $F=2.5328$ (sandy loam soil), $p<0.01$, $df=143$). As in 1989, no correlation was found between resistance evaluations and days to

Table 10.1. Ear mold ratings of 37 inbreds and F_1 single-crosses to the highly resistant inbred 'CO272'.

Inbred	Inbred			Hybrid Combination with CO272		
	Days to Silking[a]	Disease Rating[b] (1-7)	Resistance Class[c]	Days to Silking	Disease Rating (1-7)	Resistance Class
CO272[d]	75	1.3	HR	---	---	---
CO286	76	4.8	S	71	3.0	MR
CO216	67	4.7	S	69	2.8	HR
CO267[d]	76	5.5	S	68	2.9	HR
Mo17[d]	79	4.9	S	71	2.2	HR
CO273	61	4.3	S	63	2.7	HR
CO289	67	3.7	MR	68	1.3	HR
CO319	65	5.4	S	67	4.8	S
CO265[d]	67	4.2	S	62	2.4	HR
77H44	71	5.5	S	75	4.0	MR
F2[d]	64	4.3	S	58	2.9	HR
CO318	65	5.1	S	71	4.5	S
77H8[d]	70	4.6	S	69	2.6	HR
CO315	67	4.3	S	63	1.5	HR
77I82	67	5.9	S	66	1.4	HR
F7[d]	58	1.8	HR	60	2.9	HR
77K45	70	5.6	S	71	5.2	S
CK44[d]	61	3.4	MR	61	3.2	MR
78N10	66	4.1	S	71	1.7	HR
CO303	66	4.8	S	67	2.9	HR
80P14	69	3.7	MR	67	3.0	MR
80B9	72	5.1	S	66	2.2	HR
CO308	70	5.0	S	65	3.0	MR
A641[d]	68	5.0	S	63	3.1	MR

(Table 10.1 cont'd....)

Inbred	Inbred			Hybrid Combination with CO272		
	Days to Silking[a]	Disease Rating[b] (1-7)	Resistance Class[c]	Days to Silking	Disease Rating (1-7)	Resistance Class
80Z2	71	4.9	S	67	2.9	HR
81P14	64	4.3	S	68	2.9	HR
CO266[d]	59	4.8	S	61	2.0	HR
82M19	71	4.1	S	66	2.4	HR
78N9[d]	68	4.4	S	64	2.4	HR
CO314	68	5.2	S	68	1.5	HR
A634	75	3.8	MR	73	3.7	MR
B73	83	6.2	S	68	1.6	HR
CO282[d]	58	5.0	S	61	2.4	HR
CB6-9-1	75	5.4	S	74	2.7	HR
CM174	69	5.7	S	66	3.3	MR
V360	66	4.2	S	66	2.5	HR
W117	75	5.1	S	71	4.3	S

[a] The average number of days from planting to 50% of the plants with silk.

[b] Mean disease rating of two replicates (20 plants). Lower values indicate less infection and thus higher resistance.

[c] HR= highly resistant, rating <3.0; MR= moderately resistant, rating 3.0-4.0; and, S= susceptible, rating greater than 4.0.

[d] Inbred chosen for diallel cross.

silking. Silking occurred between 72 and 89 days after planting in the parents and between 69 and 84 days in the hybrids (Appendix 5).

Mean disease ratings, for each location are summarized in Tables 10.2 and 10.3. According to the resistance classification system used in Table 10.1, 'CO272' was highly resistant, 'F7' and 'F2' were moderately resistant and the other nineparental inbreds were susceptible. All hybrids involving the highly resistant line 'CO272' were resistant or moderately resistant. Most hybrids involving 'F7', 'CO325', and 'F2' were highly resistant or moderately resistant. Most hybrids involving two susceptible lines were more resistant than either of their parents.

Diallel analysis of the mean disease rating data revealed that the mean squares for general combining ability effects (GCA) and specific combining ability effects (SCA) were highly significant in both locations (Table 10.4). Reciprocal effects were significant on the sandy loam soil but were not on the clay soil. The components of variance $\sum g_i^2$, where g_i= GCA effect of the parent "i", and $\sum_i\sum_j s_{ij}^2$, where s_{ij}= SCA effect of the hybrid of parent "i" and parent "j", were estimated from the expectation of the GCA and SCA mean squares (Griffing, 1956) and expressed as the ratio $2\sum g_i^2/(2\sum g_i^2 + \sum_i\sum_j s_{ij}^2)$. The values of this ratio were 0.21 for the clay soil location and 0.16 for the sandy loam soil location.

General combining ability and specific combining ability effects are given in Tables 10.5 and 10.6. Negative effects were associated with resistance (lower disease ratings) and positive effects were associated with susceptibility (higher disease ratings). Three inbreds had significant negative GCA effects at both

Table 10.2. Mean disease ratings for the parental lines and the F_1 single-cross hybrids of a 12 x 12 diallel design[a]. Clay soil location.

Parent	Female											
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male												
CO272	2.5	2.7	2.9	3.1	2.5	2.8	3.2	2.5	3.7	2.9	3.3	2.3
CO267	2.6	5.0	3.4	5.5	3.7	3.9	4.9	3.2	3.7	4.4	3.8	3.6
F7	3.1	3.5	3.7	3.3	2.9	3.9	3.7	2.9	3.6	3.9	3.8	3.2
CK44	3.5	3.8	4.7	4.6	3.4	3.1	3.7	2.6	4.1	4.2	3.3	3.2
A641	3.5	4.6	4.2	4.0	4.1	3.4	4.7	3.3	4.7	3.5	3.9	3.4
Mo17	2.9	4.1	3.8	3.0	3.5	4.6	3.7	3.2	3.3	4.1	4.2	3.2
CO266	3.7	5.1	4.0	4.6	3.4	3.7	4.1	4.0	5.3	4.2	4.8	4.3
CO325		2.6	4.0	2.7	2.4	3.2	3.6	3.9	4.1	3.5	3.2	3.42.4
CO282	2.8	4.1	4.1	2.5	3.5	4.3	4.4	3.9	4.5	5.5	3.7	3.8
CO317	3.1	4.4	3.3	3.7	3.8	4.4	4.0	3.3	5.2	4.4	4.3	4.2
CO265	3.4	4.1	4.3	4.0	3.2	3.9	4.5	2.8	4.0	4.0	4.1	3.2
F2	3.4	2.3	2.4	2.8	2.7	3.7	4.8	4.0	2.7	3.7	3.3	3.1

[a] Disease ratings are on a scale of 1-7, where 1= no infection, and 7= 76% of the kernels infected. Each value is the mean of 80 ears averaged over two locations with four replicates each.

Table 10.3. Mean disease ratings for the parental lines and the F_1 single-cross hybrids of a 12 x 12 diallel design[a]. Sandy loam soil location.

Parent	Female CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male												
CO272	1.2	3.1	2.9	3.5	3.1	3.7	4.0	3.7	4.8	4.1	4.0	2.9
CO267	4.0	5.6	3.1	2.5	3.6	4.5	5.0	3.2	4.6	4.6	4.4	3.3
F7	3.0	3.1	3.3	3.2	3.4	4.5	3.8	2.8	2.6	3.9	4.3	2.2
CK44	3.2	4.2	2.6	4.2	3.1	4.2	4.3	2.4	2.2	4.2	.4	3.0
A641	3.5	4.0	3.8	3.6	4.5	3.1	4.6	4.0	4.0	4.2	4.0	3.3
Mo17	3.3	4.0	3.5	3.7	3.9	4.8	4.1	3.8	3.1	5.1	4.7	3.8
CO266	2.8	4.9	3.3	4.2	3.8	5.1	6.5	3.9	4.0	4.3	5.6	3.5
CO325		3.3	3.0	3.0	2.7	3.1	4.4	3.9	4.3	3.5	4.0	3.42.8
CO282	3.8	3.7	2.6	2.5	3.8	5.0	4.1	3.4	4.5	4.7	4.3	3.5
CO317	3.5	4.7	4.0	3.3	3.6	4.6	4.3	4.2	5.4	5.7	4.5	3.8
CO265	3.7	4.1	4.3	3.8	4.0	4.8	4.8	3.5	4.4	4.7	5.3	2.4
F2	3.2	3.7	2.7	2.9	2.8	3.7	4.0	2.9	3.2	4.0	3.9	3.7

[a] Disease ratings are on a scale of 1-7, where 1= no infection, and 7= 76% of the kernels infected. Each value is the mean of 80 ears averaged over two locations with four replicates each.

Table 10.4. Combining ability analysis for resistance to *Fusarium graminearum* infection via the silk. Mean disease ratings.

Source of Variation	df	Sum of Squares	Mean Square	F Value
		Clay Soil Location		
Genotypes	143	75.559	0.528	2.609**
GCA	11	35.132	3.193	15.772**
SCA	66	23.697	0.359	1.773**
Reciprocal	66	16.730	0.253	1.252ns
Error	429	86.895	0.202	
		Sandy Loam Soil Location		
Genotypes	143	91.372	0.639	6.409**
GCA	11	51.318	4.665	46.792**
SCA	66	29.155	0.442	4.430**
Reciprocal	66	10.900	0.165	1.656**
Error	429	42.789	0.100	

**,ns Significant at the 0.01 probability level, not significant respectively.

Table 10.5. General combining ability effects (diagonal) and specific combining ability effects (off diagonal) for the 12 x 12 diallel design. Mean disease ratings on clay soil location.

	Female											
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male												
CO272	-0.669**	-0.656	0.127	0.081	0.027	0.252	0.485	0.523	0.394	-0.052	-0.544	0.744
CO267		0.323*	-0.381	-0.185	-0.056	-0.415	0.085	0.023	-0.356	-0.202	0.156	0.185
F7			-0.110	-0.073	-0.335	0.740	-0.398	0.256	0.127	-0.240	-0.227	-0.294
CK44				-0.065	0.252	0.190	0.073	-0.231	-0.590	-0.127	0.194	-0.206
A641					-0.015	-0.027	-0.140	-0.281	0.023	0.073	0.231	0.394
Mo17						0.052	-0.640	-0.015	-0.427	0.140	0.131	0.923*
CO266							0.540**	-0.294	-0.610	-0.340	0.219	-0.240
CO325								-0.460**	0.010	-0.202	0.335	-0.340
CO282									0.323*	-0.202	-0.444	-0.031
CO317										0.352*	0.344	0.331
CO265											0.115	-0.131
F2												-0.385*

Standard Error:

S.E. $(g_i - g_j) = 0.130$, $(i \neq j)$

S.E. $(s_{ij} - s_{ik}) = 0.431$, $(i \neq j,k;\ j \neq k)$

S.E. $(s_{ij} - s_{kl}) = 0.411$, $(i \neq j,k,l;\ j \neq k,l;\ k \neq l)$

*,** Significantly different from zero at the 0.05 and 0.01 level respectively.

