ABSTRACT

BUSH, BRIAN JOSEPH. *Fusarium verticillioides* Infection, Fumonisin Contamination and Resistance Evaluation in North Carolina Maize. (Under the direction of Gary A. Payne and Martin L. Carson)

Fusarium ear rot and fumonisin contamination are serious problems for North Carolina maize growers. With the discovery of fumonisin toxicity to animals and humans, and the finding that no maize genotypes are resistant to *Fusarium verticillioides* infection or fumonisin contamination, management strategies for limiting fungal and toxin contamination of harvested grain are necessary. Maize ears were harvested weekly for 14 or 15 weeks after pollination and assayed for percent kernel infection and fumonisin contamination. Kernel infection and fumonisin contamination occurred before kernel maturity and increased throughout the season, with kernel infection peaking 7 to 10 weeks after pollination. Data from this experiment and data from grower's fields indicate that early harvest is necessary to limit rotten kernels and fumonisin in harvested grain.

Difficulty in identifying resistant genotypes has limited the development of more resistant hybrids. Many inoculation techniques have been employed to reproduce Fusarium ear rot with marginal results, primarily because differentially resistant and susceptible hybrids were not used to identify promising inoculation techniques. In my study, ears were treated with different inoculation techniques to reproduce ear rot and fumonisin contamination in hybrids of known resistance to Fusarium ear rot. Two inoculation techniques, Pinbar and Silk Channel, were able to separate hybrids on visible ear rot and fumonisin contamination. Addition of inoculum to ears appears important for screening hybrids for resistance to Fusarium ear rot and fumonisin contamination.

Fusarium verticillioides Infection, Fumonisin Contamination and Resistance Evaluation in North Carolina Maize.

by

BRIAN JOSEPH BUSH

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Department of Plant Pathology

Raleigh

Fall 2001

APPROVED BY:

Gary A. Payne
Co-chair of Advisory Committee

Martin L. Carson
Co-chair of Advisory Committee

Winston M. Hagler

James B. Holland

DEDICATION

The work contained in this thesis is dedicated to my wife, Karen. She gave me the strength and encouragement that I needed to keep on task each day. Thank you for all of your help through this time. I love you very much.

BIOGRAPHY

Brian was born on January 12, 1977 in Columbus Indiana. He grew up on his family's farm and worked there until graduating from high school in 1995. Upon graduation he entered Purdue University majoring in Plant Sciences in the Botany and Plant Pathology Department. In the summers between school years, he worked as a summer intern in Herbicide Development for Dupont Ag Products. He graduated from Purdue University in May 1999 with a B.S. in Agriculture.

After graduating from Purdue University, Brian entered the Plant Pathology Department at North Carolina State University. Currently he is a Masters Degree candidate under the direction of Dr. Gary Payne and Dr. Marty Carson. Future plans include a position as a Senior Research Associate with Pioneer Hi-Bred Inc. International in New Holland, Pennsylvania.

ACKNOWLEDGEMENTS

First of all, I would like to thank my major advisors, Dr. Gary Payne and Dr. Martin Carson. Dr. Payne instilled an understanding of the importance of reading the literature and was always open to listening to new ideas and problems I encountered through my graduate career. Dr. Carson provided great insight and help in laying out my experiments, in addition to explaining the nuances of industry life to me. Additionally, many thanks go to my other committee members Dr. Winston Hagler and Dr. James Holland for their help in data interpretation, advice and for serving on my committee.

Second, I need to thank Lisa Ferguson for teaching me the ways of field experimentation in Plant Pathology. She was always helpful whenever I had questions or needed advise as to how things were done at State. Dr. Marc Cubeta and Brian Cody were very helpful with arranging drying of samples, harvesting and being my eyes at the Vernon James Center. Dr. Cubeta was also encouraging me to take advantage of opportunities available, like speaking to growers. I am very grateful for the opportunities Dr. Cubeta pushed me to do. Rod Gurganus and Freddie O'Neal deserve thanks for their help with my on the farm studies.

Last, I would like to thank my Payne lab mates. They all helped rip apart my presentations to show me how unprepared I was. They were also there for me whenever I needed help with my project, which was often. Thank you all, especially Mike!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2:

CHAPTER 3:

CHAPTER 1: LITERATURE REVIEW

Fusarium verticillioides (Sacc.) Nirenberg (syn. *Fusarium moniliforme* J. Sheldon) is an anamorph of *Gibberella fujikuroi* ((Sawada) Ito in Ito & K Kimura mating population A), and is a plant pathogen that causes root, stalk, and ear rots of maize (White, 1999). Seed infection by *F. verticillioides* is of major concern because it can reduce seed quality and result in contamination of grain with mycotoxins. *Fusarium verticillioides* infection of kernels occurs at or after flowering and is favored by hot, dry conditions. Symptoms of Fusarium ear rot vary greatly depending on the crop variety, the environmental conditions, and the severity of the disease. Symptoms usually occur at the tip or in scattered areas on the ear, and in severely infected ears, whitish pink to lavender fungal mycelia can be observed. Infected kernels also may exhibit a typical "starburst symptom", which is characterized by white streaks radiating from the cap of the kernel. Many kernels infected with *F. verticillioides* may remain symptomless. The fungus overwinters in colonized plant residues. The predominant inoculum for seed infection is microconidia (White, 1999). These conidia are carried to the ear by wind or insects where they germinate and either colonize silk tissue and kernels directly or colonize damaged (insect injured, "silk-cut", or popped) kernels. Kernels also may be invaded as a result of a systemic infection of the plant, but the significance of this route of infection in Fusarium ear rot is not yet clear (White, 1999).

Fusarium ear rot of maize is responsible for significant economic loss due to decrease in yield and low grain quality. The pathogen was recently shown to produce a group of mycotoxins known as fumonisins, which cause leukoencephalomalacia in horses

and pulmonary edema syndrome in pigs. There is also evidence linking the consumption of *F. verticillioides* infected maize to the high incidences of human esophageal cancer in regions of South Africa and China (Gelderblom et al., 1988; Yoshizawa et al., 1994).

Gibberella fujikuroi is the teleomorph for many of the Fusarium species that have anamorphs in *Fusarium* section *Liseola*, such as *Fusarium verticillioides*, *F. subglutinans*, and *F. proliferatum.* However, the taxonomy of these *Fusarium* species has been under constant dispute because some have felt that the classification system is not well suited for describing the biological niches of these fungi (Toussoun, 1981). In particular, the nomenclature system for the *Fusarium* species proposed by Snyder and Hansen was confusing because some of the characteristics were not consistent with current understanding of the biology of the fungi, including fumonisin biosynthesis (Leslie, 1996). To help clarify the taxonomy of this complex genus and to develop a common taxonomic nomenclature, *G. fujikuroi* was organized into seven mating populations (or biological species) based on sexual distinctions and asexual differences (Klittich et al., 1997; Leslie, 1996). Seven mating populations with the alphabetical designations A, B, C, D, E, F, and G were proposed for *G. fujikuroi.* All seven mating populations are heterothallic, and strains within the same mating population may be either of the two mating types, "+" or "-". Two strains bearing the opposite mating types from the same mating population can be mated to generate a fertile cross. Mating populations of *G. fujikuroi* can also be characterized using criteria other than sexual compatibility, such as fumonisin production (Leslie et al., 1992), host preferences (Jardine & Leslie, 1992), isozyme production (Huss & Leslie, 1993), and DNA-sequence based differences (Xu et al., 1995).

The population of *F. verticillioides* that is the most common pathogen of maize and produces large quantities of fumonisin belongs to *G. fujikuroi* mating population A. While members of the D mating population, which includes *F. proliferatum*, are also able to produce significant amounts of fumonisins, this population is rarely found on maize. In contrast to mating population A, members of mating population F do not produce fumonisins (Leslie, 1996) and are primarily limited to sorghum as a host. Therefore, accurate identification of *Fusarium* species using the currently accepted morphological criteria may not be sufficient to differentiate between the populations of the fungus that infect grain and produce fumonisin. The suggested *G. fujikuroi* nomenclature system may be used to clarify some of the confusions that could arise when discussing *Fusarium* species and fumonisin biosynthesis.

Nirenberg pointed out in her doctoral thesis that the fungus *Fusarium moniliforme* that had been associated with Fusarium ear rot was actually misidentified and the causal agent of Fusarium ear rot was actually *Fusarium verticillioides* (Nirenberg, 1976). Because the thesis was written and published in Germany, many authors failed to take notice of the correction. Only recently have authors started using the correct classification of this fungus. In this study, we will refer to the pathogen as *Fusarium verticillioides* although many studies cited have used the older taxonomic term *Fusarium moniliforme.*

Fusarium verticillioides and *F. graminearum* are the prominent Fusarium species associated with ear rots of maize. *Fusarium verticillioides* is the causal agent of Fusarium ear rot and *F. graminearum* is the causal agent of Gibberella ear rot. *Fusarium*

verticillioides grows over a broader range of temperatures and water activities than *F. graminearum* (Reid et al., 1999). Both species are able to synthesize mycotoxins, but different mycotoxins. *Fusarium verticillioides* produces fumonisins while *F. graminearum* produces deoxynivalenol (DON) and zearalenone. DON causes feed refusal and decreased weight gain in swine, while zearalenone causes reproductive problems in swine (Prelusky et al., 1994). While both ear rots caused by these fungi are important, Fusarium ear rot caused *F. verticillioides* has come to the forefront recently because of the toxic syndromes produced by fumonisins.

Symptoms in animals associated with the consumption of maize colonized with *F. verticillioides* were described long before fumonisins were identified. Leukoencephalomalacia was described in the US as early as 1902 in horses fed contaminated maize. Sheldon identified *F. verticillioides* as the fungus associated with the moldy maize toxicosis (Marasas, 1996). Then in the 1970's leukoencephalomalacia (LEM) was reproduced using pure cultures of *F. verticillioides*, first in a donkey in Egypt, then in a horse in South Africa (Marasas, 1996). Shortly thereafter pulmonary edema (PES) in hogs and liver cancer in rats were shown to be associated with feeding these animals pure cultures of *F. verticillioides* (Marasas, 1996).

There is also concern that fumonisin contamination may pose a threat to human health in those areas where people consume large quantities of maize. Marasas (1996) found a strong correlation between the consumption of maize contaminated with fumonisins in the Transkei region of Africa and increased esophageal cancer.

The toxins associated with the mycotoxicoses resulting from eating maize contaminated with *F. verticillioides* were characterized in 1988 (Bezuidenhout et al., 1988; Gelderblom, et al., 1988) and named fumonisins. Fumonisins belong to a family of chemically related structures with the most toxic and thoroughly studied member being B1. Other commonly found structures include B2 and B3. These structures are characterized by a 20-carbon backbone with a characteristic amino group at C-2 and tricarballylic moieties at C-14 and C-15. The three structures B1, B2 and B3 differ by hydroxyl or hydrogen groups substituted at C-5 and C-10.

The association of fumonisins with the toxic syndrome produced by feeding *F. verticillioides* contaminated maize has been confirmed by a number of studies. Marasas (1988) showed that injection of FB1 intravenously into a horse induced a syndrome resembling LEM, suggesting that fumonisins are the causal agent of moldy maize toxicosis in animals. In separate studies, both LEM and PES were induced in horse and swine, respectively, by oral dosing of FB1, which proved the causative role of FB1 is the mycotoxicoses (Harrison et al., 1990; Kellerman et al., 1990).

Based on these findings, many researchers have looked for fumonisins in maize grown in nearly all maize-growing regions, and found that no region is free from fumonisin-contaminated maize (Placinta et al., 1999). Chamberlain found fumonisins and aflatoxins in the same samples (Chamberlain et al., 1993), which raised the concern for possible synergistic effects of the two mycotoxins. In field studies looking for regional differences in fumonisin production, the researchers found that June rain was negatively

correlated with fumonisin contamination in the harvested samples, indicating that dry weather prior to or at pollination may be important (Shelby et al., 1994).

Although fumonisins are found in all maize growing regions, not all ears are contaminated and fumonisin contaminated kernels are not evenly distributed within the ear. Because of the scattered distribution of fumonisins in maize samples, determining the actual amount of fumonisin in individual samples has been difficult. Whitaker et al. (1998) investigated variances involved with testing samples for fumonisin content. Results from the studies indicate that a true lot concentration of 2 ppm will vary by $\pm$ 0.85 ppm. The largest contributor to this error comes from sampling of the lot of interest. The variance associated with each step of this process, from sampling to sample preparation to analysis, increases with fumonisin concentration (Whitaker et al., 1998). Interestingly 9.7% of the variance found by Whitaker came from the analysis of the sample.

Since the discovery of fumonisins in 1988, the United States Food and Drug Administration (FDA) has been following research linking fumonisins as a promoter of cancer development. Based on coordinated studies into fumonisin toxicity, the International Agency for Research on Cancer has designated toxins derived from *F. verticillioides* as group 2B (possibly carcinogenic to humans; (Vainio et al., 1993)). Because of the possible heath risks associated with exposure to fumonisins, the FDA has issued guidance for the industry regarding fumonisin levels in human and animal foods (http://www.cfsan.fda.gov/~dms/fumongui.html). The proposed guidelines for fumonisin contamination (FB1+FB2+FB3) in degermed dry milled maize products destined for

human consumption is 2 ppm. The guideline for fumonisins in animal feeds destined for horses and rabbits is 5 ppm. The FDA has also indicated that it intend to issue a final fumonisins guidance document during 2001 (http://www.cfsan.fda.gov/~dms/cfsan101.html). This issuing should set limits for fumonisins in maize destined for interstate commerce, which could cause major problems for growers in the southern United States where fumonisins are thought to be a larger problem. Switzerland has proposed to establish the maximum fumonisin concentration in maize products at 1 part per trillion (Boutrif & Canet, 1998).

The mode of action of fumonisin in horses, swine, and rats is related to the disruption of sphingolipid metabolism by fumonisins. Fumonisins are structurally similar to sphinganine, an intermediate in the biosynthesis of complex sphingolipids. These sphingolipids are components of cerebrosides and other lipids that are found in the brain and nerve tissues that are involved in the signaling circuitry of cells that control development (Merrill et al., 1996; Merrill et al., 1996). Fumonisin B1 (FB1) and other members of the B-series fumonisins are potent, competitive inhibitors of ceramide synthase (sphinganine N-acyltransferase), which is the enzyme that is responsible for the acylation of sphinganine in the biosynthetic pathway for sphingolipids (Wang et al., 1991).

Sphingolipids are involved with membrane and lipoprotein structure, cell-cell communication, interactions between cells and the extracellular matrix, and regulation of growth factor receptors. Because of the array of important factors to cellular growth, disruption of sphingolipid biosynthesis would be expected to have major impacts on

cellular function and viability. It has been suggested that exposure to FB1 and the inhibition of ceramide synthase causes sphinganine to accumulate rapidly, resulting in inhibition of growth and to cytotoxicity. FB1 inhibits ceramide synthase by interacting with the binding site for sphinganine (Merrill, et al., 1996). Inhibition of binding leads to accumulation of sphinganine in the cells and in the blood, which can be used as an early test for fumonisin consumption. Long-chain sphingoid bases, such as sphingosine, are known to cause growth inhibition and cytotoxicity and the accumulation of sphingosine may lead to cell death (Merrill, et al., 1996). The mechanisms for the carcinogenicity of fumonisins are not clear.

Researchers only recently have begun looking at genetic control of fumonisin biosynthesis in the fungus. Desjardins et al. looked at the genetic mutants for fumonisin production. Early research resulted in isolation of three genes, Fum1, Fum2, and Fum3, which were determined to constitute a fumonisin biosynthetic gene cluster (Desjardins et al., 1996). They later characterized mutants that contained defective alleles of genes Fum1, Fum2, Fum3, and Fum5 (Proctor et al., 1999; Proctor et al., 1999). Mutations in Fum1 and Fum5 resulted in no fumonisin production, Fum2 resulted in only FB2 and FB4 produced, and mutations in Fum3 resulted in only FB3 and FB4 being produced (Proctor, et al., 1999; Proctor, et al., 1999).

Shim and Woloshuk have also conducted studies into genetic regulation of fumonisin biosynthesis. Their studies showed that nitrogen as ammonium phosphate at 10 or 20 millimolar repressed fumonisin accumulation for 75 and 120 hours, respectively (Shim & Woloshuk, 1999). This indicates that fumonisin production is controlled by

nitrogen regulation. Recently they have isolated a cyclin gene (FCC1) involved in signal transduction for both fumonisin biosynthesis and fungal development (Shim & Woloshuk, 2001).

Disease development caused by *F. verticillioides* has been studied for many years. Koehler conducted many of the initial studies on *F. verticillioides* and it's relationship with maize. He showed that kernel infection by the fungus reached a maximum at the "mature" stage (around 28% moisture) with no additional increase in infection through the "husking" stage (around 22% moisture) (Koehler et al., 1934). However, he showed that the fungus could colonize maize kernels until kernel moisture content fell below 18.4% (Koehler, 1942). In more extensive studies on the infection process, Koehler found that *F. verticillioides* enters the ear through the silk channel and infects individual kernels (Koehler, 1942). While Koehler indicated that the fungus enters the ear through the silk channel, other researchers found evidence for systemic colonization of the plant. Two researchers indicated the infection of kernels in their studies was only from the systemic infection of the plant (Lawrence et al., 1981; Kingsland & Wernham, 1962). Christensen and Wilcoxson (1966) confirmed Koehler's work showing that kernel infection resulted from inoculum entering the silk channel, but also noted that the infection they saw could have resulted from a systemic infection as well. Kedera et al. (1994) investigated the movement of *F. verticillioides* throughout the maize plant and into the kernels. Tissue from the leaves, stalk and kernels was evaluated for different vegetative compatibility groups (VCG) of *F. verticillioides*. They found multiple VCGs in the ear. Additionally, at least one VCG that colonized the stalk was also found in the ear, supporting systemic

infection hypothesis (Kedera et al., 1994). In a more comprehensive study, Munkvold and Carlton (1997) showed that silk infection is the more important route of entry into the ear. They inoculated silks of maize ears with a strain from one vegetative compatibility group (VCG) and examined the VCG of strains isolated from the ear and from stalk tissue. They was able to recover more isolates from the VCG group used to inoculate silks than from the isolate known to be systemic in stalk tissue. From these results they concluded that while there is some systemic infection, the ear is predominantly colonized by inoculum landing on silks.

Bacon, using electron microscopy, found that in asymptomatic kernels, *F. verticillioides* was limited to the tip region, while kernels associated with LEM contained hyphae in the embryo, endosperm and pericarp (Bacon et al., 1992). Interestingly, Branstetter (1927) also found *F. verticillioides* exclusively located in the tip region of kernels nearly 70 years earlier using light microscopy and a kernel assay. Briefly, kernels were cut into five pieces, with one of the pieces being the kernel tip. After surface sterilization and plating on potato dextrose agar the fungus was always found to be limited to the tip region. In all cases, if the fungus was found in other kernel tissue it was also found in the tip region (Branstetter, 1927).

Another important line of work focused on the timing of kernel infection. Koehler's (1942) work showed that while the silks and pedicels were infected shortly after silking, actual kernel infection did not occur until the kernels were near maturity. A later study showed that *F. verticillioides* could be isolated from kernels two weeks after midsilk (King & Scott, 1981). Warfield and Gilchrest (1999) took the timing of infection

a step further, looking at the effect of distinct stages of kernel maturity on fumonisin production by *F. verticillioides.* Kernels harvested from the field were brought to the lab and dried. They were rewetted to 45% moisture, inoculated with *F. verticillioides* and incubated at 25°C. for three days. The authors found that kernels in the blister stage could be infected and fumonisin could be produced, but the later stages of kernel development supported more fumonisin production and that kernels in the dent stage supported the most fumonisin contamination.

Several studies have focused on inoculation methods to reproduce disease symptoms and permit the screening of germplasm for resistance to *F. verticillioides*. Many unique methods have been employed with variable results. Koehler (1960) found that inoculation methods involving wounding caused more rot compared to nonwounding inoculation methods. Bolting (1963) evaluated many inoculation methods and found that BB pellets coated in inoculum and then shot into an ear resulted in significantly more ear rot than the other treatments examined. In separate studies, Gulya (1980) and Fajemisin (1982) found that the insertion of a toothpick contaminated with *F. verticillioides* into the middle of the ear resulted in high disease severity and the ability to differentiate between susceptible and resistant germplasm. While all of these studies indicated that wounding ears produced better results, Drepper and Renfro (1990) found that wound inoculation was better only when the environment was not favorable for disease development. With an unfavorable environment, Drepper and Renfro (1990) found that a 3.6-millimeter nail punch was best to create the high incidence and severity necessary to show differences in resistance levels for their environment (Drepper and Renfro, 1990). Koehler (1959), in

addition to showing that wounding caused more rot, found that *F. verticillioides* inoculations on the silks and tip of the ear shortly after silking significantly increased rot at harvest. Warren (1978) was the only investigator who reported that a nonwounding technique, spraying the silks with a spore suspension, resulted in high incidence of rot and differentiation of genotypes for resistance. Many of the listed studies compared both wounding and nonwounding inoculation techniques in their studies, but none of these studies looked at fumonisin production as a component of their selection criteria.

Host resistance is the most effective means of controlling Fusarium ear rot and fumonisin contamination. While working with ear rots in California, Smith and Madsen (1949) screened many available inbreds for resistance. Although no inbred they screened had complete resistance, six inbreds had consistently low ear rot scores during their trials (Smith and Madsen, 1949). Smith and Madsen felt that the inbreds with lower rot scores should be used for developing more resistant hybrids. However, kernels can contain the fungus internally without showing symptoms (Valleau, 1920), meaning an inbred could be highly colonized by the fungus but receive a score indicating little rot. An inbred in this category could be given a "resistant" rot rating but actually be highly colonized by the fungus. This could present a problem for breeders if highly colonized kernels with no visible rot more receive favorable scores for resistance to *F. verticillioides*.

Scott and King (1984) found that resistance to *F. verticillioides* was conditioned by the pericarp and not by the endosperm or embryo. Further, they found no evidence for cytoplasmic control of resistance. Hedrick and Pataky implicated persistent green silks in sweet corn genotypes with resistance to the fungus, suggesting that breeding for

silk viability may impart resistance (Headrick & Pataky, 1991). In selecting genotypes under field conditions, it was noted that selecting qualities of the host which are generally found with less ear rot, such as improved ear appearance, husk cover and grain appearance was not a guarantee that the host would possess good quality (Cardwell et al., 2000).

Because no available genotypes display acceptable levels of resistance to *F. verticillioides* or fumonisin accumulation and because the FDA is preparing to set action limits on fumonisins in grain, strategies need to be developed to manage fumonisin contamination in the field. The focus of this thesis was to obtain information on the biology and epidemiology of the fungus to aid in the development of both short and long term management strategies for control of fumonisin contamination. My specific objectives were: 1.) Determine the timing of *F. verticillioides* infection in maize kernels and subsequent fumonisin production. An integral part of any cultural practice to limit the fungal presence or fumonisin contamination in harvested grain would be to understand when these contaminants appear in the samples. Only after determining this would one be likely to recommend a cultural practice to growers that would limit the extent of *F. verticillioides* and fumonisin in harvested grain. 2.) Identify inoculation techniques that could discriminate between genotypes of known resistance to Fusarium ear rot on both visual rot symptoms and fumonisin production. Identifying such techniques would allow maize breeders to screen current genotypes for resistance, as well as any inbred lines under development. Successful identification of resistant genotypes would give breeders

material to include in future hybrids, without the need to introduce genetically engineered hybrids which have not enjoyed widespread public acceptance.

CHAPTER 2: PROFILE OF SEED INFECTION BY *FUSARIUM VERTICILLIOIDES* AND THE ACCUMULATION OF FUMONISIN IN NORTH CAROLINA GROWN MAIZE.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg. (synonym *F. moniliforme* J. Sheld, teleomorph *Gibberella fujikuroi* mating population A) is commonly associated with maize kernels and under favorable conditions causes a kernel decay known as Fusarium ear rot (White, 1999). Infection by *F. verticillioides* also can result in the contamination of seed with fumonisins, a family of mycotoxins that are toxic to animals and are suspected human carcinogens. The most toxic and thoroughly studied member of the fumonisin family is B1. Other commonly found structures include B2 and B3. Fumonisins are secondary metabolites produced by *G. fujikuroi* mating types A and D. In contrast, *G. fujikuroi* mating type F, which is also classified as *F. verticillioides* and found primarily on sorghum, does not produce fumonisins. Studies to date have failed to show any involvement of fumonisins in the pathogenicity of *F. verticillioides* and the role of fumonisins to the producing organism is unknown.

Marasas implicated the potential toxicity of fumonisins in 1988. Early studies showed a strong association with the ingestion of *Fusarium verticillioides* contaminated maize and Equine Leukoencephalamalacia in horses and Porcine Pulmonary Edema in swine (Marasas, 1996). Subsequent studies have shown that fumonisins are responsible for these diseases and there is mounting evidence that consumption of maize contaminated with fumonisin may be associated with human esophageal cancer (Marasas,

1996). As a result of studies coordinated by the International Agency for Research on Cancer showing that fumonisins can be a cancer promoter in mouse, fumonisins have been assigned to class 2B, a class that contains probable human carcinogens (Vainio, et al., 1993). Because of the health concerns associated with fumonisins, the FDA has proposed to regulate fumonisins in human and animal feeds. An extensive study coordinated by the FDA (http://www.cfsan.fda.gov/~dms/fumongui.html) on the potential harmful effects of fumonisin has resulted in the FDA proposing regulatory guidelines for fumonisins as listed in Table 1.

Concern over the quality of maize for human and animal consumption has prompted an interest in developing maize genotypes with resistance to *F. verticillioides* and subsequent fumonisin accumulation. While heritable resistance has been identified in maize (King and Scott, 1981), the selection of highly resistant genotypes and the ability to move resistance into commercially desirable lines has been difficult. Smith and Madsen (1949) were successful in identifying very susceptible genotypes in screening studies, but their studies failed to identify highly resistant lines. In part, the difficulty in developing resistant genotypes is due to the lack of understanding of the factors important to infection by *F. verticillioides* and fumonisin accumulation.

There have been several studies designed to understand the infection of maize kernels with *F. verticillioides*. Branstetter (1927) and Valleau (1920) independently concluded that seed-infecting fungi, specifically *F. verticillioides*, could colonize healthy kernels. Valleau examined hundreds of apparently healthy maize ears from the Midwest and southern states and found every ear to be infected by *F. verticillioides*. Branstetter,

in a more thorough investigation, examined the number of seeds infected by *F. verticillioides*. Surface sterilized kernels from sound ears (ears with no visible injury or rot) were plated on a medium conducive for fungal growth and examined for the presence of *F. verticillioides*. He found that nearly 80% of kernels were infected by *F. verticillioides*. Branstetter also found that kernels infected by *F. verticillioides*, were always colonized at the tip (pedicel) region. Because many (31%) of the kernels only showed fungal growth from the tip region, the author hypothesized that the tip region was more susceptible to fungi than other parts of the kernel (Branstetter, 1927). He further showed that most maize genotypes could be infected by *F. verticillioides* regardless of "resistance" to the fungus. Koehler et al. (1934), in an attempt to identify when kernels became infected in field-grown maize, harvested kernels at physiological maturity (28% moisture) and at husking stage (22% moisture). They found that the number of infected kernels did not change between the mature stage and husking stage. Warfield and Gilchrist (1999) found that the developmental stage of kernels has a significant impact on the amount of fumonisin that can be produced in the kernels. They inoculated kernels harvested at the blister, milk, dough and dent stages of development and measured the amount of fumonisin that accumulated after 15 days incubation. The more mature kernels supported the greatest amount of fumonisin production. The same trend for greater fumonisin accumulation in more mature kernels was shown for kernels adjusted to the same moisture content. To show this, Warfield and Gilchrist, (1999) lyophilized kernels at the four stages of development and rehydrated them to the 45% kernel moisture before inoculating with *F. verticillioide*s. They concluded from their

studies that the observed effect of fumonisin contamination increasing relative to kernel maturity was dependent on kernel substrate, not kernel moisture

While it is clear that *F. verticillioides* readily colonizes maize kernels, details of the infection process remain unknown. For example, there is still some controversy over the source of inoculum for kernel infection. One hypothesis is that spores land on the silks protruding from the ear, germinate and the mycelium grows down the silks and infects individual kernels. Koehler described this route of infection in some of his early studies (Koehler, 1942). There is also evidence to support a second mode of ingress into the kernels. This mode involves systemic infection of the maize plant through infected seed, root or stalk tissues. After infection, the fungal hyphae grow through the stalk tissue, into the ear and infect the developing kernels. Both Lawrence (1981) and Kingsland and Wernham (1962) found that kernels were infected by *F. verticillioides* only from mycelium systemic in the plant. On the other hand, Christensen and Wilcoxson (1966) confirmed Koehler's work of kernel infection coming from inoculum entering the silk channel, but also noted that the infection they saw could have come from a systemic origin. Kedera et al (1994) further investigated the movement of *F. verticillioides* throughout the maize plant and into the kernels by determining the vegetative compatibility groups (VCG) of *F. verticillioides* isolated from leaves, stalk and kernels. They found multiple VCGs present in the ear, and at least one VCG that colonized the stalk was also found in the ear, supporting a systemic infection hypothesis (Kedera, et al., 1994). In a more comprehensive study, Munkvold and Carlton (1997) reached the opposite conclusion. In contrast to Kedera et al, who simply identified VCGs found in

tissues, Munkvold and Carlton inoculated seed before planting with one VCG group of *F. verticillioides* and the ears produced from these seeds with a different VCG group. By using different VCG groups, Munkvold and Carlton was able to show that more isolates of the VCG inoculated on the silks were recovered from the ear than for the systemic isolate, indicating that silk infection was the predominant route of entry into developing kernels.

Over the last ten years there have been incidences of fumonisin contamination in the US exceeding 5 ppm (Marasas, 1996). The 1998 North Carolina growing season resulted in fumonisin contamination in several growing regions of the state. Several loads of grain were rejected based on arbitrary limits set by individual buying stations. Some stations were simply rejecting grain based on excessive numbers of rotted kernels, while others had purchased fumonisin ELISA test kits and rejected grain with fumonisin levels above 15 parts per million (ppm). Out of this testing, one load of grain was found to contain fumonisin contamination levels of 278 ppm.

Concerns of fumonisin accumulation prompted an interest in maize growers regarding control strategies to reduce fumonisin contamination. Certainly the development of resistant varieties is a top priority for long-term control. The focus of this project was to gain information on fumonisin contamination that would aid in the development of resistant genotypes and would also provide insight into management strategies that could be used in the short term. Two important questions were addressed in this study: when do infected kernels and fumonisin contamination first appear, and what is the profile of kernel infection by *F. verticillioides* and fumonisin accumulation

throughout the growing season. Information gained from these studies has implications in management strategies as well as in breeding programs.

Materials and Methods

Maize was grown in two locations in North Carolina, at the Central Crops Research Station in Clayton (Clayton) and the Tidewater Research Station in Plymouth (Plymouth). Pioneer hybrid 3394 was planted in 1999 and 2000, and two additional hybrids, Pioneer hybrid 34K77 and an experimental hybrid X1106D were planted in 2000. Hybrids were planted at nearly the same time each year at the recommended planting date for each location. Average midsilk dates were July 4, 1999 and July 3, 2000. A split plot experimental design was used with hybrids as the main plot and harvest date as the subplot. A 0.91 m alley separated each of the four replicates. Hybrids were planted at a population of 59000 plants per hectare as one row plots at Clayton and two row plots at Plymouth. Rows were 3.8 meters long and 0.9652 meters apart.

Each row in a replicate was randomly assigned a week that the row would be harvested. In both years of the study ten ears were harvested from the designated plot each week, starting two weeks after midsilk from each replicate plot for a total of fourteen weeks. Kernel moisture content for the ears harvested from weeks 2 through 6 was determined by comparing the weight of three representative ears of each hybrid before and after drying. Kernel moisture content of seeds harvested weeks 7 through the end of the study was determined using a Dickey John (Model GAC 2100, Auburn, IL) moisture meter. Ears from each week were rated rotted kernels. Rot ratings were based on a graded

0 to 10 scale with 0= no visible rot and 10 =100% rot. Ears were dried in a forced air drier at 35°C. for one week, shelled, and the kernels from the 10 ears in a replicate were pooled and stored at room temperature.

Incidence of kernel infection was determined using a modified protocol described by Zummo and Scott (1992). Kernels randomly selected from the pooled sample of ten ears harvested from each plot were surface disinfested using a 70% ethanol dip, followed by submersion in 20% NaOCl for three minutes. Kernels (13 per plate) were placed in 18 cm petri plates containing 15 ml of Czapeks Agar supplemented with 7% NaCl. In 1999, 390 kernels from each replicate plot were randomly sampled to detect fungal colonization. In 2000, 195 kernels were plated based on a statistical analysis showing that this was an appropriate sample size. Kernels were incubated at 28 degrees C for 7 days and the percentage of kernels with visible growth of *F. verticillioides* was recorded. Randomly selected isolates of *F. verticillioides* cultured from the plated seeds were sent to the Pennsylvania State University Fusarium Research Center for verification and identification.

In 1999 the fumonisin concentration of the kernels was quantified by Optimum Quality Grains (Des Moines, IA) using an enzyme-linked immunosorbent assay (ELISA). The protocol utilized for quantification was a proprietary modification to the protocol described in Kulisek and Hazebroek (2000). In 2000, fumonisin concentrations were quantified by Dr. Winston Hagler, director NCSU Mycotoxin Lab, using the Romer Labs, Inc. (Union, MO) fumonisin protocol FUM-LC1. Briefly, a 454-gram subsample of harvested kernels was randomly selected from each replicate and then individually ground

to mesh size 20. A 25-gram sub-sample was extracted for 1 hr with 100 ml of CH_3CN/ H_2O (50/50), and a 2 mL of the extract was diluted with 8 ml MeOH/H_2O (3/1) and added to a column conditioned with 5 ml MeOH followed by 5 ml 3:1 MeOH/H_2O . Then the column was washed with 8 ml of MeOH/H_2O (3/1) followed by 3 ml of MeOH and the wash solvent was discarded. The sample was eluted with 10 ml MeOH/HOAC (99/1), then dried overnight on a Speedvac System SS3 (Savant, Holbrook, NY).

For derivatization, the residue was dissolved in 1 mL of MeOH. Then 1 ml of 0.05M sodium borate buffer (pH = 9.5), 0.5 ml of sodium cyanide reagent (13 mg/L H_2O), and 0.5 ml of NDA reagent were added to the sample in stated order. The sample was sealed and heated for 15 minutes at 60 degrees C, then cooled to room temperature and diluted with 7 ml of 0.05M phosphate buffer (pH 7)/ CH_3CN (40/60). Twenty µl of the sample was placed in the HPLC System. The HPLC system consisted of a model LC-600 HPLC pump and a SIL-9A auto injector (Shimadzu Corporation, Norcross, GA), a Brownlee column (0.4 by 10 cm, Perkin-Elmer Corp, Norwalk, CT) and a model RF-551 programmable and scanning fluorescence HPLC monitor (Shimadzu) set at 420 nm excitation and 500 nm emission.

All data were analyzed using the General Linear Models (GLM) procedure of SAS version 8.1 (SAS Institute, Cary, NC). Kernel infection data were transformed using a Logit transformation and fumonisin B1 was transformed using a log transformation.

Results

In 1999, kernels of maize hybrid 3394 became infected 4 weeks and 5 weeks after the midsilk stage of development at Plymouth (Fig 1) and Clayton (Fig 2), respectively. The number of infected kernels increased at each location to a peak at 7 weeks at

Plymouth and 9 weeks at Clayton. These two peaks in the number of infected kernels occurred at kernel moistures of 17% and 21.5% at Plymouth and Clayton, respectively. A hurricane on September 16, 1999 destroyed the plots in Plymouth and delayed harvest of the plots at Clayton for two weeks.

Detectable fumonisin contamination occurred 4 and 5 weeks after pollination at Plymouth and Clayton, respectively (Fig. 1 and 2). At each location, the concentrations of fumonisin peaked within 1 week of the time when the maximum number of infected kernels was observed. The number of infected kernels and the concentration of fumonisins decreased at both locations after initial peaks, but both fumonisin contamination and the number of infected kernels increased again late in the season.

In 2000, three maize hybrids were compared during the growing season for number of kernels infected with *F. verticillioides* and the concentration of fumonisin. In addition to Pioneer hybrid 3394, which is designated as moderately resistant to Fusarium ear rot (intermediate), a more resistant hybrid, Pioneer 34K77 and a more susceptible experimental hybrid, Pioneer X1106D were compared. Weather conditions for the two years of this study were markedly different. In contrast to 1999, which was characterized by above average temperatures and below average rainfall until a hurricane in mid September, weather conditions in the 2000 growing season were characterized by below average temperatures and above average rainfall. Despite these inter-seasonal differences, initial infection of kernels with *F. verticillioides* in 2000 occurred within one week of the time observed in 1999. The peak for maximum number of infected kernels, however, occurred later in 2000 than in 1999. The maximum number of infected kernels

of hybrid 3394 occurred at 10 weeks at Plymouth (Fig 3) and 11 weeks at Clayton (Fig 4). For hybrid 3394, the maximum percentage infected kernels of 38% at Plymouth in 2000 was similar to the 42% observed for 1999 and maximum percentage of kernels infected of 58% at Clayton was similar to the 62% observed in 1999.

The profile for percent kernel infected was similar for all three hybrids at Plymouth, regardless of resistance levels. Pioneer hybrid 34K77 had fewer infected kernels than the other two hybrids throughout the growing season, but the difference between the three hybrids in percent infected kernels was within 20% for the duration of the study. The profile for percent kernel infection between the three hybrids at Clayton was similar until Week 9. After Week 9, the more susceptible hybrid X1106D had a higher percentage of infected kernels than the more resistant hybrids.

Fumonisin concentrations of maize grown at Plymouth in 2000 were lower than for 1999 and remained fairly constant between weeks 8 and 13 after pollination (Fig 1,5). Fumonisin first appeared 5 weeks after pollination in the intermediate and susceptible hybrids and at 6 weeks in the resistant hybrid (Fig. 5). The maximum fumonisin concentration never exceeded 5 ppm in the resistant hybrid, but reached a maximum concentration of 11 ppm in the susceptible hybrid.

In contrast to Plymouth, fumonisin concentrations at Clayton in 2000 were greater than levels observed in 1999 with peaks at 18 ppm at 10 weeks after pollination (Fig 2, 6). The profile for fumonisin accumulation was very similar for the three hybrids until 9 weeks after pollination. After 10 weeks, however, the resistant hybrid had a peak

of fumonisin contamination of 18 ppm. A second peak at 13 weeks post pollination followed this initial peak.

Discussion

The question that directed the design of this study was how soon and at what rate do kernels become infected with *F. verticillioides* and contaminated with fumonisin. This question is important because the answer has a direct bearing on management strategies. For example, if the number of infected kernels and the concentration of fumonisin continued to increase during the growing season, a feasible control strategy may be to harvest maize as early as possible. Information on the profile of fumonisin accumulation may also be important for maize breeders in choosing an appropriate time to compare maize genotypes for resistance to fumonisin accumulation. Although several studies have focused on the infection of maize by *F. verticillioides*, I am unaware of study that followed infection and fumonisin accumulation throughout the growing season.

I found that kernels become infected with *F. verticillioides* within 4-5 weeks after pollination and that the number of infected kernels increases during the season, at least until the average harvest date for maize in North Carolina. The average date at which 50% of the maize is harvested in North Carolina is September 12 (Meadows, 2000). This approximately corresponds to harvest date 9 in both years of this study.

The increase of infected kernels 4 to 5 weeks after pollination was also observed in artificially inoculated ears (Fig 7). These rows were planted in adjacent plots to the naturally infected ears. The ears were inoculated 2 weeks after pollination and assayed for kernel infection under the same protocol as previously described. Even with

additional inoculum added to the ear, significant kernel infection was not detected until 4 to 5 weeks after pollination. Inoculation increased overall kernel infection levels, with peak kernel infection approximately 20% greater than the naturally infected kernels. The delay in increase of kernel infection may indicate that host defenses are limiting kernel infection until 4 to 5 weeks after pollination.

Overall the profile of fumonisin accumulation followed that for the incidence of seed infection. A surprise finding was how soon fumonisin appeared in maize kernels. It appeared within one week of when a significant number of infected kernels was detected and peaked within 2-3 weeks after its appearance. Interestingly, the pattern of fumonisin accumulation and the number of seeds infected fluctuate late in the season. Either may decrease only to increase again later in the season. It is clear from these studies that delaying maize harvest after the average harvest date increases the risk of fumonisin contamination.

The reasons for the late season increase in fumonisin contamination is unclear. Late season rains such as occurred in Clayton in 1999 likely contributed to the increase that year. However, we were unable to correlate seed moisture with an increase in fumonisin contamination. Similarly, we were unable to correlate the time of seed infection with kernel moisture. The 1999 and 2000 growing seasons were quite different and the rate at which the kernels dried differed greatly between the two years (Fig. 1,2,3,4). Kernel moisture content 6 weeks after pollination was approximately 20 % in 1999 and 40% in 2000. Regardless, maximum kernel infection occurred around 20% moisture content in both years of the study.

The profile of kernel infection and fumonisin accumulation in maize was similar to that found for *Aspergillus flavus* infection and aflatoxin accumulation (Payne et al., 1988). In their study, Payne et al. (1988) found maximum seed infection and aflatoxin contamination to occur at 16% moisture. They also observed a decrease in kernel infection and in aflatoxin contamination later in the season followed by a second peak in both seed infection and aflatoxin contamination. Miller et al (1983) also found a similar profile when investigating *Fusarium graminearum* isolated from inoculated maize cobs. Ears were harvested weekly, ground in a mill and a subsample was diluted and plated on nutrient agar plates to determine the colony forming units present in the ear. The colony forming unit counts of *F. graminearum* increased until 6 weeks after inoculation, then decreased, which is similar to the trend to what I observed at Plymouth in 1999. The timing of *F. verticillioides* appearance in my study was very similar to that of 2 weeks post pollination reported by Scott (1981).

Similar to Warfield and Gilchrist (1999), I found more fumonisin produced on kernels in the later stages of development. Under the conditions of this study, I did not find any fumonisin contamination in kernels until at least the dent stage of kernel development (approximately 45-35% moisture). It is unclear from this study if the appearance of fumonisin is related to kernel development or to kernel moisture.

In 2000, I compared three maize hybrids that differ in susceptibility to Fusarium ear rot. All three hybrids showed a similar profile of infection at the two locations but their relative ranking for number of seeds infected differed between the two locations (Fig 3, 4). Resistance to Fusarium ear rot as expressed in the hybrids tested did not appear to

be necessarily related to the number of infected kernels (Fig 3, 4). At Clayton, the resistant hybrid had more infected kernels than the intermediate hybrid from Week 8 until the end of the study (Fig 4). At Plymouth this trend is not as evident, but there were weeks in which the hybrid with greater resistance had more infected kernels than the more susceptible hybrid (Fig 3, weeks 6, 8 and 10).

In addition to comparing hybrids based solely on kernel infection levels, I wanted to see if rates of infection differed between the three resistance levels. For this comparison, I restricted the dataset to the data points between Week 4 and Week 11 because it appeared that this was the time that kernel infection generally increased. After logit transformation of the data and combining the data from the Plymouth and Clayton locations, I found that the susceptible hybrid had a significantly greater infection rate than the resistant or intermediate hybrids, which is what one might expect.

The profile of fumonisin contamination was similar for all three hybrids for the early parts of the study. At both Clayton and Plymouth a similar trend between fumonisin contamination is observed until Week 9. After Week 9 the differences between hybrids became apparent. At Plymouth, the susceptible hybrid had more fumonisin contamination than the other hybrids (Fig 5). Surprisingly, at Clayton the resistant hybrid had the highest fumonisin contamination levels after Week 9.

After a log transformation of the fumonisin data, the results reinforced the trends observed in the untransformed data. At Plymouth, the susceptible hybrid was found to contain significantly more fumonisin than either the intermediate or resistant hybrids. But at Clayton, the resistant hybrid had significantly greater fumonisin contamination

than either the intermediate or susceptible hybrid. There was no indication from the percent kernel infection data to suggest that the resistant hybrid would have greater fumonisin contamination than the other hybrids. This information indicates that there are two different processes controlling resistance to infection and fumonisin contamination. Based on this insight, knowing information about a hybrids resistance to Fusarium ear rot would not necessarily tell you about the hybrid's resistance to fumonisin accumulation.

In addition to *F. verticillioides*, several other fungi were isolated from the surface sterilized kernels. Three fungi, *Aspergillus flavus, Fusarium semitecticum* and a *Penicillium* spp. were the most common. Of these three, the *Penicillium spp.* was the most common and *Aspergillus flavus* was rarely found. Each of these fungi was found to coinfect kernels with *Fusarium verticillioides*. There may have been other fungi present in the kernels, but the protocol used was optimized for *A. flavus* and *F. verticillioides* and the conditions present may have inhibited growth of other fungi and bacteria present.

Results from this study show that early harvest can be employed as a control strategy for fumonisin contamination. Fumonisin concentrations appear to increase over the growing season up to the average harvest date for maize in North Carolina. Beyond this date, the concentrations of fumonisin fluctuate. Under years conducive for fumonisin contamination, early harvest may help reduce the level of contamination. Certainly, growers should avoid late harvesting of maize. These data also have implications for breeding programs. Because the levels of fumonisin can vary late in the season, a comparison of hybrids at a recommended harvest date may give a more accurate assessment of their susceptibility to fumonisin accumulation. Based on our studies the

ideal time to screen hybrids for kernel infection and fumonisin production appear to be between weeks 7 and 10, since the peak in infected kernels and fumonisin was within this range during both years of our study.