Table 10.6. General combining ability effects (diagonal) and specific combining ability effects (off diagonal) for the 12 x 12 diallel design. Mean disease ratings on sandy loam soil location.

	Female											
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male												
CO272	-0.494**	0.024	0.144	0.074	0.515	0.257	0.349	-0.014	0.532	0.211	-0.197	-0.118
CO267		0.214*	0.424	-0.222	0.911*	-0.414	-0.593	0.386	-0.330	0.049	0.211	-0.105
F7			-0.507**	-0.493	-0.085	-0.285	0.053	0.290	0.015	-0.630	0.257	0.049
CK44				-0.386**	0.070	-0.135	0.057	-0.330	0.120	-0.101	0.061	-0.497
A641					-0.086	-0.180	-0.239	-0.072	0.249	0.157	0.065	-0.110
Mo17						0.410**	0.001	-0.776*	-0.543	0.103	0.053	0.594
CO266							0.581**	0.078	-0.547	-0.393	-0.160	-0.001
CO325								-0.328**	-0.243	-0.189	-0.414	-0.014
CO282									0.077	-0.151	-0.660	-0.247
CO317										0.572**	0.344	0.040
CO265											0.468**	-0.605
F2												-0.519**

Standard Error:

S.E. $(g_i - g_j) = 0.091$, $(i \neq j)$

S.E. $(s_{ij} - s_{ik}) = 0.302$, $(i \neq j,k;\ j \neq k)$

S.E. $(s_{ij} - s_{kl}) = 0.288$, $(i \neq j,k,l;\ j \neq k,l;\ k \neq l)$

*,** Significantly different from zero at the 0.05 and 0.01 level respectively.

locations: 'CO272', 'F2', and 'CO325'. Two additional inbreds, 'F7' and 'CK44', had significant negative GCA effects at the sandy loam soil location. The inbred 'CO266' had the largest positive GCA effect in both locations. GCA effects were significantly correlated ($p<0.01$) with the mean disease ratings of the inbreds in both locations ($r=0.73$, clay soil; $r=0.82$, sandy loam soil). Only one hybrid, 'F2'X 'Mo17' had an SCA effect significantly different from zero in the clay soil location (Table 10.5). At the sandy loam soil location, only the hybrids 'A641' X 'CO267' and 'CO325' X 'Mo17' had SCA effects significantly different from zero (Table 10.6). Many of the reciprocal effects in the sandy loam location were significantly different from zero (Table 10.7). None of the parental inbreds appeared to be consistently better as a maternal parent than as a paternal parent, or vice versa.

For the disease incidence data, location effects were not significant. Of all parents, 'CO272' had the lowest disease incidence followed by 'F2' (Table 10.8). All other parents had 70% or more ears infected. Most of the hybrids had lower disease incidence than their parents but none had lower incidence than 'CO272'. The average disease incidence of hybrids was lowest when 'CO272' or 'F2' were one of the parents.

Both GCA and SCA were highly significant sources of variation in the diallel analysis of disease incidence (Table 10.9). The ratio of the components of variance ($\sum g_i^2$ and $\sum_i \sum_j s_{ij}^2$) was 0.15. Reciprocal effects were not significant.

The inbred 'CO272' had the largest negative GCA effect (Table 10.10) for disease incidence. The inbreds 'F2', 'CO325', and 'CK44' also had significant

Table 10.7. Reciprocal effects for the 12 x 12 diallel design. Mean disease ratings on the sandy loam soil location.

	Female										
	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male											
CO272	0.45**	0.05	0.20	-0.20	0.15	-0.15	-0.30*	0.00	-0.30*	0.50**	0.25*
CO267		-0.15	-0.20	-0.50**	0.00	-0.10	0.20	0.25*	-0.05	0.30*	-0.05
F7			-0.60**	-0.30*	0.85**	-0.45**	-0.50**	-0.25*	0.40**	0.45**	0.10
CK44				-0.15	0.20	0.05	-0.25*	-0.25*	-0.40**	-0.25*	0.05
A641					-0.25*	-0.15	0.10	-0.05	-0.45**	0.05	0.05
Mo17						0.20	0.00	0.15	-0.10	-0.05	0.35**
CO266							0.05	0.15	-0.30*	0.00	0.05
CO325								-0.45**	0.00	0.05	-0.15
CO282									-0.25*	0.00	0.10
CO317										0.40**	0.10
CO265											0.75**

Standard Error:
S.E. $(r_{ij}-r_{kl})$= 0.100, $(i \neq j;\ k \neq l)$
*,** Significantly different from zero at the 0.05 and 0.01 level respectively.

Table 10.8. Disease incidence (averaged over both locations) for the parental lines and the F_1 single-cross hybrids of a 12 x 12 diallel design[a].

	Female											
	C0272	C0267	F7	CK44	A641	Mo17	C0266	C0325	C0282	C0317	C0265	F2
Male												
C0272	21.6	47.6	68.3	70.5	59.3	65.4	77.3	65.9	90.3	70.8	83.5	52.3
C0267	61.1	90.5	65.2	71.8	77.8	86.2	96.3	65.5	77.9	95.8	89.4	67.0
F7	66.6	65.5	83.8	74.1	67.1	98.3	90.4	66.0	67.9	81.1	92.8	48.6
CK44	72.0	89.3	75.4	88.5	72.5	80.3	74.5	24.1	76.3	91.3	85.9	67.0
A641	75.9	91.1	81.9	78.3	94.8	69.0	97.5	85.0	88.8	81.9	86.4	64.0
Mo17	68.8	77.1	89.7	72.4	84.3	93.3	90.4	79.0	92.3	98.8	97.6	75.4
C0266	66.4	98.8	89.4	93.5	80.0	86.6	82.1	87.0	92.9	87.6	97.9	80.3
C0325	69.1	75.9	59.4	49.3	74.9	87.6	84.4	87.5	78.1	78.5	74.5	52.4
C0282	63.4	76.0	67.5	38.5	84.8	94.4	91.6	83.1	98.5	92.1	83.9	74.8
C0317	68.3	98.8	83.0	66.8	83.1	90.4	90.0	83.9	95.0	80.6	98.8	79.5
C0265	80.9	81.0	91.4	77.9	86.9	92.4	91.3	74.5	83.3	98.8	89.5	53.9
F2	56.6	60.1	48.5	58.9	54.5	79.4	93.8	64.9	64.2	87.5	77.9	68.6

[a] Infected ears are those with disease ratings greater than 2.

Table 10.9 Combining ability analysis for resistance to *Fusarium graminearum* infection via the silk. Disease incidence.

Source of Variation	df	Sum of Squares	Mean Square	F Value
Genotypes	143	30662.24	214.42	4.30**
GCA	11	16084.13	1462.19	29.32**
SCA	66	10610.08	160.76	3.22**
Reciprocal	66	3968.03	60.12	1.20^{ns}
Error	1001	49908.50	49.86	

**,ns Significant at the 0.01 probability level, not significant respectively.

Table 10.10. General combining ability effects (diagonal) and specific combining ability effects (off diagonal) for the 12 x 12 diallel design analyzed for disease incidence (averaged over both locations).

	Female											
	CO272	CO267	F7	CK44	A641	Mo17	CO266	CO325	CO282	CO317	CO265	F2
Male												
CO272	-13.67**	-10.99	5.88	1.50	8.75	1.51	8.92	5.10	9.02	1.64	-6.20	10.82
CO267		1.04	12.52	-4.47	9.09	-10.93	-2.76	-2.52	-15.31**	1.91	3.77	4.73
F7			-2.73	-2.07	-2.74	7.11	-5.51	11.67	1.20	-10.24	4.81	0.80
CK44				-5.57*	10.04	3.64	10.30	5.07	-3.17	-0.64	1.52	-5.78
A641					1.81	-4.48	-1.67	-6.96	1.98	5.73	2.05	-2.41
Mo17						7.12**	-4.10	-10.97	-30.15**	3.58	3.67	4.14
CO266							9.62**	-1.15	-18.45*	-5.26	3.67	-5.68
CO325								-5.55*	-1.34	-0.98	1.21	-0.56
CO282									3.45	-9.17	-6.78	-10.32
CO317										7.99**	-0.85	14.90*
CO265											7.86**	-8.57
F2												-11.36**

Standard Error:

S.E. $(g_i - g_j) = 2.04$, $(i \neq j)$

S.E. $(s_{ij} - s_{ik}) = 6.76$, $(i \neq j,k;\ j \neq k)$

S.E. $(s_{ij} - s_{kl}) = 6.45$, $(i \neq j,k,l;\ j \neq k,l;\ k \neq l)$

*,** Significantly different from zero at the 0.05 and 0.01 level respectively.

negative GCA effects for disease incidence. Estimates of GCA effects were significantly correlated to the percentage of infected ears of the inbreds ($r=0.64$, $p<0.05$). Four hybrids had SCA effects significantly different from zero: 'CO282' X 'CO267', 'CO282' X 'Mo17', 'CO282' X 'CO266', and 'F2' X 'CO317'.

10.5 Discussion

The preliminary screening of inbreds suggested that a high degree of susceptibility to *F. graminearum* existed. The evaluation of hybrids showed that hybrids between highly resistant and susceptible inbreds can be moderately to highly resistant. The relatively high resistance of the hybrids suggests that dominance was involved in the inheritance of the resistance from 'CO272'.

The lack of correlation between silking date and resistance evaluations suggests that the low level of infection in 'CO272' is not attributable to lack of infection due to late silking. Other late-silking inbreds were rated as susceptible. This lack of correlation between resistance evaluations and days to silking is similar to the results obtained by Hart et al. (1984) who reported that disease ratings obtained with the toothpick method of inoculation were not associated with early maturity. Although 'CO272' was relatively late-maturing for the region in which these experiments were conducted, it is encouraging to note that all of the hybrid combinations tested silked earlier than 'CO272' itself.

General combining ability is used to indicate the average performance of a line in hybrid combination. Specific combining ability indicates which

combinations (crosses) do better or worse than expected based upon the GCA of the lines involved (Griffing, 1956). The presence of both significant GCA effects and significant SCA effects suggested that both additive and non-additive gene action may be important in the inheritance of resistance to ear rot infection via the silk.

The results of the diallel analyses of mean disease ratings and disease incidence were similar to each other. Both had significant general and specific combining abilities. Both identified the inbreds 'CO272', 'CK44', 'CO325', and 'F2' as having good general combining ability for resistance to *F. graminearum*. Disease incidence was apparently less sensitive to environmental effects, as evidenced by the lack of location effects and the homogeneity of error variances. Reciprocal effects, which were observed at one location in the analysis of disease ratings, were not evident at the other location or in the analysis of disease incidence.

The relative importance of general and specific combining ability effects in determining progeny performance was assessed by calculating the ratio of the components of variance as outlined by Baker (1978). Since this ratio was not close to unity for either mean disease ratings or disease incidence data, one could not predict the performance of a hybrid based solely upon the GCA of its parents or the parental inbred reaction to infection. The identity of the hybrids with significant SCA effects differed between the two measures of resistance, and more hybrids had significant SCA effects for disease incidence than for mean disease

rating. However, in all of the hybrid combinations with significant SCA effects, one or both of the parents had poor general combining ability. Resistance in these hybrids was better than would have been expected based upon the combining abilities of their parents, but still was relatively poor. Specific combining ability effects were not significant for any of the crosses in which both parents had good combining ability. Thus, parental GCA effects may be useful for predicting which hybrids will have the best resistance, even though they could not be used to predict the resistance of all crosses within this diallel.

Previous diallel studies on the inheritance of resistance to ear rot using wound inoculation techniques have reported GCA effects to be significant (Hart et al., 1984; Odiemah and Manninger, 1982; Szel, 1984; Odiemah and Kovacs, 1990). These studies, except for that of Hart et al. (1984), also found significant SCA effects.

The significant correlations of GCA effects with the resistance evaluations of the parental inbreds suggests that the resistance of inbreds gives some indication of their value as parents. The inbred 'CO272' was the most resistant per se, and had the best GCA in all three diallel analyses. However, not all of the other inbreds with good GCA for resistance would have been identified on the basis of their performance. Thus the evaluation of a diallel arrangement of crosses has provided more informaticn than could have been obtained simply by screening inbreds.

In conclusion, the inbred genotypes studied were mostly susceptible to *F.*

graminearum infection via the silk and silk channel, but susceptibility was largely overcome in hybrid combinations with resistant inbreds. Inheritance of this resistance is not entirely additive.

Preface to Chapter 11

The previous chapters have demonstrated that only a few inbred maize lines looked at have high resistance to *F. graminearum* infection via the silk and/or silk channel. The following chapter, Chapter 11, was designed to find other sources of resistance with emphasis on exotic corn genotypes already shown to possess resistance to other ear-rotting pathogens with similar modes of entrance into the corn ear, or to insects which feed upon the silks. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table is presented on the page following the first referral to it.

CHAPTER 11

RESISTANCE OF SOME EXOTIC MAIZE GERMPLASM TO FUSARIUM GRAMINEARUM SILK INFECTION

11.1 Abstract

In a search for sources of resistance to *Fusarium graminearum* ear rot, twelve accessions of exotic maize germplasm were evaluated for resistance to infection via the silk and/or silk channel. Resistance evaluations were made: (1) in the field after artificial inoculation of the silk channel with a spore suspension; and (2), by an *in vitro* test of detached silk tissue. Six earworm-resistant maize inbreds from Georgia varied in ear-rot resistance and in the ability to mature in a northern-temperate region. One of these, 'GT117', was more resistant than the resistant inbred check and was able to mature. Two *Aspergillus flavus* resistant lines , 'Mp313E' and 'Tx601', exhibited high resistance to *F. graminearum* but were unadapted; however, hybrids of these lines with adapted resistant inbreds were moderately resistant and earlier flowering. The Mexican landrace Zapalote Chico was both moderately resistant and photo-insensitive. Some of these exotic genotypes have potential for use in breeding programs to increase resistance to *F. graminearum* ear rot.

11.2 Introduction

Fusarium graminearum Schwabe [sexual state: *Gibberella zeae* (Schw.)

Petch] ear rot of maize is a disease of large concern in many parts of the world, especially Canada (Sutton, 1982). Most of the concern centers around the fact that *F. graminearum* produces mycotoxins which can contaminate livestock feed. Entrance into the maize ear is primarily via the silk or silk channel especially in epidemics. The infection then spreads down the ear from the tip (Koehler, 1942; Hesseltine and Bothast, 1977).

Various management strategies have been tried to control this pathogen (Martin and Johnston, 1982; Shurtleff, 1984; Enerson and Hunter, 1980), but the most feasible solution is the development of resistant hybrids. However, very few genetic sources of resistance to silk infection have been identified. Most of the historically important and commonly used inbred lines such as 'B73', 'Mo17', and 'A619' are very susceptible to *F. graminearum* ear rot (Drs. A.T. Bolton and R.I. Hamilton, unpublished data). Thus far only a limited number of resistant lines such as 'CO272' and 'F7' have been used in breeding programs.

Exotic genotypes are often very useful sources of genes and gene combinations (Robinson, 1978). These consist of germplasm that may not be fully acclimatized and has to be crossed with adapted germplasm in a tropical nursery or photoperiod chamber. Some exotic lines may be resistant to pathogens that are predominant in the geographical area where they were developed but not new environments. However, if two pathogens have similar modes of infection on the same organs, then possibly genes that condition resistance to one pathogen also condition resistance to the other pathogen.

The ear-rotting pathogen *Aspergillus flavus* Link ex. Fries which produces the mycotoxin aflatoxin, enters the ear via the silk (Marsh and Payne, 1984a, 1984b). It might be hypothesized that *A. flavus* resistant inbreds such as Mp313E (Scott and Zummo, 1990) and Tx601 (Scott and Zummo, 1988) are potential sources of resistance to *F. graminearum* ear rot.

Similarly, it might be hypothesized that maize genotypes with resistance to insects which feed upon the silks, such as the corn earworm (*Heliothis zeae* Boddie), also may have resistance to fungal pathogens which enter the ear via the silk. Resistance to *H. zeae* has been associated, in part, with the presence of the flavone C-glycoside maysin which was first discovered in the silks of the Mexican landrace Zapalote Chico (Waiss et al., 1979; Reese et al., 1982; Wiseman et al., 1985). *H. zeae* germplasm resistant lines, some of which have high maysin levels, have been developed and released by Widstrom et al. (1988).

In view of the need for additional sources of resistance to *F. graminearum*, this study was designed to examine the resistance characteristics of some exotic maize germplasm with *A. flavus* resistance and *H. zeae* resistance.

11.3 Materials and Methods

In 1990, a randomized complete block design with two blocks was conducted on a clay soil location at the Plant Research Centre of Agriculture Canada, Ottawa. In each block 15 genotypes were planted each in four-row plots. Each row (3.8 m long and 76 cm between rows) consisted of 12 plants. The

centre two rows were used to evaluate resistance to silk infection by *F. graminearum* while the outside two rows were border rows.

Days to silking, taken as the number of days from planting until 50% of the plants of a genotype have silk protruding 1-2 cm from the husk, was recorded for each genotype.

The exotic maize genotypes evaluated included: six recently released corn earworm, resistant germplasm lines from Georgia ('GT113', 'GT114', 'GT115', 'GT117', 'GT118', and 'GT119') some of which are believed to have high maysin levels (N.W. Widstrom, personal communication); two *A. flavus* resistant lines ('Mp313E' and 'Tx601') and three F_1 hybrids of these lines to the highly resistant adapted inbred line 'CO272' and the moderately resistant adapted inbred line 'F7' ('Mp313E' X 'F7'; 'Tx601' X 'F7'; and, 'Tx601' X 'CO272'); and the Mexican landrace, Zapalote Chico. The remaining three lines consisted of 'CO272', 'F7', and the highly susceptible line 'CO266' used for purposes of comparison. Additional plots of the latter three lines were inoculated with sterile water to serve as water controls.

F. graminearum cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in

500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1-hour periods at 4 hour intervals for eight days. Spore counts were approximately 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Plants in one of the centre two rows were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear when the silks were elongated, pollinated, and had some tip browning (approximately 6 days post-silk emergence). After inoculation, plots were irrigated for four weeks at a rate of 2-5 mm twice daily. Evaluations for resistance to spread of infection were made in mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected.

Resistance evaluations of the other row of the center two rows were made using an *in vitro* test of detached silk tissue. The method consisted of taking silks from the primary ears of each plant at the same stage as above. From each ear, two 1-g samples of fresh silk tissue were cut from the silk channel and placed in each of two petri dishes containing two pieces of filter paper moistened with 3 ml

of sterile water. Three millilitres of a *F. graminearum* spore suspension (5 X 10^5 spores/ml) were sprayed evenly over the silk of one petri dish with an atomizer. The second petri dish was sprayed with 3 ml of sterile water. The petri dishes were placed in a randomized arrangement in a growth chamber and incubated for 24, 72, and 168 hrs at 25°C and 95% relative humidity. Following incubation, samples were dried in an oven at 80°C for 48 hrs and weighed. Results were expressed as percentage dry weight reduction from the water control due to fungal degradation of silk tissue. This method is similar to that used by Naik and Busch (1978) for evaluating the effect of maize pollen on *F. graminearum* growth.

Analysis of mean disease ratings was carried out using Friedman's two-way analysis of variance by ranks and the corresponding multiple comparison test for non-parametric data (Daniel, 1990). Analysis of *in vitro* test results was carried out using parametric analysis of variance and Duncan's multiple range test (Steel and Torrie, 1980).

11.4 Results and Discussion

Genotypic differences were significant for both silk channel inoculation evaluations and the *in vitro* test of detached silk tissue (Table 11.1). The two resistance evaluation techniques were correlated ($r = 0.69$, $p = 0.0041$, Spearman's rank correlation coefficient). No correlation was found between days to silking and resistance evaluations.

Table 11.1. Resistance evaluations and days to silking of some exotic and adapted genotypes.

Genotype	Mean Disease Rating[a]	Tissue Degradation (% Reduction in Dry Wt.[b])	Days to Silking
GT113	5.2ab	29.0a	82
GT114	5.0a	24.2a	81
GT115	2.1d	8.1c	123
GT117	1.5d	9.3bc	89
GT118	1.1d	9.6b	124
GT119	5.2a	26.3a	84
Mp313E	1.0d	1.7d	124
Tx601	1.6d	1.3d	123
Mp313E X F7	3.1bc	3.1d	85
Tx601 X F7	2.9bc	9.7b	82
Tx601 X CO272	2.6cd	9.2bc	83
Zapalote Chico	3.0b	3.5d	82
CO272	1.9d	7.2c	85
F7	3.2ab	23.9a	69
CO266	5.0a	32.1a	79

[a] Mean of 20 plants, based on a scale of 1-7 where 1= no infection and 7= >76% of the ear infected. Means followed by the same letter are not significantly different at the 0.025 pairwise error rate.