Table 1. Guidance levels for fumonisins in human foods and in animal feeds
(Adapted from the FDA web site http://www.cfsan.fda.gov/~dms/fumongui.html)

Corn Products	Total Fumonisins (FB1+FB2+FB3)
Human Foods	
Degermed dry milled corn products (flaking grits, corn grits, corn meal, corn flour with fat content of <2.25%, dry weight basis)	2 part per million (ppm)
Whole or partially degermed dry milled corn products (flaking grits, corn grits, corn meal, corn flour with fat content of <2.25%, dry weight basis)	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for masa production	4 ppm
Cleaned corn intended for popcorn	3 ppm
Animal Feeds: corn and corn by-products intended for;	
Equids and rabbits	5 ppm (no more than 20% of diet)*
Swine and catfish	20 ppm (no more than 50% of diet)*
Breeding ruminants, breeding poultry, and breeding mink	30 ppm (no more than 50% of diet)*
Poultry being raised for slaughter	100 ppm (no more than 50% of diet)*
All other species or classes of livestock and pet animals	10 ppm (no more than 50% of diet)*

* dry weight basis

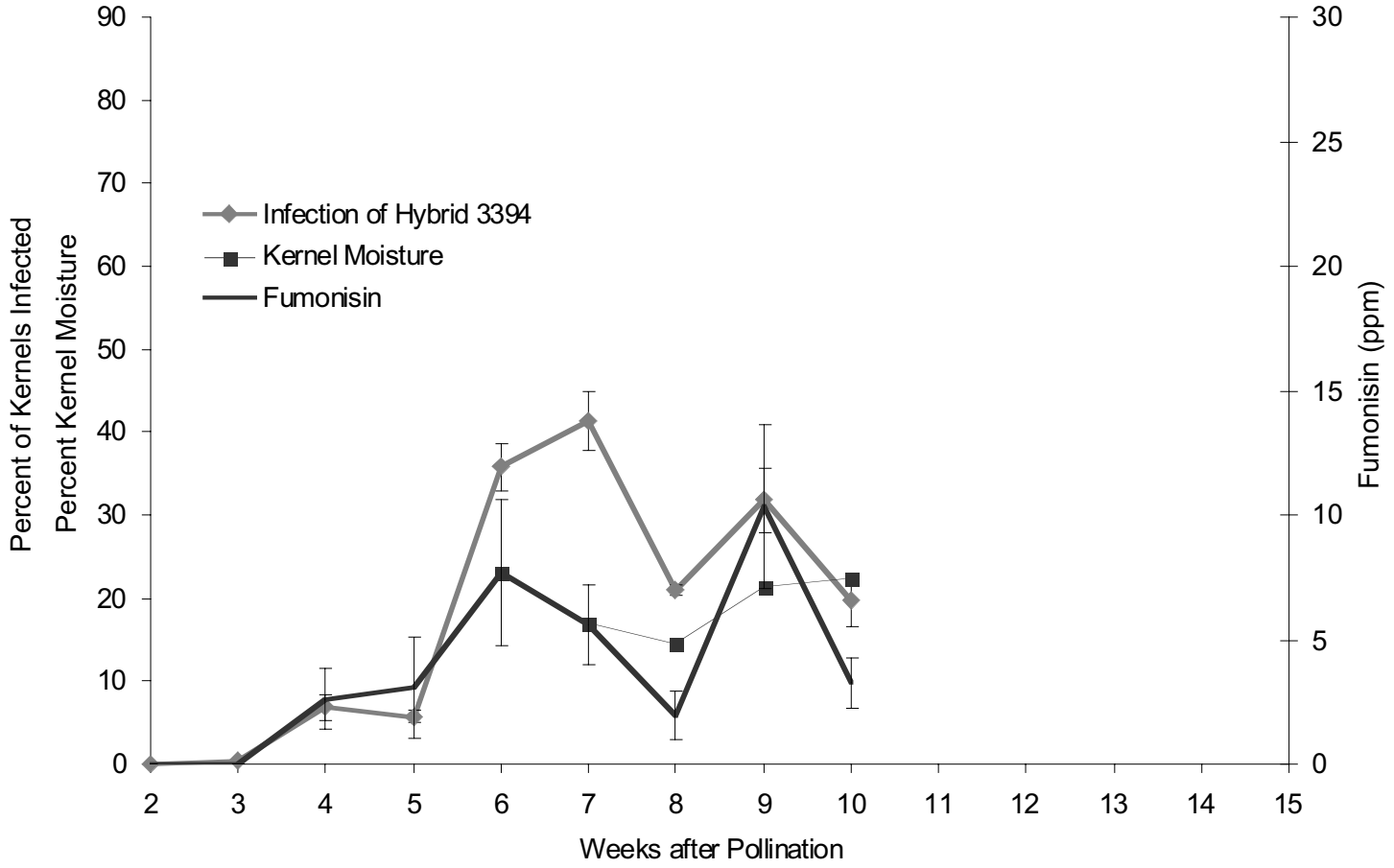

Figure 1. Infection by *Fusarium verticllioides* in corn kernels and fumonisin contamination based on weekly harvests after pollination at Plymouth, 1999. Bars show stantard error. Fumonisin contamination closely follows kernel infection by *F. verticillioides* .

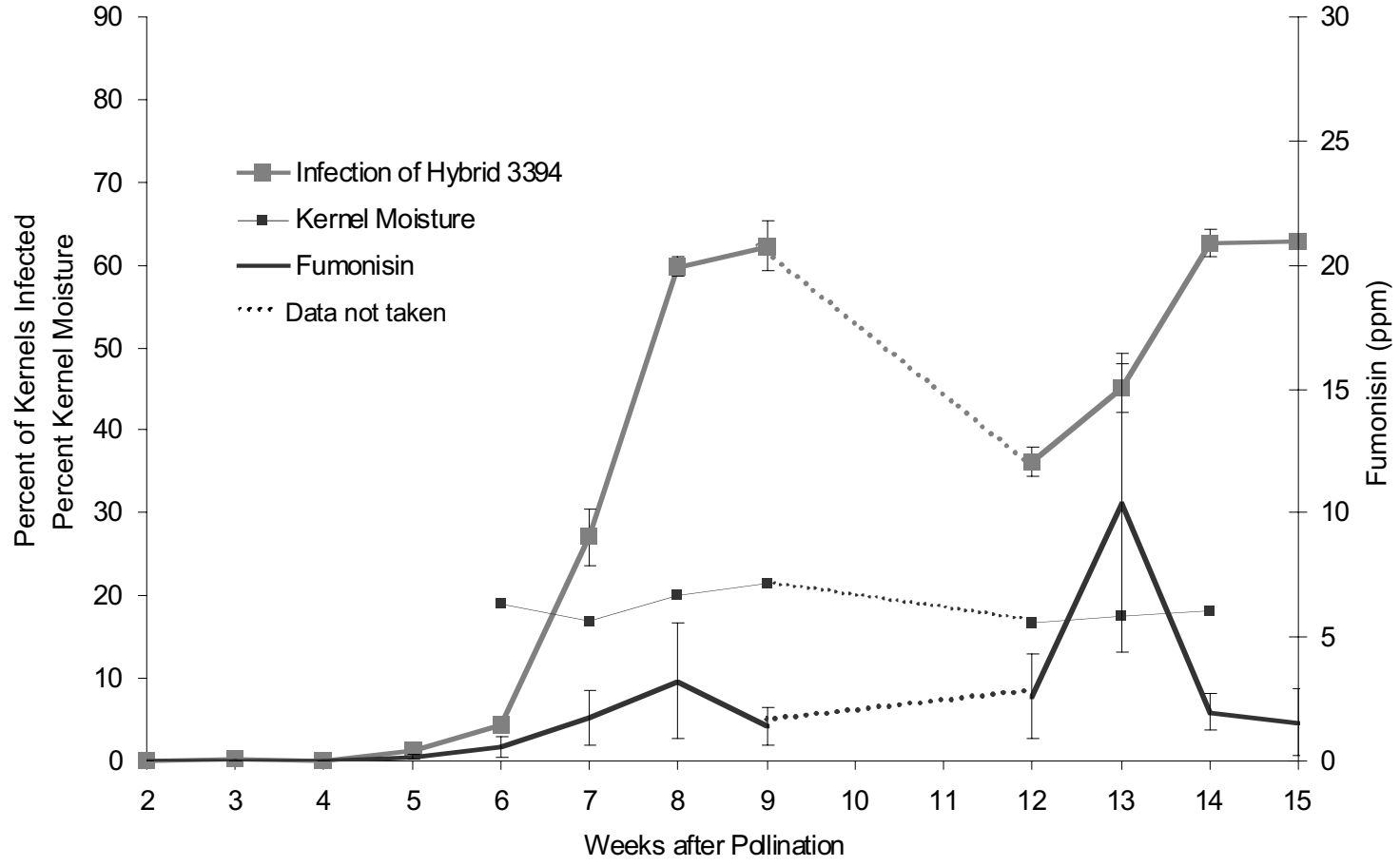

Figure 2. Infection by *Fusarium verticilioides* in corn kernels and fumonisin contamination based on weekly harvests after pollination at Clayton, 1999. Bars show standard error. Fumonisin contamination closely follows kernel infection by *F. verticillioides* . ··· - Missing data points because of a hurricane.

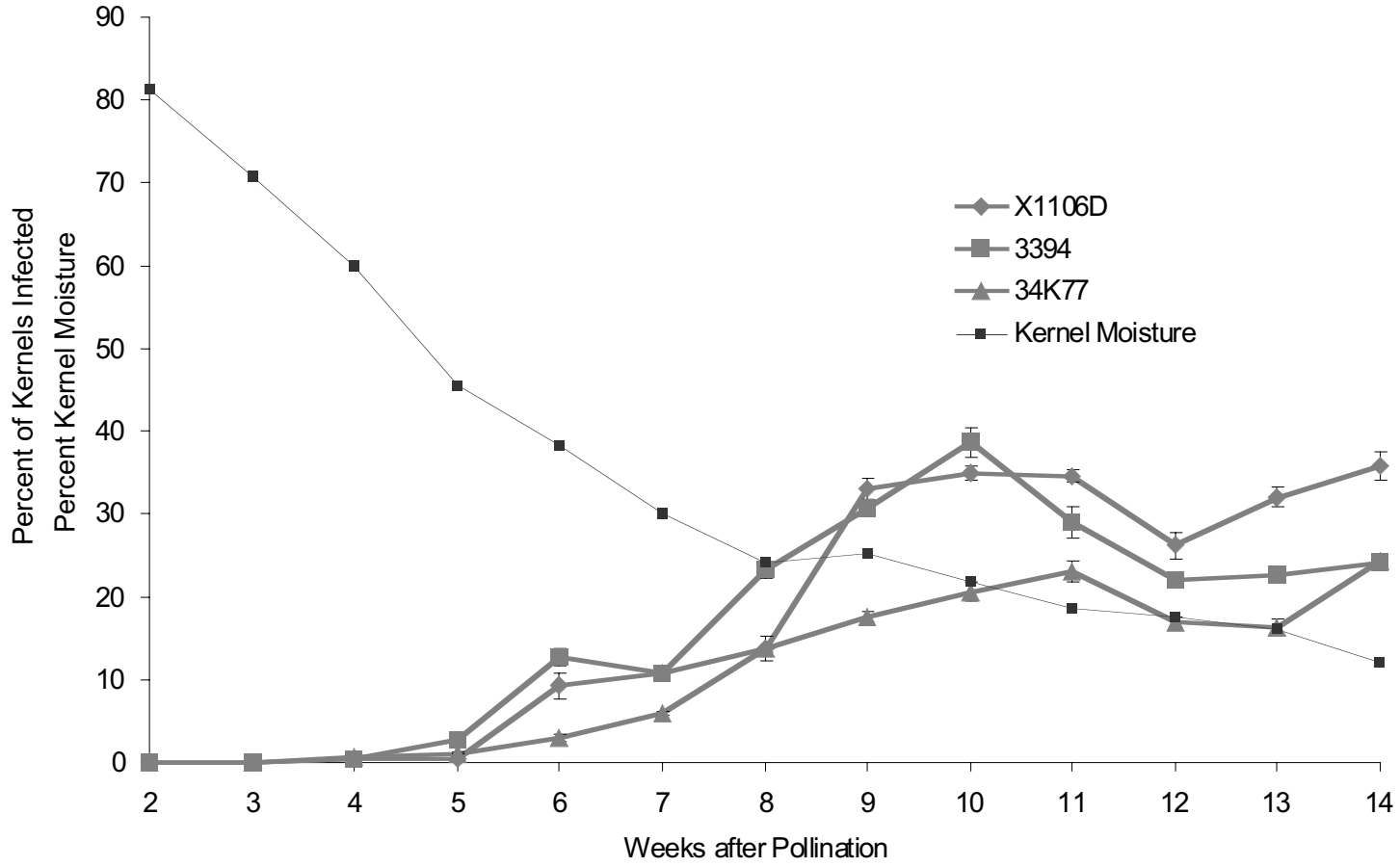

Figure 3. Kernel Infection by *Fusarium verticilloides* in three corn hybrids that differ in resistance to Fusarium ear rot at Plymouth, 2000. Bars show standard error Infection levels are much lower than levels observed at Clayton, 2000.

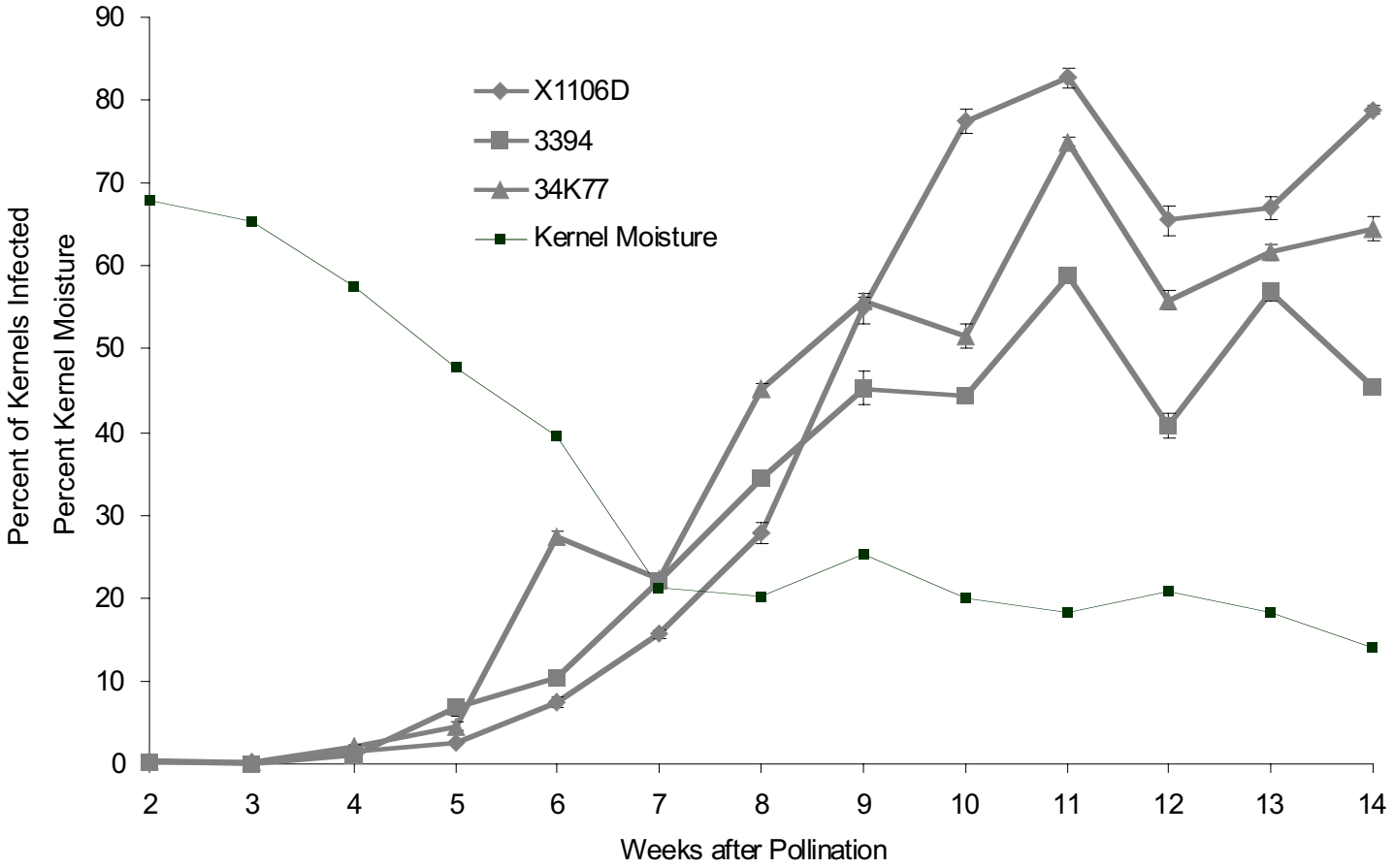

Figure 4. Kernel Infection by *Fusarium verticilloides* in three corn hybrids that differ in resistance to Fusarium ear rot at Clayton, 2000. Profile of infection appears similar for all hybrids regardless of resistance level.

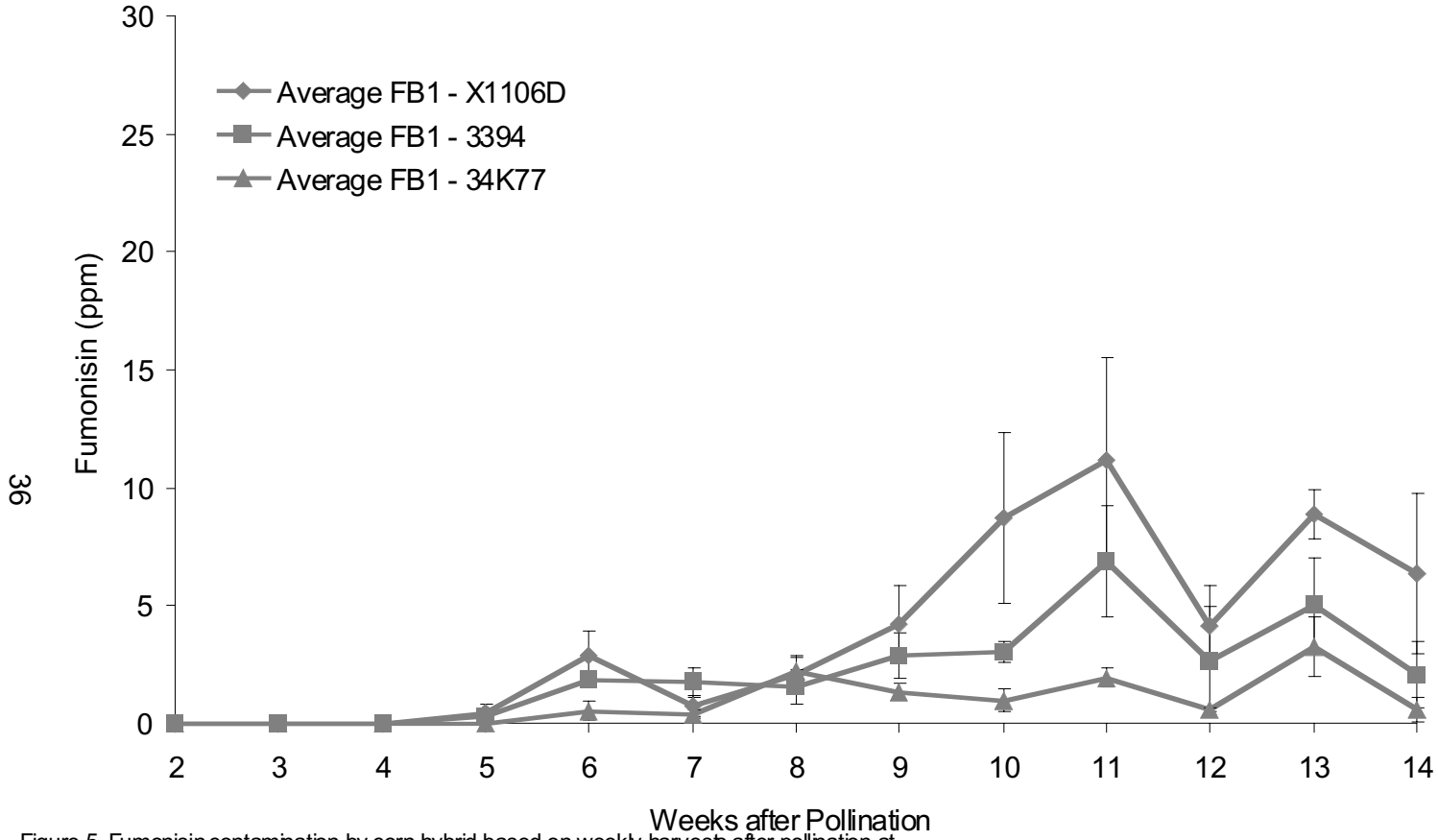

Figure 5. Fumonisin contamination by corn hybrid based on weekly harvests after pollination at Plymouth, 2000. Bars show standard error. Through the first 8 weeks, fumonisin contamination by hybrid does not appear to differ. After 8 weeks, the more susceptible hybrid to Fusarium ear rot showed the highest amount of fumonisin contamination.

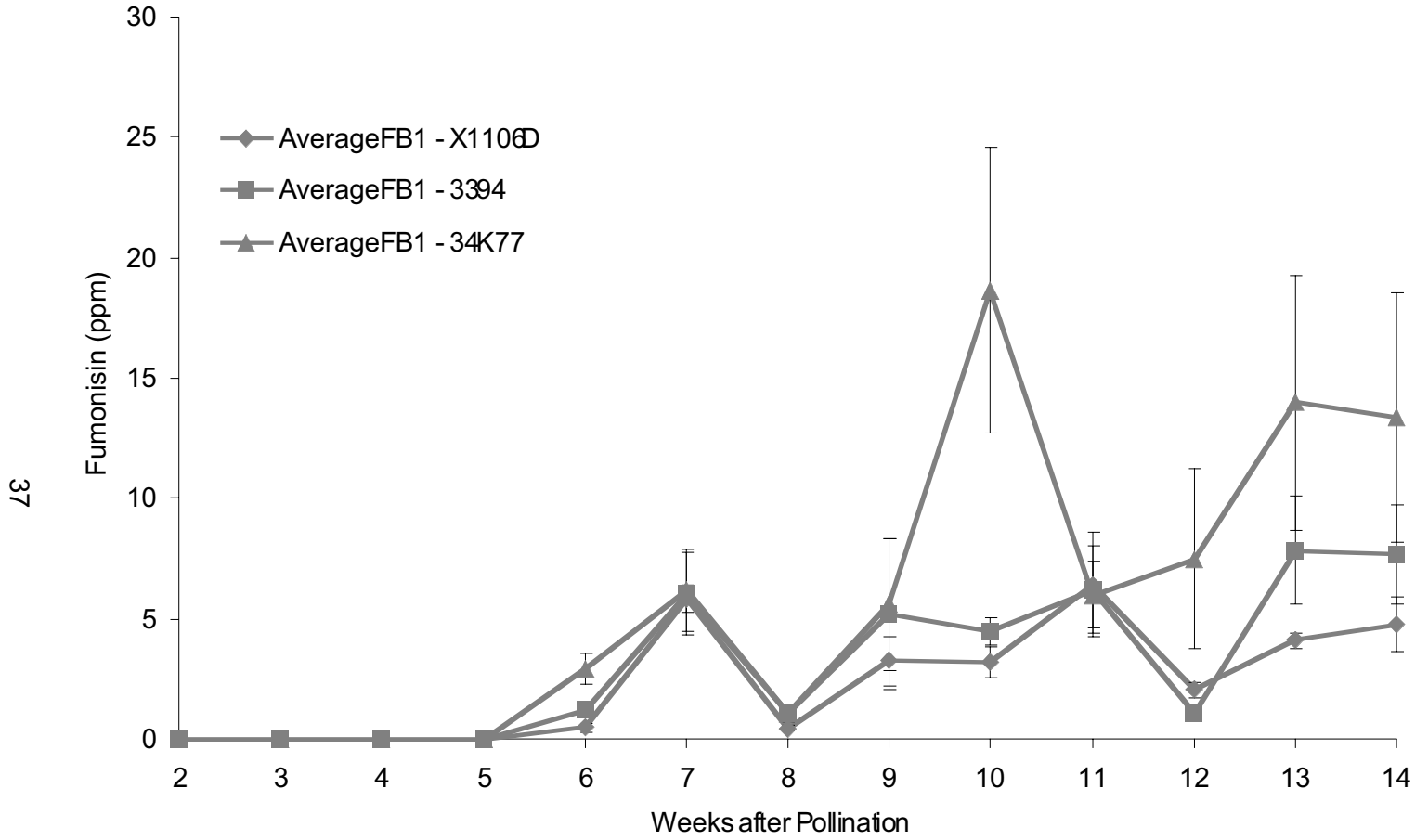

Figure 6. Fumonisin contamination by corn hybrid based on weekly harvests after pollination at Clayton, 2000. Bars show standard error. Through the first 8 weeks, fumonisin contamination by hybrid does not appear to differ. After 8 weeks, the hybrid with more resistance to Fusarium ear rot showed the highest amount of fumonisin contamination.