[b] Mean of five replicates of percentage reduction in dry weight relative to a sterile water control due to fungal degradation of silk tissue. Means followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test.

Based upon mean disease ratings, seven exotic genotypes ('GT115', 'GT117', 'GT118', 'Mp313E', 'Tx601', 'Tx601' X 'F7', and 'Tx601' X 'CO272') were highly resistant (rating <3.0); two ('Mp313E' X 'F7', and Zapalote Chico) were moderately resistant (rating 3-4); and three ('GT113', 'GT114', and 'GT119') were susceptible (rating >4). The disease ratings of the three susceptible genotypes were similar to that of the highly susceptible inbred 'CO266'.

In some of the exotic genotypes, there was very little percentage reduction in dry weight due to fungal assimilation of the silk tissue. There was less assimilation of silk tissue in 'Mp313E', 'Tx601', 'Mp313E' X 'F7', and Zapalote Chico than in the highly resistant inbred 'CO272'.

The majority of the exotic genotypes were highly resistant indicating that they could be important sources of resistant germplasm and should be further studied. Four of the exotic genotypes ('GT117', 'GT118', 'Mp313E', and 'Tx601') had lower mean disease ratings than the highly resistant inbred 'CO272'. However, only one of these four, 'GT117', was adapted to a northern-temperate photoperiod Introgression of the less adapted lines would probably require a southern nursery location possibly involving cyclical selection and recombination to adapt and acclimatize the exotic germplasm.

The high frequency of *F. graminearum* resistance found in both *A. flavus* and *H. zeae* resistant lines suggested that there may be an association between *A. flavus* and *H. zeae* resistance and *F. graminearum* resistance. This needs to be further explored.

The Mexican landrace Zapalote Chico was adapted and photoperiod-insensitive as well as moderately resistant. The photoperiod-insensitivity of this race is important since it means that it can be readily used in current northern breeding programs. Although Zapalote Chico had a moderately resistant rating (3.0), there were no ears with ratings of 3 to 5. The moderately resistant rating was a result of the majority of the ears rating 1 or 2 and the remaining ears rating 6 or 7. This may reflect genetic segregation for useful resistance to infection.

In conclusion, this initial screening of one Mexican landrace and a very small group of exotic inbreds with resistance to *A. flavus* or *H. zeae* has identified several possible sources of resistance to *F. graminearum* infection via the silk. It may be worthwhile to exploit these sources of resistance in maize breeding and to evaluate the resistance potential of additional exotic germplasm.

Preface to Chapter 12

Throughout the previous chapters a visual rating scale was used to evaluate resistance to *F. graminearum* infection via the silk and/or silk channel. Since this pathogen produces mycotoxins which are a threat to livestock production, it was important to determine if visual evaluations of resistance were associated with toxin levels found in the infected ears. Chapter 12 was designed to test this to determine whether the use of visual evaluations in breeding programs is sufficient to reduce both the amount of infection on the ear and the amount of toxin in the kernels or the ear. As well, the distribution of toxin within the ear itself was examined. All literature cited in this chapter is listed in Chapter 17 of this thesis. Each table is presented on the page following the first referral to it.

CHAPTER 12

ASSOCIATION BETWEEN DEOXYNIVALENOL CONTENT AND VISUAL EVALUATIONS OF RESISTANCE OF MAIZE TO FUSARIUM GRAMINEARUM SILK INFECTION

12.1 Abstract

To determine if visual evaluations of resistance to *Fusarium graminearum* infection via the silk and/or silk channel are correlated to deoxynivalenol (DON) content of the ear, six maize inbreds and two hybrids were artificially inoculated with a spore suspension injected into the silk channel. Visual evaluations used a scale of 1-7 where 1= no infection and 7= >76% of the kernels moldy. DON levels were determined from three tissue fractions: symptomless kernels, symptomatic kernels, and the rachis. Total ear DON levels were calculated as well as total kernel levels. Visual evaluations were correlated to total ear DON levels, total kernel levels, symptomatic kernel levels, and rachis levels. There was no correlation between the toxin levels of symptomatic and symptomless kernels from the same genotype. Levels of DON in the rachis were correlated to those in both kernel fractions. Of the three tissue fractions, the largest concentration of DON was found in the rachis. Visual evaluations of resistance should be sufficient for routine screening of germplasm.

12.2 Introduction

Fusarium graminearum Schw. [sexual state: *Gibberella zeae* (Schw.) Petch.] causes ear rot of maize or corn (*Zea mays* L.) in Canada and many other maize producing areas of the world with cool, wet seasons (Sutton, 1982). Although *F. graminearum* ear rot occurs only sporadically, it can represent a serious problem due to mycotoxins which are produced by the pathogen. This is of considerable concern for livestock producers. Swine are particularily sensitive to *F. graminearum* toxins. Two major mycotoxins are produced: zearalenone and deoxynivalenol (DON).

Zearalenone is an estrogen-like metabolite which tends to develop in storage rather than in the field (Urry et al., 1966; Stob et al., 1962; Mirocha and Christensen, 1974). This mycotoxin causes swine estrogenic syndrome, as well as male infertility, reduced litter size, feed refusal, and haemorrhagia (Mirocha and Christensen, 1974).

The trichothecene toxin, deoxynivalenol (DON, vomitoxin), is more likely to be produced in the field than in storage (Vesonder et al., 1981; Greenhalgh et al., 1983). DON induces emesis in swine which is characterized by vomiting, feed refusal, and decreased weight gain (Vesonder et al., 1981).

Plant breeding for increased resistance to *F. graminearum* usually involves artificial inoculation followed by visual evaluations of disease resistance. Whether resistance evaluated in this way is correlated to the ultimate toxin content of infected ears has been of concern. Cullen et al. (1983) reported positive

correlations between zearalenone production and the extent of fungal invasion from the point of a wound inoculation in the center of the ear with a toothpick overgrown with mycelium. Using the same inoculation technique, Atlin et al. (1983) found correlations of 0.72 and 0.83 for zearalenone and deoxynivalenol respectively with ear mold ratings. However, no studies have examined the relationship between toxin content and infection of maize ears via the silk and/or silk channel, a major mode of entrance of *F. graminearum* during epidemics. With silk infection mycelial growth spreads down the silk to the tip of the ear then towards the butt (Koehler, 1942; Hesseltine and Bothast, 1977). Visual evaluations have used a rating of spread of infection from the ear tip.

The purpose of this study was to determine: (1) whether levels of DON correlate to visual evaluations of the spread of infection after silk channel inoculation and (2) the distribution of DON in three ear tissue fractions: symptomless kernels, symptomatic kernels, and the rachis.

12.3 Materials and Methods

In 1990, six inbreds of varying susceptibility to *F. graminearum* ('CO272', 'F7', 'F2', 'A641', 'CO282', and 'CO266') and two hybrids ('CO272' X 'CO266' and 'F7' X 'CO266') were artificially inoculated in a randomized complete block design with three blocks at the Plant Research Centre, Agriculture Canada, Ottawa. Each block consisted of ten single-row plots, one for each genotype, and two which were used for sterile water inoculations of 'CO272' and 'CO266'. Each plot was

a 3.8 m long row (76 cm between rows) with 12 plants, the center 10 of which were artificially inoculated.

F. graminearum cultures were maintained on potato dextrose agar (PDA). Inoculum was prepared using a modified Bilay's liquid medium consisting of 2 g potassium dihydrogen phosphate (KH_2PO_4), 2 g potassium nitrate (KNO_3), 1 g magnesium sulfate ($MgSO_4$), 1 g potassium chloride (KCl), 0.2 ppm ferric chloride ($FeCl_3$), 0.2 ppm manganese sulfate ($MnSO_4$), 0.2 ppm zinc sulfate ($ZnSO_4$), and 1 g dextrose in 1 litre of water. The fungus was grown in the liquid medium in 500-ml Erlenmeyer flasks by dispensing 200 ml of autoclaved medium into the flask and adding a 1-cm square piece of PDA containing mycelium and spores. These flasks were placed on a culture shaker under natural light supplemented with cool white fluorescent lights and shaken for 1 hour periods at 4 hour intervals for eight days. Spore counts were approximately 2 million/ml. After filtering through four layers of cheesecloth, this undiluted suspension was used as inoculum.

Plants were artificially inoculated with a single isolate of *F. graminearum* (ARC2, obtained from diseased maize near Ottawa) by injecting 2 ml of spore suspension into the silk channel (the area between the cob tip and the point where the silks emerge from the husk) of each ear when the silks were elongated, pollinated, and had some tip browning (approximately 6 days post-silk emergence). After inoculation, plots were irrigated for four weeks at a rate of 2-5 mm twice daily. Evaluations for resistance to spread of infection were made in

mid-October using a 7-class rating scale where 1= no infection, 2= 1-3%, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= >76% of the kernels infected. Ears were then bagged in onion sacks and stored at 10°C under dry conditions until further analysis.

For DON analysis, eight of the 10 ears of each row were combined as one replicate. These ears were then separated into three tissue fractions: symptomless kernels with no visible signs of infection, symptomatic kernels with visible signs of infection such as rot and mycelial growth, and rachis. Each fraction was ground to a fine powder in a mill and passed through a 0.75 mm wire mesh. A sample of 50 g of each was sent to Paracel Laboratories, Ottawa, for DON analysis. The latter involved extraction in an acetonitrile-water solvent followed by cleaning of extracts on Al-charcoal columns and quantification of DON levels with high performance liquid chromatography (Dr. W.G. Craig, Paracel Laboratories, person. comm.).

Statistical analysis of disease ratings was made using Friedman's two-way analysis of variance by ranks and the corresponding multiple comparison test (Daniel, 1990). Analysis of DON results was made using parametric statistics and Duncan's multiple range test (Steel and Torrie, 1980). Spearman's rank correlation coefficient was used to test correlations between disease ratings and DON levels.

12.4 Results and Discussion

Significant genotypic differences were found for both disease ratings and

DON content. The inbred 'CO272' had the lowest mean disease rating, while 'CO266' was the most susceptible with the highest disease rating (Table 12.1). Both of these inbreds had natural infection as evidenced by the water-inoculated mean disease ratings of 1.2 and 2.1 for 'CO272' and 'CO266', respectively. Re-isolation of symptomatic kernels from these ears revealed that *F. graminearum* was present (data not shown). After 'CO272', the genotype with the next lowest disease rating was 'F2' followed by 'CO272' X 'CO266', 'F7', 'F7' X 'CO266', 'A641', and 'CO282'.

The total ear DON level (all three tissue fractions) was correlated with mean disease ratings ($r=0.733$, $p<0.05$). The largest concentration of DON, 4060.5 ppm, was found in the susceptible inbred 'CO282'. This concentration and the others found in this study were much higher than those found by other researchers. For example, Bennet et al. (1988) found a maximum of 162.3 ppm DON in infected kernels. Lower levels were found only in the 'CO272' water control of the present study. All levels were more than sufficient to cause emesis in swine. Concentrations of DON greater than 2 ppm will make cereal grains unfit for both food and feed (Scott et al., 1983; van Egmond, 1989). Higher levels could be due to a number of factors including different environments (Miller et al., 1983a; Greenhalgh et al., 1983). Different isolates also produce different levels of mycotoxins (El-Bahrawy et al., 1985; Megalla et al., 1987; Miller et al., 1983a). We also have noted lower DON levels in ears infected with different *F. graminearum* isolates (data not shown).

Table 12.1. Deoxynivalenol (DON) content and visual evaluations of resistance of ears infected via the silk with *F. graminearum*.

Geno-type	Visual Disease Rating[a]	DON Content (ppm)[b]				
		Total Ear	Kernels			Rachis
			Total	Symptomless	Symptomatic	
CO272, water	1.2a	62.6a	52.8a	0.3a	52.5a	9.8a
CO266, water	2.1ab	1807.9b	1018.4c	23.4b	995.0c	789.5c
CO272	1.9a	1634.0b	811.0b	41.5c	769.5b	823.0c
CO266	5.3d	2216.6c	1025.3c	40.6c	984.7c	1191.3d
F7	3.5b	2873.2d	1251.5d	24.8b	1226.7d	1621.7e
F2	3.3b	2127.6c	793.9b	17.6b	776.3b	1333.7d
A641	4.3c	3969.0e	1697.7e	134.0d	1563.7e	2271.3f
CO282	4.5c	4060.5e	1835.5e	815.5e	1020 0c	2225.0f
CO272 X CO266	3.4b	1601.0b	1271.0d	6.5a	1264.5d	330.0b
F7 X CO266	3.7bc	1893.5b	998.0bc	12.0ab	986.0c	895.5c

[a] Mean of 40 plants. Based on a scale of 1-7, where 1= no infection and 7= >76% of the ear infected. Means followed by the same letter are not significantly different at the 0.025 pairwise error rate by the multiple comparison test to follow Friedman's two-way analysis of variance by ranks (Daniel, 1990).

[b] Mean of three replicates. Means followed by the same letter for a given cob fraction(s) are not significantly different at the 0.05 level of probability by Duncan's multiple range test (Steel and Torrie, 1980).

DON content of symptomless kernels was not correlated with visual evaluations of resistance of the genotypes they came from. However, DON levels of symptomatic kernels and the rachis were both significantly ($p<0.05$) correlated to visual evaluations of resistance ($r= 0.70$ and $r=0.68$, respectively). Total kernel DON level (symptomatic kernels + symptomless kernels) was correlated to visual evaluations ($r=0.72$, $p<0.05$).

Of the three tissue fractions, the lowest level of DON was found in the symptomless kernels as would be expected (Table 12.1). However, in the susceptible line 'CO282', the level in symptomless kernels exceeded that of symptomatic kernels from more resistant lines. High levels of DON were found in both symptomatic kernels and rachis tissue fractions of all the genotypes except the water- inoculated plants of the resistant inbred 'CO272'. Bennet et al. (1988) also found higher levels of both DON and zearalenone in infected kernels and rachis when ears were wound-inoculated by inserting a colonized toothpick into the tip of the ear. High levels of DON in the rachis would be expected when ears are toothpick-wound inoculated since the toothpick is inserted through the rachis; however, in the present study the ears were silk inoculated and the high levels found in the rachis were not expected. In the inoculated inbreds, DON levels were higher in the rachis than in symptomatic or symptomless kernels. Although no measurement of the extent of infection in the rachis was taken, it was noted that in susceptible genotypes the rachis was severely rotted even at the base of symptomless kernels. This may mean that a large proportion of the toxin will be

removed during the harvest of grain corn since heavily infected kernels have low densities and pass through the combine along with the rachis.

There was no correlation between the DON levels of symptomatic and symptomless kernels. DON levels in the rachis were positively correlated to DON levels in the symptomless kernels ($r=0.607$, $p<0.05$) and the symptomatic kernels ($r=0.625$, $p<0.05$) and thus the total kernel fraction ($r=0.804$, $p<0.01$). Translocation of mycotoxins including DON has been found to occur in maize (Sutton et al., 1976; Miller et al., 1983; Young and Miller, 1985). The appearance of DON in clean kernels may be due to translocation from the rachis with the flow of photosynthate. It is unlikely that any kernel to kernel movement of toxin occurs. Thus, although the removal of contaminated grain will substantially lower the amount of toxin, some may still remain especially in heavily infected susceptible ears. However, the latter would probably be discarded before use.

The hybrid 'CO272' X 'CO266' had a disease rating intermediate to that of its parents. The DON levels of this hybrid were higher than the parents in the total kernel fraction, but much lower in the rachis fraction therefore resulting in a total ear DON level lower than that of the resistant parent. Similar results were obtained when the moderately resistant line 'F7' was crossed to 'CO266'. The disease rating of this hybrid was much closer to that of the resistant parent and the DON levels were lower than that of both parents for all tissue fractions. Thus crossing of susceptible inbreds to more resistant inbreds reduced the amount of infection and subsequently reduced the DON levels relative to the susceptible parent.

In conclusion, DON levels were correlated to visual evaluations of *F. graminearum* infection of maize ears via the silk. Thus analysis of mycotoxin levels in addition to visual evaluations of resistance would not be required for the purposes of screening for resistance. This is important because of the high cost of such analyses. Breeding for increased resistance to fungal infection should effectively also lower contamination of feedstuffs with mycotoxins.

CHAPTER 13

GENERAL DISCUSSION

This research has indicated that the silks of maize play an important role in resistance to *F. graminearum* ear rot. Since silk is the first tissue to come into contact with the pathogen, when invasion of the ear occurs via the silk and/or silk channel (Koehler, 1942; Hesseltine and Bothast, 1977; Sutton, 1982), silk resistance probably acts as the initial barrier to infection. If the silk can act as a natural resistance barrier then the kernels will be free of contaminating mycotoxins such as deoxynivalenol and zearalenone, providing the pathogen does not enter the ear through bird and/or insect wounds.

In this research, genotypic silk differences were found in resistance to *F. graminearum*. These were expressed as both differences in the ability of the pathogen to degrade detached silk tissue (*in vitro*) of different genotypes (Fig. 5.1) and in infection of ears of different genotypes by artificial inoculation with a spore suspension injected into the silk channel. A sample of 12 inbred lines were found to differ significantly for both characteristics. Moreover, degradation of silk tissue and resistance to artificial inoculation were significantly correlated (Fig. 5.2). These results suggested that silk tissue of resistant genotypes possessed some resistance mechanism(s). Thus, as originally hypothesized, differences in infection from artificial inoculation of the silk channel can be explained, in part, by differences in silk resistance.

The existence of genotypic differences in silk resistance suggested that it should be possible to use this resistance in breeding programs. Screening for this resistance currently involves field evaluations of the amount of infection after artificial inoculation. However, the results of the *in vitro* test of detached silk tissue were quite promising and indicated the potential use of this method as an additional screening technique. This test also could be modified to allow removal of some silks after pollination while still allowing kernels to develop and be available for seed generation and production. For example, the removal and testing of exposed silk would be much less destructive. Although exposed silk was less degraded than silk channel silk, resistant lines could still be distinguished from susceptible lines. Also, with this technique, ears could still be inoculated in the silk channel, thereby allowing two evaluations of resistance on the same ear. The *in vitro* test of detached silk tissue also could be carried out in the off-season since results with winter greenhouse silk were highly correlated to summer field silk results.

The above discussion is based on silk that was tested or inoculated when the ears were approximately 6 days post-silk emergence. At this time, silks were elongated, pollinated, and may have had some tip browning. Unless anthocyanin pigmentation is present, most silks at this age are yellow-green in colour. Unlike most tissues, silk senesces very rapidly especially after pollination (Heslop-Harrison, 1979). This means that the tissue upon which pathogens such as *F. graminearum* enter the ear is rapidly undergoing physiological change. It was

originally hypothesized that such changes in silk with age would affect the resistance of a given genotype to infection. This was found to be true. When both hybrids and inbreds were artificially inoculated with *F. graminearum* spores injected into the silk channel at either 0 to 18 days post-silking (Fig. 6.1) or at three stages of silk development (early, intermediate, and late) (Fig. 6.2) respectively, susceptibility to infection decreased with silk age or senescence. Thus, again these results show the importance of silk as a resistant barrier to *F. graminearum*. These results also have large implications on field screening techniques for resistance. If inoculations were conducted on older silk, inaccurate assessments of resistance could be made. Care must be taken when screening diverse groups of germplasm containing genotypes of varying maturities to ensure that all are inoculated at the same stage or chronological time of silk development. Based upon these results it would be recommended that inoculations be made at the intermediate stage of silk development or approximately 5-7 days post-silk emergence. This would allow for maximum differentiation between genotypes and sufficient infection to correctly assess resistance.

Why silk age affects resistance is not known. Although decreases in silk channel length and exposed silk water content were observed, these did not account for all of the change in resistance because *in vitro* tests of early, intermediate, and late stages of silk development also showed a decrease in degradation with age for both silk channel silk and exposed silk (Fig. 6.3). Also, differences in water content were not associated with genotypic differences in

resistance. Other mechanism(s) would be necessary to explain the resistance in the silk (e.g. chemical).

Resistance of a chemical nature was hypothesized. Since silk contains a wide variety of secondary metabolites including phenolics, it was also hypothesized that phenolic compounds played a role in resistance. Such a role could involve higher concentrations of phenolics in resistant lines. The results of this research suggested that phenolics do play a role in resistance but not due to higher concentrations in resistant lines.

Surprisingly, total phenolics of the silk channel silk of five inbred lines of varying resistance to *F. graminearum* revealed that the most resistant line 'CO272' had lower total phenolic levels than all but one of the four more susceptible lines. Since such a significant increase in resistance occured with silk age, it was hypothesized that levels of phenolic compounds also may increase with age as in the case of other species (Nozzolillo et al., 1990). However, significant increases in total silk phenolics with age were found only for two of the five inbreds (Fig. 7.1). Thus it was apparent that pre-inoculation phenolic levels were not important in resistance.