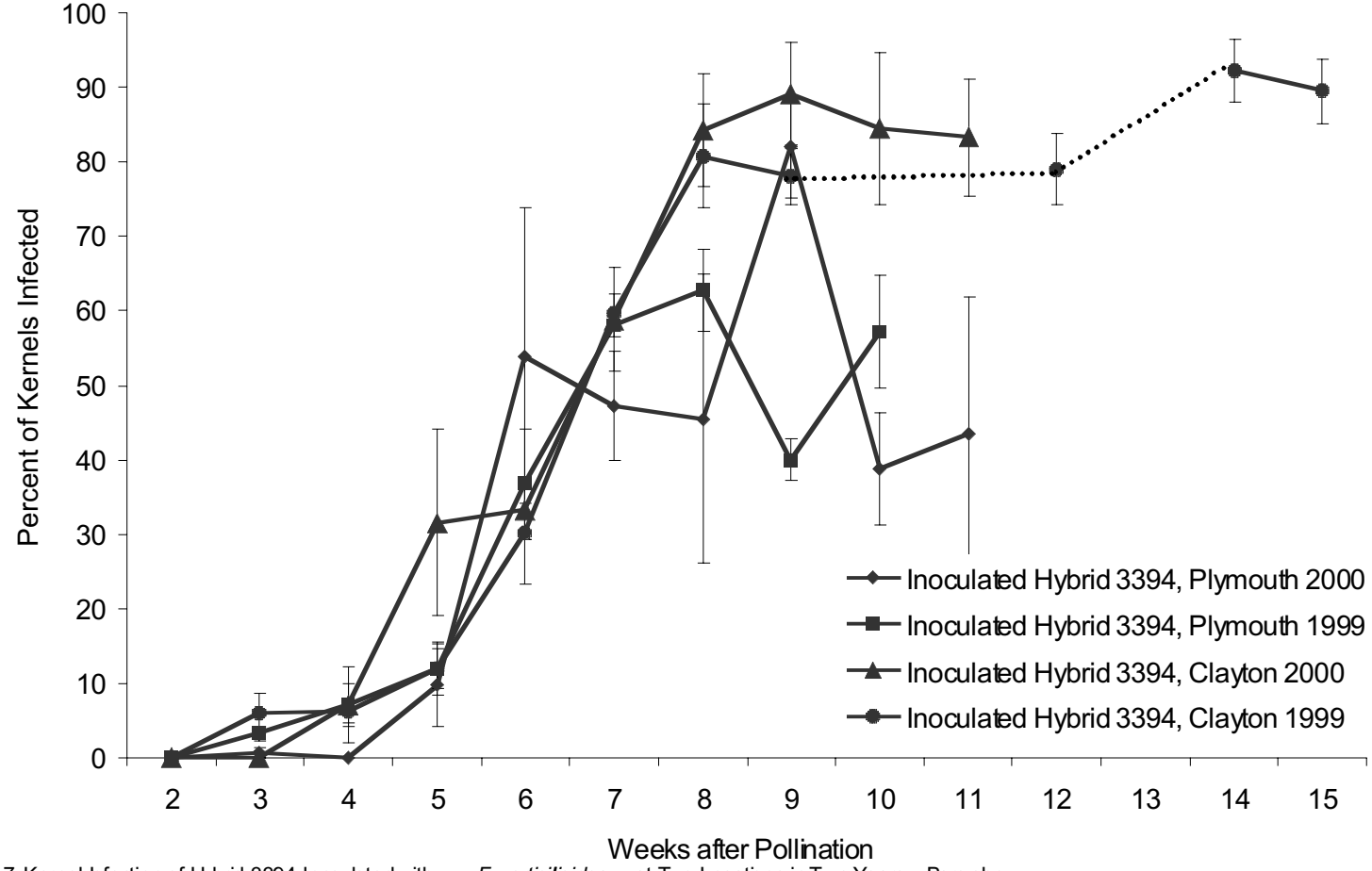

Figure 7. Kernel Infection of Hybrid 3394 Inoculated with *F. verticillioides* at Two Locations in Two Years. Bars show standard error. Kernel infection significantly increases approximately five weeks after pollination. ···- Missing data points

CHAPTER 3: EVALUATION OF INOCULATION TECHNIQUES FOR THEIR ABILITY TO DIFFERENTIATE BETWEEN HYBRIDS OF KNOWN RESISTANCE FOR FUSARIUM EAR ROT AND FUMONISIN PRODUCTION.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg. (synonym *F. moniliforme* J. Sheld) is commonly associated with maize kernels and under favorable conditions causes an ear rot known as Fusarium ear rot (White, 1999). The disease reduces seed quality and recently the fungus has been shown to produce the mycotoxin fumonisin, which is toxic to animals and an implicated human carcinogen. Equine Leukoencephalamalacia in horses and Porcine Pulmonary Edema in swine have been shown to be caused by the ingestion of *F. verticillioides* contaminated maize and by dosing animals with purified fumonisin (Marasas, 1996). Because of the health concerns associated with fumonisins, the FDA has proposed to regulate fumonisins in human and animal feeds. An extensive study coordinated by the FDA (http://www.cfsan.fda.gov/~dms/fumongui.html) on the potential harmful effects of fumonisin has resulted in the FDA proposing regulatory guidelines for fumonisin. The suggested guidelines are listed in Table 1.

With the discovery of fumonisins and their toxin effects to humans and animals, there has been a renewed interest in breeding for resistance to Fusarium ear rot. Currently there are no commercially available maize genotypes that contain adequate levels of resistance to the fungus or toxin accumulation. Assessing resistance to the fungus and transferring resistance to elite germplasm has been difficult. Several inoculation techniques are being used to evaluate germplasm for resistance to Fusarium. These

techniques inclyde those that involve applying inoculum to the ear with or without wounding, simultaneous inoculation of several species of Fusarium into the ear to select for broad resistance to *Fusarium* species, and indirect selection for more favorable ear qualities. One common thread with all of these approaches is the conservation of time. Because maize breeders often include hundreds of genotypes in their nurseries, techniques are needed that can be applied quickly and provide accurate and reproducible results. Passive selection for more favorable ear qualities has not necessarily lead to superior genotypes with resistance to Fusarium (Cardwell, et al., 2000), and techniques that combine several Fusarium species in an inoculum mixture are probably not appropriate, due to differences in growth requirements between different species of Fusarium (Reid, et al., 1999). Many studies have attempted to identify a technique that will give reproducible and accurate rot ratings. The problem with many of these studies is that they do not start with material with known levels of resistance or susceptibility to Fusarium ear rot. Without having known differentials, if is difficult to evaluate the reliability of the technique.

While no inbred with absolute resistance has been identified, there are inbreds that differ in their resistance to the fungus and the toxin. Such resistance has been shown to be genetic and heritable (King and Scott, 1981). Several studies have investigated inoculation techniques for their ability to reproduce Fusarium ear rot symptoms. Koehler (1960) found that inoculation methods involving wounding caused more rot compared to nonwounding inoculation methods. Bolting (1963) evaluated many inoculation methods and found that BB pellets coated in inoculum and then shot into an ear resulted in

significantly more ear rot than the other treatments examined. Salazar and Vargas (1977) found that a toothpick inserted into maize ears was best at reproducing disease symptoms. In separate studies, Gulya (1980) and Fajemisin (1982) found that the insertion of a toothpick contaminated with *F. verticillioides* into the middle of the ear resulted in high disease severity and the ability to differentiate between susceptible and resistant germplasm. While all of these studies indicated that wounding ears produced better results, Drepper and Renfro (1990) found that wound inoculation was better only when the environment was not favorable for disease development. With an unfavorable environment, Drepper and Renfro (1990) found that a 3.6-millimeter nail punch was best to create the high incidence and severity necessary to show differences in resistance levels for their environment. Koehler (1959), in addition to showing that wounding caused more rot, found that *F. verticillioides* inoculations on the silks and inoculating the tip of the ear shortly after silking significantly increased rot at harvest. Warren (1978) was the only investigator who reported that a nonwounding technique, spraying the silks with a spore suspension, resulted in high incidence of rot and differentiation of genotypes for resistance. Many of the listed studies compared both wounding and nonwounding inoculation techniques in their studies, but none of these studies looked at fumonisin contamination as a component of their selection criteria.

The objective of this study was to evaluate inoculation techniques at different locations for the ability to reproduce differences in visual Fusarium ear rot symptoms and fumonisin production on hybrids of known resistance to Fusarium ear rot. The goal of

the experiments was to identify a technique that can be used to screen germplasm for resistance to Fusarium ear rot and/or fumonisin accumulation.

Materials and Methods

In 1999 and 2000, four hybrids known to differ in resistance were planted at three locations, Central Crops Research Station, Clayton, NC (Clayton), Pioneer Hi-Bred research station in Winterville, NC (Winterville) and Dekalb Genetics Research Station, Mt. Olive, NC (Mt. Olive). Pioneer hybrid 34K77 and experimental X1106D were planted at Winterville. Pioneer considers X1106D as susceptible and 34K77 as more resistant to Fusarium ear rot (http://www.pioneer.com/usa/index.htm.). Dekalb hybrid 635 and Dekalb experimental hybrid 663B were planted at Mt. Olive. Dekalb hybrid 635 is considered more resistant than Dekalb hybrid 663B to Fusarium ear rot (Dale Dowden, personal communication). All four hybrids were planted at Clayton. All hybrids were planted at the recommended date of planting for their location.

Hybrids were planted in a Randomized Complete Block design with rows at all locations approximately 3.8 m long and 0.9652 m apart. The four replicates were separated by 0.91 m alleys. At Clayton, hybrids were planted as one row plots containing approximately 22 plants per plot. At Mt. Olive, hybrids were planted as two row plots with plants thinned to 12 plants per plot. At Winterville, hybrids were planted as two row plots containing approximately 25 plants per row.

Five inoculation techniques were evaluated during the first year of the study. They were 1) a *F. verticillioides* colonized toothpick inserted mid ear (Toothpick), 2) 2 ml of a spore suspension (1x10^5 conidia/ml) injected down the silk channel (Silk

Channel), 3) a plastic shoot bag placed over the ear (Plastic Bag), 4) a pinbar inoculation with 2ml of a spore suspension (1x10^5 conidia/ml) sprayed over the injured surface (Pinbar), and 5) no inoculation (Control). Additionally, a silk channel plus plastic shoot bag (SC + PB) treatment (treatment 6) was included at Central Crops during the first year. This treatment was included with the previously listed five treatments at all locations for the second year. Because of a mistake in replication at Clayton in 2000, treatments were averaged over replicates within a test for all locations. During the first year, the plastic bags were left on the ears for 30 days. After noticing exaggerated ear rot symptoms after the first year, the plastic bags were left on the ear for only one week in 2000. The silk channel and toothpick treatments were applied one week after midsilk both years, while the pinbar, plastic bag and SC + PB treatments were applied two weeks after midsilk.

Toothpicks for the inoculation technique were prepared by placing round toothpicks vertically in a 250 ml beaker filled with distilled water and allowing them to soak overnight. The next morning the distilled water was removed and replaced with fresh distilled water. Later the same day, the water was removed and replaced by Potato Dextrose Broth (Difco Laboratories, Detroit, MI). The beaker was covered and autoclaved for one hour. After the broth cooled, a spore suspension (1x10^5) was added to the beaker and allowed to incubate for two weeks at room temperature under a 12 hr light/dark regime. After two weeks time, the toothpicks were removed from the beaker and dried in a disinfested fume hood.

Conidia for the silk channel and pinbar treatments were prepared by placing a conidial suspension onto petri plates containing Carnation Leaf Agar and allowing the plates to incubate for 7 days at 28 degrees C under a 12 hour light/dark regime. Conidia were harvested with the aid of a glass rod and resuspended in 0.05% Triton X-100. The concentration of conidia was quantified using a hemacytometer, adjusted to the desired concentration with sterile distilled water and the suspension was placed on ice until used.

All ears within each plot were inoculated. Approximately 15 weeks after pollination, 10 randomly chosen ears were harvested and forced air dried at 35 degrees C for one week. Dried ears were rated for rotten kernels, shelled and stored at room temperature. Rot ratings were based on a graded 0 to 10 scale with 0= no visible rot and 10 =100% rot.

The fumonisin concentration of maize grown the Clayton and Winterville locations in 1999 was quantified by Optimum Quality Grains (Des Moines, IA) using an enzyme-linked immunosorbent assay (ELISA). A 454-gram sample of maize from each replicate was sent for fumonisin quantification. The protocol utilized for quantification was a proprietary modification to the protocol described in Kulisek and Hazebroek (2000). The fumonisin concentration of the samples from Mt. Olive and all samples in 2000 was quantified by Dr. Winston Hagler, director NCSU Mycotoxin Lab, using the Romer Labs, Inc. (Union, MO) fumonisin protocol FUM-LC1. Briefly, a 454-gram subsample of harvested kernels was randomly selected from each replicate and then individually ground to mesh size 20. A 25-gram sub-sample was extracted for 1 hr in 100 ml of CH_3CN/ H_2O, (50/50). A 2 ml sample of the extract was added to 8 ml

MeOH/H_2O (3/1) and the resulted solution applied to a column conditioned with 5 ml MeOH followed by 5 ml 3:1 MeOH/H_2O. The column was washed with 8 ml of MeOH/H_2O (3/1) followed by 3 ml of MeOH. The sample was eluted with 10 ml MeOH/HOAC (99/1), and dried overnight on a Speedvac System SS3 (Savant, Holbrook, NY).

For derivatization, the residue was dissolved in 1 ml of MeOH. Then 1 ml of 0.05M sodium borate buffer (pH = 9.5), 0.5 ml of sodium cyanide reagent (13 mg/L H_2O), and 0.5 ml of NDA reagent were added to the sample in stated order. The sample was sealed and heated for 15 minutes at 60 degrees C, then cooled to room temperature and diluted with 7 ml of 0.05M phosphate buffer (pH 7)/ CH_3CN (40/60). Twenty µl of the sample was applied to a Brownlee HPLC column (0.4 by 10 cm, Perkin-Elmer Corp, Norwalk, CT) and a model RF-551 programmable and scanning fluorescence HPLC monitor (Shimadzu) set at 420 nm excitation and 500 nm emission.

Analyses of variance were conducted on Fusarium ear rot ratings and fumonisin contamination of samples. Fusarium ear rot data was transformed using a square root transformation and the fumonisin contamination was transformed using a log transformation. The general linear models procedure of SAS (SAS Institute, Cary, NC) was used for the analyses.

Results

Statistical analysis of the data from the two years indicated that it was valid to pool data from all years and locations for comparison by hybrid. The resistant and susceptible hybrids from each company were compared by inoculation technique using

the slice procedure of GLM. Higher order interactions were pooled and used as error after determining that the interactions were not significant (α=0.25).

In the pooled analysis several interesting findings emerged. Only the Silk Channel inoculation technique was able to discriminate between the two Dekalb hybrids for resistance to Fusarium ear rot (Fig. 13) and only the Pinbar technique was able to differentiate between the two Pioneer hybrids for Fusarium ear rot (Fig. 11). Several other techniques had p values just exceeding the 0.05 significance level (Table 2). The Silk Channel plus Plastic Bag and Pinbar techniques, for example, were almost significant at the p=0.05 for separating the Dekalb hybrids and the Plastic Bag, Silk Channel and SC + PB techniques all had p values just exceeding 0.05 for the Pioneer Hybrids.

The comparison of inoculation techniques for the ability to identify resistance to fumonisin contamination showed a different trend than for resistance to Fusarium ear rot. For example, every technique was able to differentiate between the two Dekalb hybrids for resistance to fumonisin accumulation (Fig. 12). Conversely, none of the techniques was able to separate the two Pioneer hybrids for resistance to fumonisin accumulation (Fig. 14).

The location of the study appeared to have a major role in the results obtained from the individual locations. The effectiveness of each inoculation technique for separating the resistant and susceptible hybrids depended on the location, a point that is lost in the pooled analysis. Both the severity of Fusarium ear rot and the concentrations of fumonisin found in the kernels were greater in 1999 than in 2000 (Figures 15-30).

Location appeared to have a greater effect on individual inoculation techniques than the seasonal effects. Within a location, the same techniques were effective in both years. Within these graphs the effectiveness of inoculation techniques to differentiate between the resistant and susceptible hybrids is evident. With the exception of the Pinbar and Silk Channel techniques that were previously described to work for Pioneer and Dekalb hybrids, respectively, across locations, many of the techniques were able to differentiate between the hybrids at more than one location. The SC + PB and Pinbar treatments were able to separate the Dekalb hybrids at the Mt. Olive and Clayton locations in 1999 and 2000 (Fig. 17,21, 25, 27). The Silk Channel and Control treatments were able to separate the Pioneer hybrids at the Winterville and Clayton locations in 1999 and 2000 (Fig. 15, 17, 27).

Discussion

The recent interest in food quality along with emerging information on the toxicity of fumonisins add importance to develop maize genotypes with resistance to Fusarium ear rot and fumonisin contamination. Currently no maize hybrids that are commercially available are known to be resistant to fumonisin accumulation. Genotypes do differ in resistance to Fusarium ear rot and fumonisin accumulation and it is important to have accurate and reliable methods of evaluating this resistance. Two inoculation techniques were identified that could reliably identify hybrids known to differ in resistance to Fusarium ear rot and fumonisin contamination. These techniques were effective over different locations and across years. Many of the techniques were close to being significant, as seen in Table 2.

It is important to note that the neither the Pioneer hybrids nor the Dekalb hybrids are designated as being resistant to fumonisin accumulation; rather they are designated as having some resistance to Fusarium ear rot. For the Dekalb hybrids, our techniques were also able to show some level of resistance to fumonisin contamination in addition to resistance to ear rot. The techniques were not as effective for showing resistance to fumonisin contamination with the Pioneer hybrids. I am uncertain why this is so, but it may be indicative of the screening procedures utilized by the respective companies.

The hybrids tested in this study would be representative for the acceptable limits of rot that companies would be able to market to their customers. Based on this assumption, the inoculation techniques that were able to differentiate between our differentials would also be able to separate more resistant germplasm from more susceptible germplasm in a breeding nursery. This means the two inoculation techniques, Pinbar and Silk Channel, should be able to identify more susceptible maize germplasm if the resistant and susceptible hybrids were included as checks to compare the resistance of new genotypes. These techniques were able to increase Fusarium rot to levels above that of the uninoculated control. This would give one the ability to screen maize lines under conditions that would not be considered ideal for ear rot screening.

It was difficult to compare inoculation techniques across the different company's hybrids because each company utilizes different screening methods to determine resistance to Fusarium ear rot. Because of this, one might expect the inoculation technique that was closest to the technique used by the company to stand out as the best technique to differentiate their hybrids. It appears from our results that each company

uses different inoculation techniques or conditions to screen their hybrids for Fusarium ear rot. Because of this obvious difference, we felt it was important to only compare resistance levels within a company and not between companies. With this point understood, we found inoculation techniques that could differentiate between resistance levels that can be easily marketed in today's hybrid maize market. Using the techniques identified, one would be able to easily screen out material more susceptible to ear rot than our susceptible hybrid, which is the basis of breeding nurseries. One rarely identifies resistant lines, rather the more susceptible inbreds are dropped and the lines with more resistance are crossed to gain more resistant lines.

From a practical point of view, it may be informative to look at the arithmetic means rather than transformed means because this data holds many interesting and important details that are lost from figures 11, 12, 13 and 14. For example, under conditions favorable for Fusarium ear rot, no inoculation was necessary to differentiate between the hybrids (Fig 15, 17, 19, 21). The two hybrids could be separated for resistance without any inoculation, like the un-inoculated control. In contrast, under conditions unfavorable for Fusarium ear rot, the control treatment (no inoculation) was unable to separate the hybrids at most locations (Fig 23, 25, 29). These data indicate that an effective screening program could be developed in an area conducive for ear rot without inoculation with *F. verticillioides*. Such a program, however, would not work at all under unfavorable years. The addition of inoculum does appear to aid in the separation of lines that differ in resistance. Figures 21, 23 and 29 show the Plastic Bag and SC + PB treatments which differ only in addition of inoculum to the ear. In these cases, differences

in resistance between the screened lines could only be observed when inoculum was applied to the ear.

Drepper and Renfro (1990) noted that when conditions are not favorable for Fusarium ear rot, wounding inoculation techniques are superior for reproduction of ear rot. Our results do not wholly support these findings. While we found one wounding technique, the pinbar technique, that was effective, we also found a nonwounding technique, the silk channel technique, which was effective in unfavorable conditions. Within this study, two inoculation techniques seemed to consistently work for separating the resistant and susceptible maize lines based on both Fusarium ear rot and fumonisin contamination. Both the Silk Channel and Pinbar techniques tended to work across locations and years tested. Because of the demanding labor required to complete the pinbar inoculations, our recommendations would be to use the Silk Channel technique when screening for resistant lines.

Table 2. Statistical Comparison of Inoculation Techniques after Square Root Transforming the Fusarium Ear Rot Data and Log Transforming the Fumonisin Data

Dekalb Rot Data (Square Root Transformed)		Dekalb Fumonisin Data (Log Transformed)	
Treatment	P value	Treatment	P value
Plastic Bag	0.2636	Plastic Bag	0.0092
Silk Channel	0.0391	Silk Channel	<.0001
SC + PB[A]	0.0587	SC + PB	0.0015
Control	0.1404	Control	0.0023
Toothpick	0.08	Toothpick	<.0001
Pinbar	0.0659	Pinbar	0.0016

Pioneer Rot Data (Square Root Transformed)		Pioneer Fumonisin Data (Log Transformed)	
Treatment	P value	Treatment	P value
Plastic Bag	0.0524	Plastic Bag	0.4354
Silk Channel	0.0571	Silk Channel	0.5238
SC + PB	0.0577	SC + PB	0.7856
Control	0.1149	Control	0.1894
Toothpick	0.4817	Toothpick	0.8646
Pinbar	0.0172	Pinbar	0.7185

A= Silk Channel plus Plastic Bag

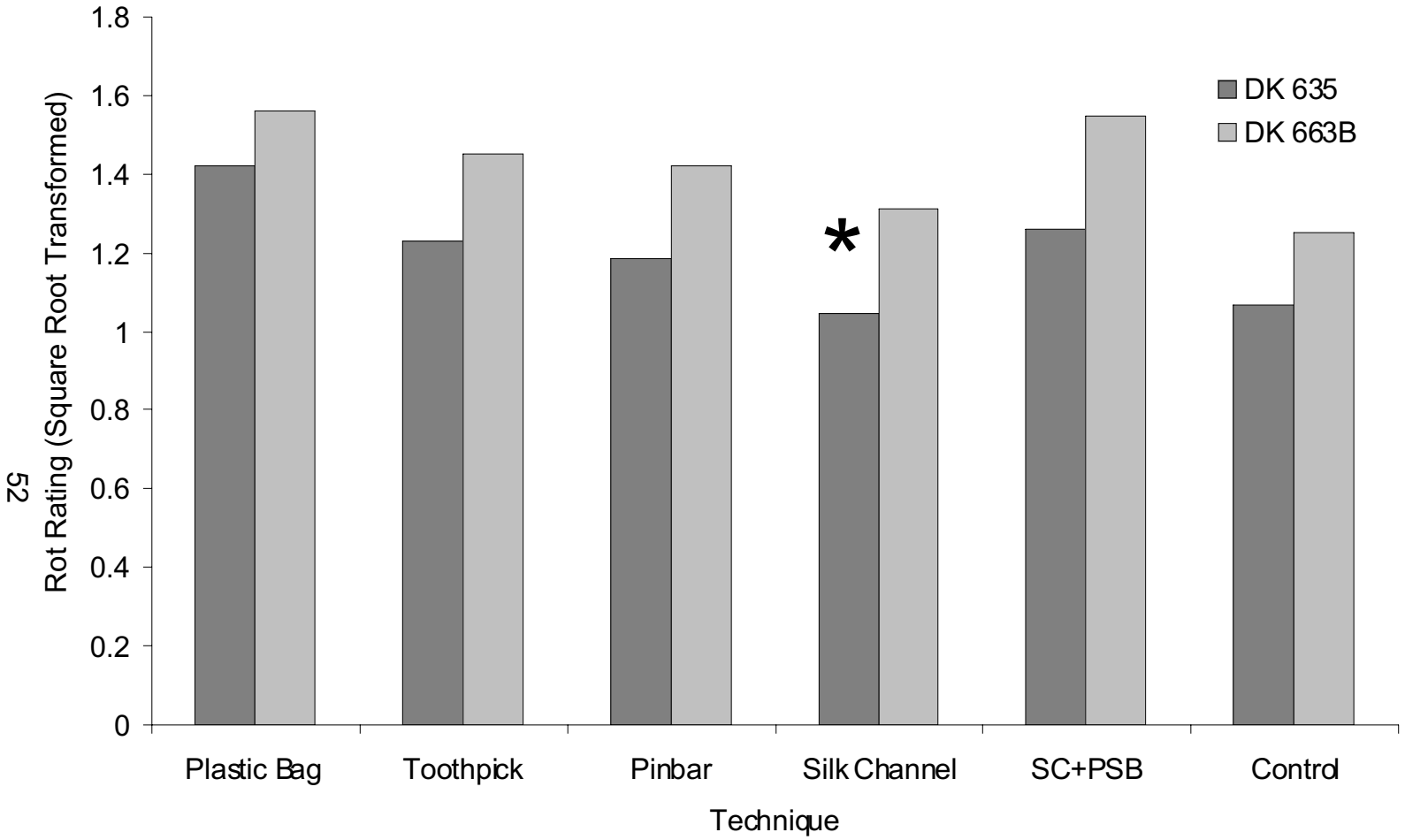

Figure 8. Comparison of Dekalb Hybrids based on Fusarium Ear Rot induced by Inoculation Techniques. Data shown is square root transformed. Techniques with an asterisk (*) are significantly different at the 0.05 level.

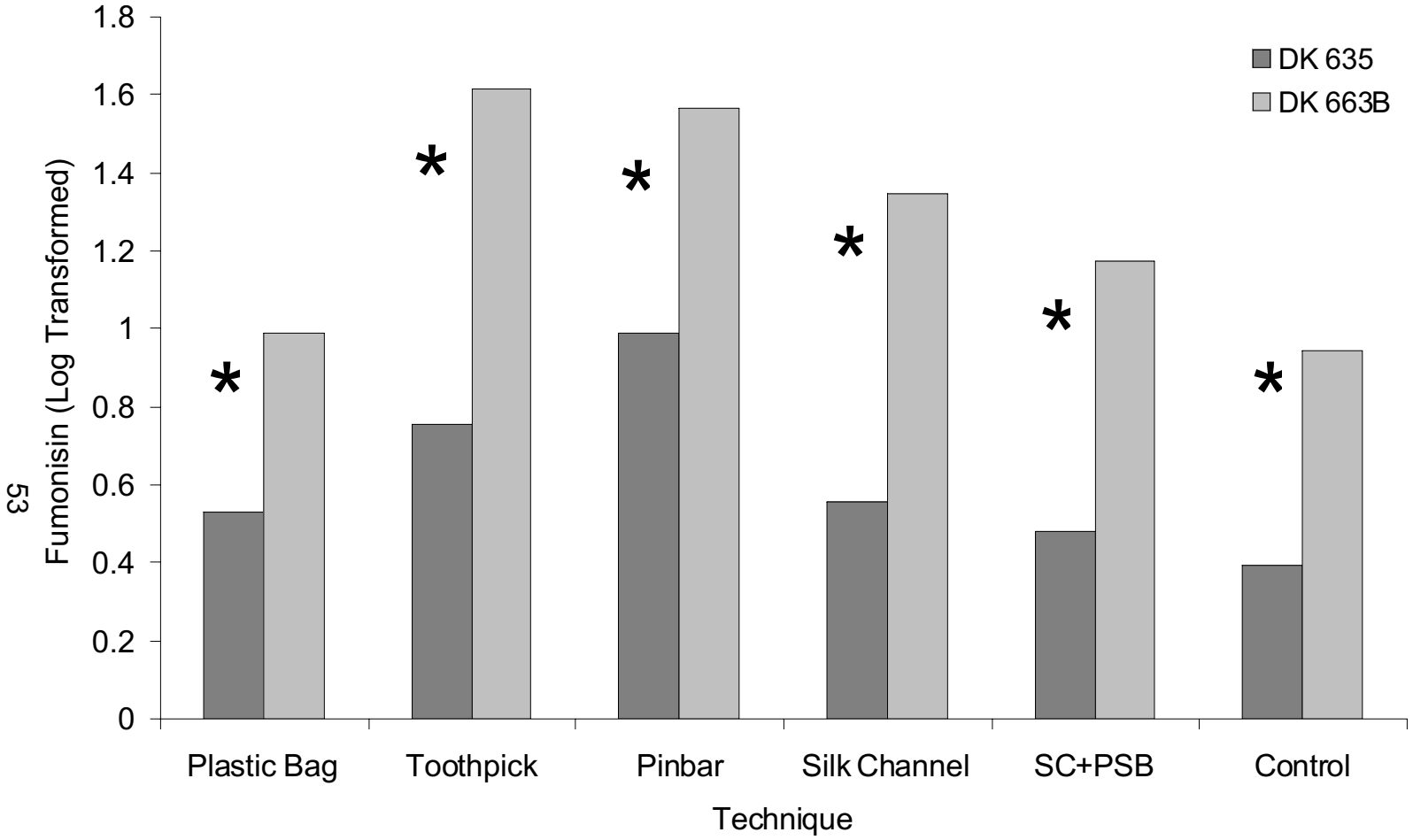

Figure 9. Comparison of Dekalb Hybrids based on Fumonisin Contamination induced by Inoculation Technique. Data shown is log transformed. Techniques with an asterisk (*) are significantly different at the 0.05 level.

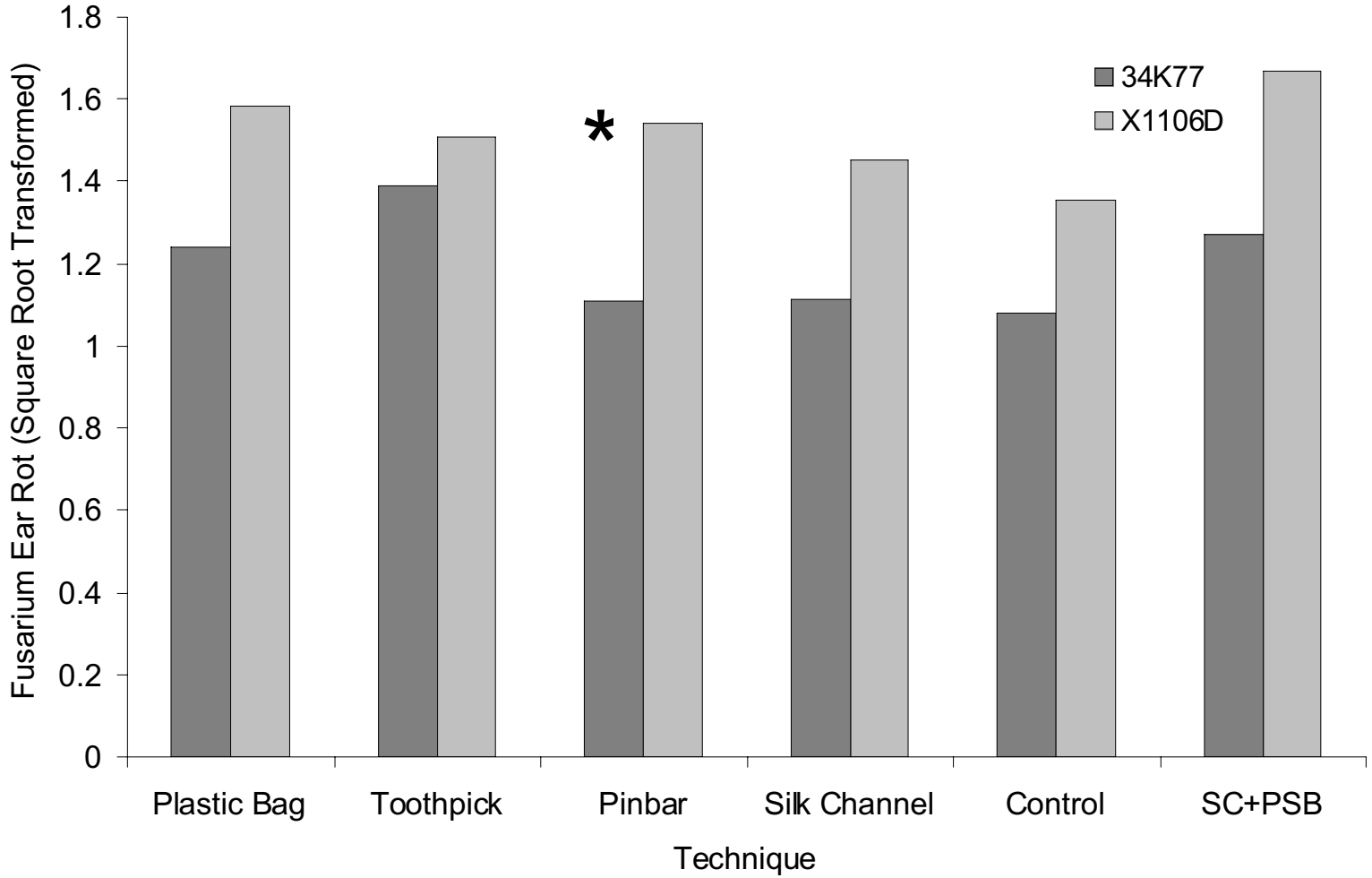

Figure 10. Comparison of Pioneer Hybrids based on Fusarium Ear Rot induced by Inoculation Techniques. Data shown is square root transformed. Techniques with an asterisk(*) are significantly different at the 0.05 level.

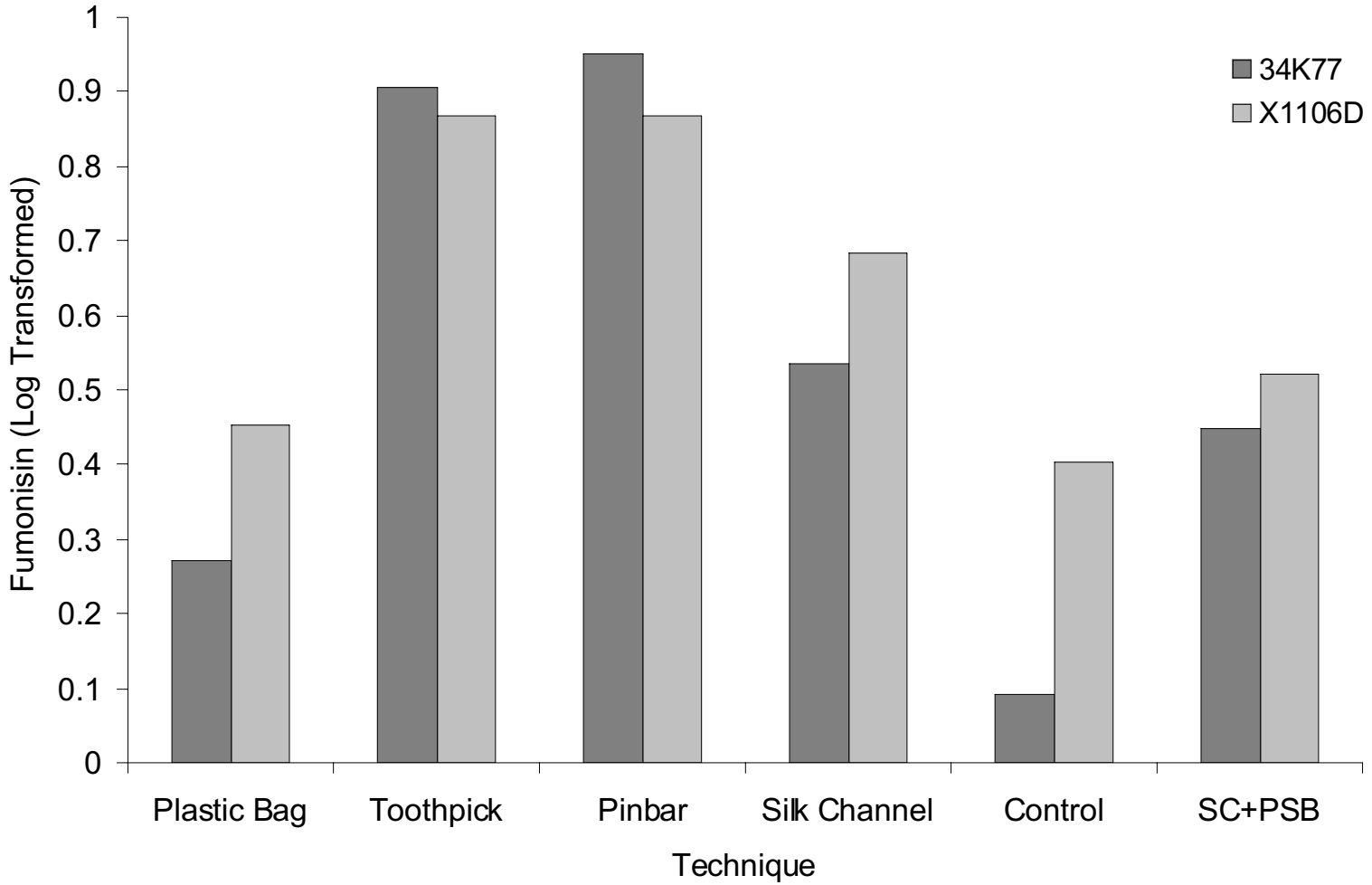

Figure 11. Comparison of Pioneer Hybrids based on Fumonisin Contamination induced by Inoculation Technique. Data shown is log transformed. Techniques with an asterisk (*) are significantly different at the 0.05 level.

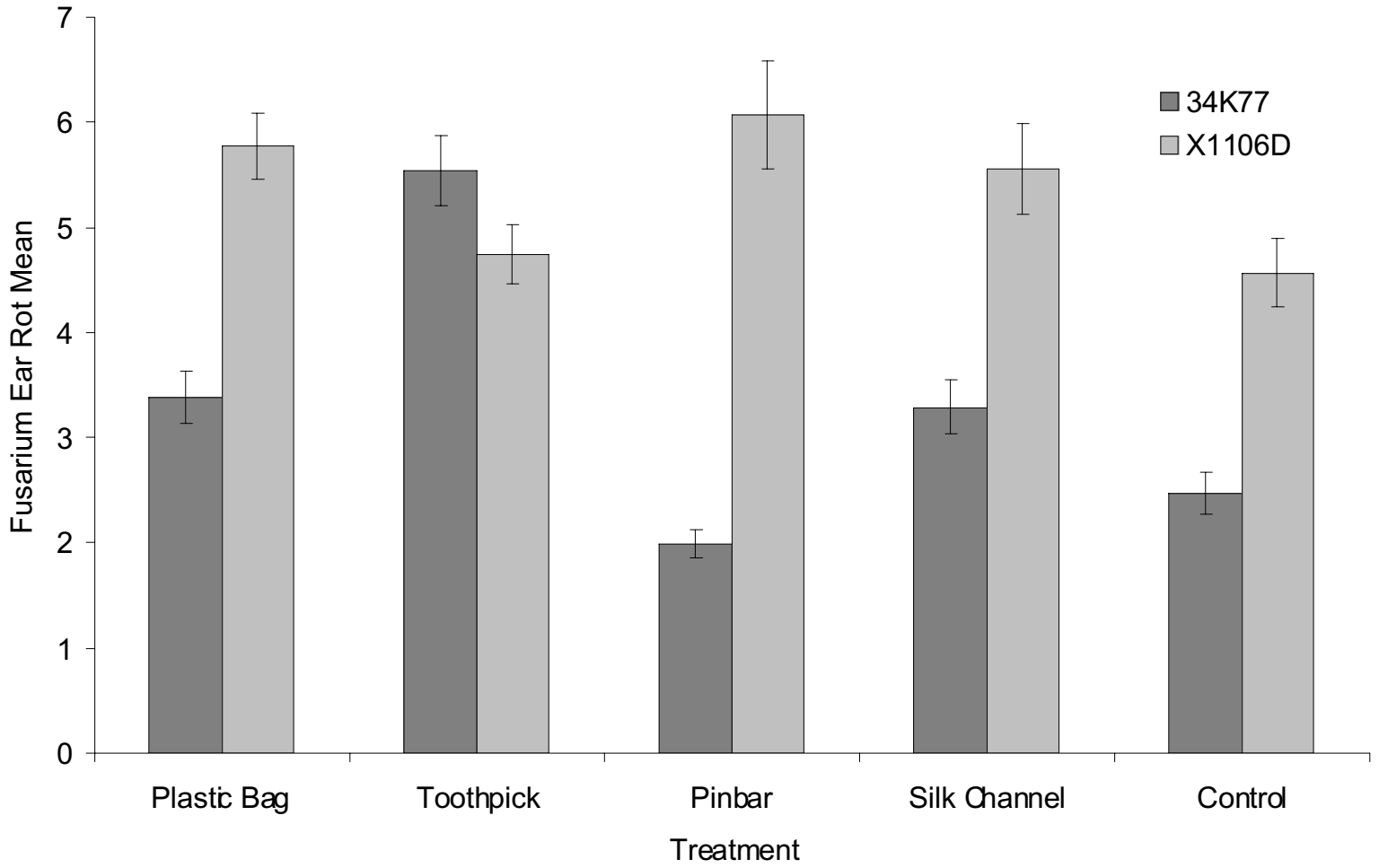

Figure 12. Comparison of Pioneer Hybrids based on Fusarium Ear Rot at Winterville, 1999. Bars show standard error.

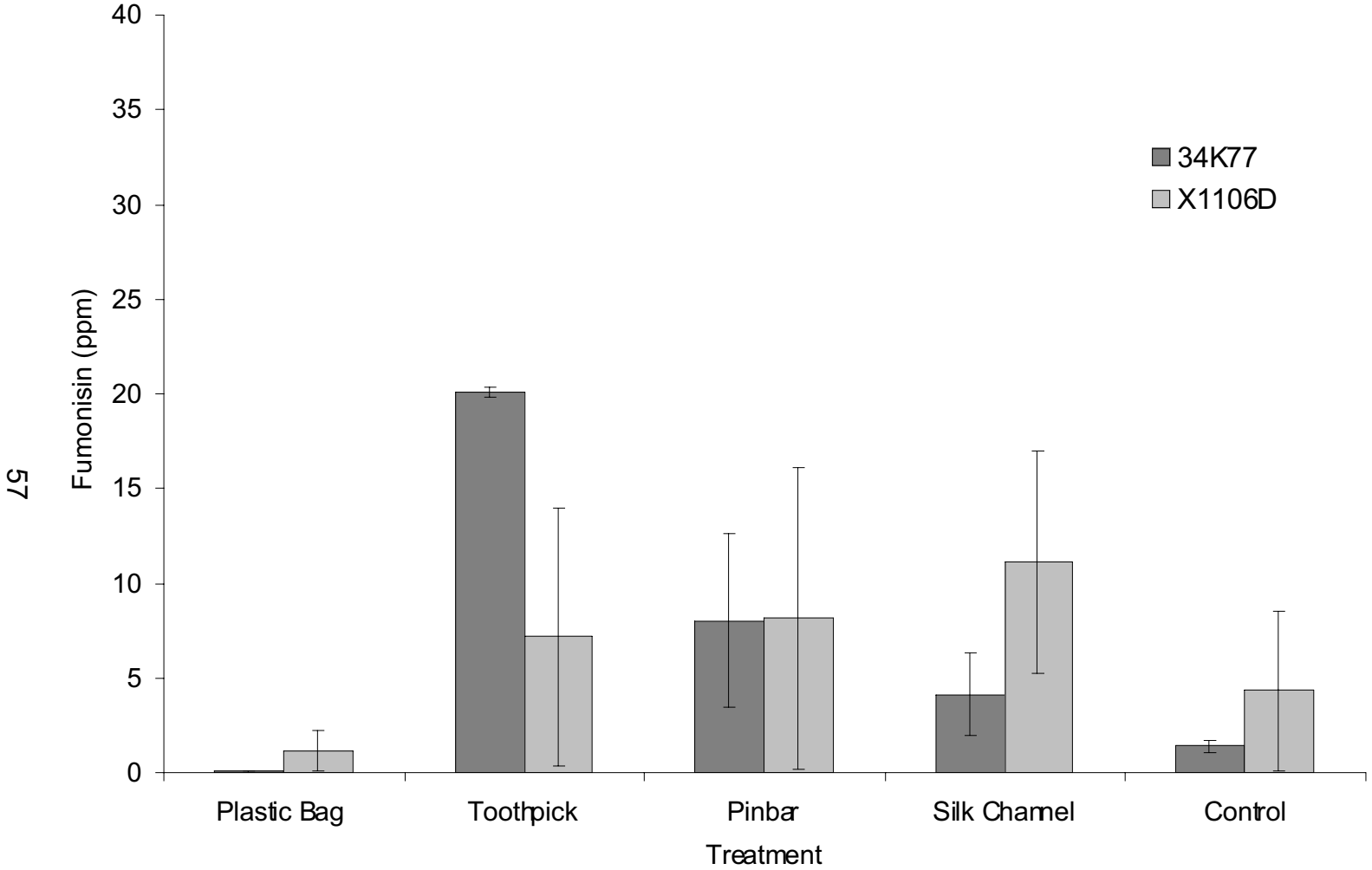

Figure 13. Comparison of Pioneer Hybrids based on Fumonisin Contamination at Winterville, 1999. Bars show standard error.