However, the change in total phenolic levels of the same five inbreds after inoculation of the ears by spraying a spore suspension on the exposed silks, revealed that phenolic compounds may play a role in resistance. An increase in total phenolic levels in response to inoculation or infection was observed in the highly resistant line 'CO272' and the moderately resistant line 'F7', and a decrease

was observed in more susceptible lines. Such a reaction to infection, as well as lower initial levels in more resistant plants, has been observed in maize in response to infection by anthracnose (Hammerschmidt and Nicholson, 1977). This result is significant for two reasons. First, it indicates that resistant lines were capable of carrying out induced responses to infection despite the possible presence of protein synthesis inhibiting fungal toxins such as deoxynivalenol. Second, it was quite possible that phenolics were involved in resistance and that the response occurred in advance of actual infection, i.e. in the silk channel silk of ears in which only the exposed silk was inoculated.

Methodology to conduct bioassays of silk extracts and identified phenolic compounds still needs to be developed to assess if they have antifungal activity and would thus directly be involved in resistance. However, this may not be the case. It is possible that these compounds were substrates for a phenolase reaction since they appeared to have the proper substrate structure and phenolase enzymes have been found in the silk. In addition, silk undergoes rapid browning after wounding or infection, a common indicator of phenolase activity (Levings and Stuber, 1971). All of these aspects require further study.

Silk age also affected changes in total phenolics in response to inoculation. Silk inoculated at an early stage had similar responses as that inoculated at an intermediate stage (Fig. 7.3). However, silk inoculated at a late stage when the exposed silk was brown and dry had much less response to infection. This was not unexpected since silk in an advanced stage of senescence would be less likely

able to respond to infection.

Thus it was apparent that a combination of silk factors and resistance mechanisms were responsible for resistance to *F. graminearum* infection. These studies have shown that silk resistance was definitely present and important, but what actually conditioned resistance still requires more extensive study.

The second major goal of this research was to study the mode of inheritance of resistance to silk infection. Since genotypic differences in resistance exist, a knowledge of the inheritance of resistance would facilitate the selection of breeding strategies to produce genotypes with high resistance to infection and thus low levels of toxins.

Previous work by Drs. A.T. Bolton and R.I. Hamilton revealed that some inbred lines such as 'CO272' had very high resistance to *F. graminearum* silk infection (personal comm.). This was confirmed in all of the experiments involving 'CO272' in this thesis. However, resistance to pathogens has sometimes been isolate-specific. This would necessitate the use of more than one isolate of the pathogen when screening for resistance. It was hypothesized that resistance to silk infection was not isolate-specific. This was found to be the case when 13 inbred lines were inoculated with three different *F. graminearum* isolates. Thus, it should not be necessary to use more than one isolate when screening for resistance to silk infection. A sufficiently aggressive isolate would ensure adequate infection to differentiate genotypes. Although these results suggested that it was not necessary, the use of a mix of three or more isolates would more closely mimic

nature and avoid the possibility of intermediate symptoms caused by a less aggressive isolate.

It was also hypothesized that the high resistance of 'CO272' and other lines with high resistance was heritable and due to one or a few dominant genes. This is different than the quantitative inheritance that has been previously reported for resistance to infection after wounding (Odiemah and Manninger, 1982, Chiang et al., 1987; Hart et al., 1984; Boling and Grogan, 1965; Odiemah and Kovacs, 1990). The original plan of this research was to evaluate the resistance of six generations (P1, P2, F_1, F_2, BC1, and BC2) of two resistant X susceptible parental combinations ('CO272' X 'CO266' and 'F7' X 'CO266'), and carry out a generation means analysis of the data and/or look for Mendelian ratios of simple inheritance. The results of this study indicated that resistance to silk infection is heritable and dominance is involved, but no conclusions could be made as to the number of genes involved. Analysis of the data revealed that the observed ratios of infected:uninfected plants within the F_2 and backcross generations were not consistent with those expected for simple qualitative models involving one or two dominant genes. Quantitative genetic analyses were not conducted since the data failed to satisfy certain assumptions of the analyses such as normal distributions and fitting to a simple additive-dominance model.

The results of this study, particularly the bimodal distributions found in the F_1, F_2, BC1, and BC2 generations (Fig. 9.1), suggested that resistant lines such as 'CO272' and 'F7' have resistance in the silk which can impede the progress of

the pathogen down the silk channel. However, infections can still occur. The F_1, F_2, and backcross progeny of these lines also have this resistance but the incidence of infection reaching the cob is much higher. After infection has breached the silk barrier the spread of infection on the ear is due to mostly environmental factors in the cross involving 'CO272' and to a combination of environmental and genetic factors in the cross involving 'F7'. Under natural conditions, which do not favour the pathogen as much as the artificial inoculation and irrigation used in this study, much less disease incidence or breaching of the silk barrier would probably occur. In breeding programs, both disease incidence and disease severity should be evaluated when making selections. Consideration of disease incidence would ensure that lines with high silk resistance such as 'CO272' would not be discarded because of a few ears which were heavily infected due to environmental factors.

The inheritance of resistance to *F. graminearum* silk infection was also investigated by conducting a 12 X 12 complete diallel cross of maize inbreds of varying susceptibilities Similar results were obtained whether disease incidence or mean disease ratings (disease severity) were used in the analysis. Reciprocal effects were not significant, however both general combining ability and specific combining ability effects were significant. Thus both additive and non-additive gene action are important in the inheritance of resistance. The performance of a hybrid can not be predicted based solely upon the performance and general combining ability effect of its parents.

The hypothesis that the highly resistant inbred 'CO272' readily transmitted its resistance to its progeny proved to be true as seen in both the generation and diallel studies. In the latter, 'CO272' had the highest negative general combining ability effect and all hybrids involving this parent were moderately to highly resistant. Thus 'CO272', along with some of the other inbreds identified in this study, would be useful for increasing resistance in a breeding program.

In the diallel study, the number of days to silking of each genotype was recorded was found to be not correlated with mean disease ratings Thus the resistance of one of the later silking lines ('CO272') is not attributable to lack of infection due to late silking. Some late-silking lines were found to be highly susceptible.

In all of the studies in this research, 'CO272' was consistently the most temperately-adapted resistant line and most other genotypes were susceptible. Other studies have come to the similar conclusion that very few sources of resistance to silk infection by *F. graminearum* are available (Drs. R I. Hamilton and A.T. Bolton, person. comm.). However, new sources of resistance may exist in exotic germplasm. It was hypothesized at the start of this research that resistance to *F. graminearum* may be found in germplasm with resistance to other ear-rotting pathogens that enter the ear via the silk. Two *A. flavus* resistant lines, 'Mp313E' and 'Tx601', exhibited high resistance in both *in vitro* and field evaluations. When crossed to the highly resistant line 'CO272' or the moderately resistant line 'F7', the progeny were moderately resistant, much earlier silking and therefore more

adapted than their exotic parent. These results suggested that there may be an association between *F. graminearum* resistance and *A. flavus* resistance. This could be due to the fact that *A. flavus* uses the same mode of entry into the ear and also produces a mycotoxin, aflatoxin.

It was also hypothesized that germplasm with resistance to insects which feed upon the silks such as the corn earworm, *H. zeae*, also may have resistance to *F. graminearum*. This was hypothesized since one of the factors conditioning resistance to *H. zeae* has been found to be presence of the flavone C-glycoside maysin in the silk of the more resistant lines. *F. graminearum* resistance evaluations, both in the field and *in vitro*, of six *H zeae* resistant germplasm lines from Georgia revealed that three of these lines were very resistant to *F. graminearum* but the remaining three were highly susceptible. One line in particular, 'GT117', was very resistant to *F. graminearum* and adapted to northern-temperate growing conditions. Thus there may be some association between *H. zeae* resistance and *F. graminearum* resistance

It is possible that maysin may play a role in *F. graminearum* resistance. However, the results of the flavone analysis of five inbreds ranging from very susceptible to very resistant suggested that maysin was not essential for resistance since it was not found in the silk of the most resistant line 'CO272'. The Mexican landrace Zapalote Chico in which maysin was first discovered was moderately resistant. However it was heterogeneous, containing both highly resistant and highly susceptible plants.

Throughout this research a visual rating scale from 1-7 was used to evaluate resistance to *F. graminearum* ear rot in the field after artificial inoculation of the silk channel with a spore suspension. Whether such evaluations are correlated to mycotoxin content of the ears has been of concern. It was hypothesized at the start of this research that deoxynivalenol (DON) contents of *F. graminearum* infected ears were correlated to visual evaluations of resistance, in particular to the rating scale used in this study. DON levels were analyzed from three ear fractions (symptomless kernels, symptomatic kernels, and the rachis) from six inbreds and two hybrids. Visual evaluations of resistance were correlated to total ear DON levels (all three fractions), total kernel levels (symptomatic + symptomless kernels), symptomatic kernel levels, and rachis levels. This data suggested that visual evaluations of resistance should be sufficient in screening programs and there was no need to carry out additional costly mycotoxin analyses. However, it should be noted that the highest levels of DON were found in the rachis and that symptomless kernels did contain DON, possibly from translocation from the rachis. This implies that if ears were infected, even though the total ear DON level was correlated to visual evaluations, there was still a chance that mycotoxins may occur in symptomless kernels. Thus even if contaminated kernels and the rachis are removed at harvest during combining, feed may still have to be tested for mycotoxins if *F. graminearum* infection were suspected. However, the use of visual evaluation systems in breeding for resistance to fungal infection should have the effect of also lowering contamination of feedstuffs with mycotoxins.

CHAPTER 14

SUMMARY AND CONCLUSIONS

The major goals of this research were to determine if silk played a role in resistance to *F. graminearum* ear rot and to investigate the mode(s) of inheritance of resistance to infection via the silk in maize, with emphasis on the highly resistant line 'CO272'. Results showed that silk did play a role in resistance, but we are still a long way from understanding what this role is. The inheritance of resistance to silk infection was found to be more complicated than what was first hypothesized and still requires more study.

Comparisons of the resistance to *F. graminearum* infection via the silk and/or silk channel under artificial inoculation of several maize genotypes were conducted in the field. An *in vitro* test of detached silk tissue was carried out to compare degradation of silk tissue of different genotypes when incubated with *F. graminearum*. It was concluded that genotypic differences existed for both measurements and that *in vitro* results were correlated to field evaluations of resistance. Thus differences in silk resistance did exist and could be used in breeding programs for increasing resistance.

Field evaluations of resistance with inoculation at different stages of silk development and *in vitro* tests revealed that resistance to *F. graminearum* increased with silk age. It was shown that maximum differentiation between genotypes would be achieved if inoculations were made 5-7 days post-silk

emergence. Inoculations made later than 10 days could result in inaccurate assessments of resistance due to insufficient infection.

Although initial levels of total phenolics in the silk did not appear to be associated with resistance, total phenolic levels increased in the silk channel silk of resistant lines when exposed silks were sprayed with a spore suspension of *F. graminearum*. Flavone compounds were found to be the major phenolic constituents of maize silks. Five flavones were identified: iso-orientin, iso-vitexin, maysin, luteolin, and apigenin. It was concluded that a chemical resistance mechanism to *F. graminearum* infection may be present in the silk.

Inoculation of the silk channel of maize ears with three different *F. graminearum* isolates resulted in no major isolate effects. It was concluded that resistance to silk infection was not isolate-specific and that the use of more than one isolate in a screening program was not necessary.

Inheritance of resistance to silk infection in two resistant X susceptible parental combinations revealed that simple inheritance models and additive-dominance models were not adequate to explain the inheritance of resistance in these lines. The data of F_1, F_2, and backcross generations was bimodally distributed. It appeared that dominance was involved in resistance. Also, resistance in the silk could be overcome and infection of the ear could occur, especially when the environment favoured the pathogen as under artificial inoculation and irrigation conditions. In some genotypes there does not appear to be much variation for resistance to the spread of infection once the kernels and

rachis are infected.

A complete diallel cross of 12 inbred lines including the highly resistant line 'CO272' revealed that both general combining ability and specific combining ability effects were significant but reciprocal effects were not. It was concluded that both additive and non-additive gene action were important in the inheritance of resistance to silk infection and that the performance of a hybrid could not be predicted solely on the general combining ability or the performance of the parents. However, the most resistant lines did have the largest negative combining abilities with 'CO272' having the largest of all. Thus 'CO272' can be expected to transmit most of its resistance to its progeny, and would be a good line to use in breeding programs. It was also concluded from the genetic studies that both incidence of infection and severity of infection should be considered in screening for resistance.

Both *A. flavus* and *H. zeae* resistant exotic germplasms with high resistance to *F. graminearum* silk infection were found. It was concluded that there may be an association among resistances to these three organisms.

Visual evaluations of resistance to silk infection were correlated to deoxynivalenol levels in the ear. Thus, in screening programs, it may not be necessary to carry out costly toxin analyses since visual evaluations may be sufficient.

The major conclusions from this research were that resistance to silk infection in maize by *F. graminearum* does exist, is measurable, has genotypic

differences, has resistance mechanisms located in the silk itself, was not isolate specific, and was heritable. Thus the breeding of new maize lines with resistance to infection via the silk and/or silk channel would reduce the amount of *F. graminearum* mold on ears and in turn would decrease the contamination of feedstuffs with mycotoxins.

CHAPTER 15

CONTRIBUTIONS TO KNOWLEDGE

Resistance of maize to *F. graminearum* ear rot and the inheritance of this resistance has been researched previously. However, this was the first study to concentrate extensively on the role of silk in resistance and the inheritance of silk resistance as opposed to resistance to infection via wounds. The following would be considered to be specific contributions to original knowledge made in this research:

1. Using an *in vitro* test of detached silk tissue, genotypic differences in the assimilation of silk by *F. graminearum* were found and were correlated to field evaluations of resistance. This was the first research to report that silk played a role in resistance and that genotypic differences existed for this resistance.

2. When the silk channels of maize genotypes were artificially inoculated with *F. graminearum* spore suspensions at different stages of silk development or age, susceptibility to infection was found to decrease as silk aged. *In vitro* results also showed a decrease in degradation of older silk. This was the first experiment to test the effect of silk age on resistance to *F. graminearum* ear-rot. Results of this study have large implications for screening programs which must inoculate a number of genotypes of varying maturities.

3. When a spore suspension of *F. graminearum* was sprayed upon the exposed silks of maize ears, the levels of total phenolics in the silk channel silk were found to increase above a water control in resistant genotypes and decrease below a water control in susceptible genotypes. This was the first research to report a possible chemical resistance mechanism in maize silks. It was also the first to report an induced response to *F. graminearum* infection in this manner.

4. Analysis of the phenolic constituents of maize silk revealed the presence of many flavone/flavonol compounds, five of which were identified: maysin, iso-orientin, iso-vitexin, luteolin, and apigenin. This was the first study to consider the possible role of these compounds in resistance to *F. graminearum*.

5. Inoculation of 13 maize inbreds with three different *F. graminearum* isolates revealed that resistance to silk infection was not isolate-specific. Although other studies have come to similar conclusions, this was the first study to test isolate effects in silk resistance. The implication of this study was that it was not necessary to use more than one isolate when screening for resistance.

6. Analysis of the P1, P2, F_1, F_2, BC1, and BC2 generations of two resistant X susceptible crosses revealed that for resistance to silk infection disease severity ratings in F_1, F_2 and BC generations were bimodally distributed and

a simple additive-dominance model was not adequate to explain the variations among generations nor were simple one- and two-gene Mendelian models. This was the first study to make a detailed investigation into the inheritance of resistance to silk infection. It also was the first to reveal that genetic differences existed in the incidence of disease, i.e. whether or not infection spread down the silk channel, and that this appeared to be a separate process from the severity of disease which may not have had as much genetic variation as disease incidence in some crosses.

7. A diallel analysis of 12 inbred lines revealed that both general combining ability and specific combining ability effects were significant for resistance to infection via the silk and/or silk channel. Reciprocal effects were not significant. It also revealed that inheritance was not entirely additive. Although this was not the first diallel analysis carried out on resistance for *F. graminearum*, it was the first to consider resistance to infection via the silk. Inbreds were identified which have high general combining abilities and potential for future breeding programs. As well, certain combinations of inbreds with high specific combining abilities were identified.

8. No correlation between resistance and days to silking was found in any of the studies. This was the first research to report that relative maturity had no effect on resistance to silk infection.

9. Some genotypes with resistance to *A. flavus* or to the earworm, *H. zeae*, had high resistance to *F. graminearum* silk infection. This was the first study to look at exotic germplasm for sources of resistance to *F. graminearum*. It also was the first to suggest that there may be a relationship between *A. flavus* resistance and *F. graminearum* resistance.

10. Deoxynivalenol levels were correlated to visual evaluations of resistance to silk infection. This was the first research to show a correlation between deoxynivalenol levels and visual evaluations of resistance after inoculation of the silk channel. The implication of this was that in screening programs, visual evaluations of resistance should be sufficient.

CHAPTER 16

SUGGESTIONS FOR FUTURE RESEARCH

1. Breeding strategies should be developed to utilize the sources of resistance identified in this research.

2. Further investigations into the inheritance of resistance to silk infection should be made with emphasis on the difference between resistance to disease incidence and resistance to the spread of infection (disease severity).

3. Associations between resistance to *F. graminearum* and resistance to other predominant *Fusarium* species needs to be examined to determine if common resistance mechanisms exist and the degree of competition and synergism that may be occuring between species.

4. The role of the rachis in infection and perhaps resistance needs to be investigated.

5. Deoxynivalenol levels should be traced from the point of infection to where they are in the ear upon harvest. This should be done on an individual kernel basis.

6. Additional accessions of exotic germplasm should be screened for resistance, with emphasis on germplasm with resistance to other ear fungal pathogens and insect pests.

7. The relationships among *A. flavus* resistance, *H. zeae* resistance and *F. graminearum* resistance should be investigated.

8. Further evaluations of the *in vitro* test of detached silk tissue should be made to determine if this technique can be used as an additional screening method for *F. graminearum* resistance. This work is in progress.

9. The antifungal activity of the flavone compounds found in the silk should be tested. Bioassays also should be developed to test crude extracts of silk. These studies are currently in progress.

10. The possible role of silk browning in resistance should be investigated. Enzyme assays of polyphenol oxidase activity should be conducted in the silk to see if levels increase with infection in resistant genotypes.

11. The flavone compounds found in the silk should be tested to see if they could act as substrates for phenolase enzymes.

12. The increase in total phenolics response of resistant silk should be tested to determine if similar responses occur when inoculations are made with other ear-rotting pathogens, especially other *Fusarium* species such as *F. moniliforme* and *F. culmorum*.

13. The phytochemical properties of maize kernels should be investigated.

14. The histopathology of silk infection should be studied with emphasis on any differences between resistant and susceptible genotypes. This could also involve microscopy with stains specific for phenolic compounds to allow an examination of changes in phenolics at the point of infection and further down the silk.

15. More detailed studies should be made on the role of silk length and silk channel length in resistance.

16. The water potential of silk should be examined to see if differences exist among genotypes and with silk age in association with resistance. Also, the rate of silk drying and senescence should be further examined with respect to resistance.