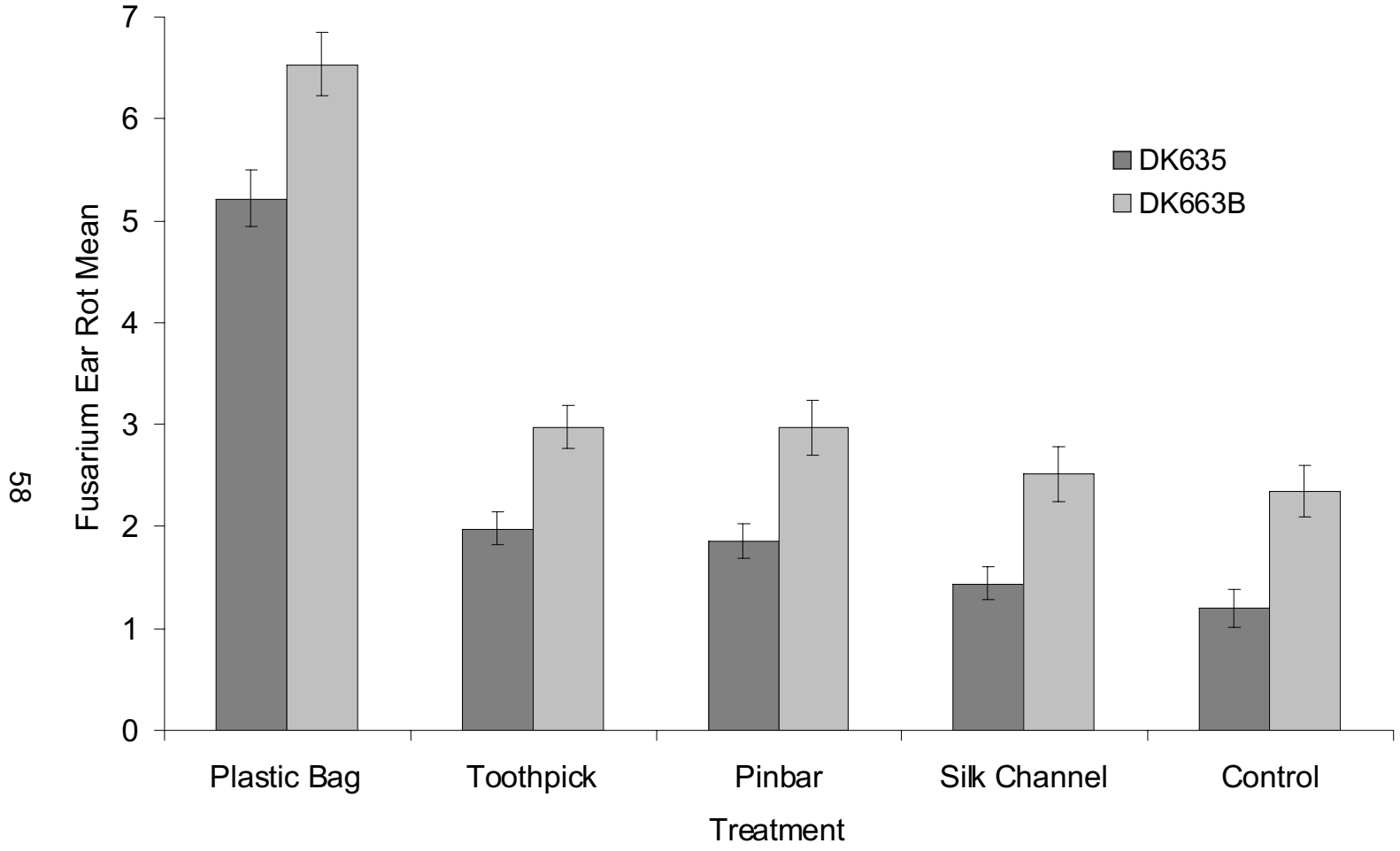

Figure 14. Comparison of Dekalb Hybrids based on Fusarium Ear Rot at Mt.Olive, 1999. Bars show standard error.

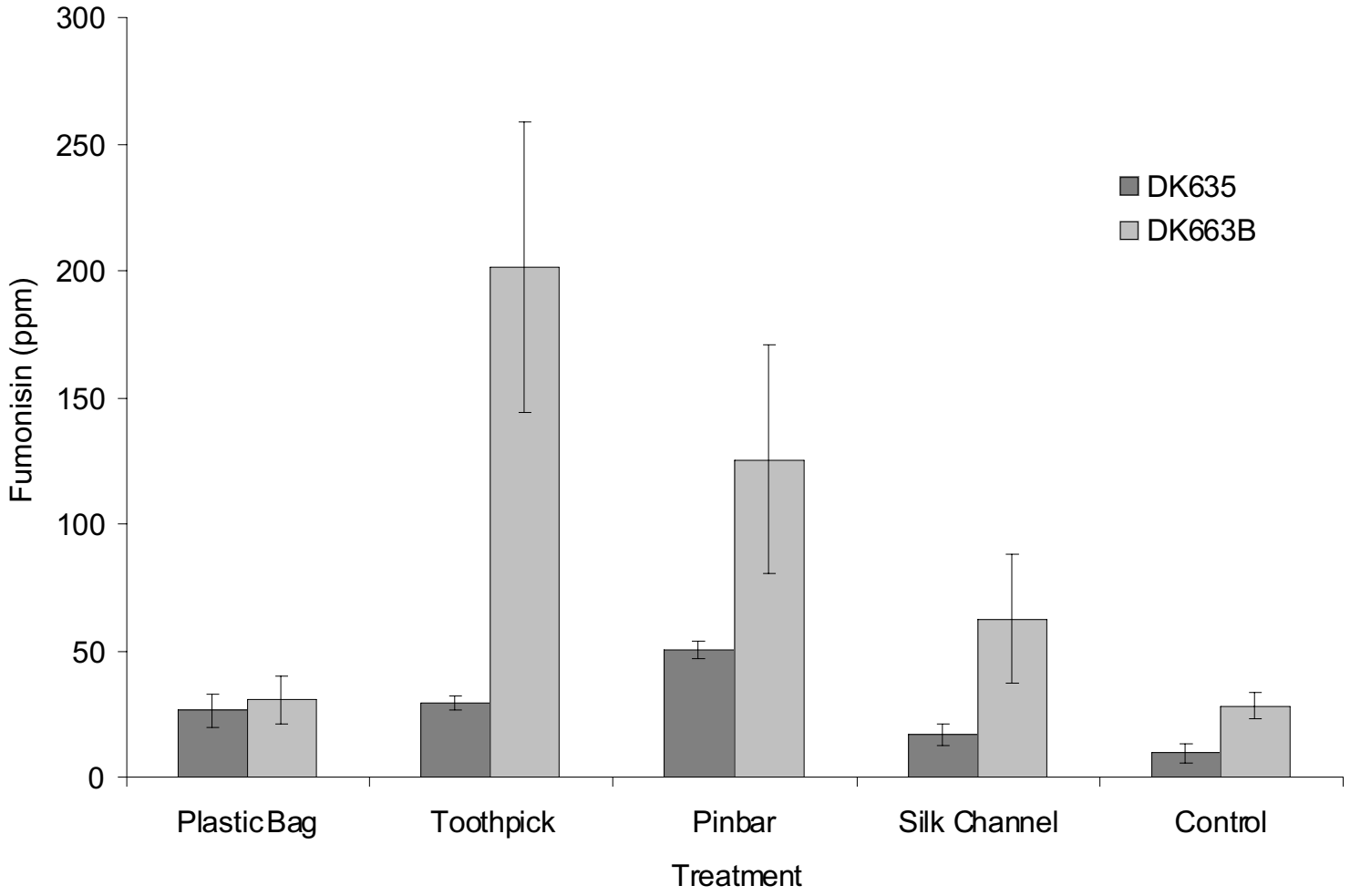

Figure 15. Comparison of Dekalb Hybrids based on Fumonisin Contamination at Mt Olive, 1999. Bars show standard error.

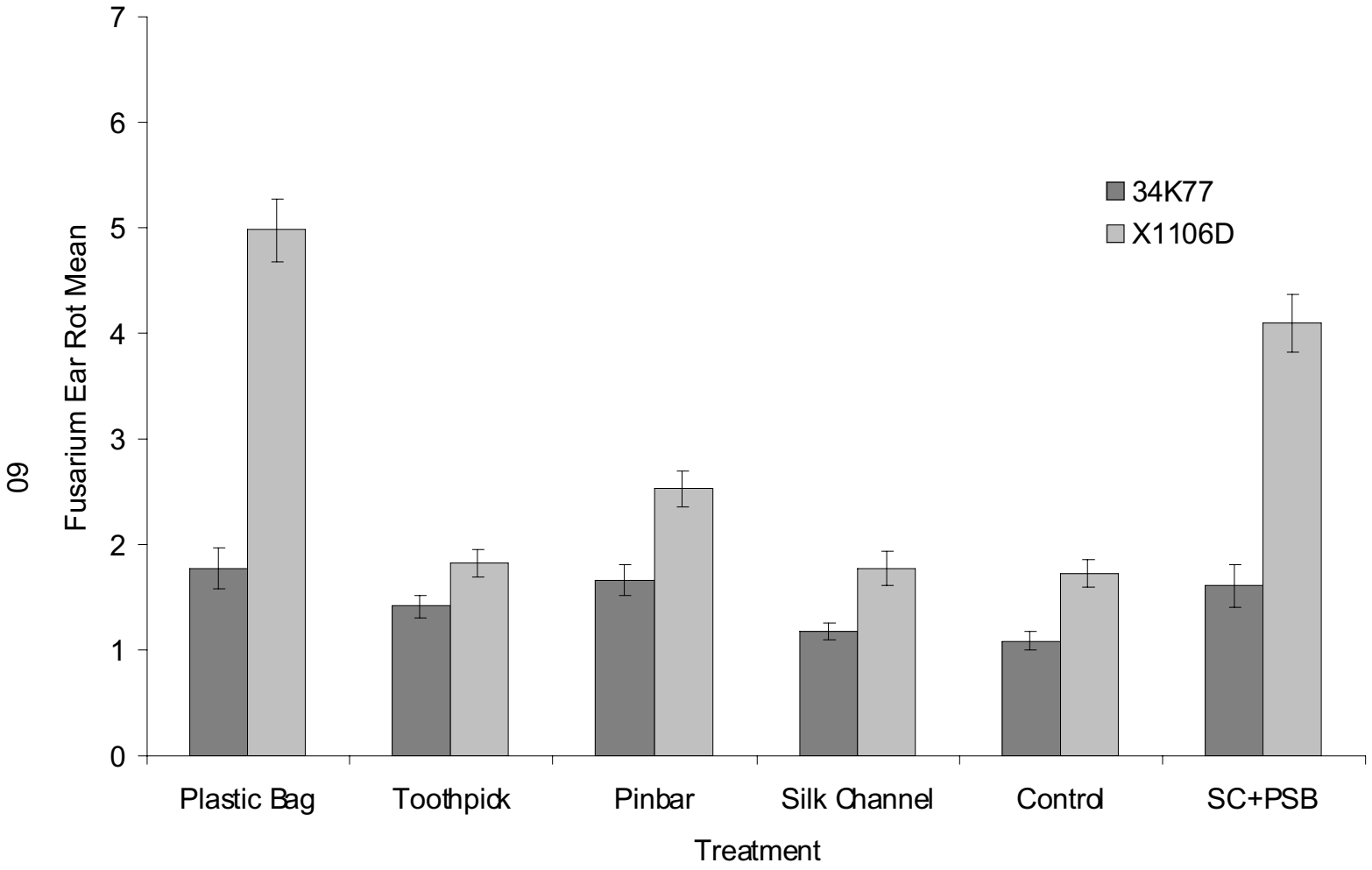

Figure 16. Comparison of Pioneer Hybrids based on Fusarium Ear Rot at Clayton, 1999. Bars show standard error.

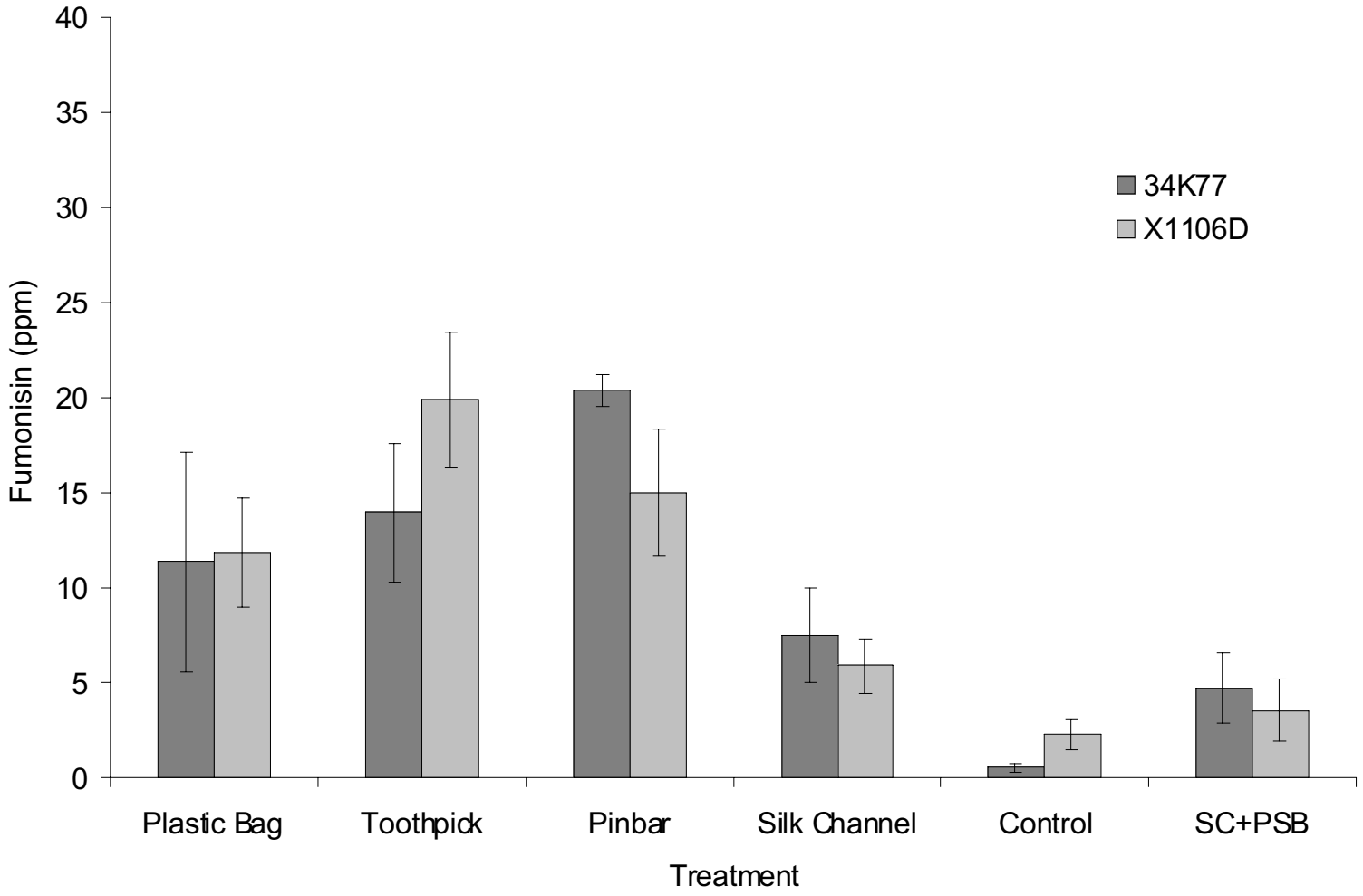

Figure 17. Comparison of Pioneer Hybrids based on Fumonisin Contamination at Clayton, 1999. Bars show standard error.

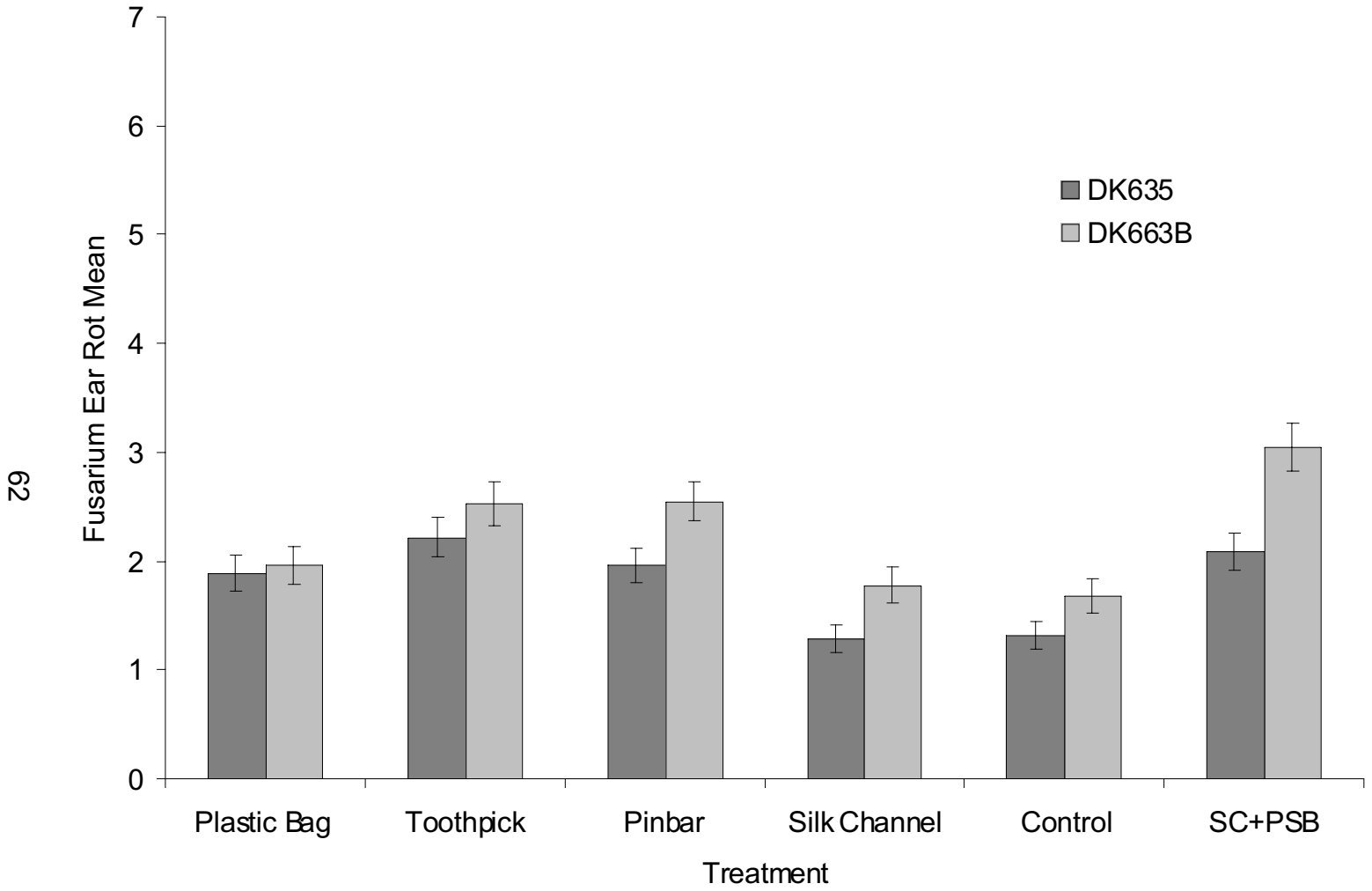

Figure 18. Comparison of Dekalb Hybrids based on Fusarium Ear Rot at Clayton, 1999. Bars show standard error.

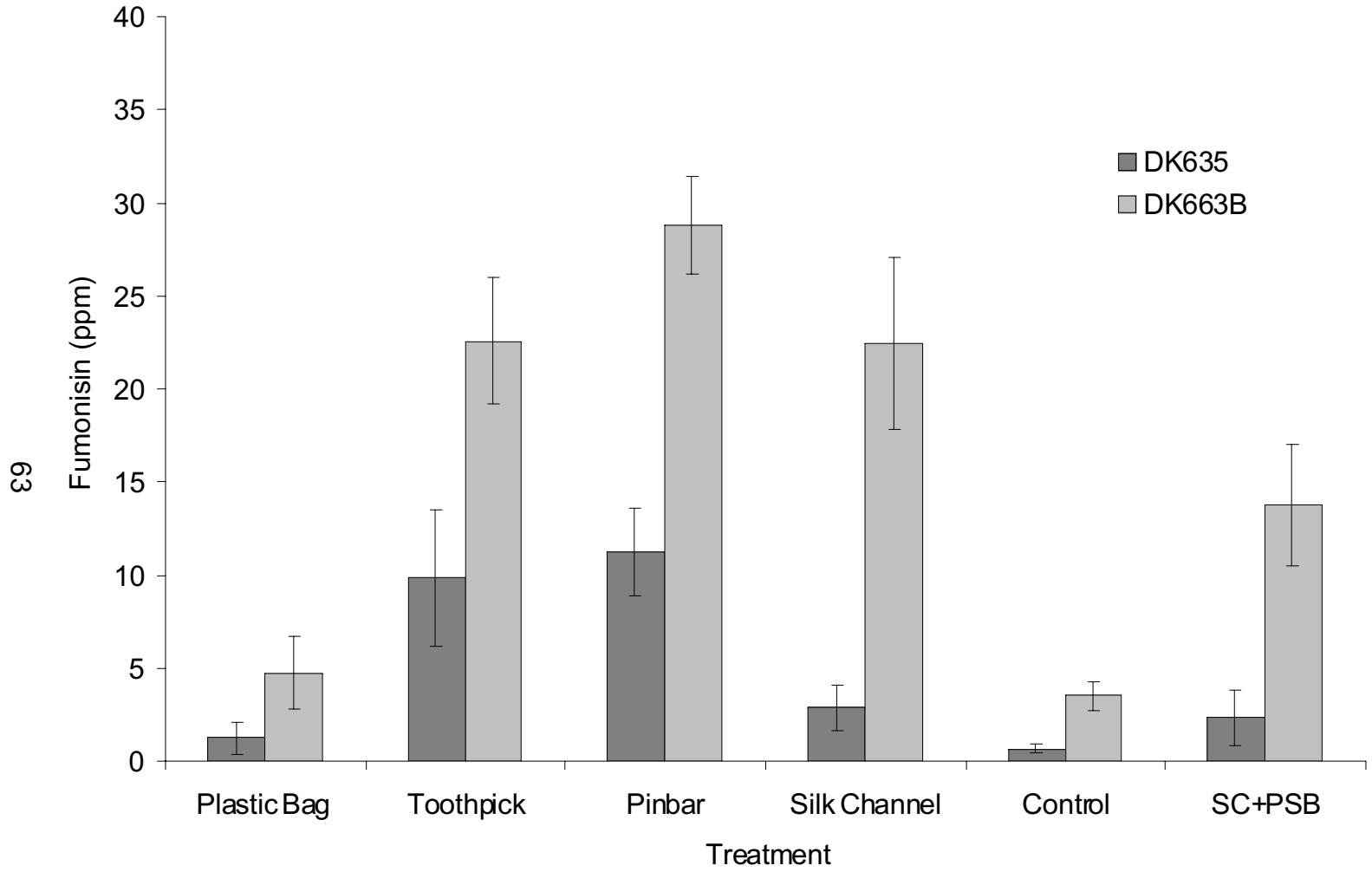

Figure 19. Comparison of Dekalb Hybrids based on Fumonisin Contamination at Clayton, 1999. Bars show standard error.

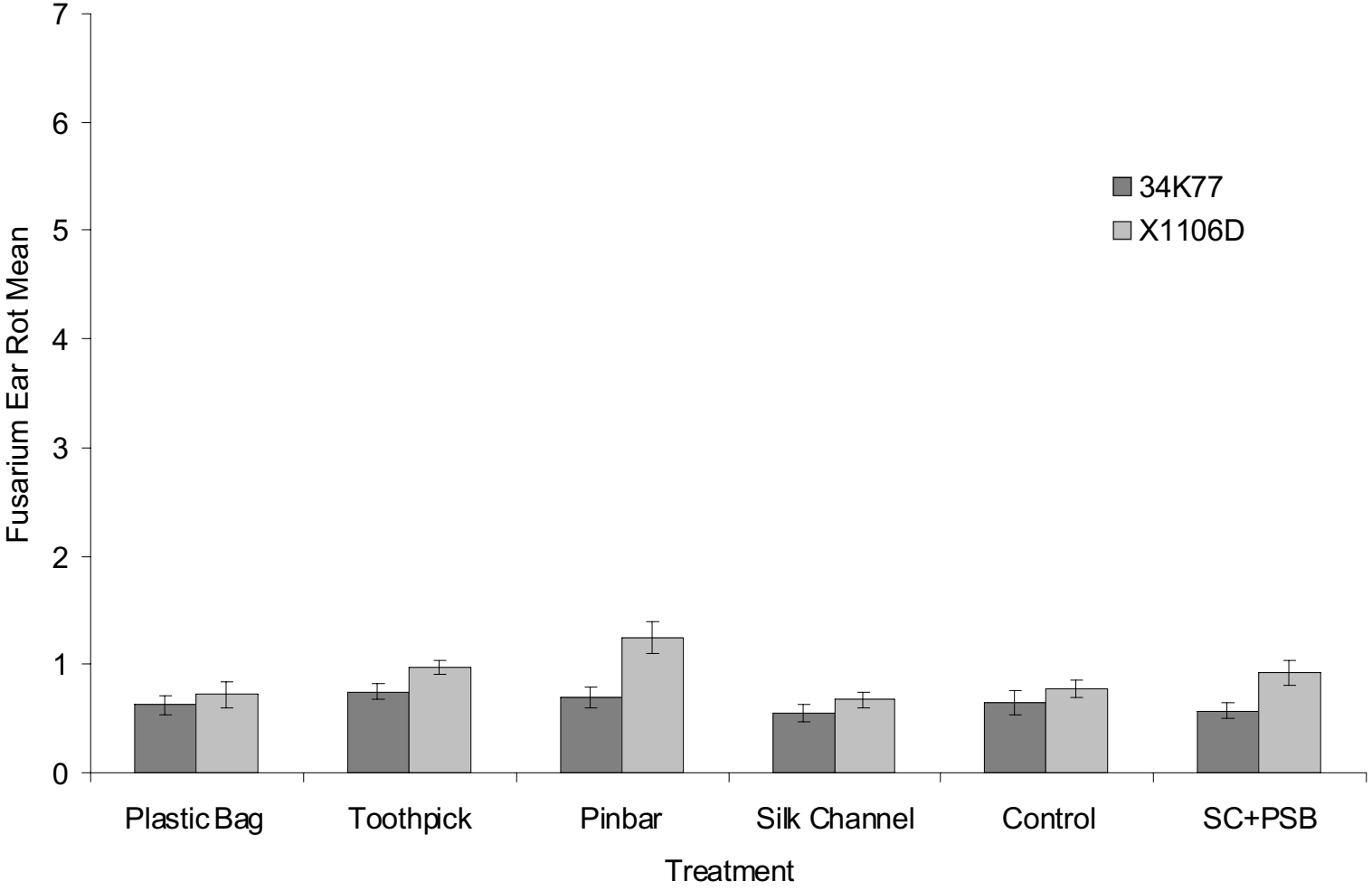

Figure 20. Comparison of Pioneer Hybrids based on Fusarium Ear Rot at Winterville, 2000. Bars show standard error.

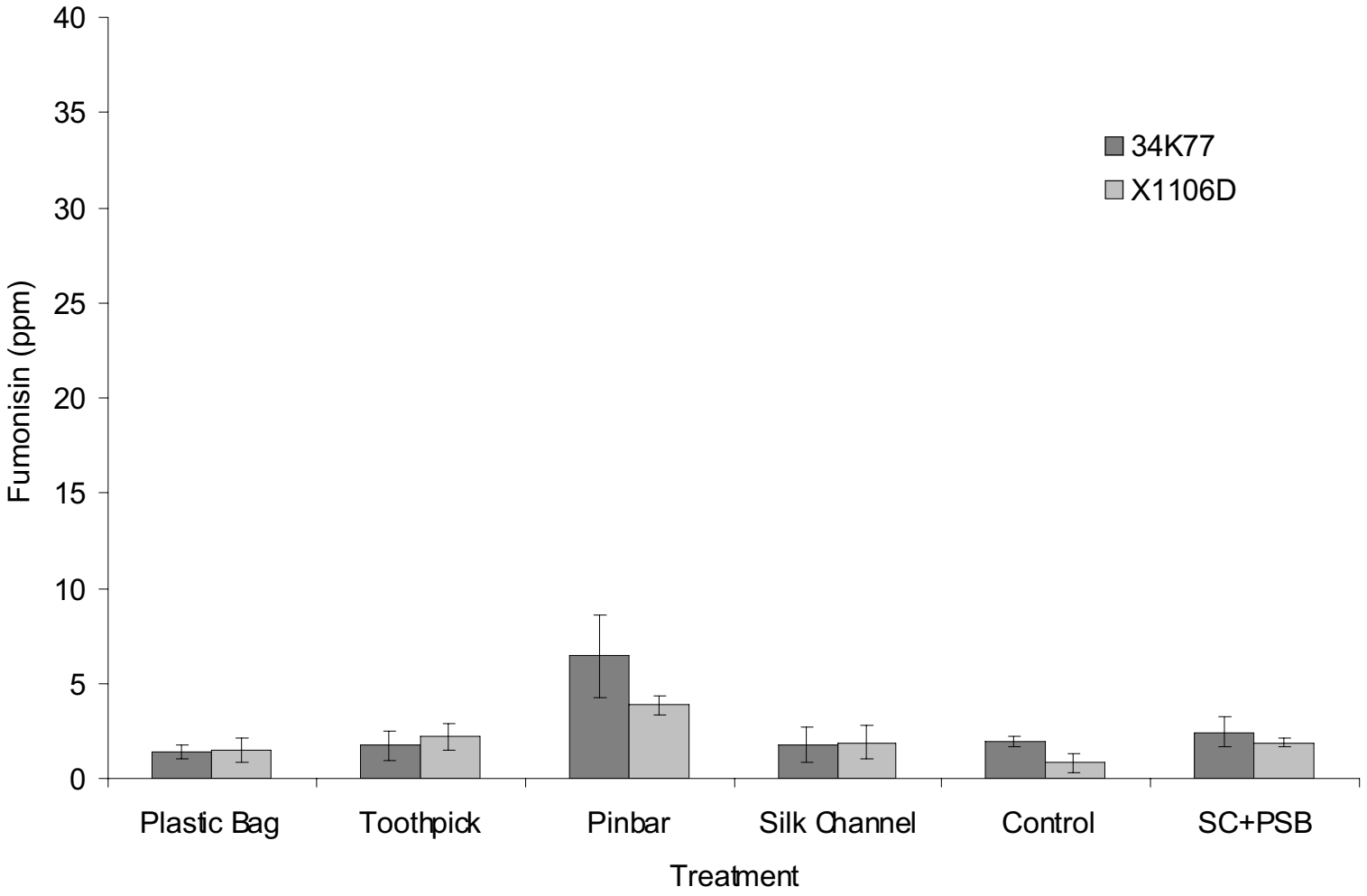

Figure 21. Comparison of Pioneer Hybrids based on Fumonisin Contamination at Winterville, 2000. Bars show standard error.

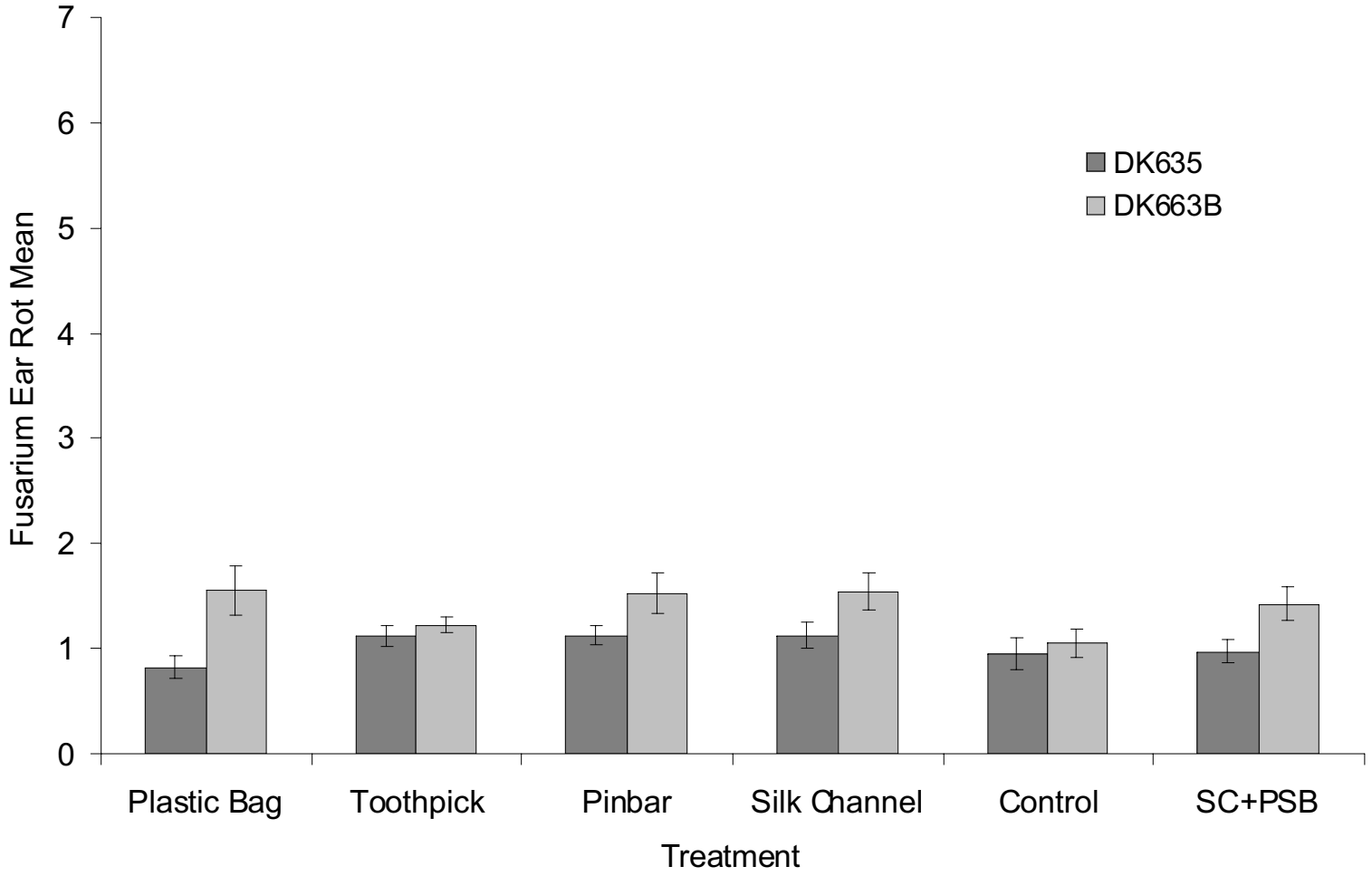

Figure 22. Comparison of Dekalb Hybrids based on Fusarium Ear Rot at Mt. Olive, 2000. Bars show standard error.

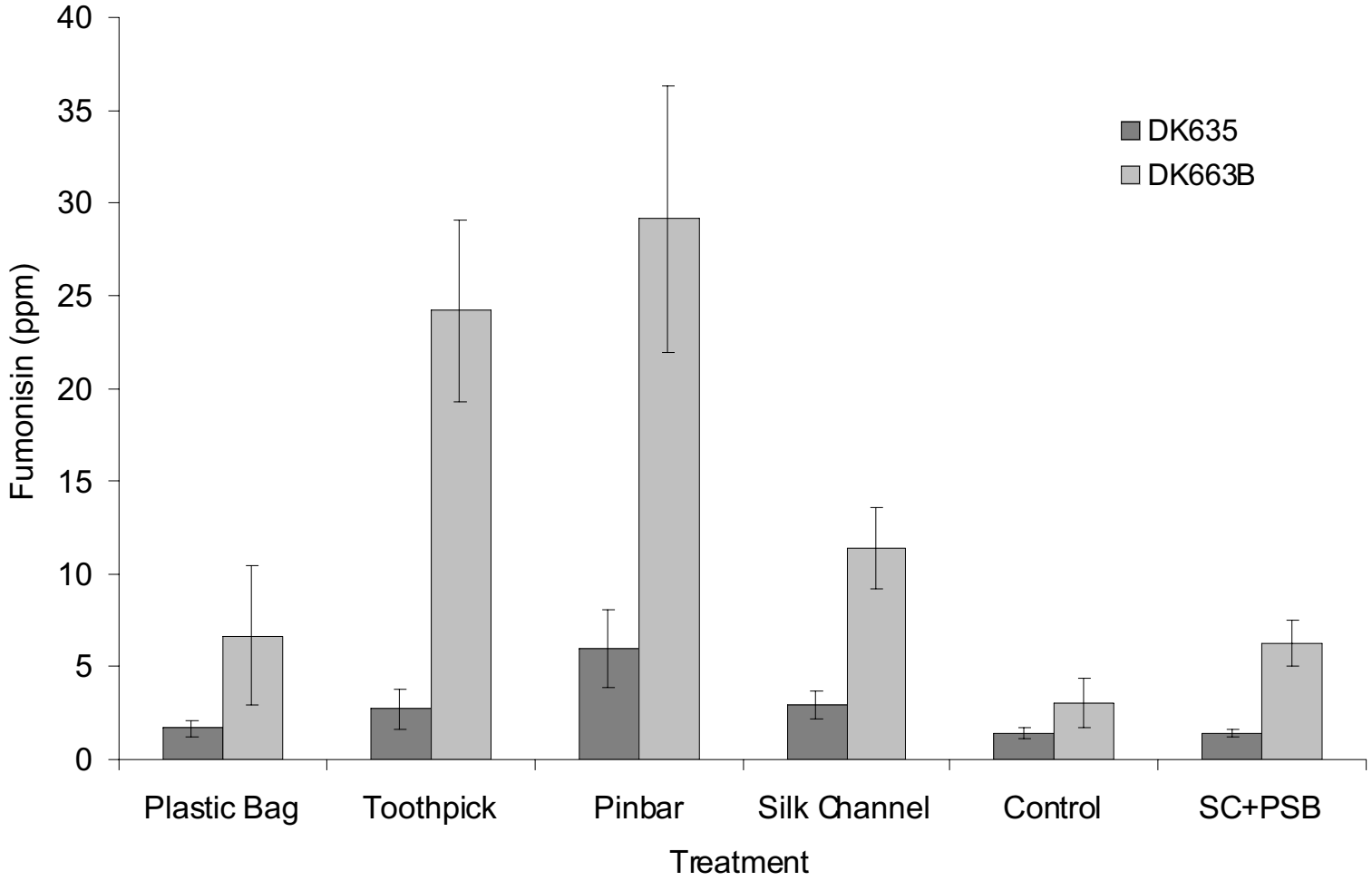

Figure 23. Comparison of Dekalb Hybrids based on Fumonisin Contamination at Mt. Olive, 2000. Bars show standard error.

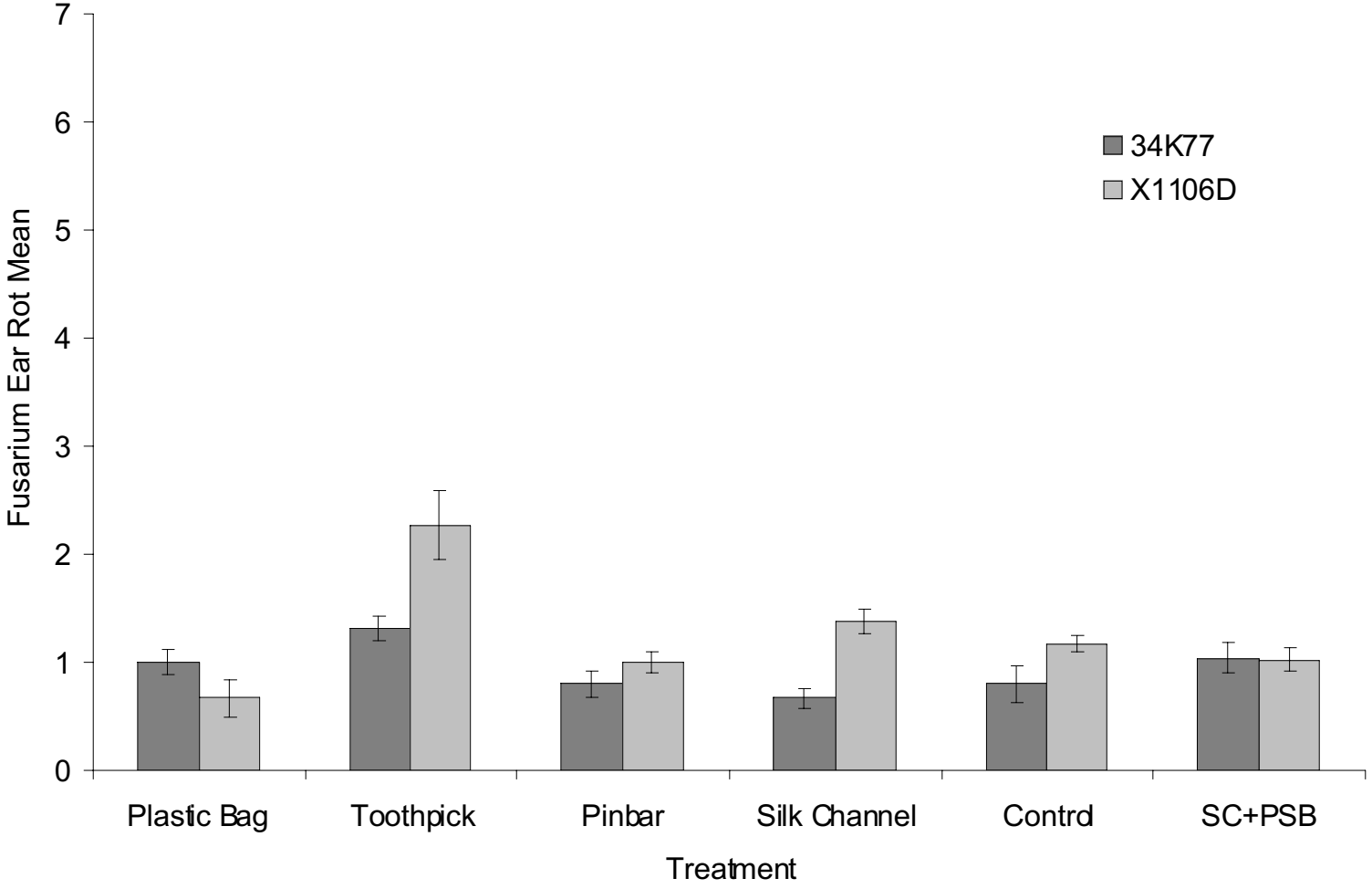

Figure 24. Comparison of Pioneer Hybrids based on Fusarium Ear Rot at Clayton, 2000. Bars show standard error.

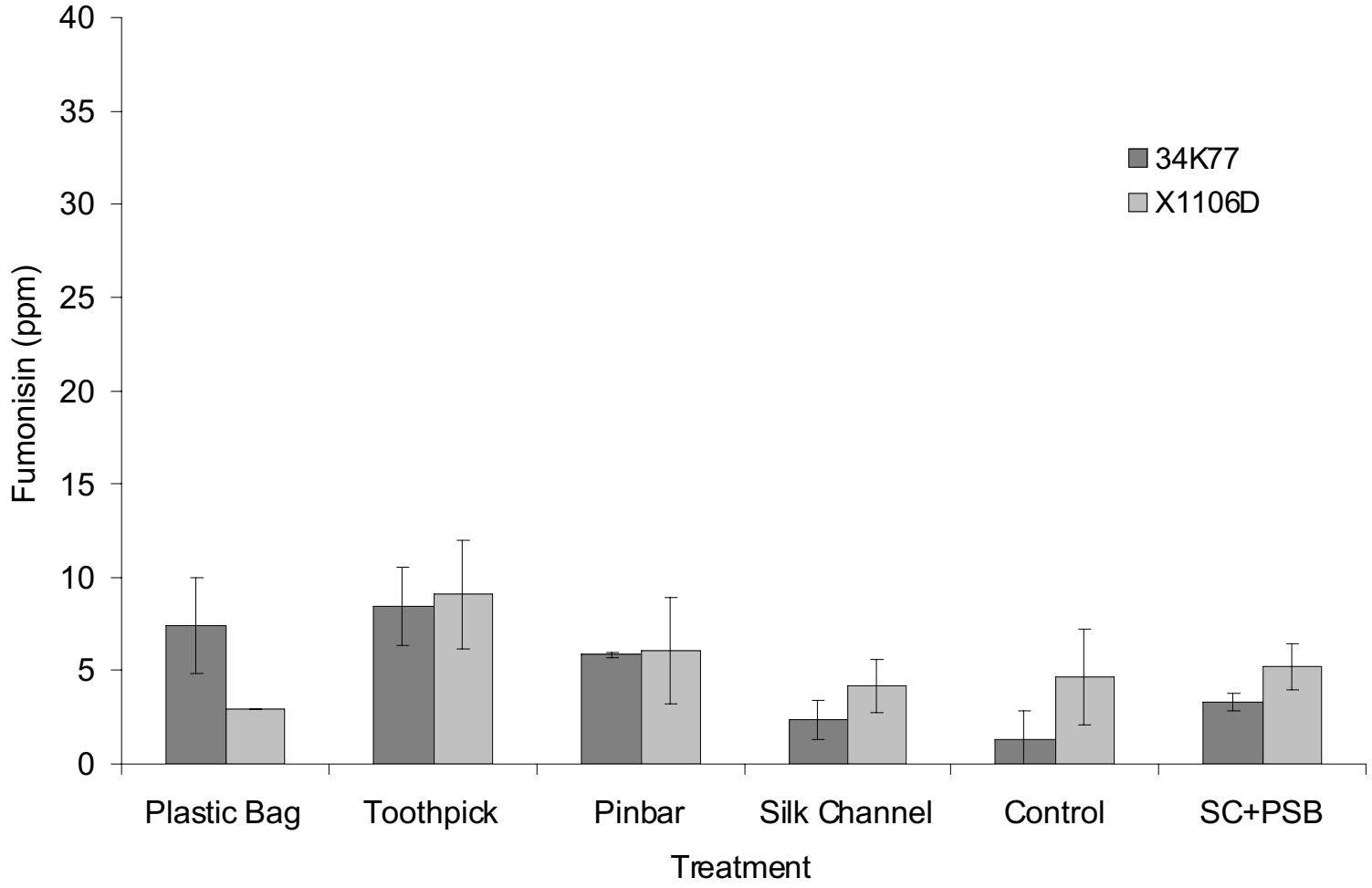

Figure 25. Comparison of Pioneer Hybrids based on Fumonisin Contamination at Clayton, 2000. Bars show standard error.