CHAPTER 17

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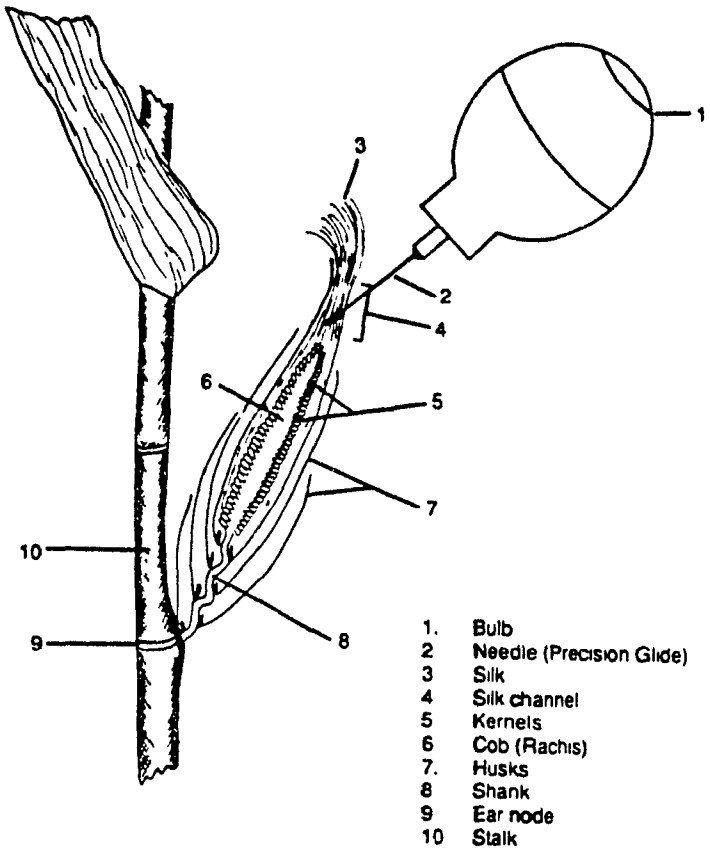
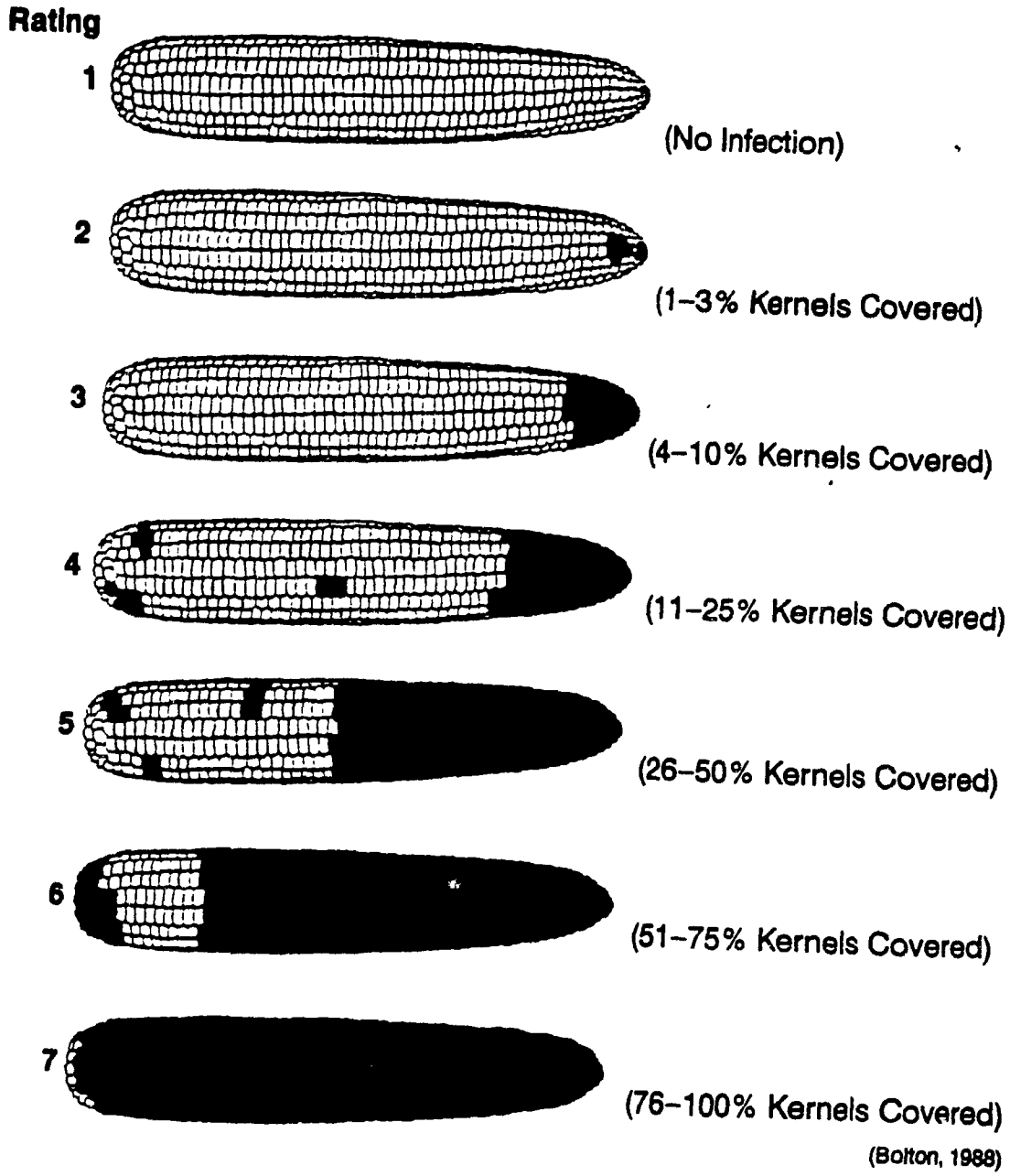
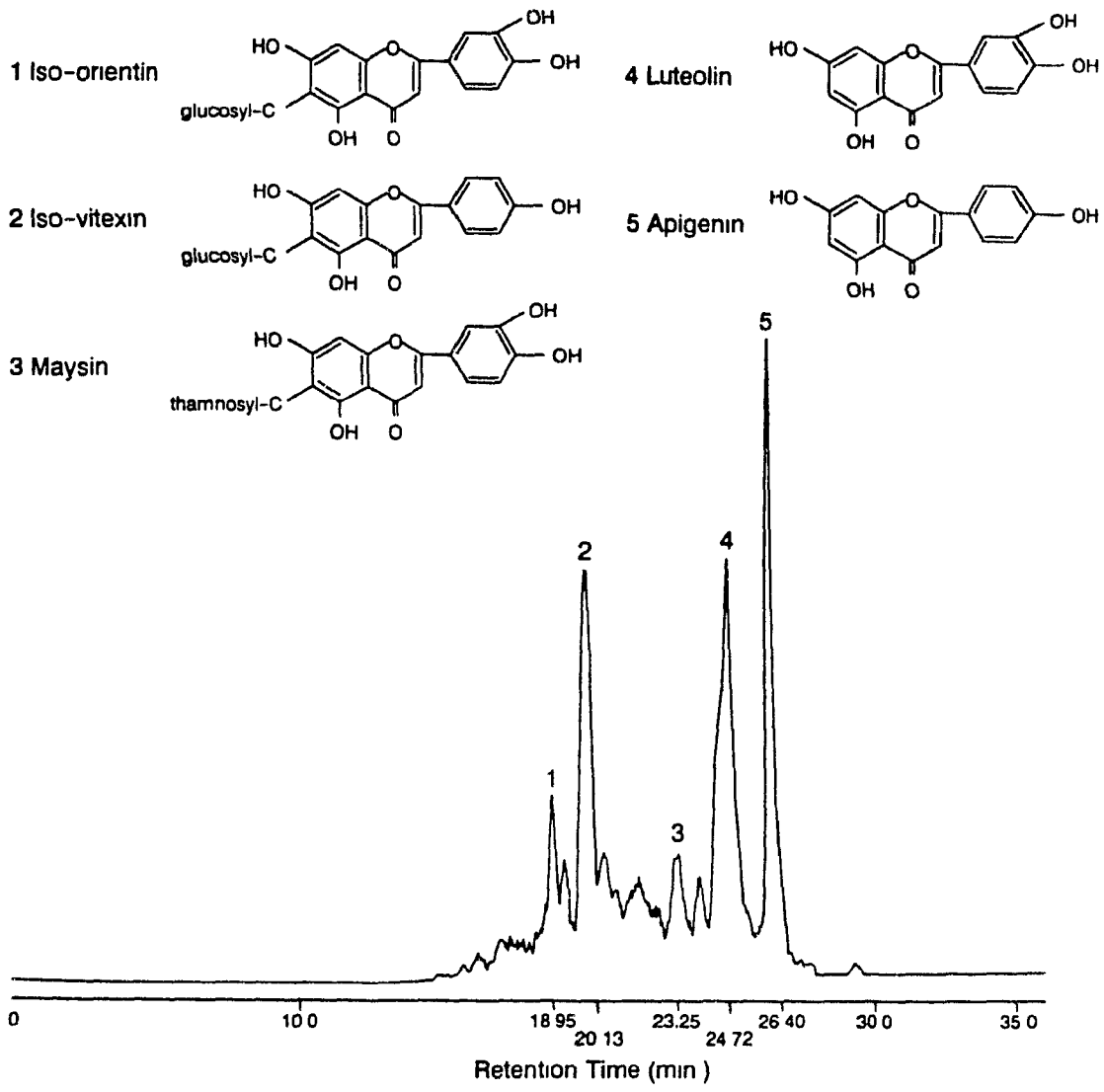


Appendix 1. Diagram to illustrate the artificial inoculation of the silk channel with *F. graminearum*. Two milliliters of a spore suspension is injected into the silk channel of individual ears using a 25 ml pipette bulb and a 16 gauge hypodermic needle.



Appendix 2. Rating scale used to evaluate the spread of *F. graminearum* infection from the tip of a maize ear after artificial inoculation in the silk channel with a spore suspension.



Appendix 3. HPLC trace and chemical structure of the five flavone compounds identified in maize silk.

Appendix 4. Number of ears in each rating class for the two resistant X susceptible parental combinations.

Generation	Rating Class[a]							
	1	2	3	4	5	6	7	Total
				C0272 X C0266 (1989)				
P1	25	0	2	9	0	0	0	36
P2	0	0	0	2	15	8	10	35
F_1	14	3	5	12	3	0	0	37
F_2	10	1	3	13	3	2	4	36
BC1	15	2	3	9	1	4	3	37
BC2	6	1	2	10	8	3	2	32
				C0272 X C0266 (1990)				
P1	57	30	14	13	7	0	0	121
P2	6	5	1	18	47	34	54	165
F_1	63	18	44	60	16	11	14	226
F_2	372	150	243	575	230	30	75	1675
BC1	109	41	56	147	63	5	21	442
BC2	63	20	58	209	96	21	39	506
				F7 X C0266 (1990)				
P1	79	19	31	21	2	1	1	154
F_1	85	56	82	46	9	1	0	279
F_2	248	188	249	492	115	15	47	1354
BC1	140	80	119	77	19	1	9	515
BC2	28	13	23	95	35	2	16	212

[a] Rating classes: 1= no infection, 2= 1-3 %, 3= 4-10%, 4= 11-25%, 5= 26-50%, 6= 51-75%, and 7= 76-100% of the kernels moldy. 1990 data combined over both locations. C0272 is highly resistant, F7 is moderately resistant, and C0266 is highly susceptible.

Appendix 5. Days to silking for the parental lines and F_1 single-cross hybrids of a 12 x 12 diallel design (averaged over both locations)[a].

Parent	Female											
	C0272	C0267	F7	CK44	A641	Mo17	C0266	C0325	C0282	C0317	C0265	F2
Male												
C0272	88	80	74	77	78	74	80	80	79	81	80	72
C0267	80	84	76	74	77	79	77	76	72	74	78	71
F7	77	74	77	74	75	79	75	72	69	80	74	71
CK44	76	76	72	79	73	80	77	73	72	73	77	71
A641	79	73	75	76	80	81	74	78	77	75	75	72
Mo17	84	80	80	79	80	89	80	76	78	80	81	82
C0266	79	73	74	78	74	80	79	75	72	78	76	73
C0325	80	77	73	73	77	76	73	79	75	77	78	74
C0282	81	73	73	74	77	81	73	72	73	77	75	71
77H8	80	77	79	73	80	80	77	78	74	81	77	79
C0265	79	75	76	76	76	80	74	71	78	79	76	72
F2	78	74	71	69	73	78	71	72	75	73	75	72

[a] Days to silking taken as the average number of days from planting to 50% of the plants with silk. Each value is the mean of 20 ears averaged over two replicates.