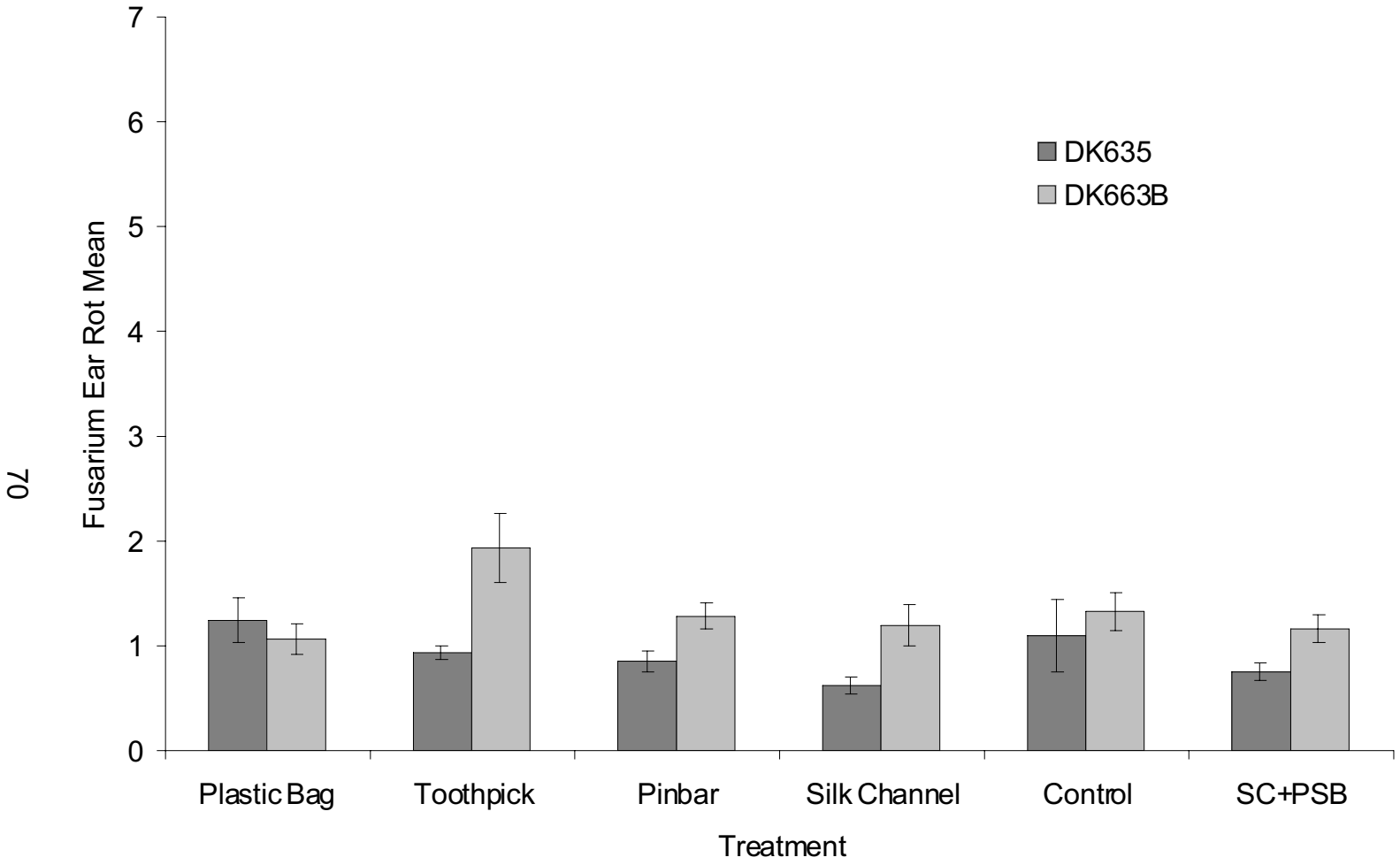

Figure 26. Comparison of Dekalb Hybrids based on Fusarium Ear Rot at Clayton, 2000. Bars show standard error.

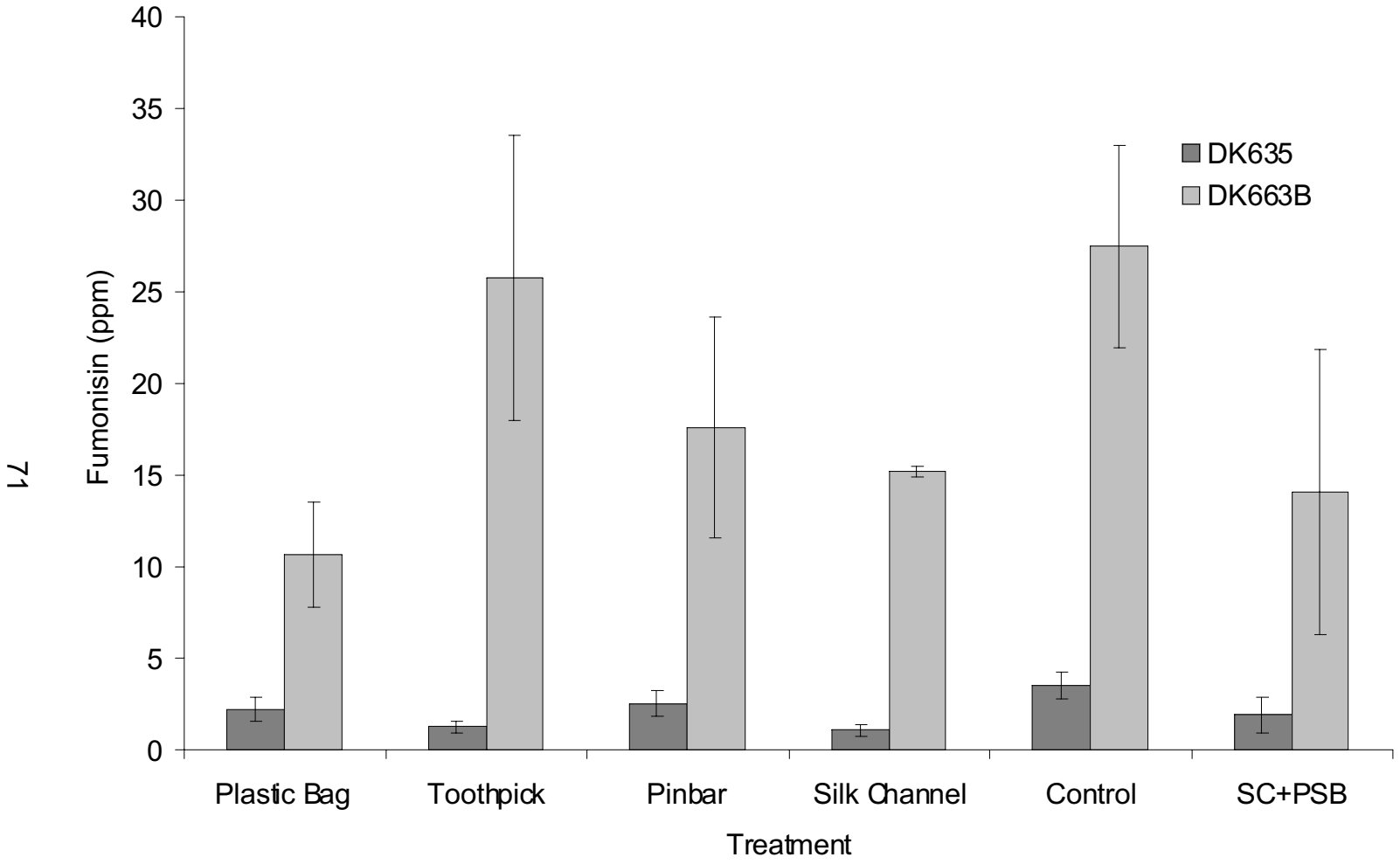

Figure 27. Comparison of Dekalb Hybrids based on Fumonisin Contamination at Clayton, 2000. Bars show standard error.

Appendix: On Farm Studies of Fumonisin Contamination of Maize from Camden and Beaufort Counties, 2000.

Introduction *Fusarium verticillioides* (Sacc.) Nirenberg. (synonym *F. moniliforme* J. Sheld) is commonly associated with maize kernels and under favorable conditions causes an ear rot known as Fusarium ear rot (White, 1999). The disease reduces seed quality and recently the fungus has been shown to produce the mycotoxin fumonisin, which is toxic to animals and an implicated human carcinogen. Maize growers in North Carolina have experienced elevated fumonisin levels in harvested grain in the past 5 years. This problem came to the forefront in 1998 when loads of maize were rejected at the grain-buying stations. Some stations had purchased ELISA tests to determine fumonisin content of the grain and set an arbitrary limit at 15 ppm for the grain they would purchase. Several loads of maize were rejected and one load was found to contain fumonisin contamination of 278 ppm. Other stations were assaying maize and rejecting loads of grain based on elevated levels of rotten kernels. While fumonisin contamination has not been as severe in recent years as compared to 1998, maize growers have been requesting information on seed resistance to *F. verticillioides*. Because no hybrids contain adequate levels of resistance to the fungus, cultural methods may be the only way to limit fumonisin in harvested grain.

The objective of this study was to determine the amount of fumonisin contamination in harvested grain at three distinct times when a grower would be able to harvest the grain.

Materials and Methods

In 2000, Pioneer brand hybrids 34K77, 3394 and 32Z18 were planted near Pantego in Beaufort County and near South Mills in Camden County at a normal planting time for each area. Midsilk occurred near June 25 at both locations. Hybrids were planted in a Split Plot design with hybrid as the main plot and harvest date as the subplot. Rows at all locations were at least 3.8 m long and 0.9652 m apart. The four replicates were separated by 0.91 m alleys. All hybrids were naturally infected by the fungus *Fusarium verticillioides*. Kernel moisture was monitored weekly and the first harvest was conducted when kernel moisture reached approximately 30%. The second harvest was two weeks later and the third harvest was six weeks after the initial harvest. Kernel moisture was determined on the harvested ears and the ears were immediately dried.

Time one was designated “Early”, which corresponded to the earliest time a grower could harvest grain, which was approximately 30% kernel moisture. Time two was designated “Normal” and corresponded to the historical date when half of the maize crop is harvested in North Carolina, which was September 1. Time three was designated “Late” and was designated to represent unfavorable conditions that would delay harvest of grain in a timely manner. The three hybrids chosen differed in their resistance to Fusarium ear rot. Pioneer Hybrid 34K77 is more resistant, Pioneer Hybrid 3394 is moderately resistant, and Pioneer Hybrid 32Z18 is more susceptible.

The fumonisin concentration of the samples was quantified by Dr. Winston Hagler, director NCSU Mycotoxin Lab, using the Romer Labs, Inc. (Union, MO)

fumonisin protocol FUM-LC1. Briefly, a 454-gram subsample of harvested kernels was randomly selected from each replicate and then individually ground to mesh size 20. A 25-gram sub-sample was extracted for 1 hr in 100 ml of CH_3CN/ H_2O, (50/50). A 2 ml sample of the extract was added to 8 ml MeOH/H_2O (3/1) and the resulted solution applied to a column conditioned with 5 ml MeOH followed by 5 ml 3:1 MeOH/H_2O. The column was washed with 8 ml of MeOH/H_2O (3/1) followed by 3 ml of MeOH. The sample was eluted with 10 ml MeOH/HOAC (99/1), and dried overnight on a Speedvac System SS3 (Savant, Holbrook, NY).

For derivatization, the residue was dissolved in 1 ml of MeOH. Then 1 ml of 0.05M sodium borate buffer (pH = 9.5), 0.5 ml of sodium cyanide reagent (13 mg/L H_2O), and 0.5 ml of NDA reagent were added to the sample in stated order. The sample was sealed and heated for 15 minutes at 60 degrees C, then cooled to room temperature and diluted with 7 ml of 0.05M phosphate buffer (pH 7)/ CH_3CN (40/60). Twenty µl of the sample was applied to a Brownlee HPLC column (0.4 by 10 cm, Perkin-Elmer Corp, Norwalk, CT) and a model RF-551 programmable and scanning fluorescence HPLC monitor (Shimadzu) set at 420 nm excitation and 500 nm emission.

Results and Discussion

Trends at both locations were similar during the study. Fumonisin was found in all samples starting from the beginning of this study and increased until the last harvest. Fumonisin levels for all three hybrids at both locations generally increased between harvest dates, with fumonisin peaking during the last harvest for all three hybrids (Figure 31, 32).

Fumonisin levels were dramatically different between the two locations. At the Beaufort location, fumonisin levels were at least three times greater than the levels found at the Camden location. While individual fields may be expected to differ in fumonisin contamination, this was much more variation than we expected to see. We would have predicted to find more fumonisin at the Camden location based on historical records from the location. In 1998, the same field in Camden County had a sample of maize with a fumonisin content of 278 parts per million (ppm). While this sample was not obtained from a designed experiment, the results indicated that this location had the potential for high levels of fumonisin contamination in harvested grain. The difference in fumonisin levels between the samples from 1998 and 2000 would lead us to believe that fumonisin contamination is highly dependent on local environmental conditions that have not yet been determined. Environmental conditions did not appear to be notably different between the two locations studied during 2000, but evidently there was a difference between the two locations to account for this difference in fumonisin contamination.

Regardless of the levels of fumonisin at each location, trends within and between hybrids were very similar. Hybrid 34K77 had lower fumonisin levels at the Early harvest date when compared to both the Normal and Late harvest timings, with fumonisin levels increasing two fold between the Early and Late harvest dates (Figures 31, 32). Trends observed with hybrid 32Z18 were very similar to hybrid 34K77. The major difference between the two hybrids was the difference between fumonisin at the Early and Late harvest date. With hybrid 32Z18 we found a five-fold difference between Early and Late harvests at one location and a ten fold difference at the other location. Hybrid 3394 was

the only hybrid in which we noticed a decrease in fumonisin levels between harvest dates. At the Beaufort County location, fumonisin levels increased four fold between the Early and Normal harvest dates but then decreased to approximately three times the initial fumonisin concentration at the Late harvest date. This drop in fumonisin concentration among harvest timings has been seen in other fumonisin studies (Chapter Two of this thesis). At the other location, fumonisin levels increased four fold between the Early and Late harvest dates.

While the hybrids tested were not designated as being resistant to fumonisin accumulation, it appears that some level of resistance to fumonisin is associated with ear rot resistance. This resistance appears to be related to the rate of fumonisin accumulation. Fumonisin appears to increase two fold over a harvest season with a resistant hybrid, four fold with a moderately resistant hybrid and five to ten-fold with a susceptible hybrid. The actual amount of accumulation for a susceptible hybrid seems to be more closely dependent on environmental conditions than the other two hybrids, meaning when conditions are favorable for fumonisin accumulation, the susceptible hybrid will accumulate fumonisin to a higher level than when compared to conditions that are not favorable for fumonisin accumulation. A resistant hybrid in my study was found to increase in fumonisin two fold in favorable or unfavorable conditions for fumonisin accumulation.

In summary, fumonisin can be expected to increase over the course of a harvest season. Fumonisin levels appear to be lowest directly after kernel maturity and increase until harvest. This would indicate that grain should be harvested as soon as possible and

dried to levels below that for fungal growth to limit fumonisin contamination in harvested grain. The problem with this scenario is that it may not be economically feasible to harvest grain at 30% moisture and dry the maize to an acceptable level for selling. Compounding this issue is the fact that fumonisin levels may already be above the FDA recommended levels, meaning the extra costs associated with trying to limit fumonisin may not yield a marketable product. This scenario would be most common in the high risk areas of Eastern North Carolina where fumonisin contamination appears to occur more frequently than in other maize growing regions.

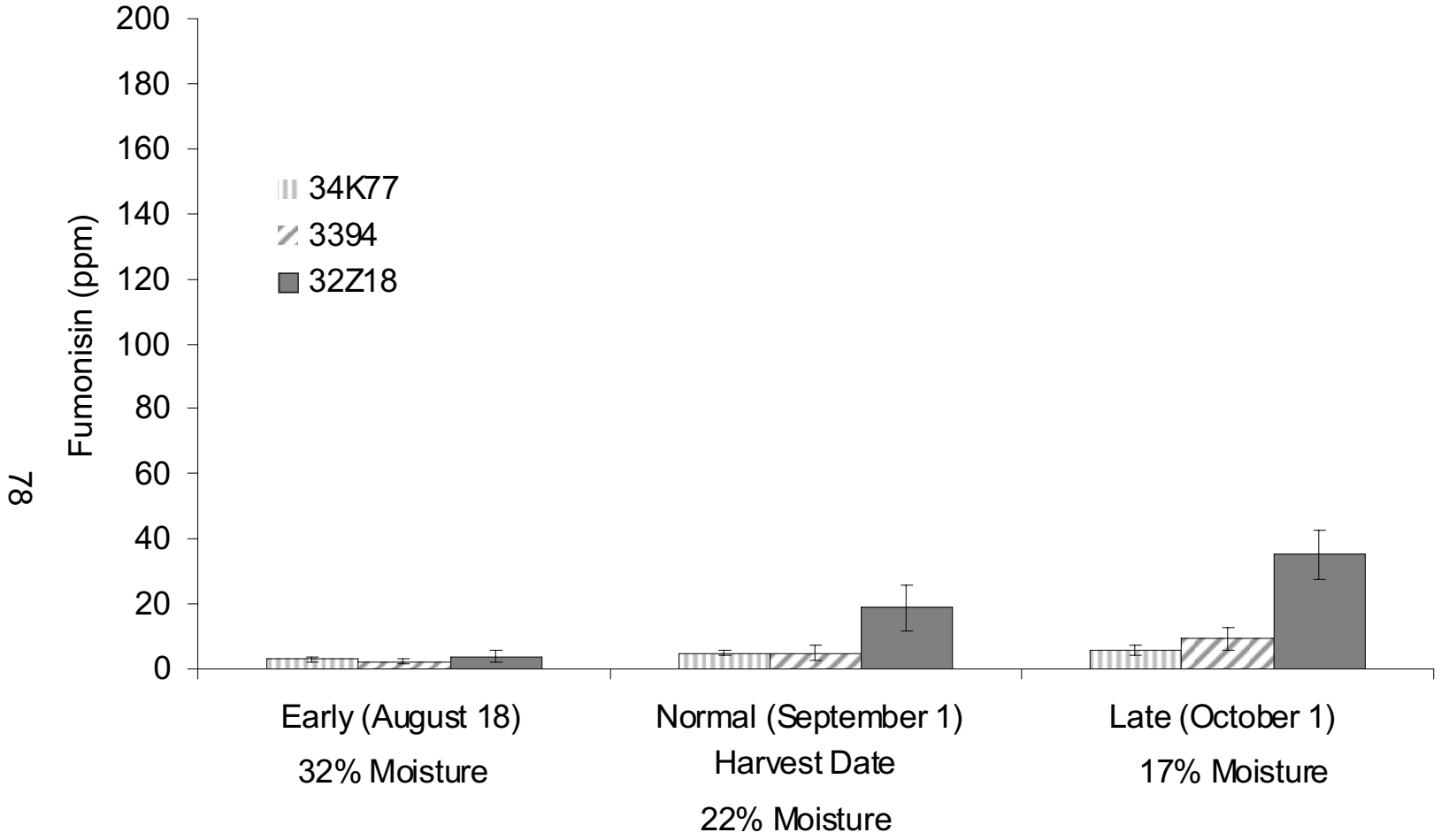

Figure 28. Fumonisin Contamination of Pioneer Hybrids at Camden County, NC, 2000. Bars show standard error. The three hybrids, 34K77, 3394 and 32Z18 are resistant, intermediate and susceptible to Fusarium ear rot, respectively.

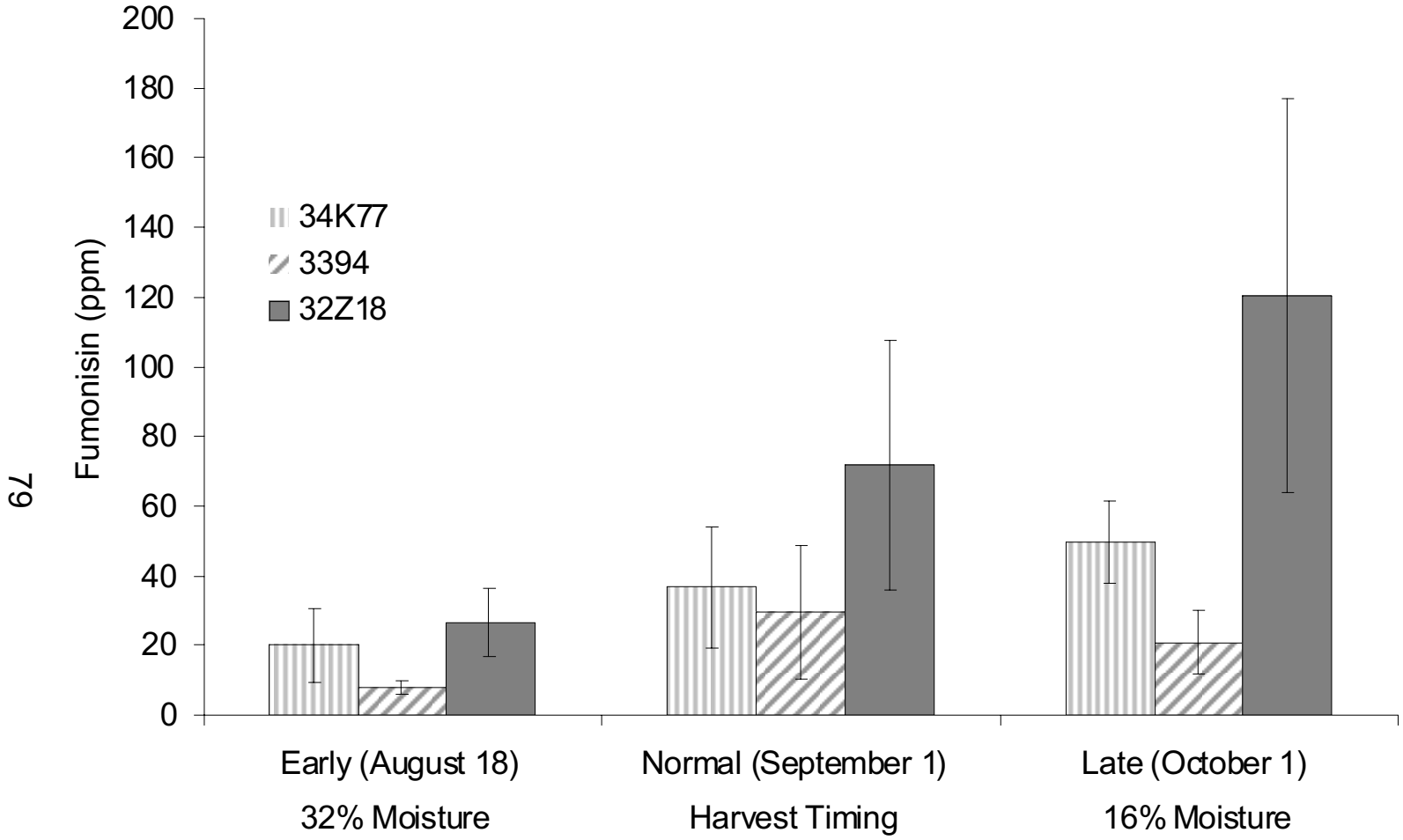

Figure 29. Fumonisin Contamination of Pioneer Hybrids at Beaufort County, NC, 2000. Bars show standard error. The three hybrids, 34K77, 3394 and 32Z18 are resistant, intermediate and susceptible to Fusarium ear rot, respectively.

Bacon, C. W., Bennett, R. M., Hinton, D. M. & Voss, K. A. (1992). Scanning Electron-Microscopy of Fusarium-Moniliforme Within Asymptomatic Corn Kernels and Kernels Associated With Equine Leukoencephalomalacia. *Plant Disease* **76**, 144-148.

Bezuidenhout, S. C., Gelderblom, W. C. A., Gorstallman, C. P., Horak, R. M., Marasas, W. F. O., Spiteller, G. & Vleggaar, R. (1988). Structure Elucidation of the Fumonisins, Mycotoxins From Fusarium-Moniliforme. *Journal of the Chemical Society-Chemical Communications* , 743-745.

Boling, M., Grogan, C. & Broyles, J. (1963). A new method for artificially producing epiphytotics of Fusarium ear rot of maize. *Plant Disease Reporter* **47**, 315-317.

Boutrif, E. & Canet, C. (1998). Mycotoxin prevention and control: FAO programmes. *Revue Med. Vet* **149**, :681-694.

Branstetter, B. (1927). Corn Root Rot Studies. *Missouri Agricultural Experiment Station Research Bulletin 113* , 80.

Cardwell, K. F., Kling, J. G., Maziya-Dixon, B. & Bosque-Perez, N. A. (2000). Interactions between Fusarium verticillioides, Aspergillus flavus, and insect infestation in four maize genotypes in lowland Africa. *Phytopathology* **90**, 276-284.

Chamberlain, W. J., Bacon, C. W., Norred, W. P. & Voss, K. A. (1993). Levels of Fumonisin B-1 in Corn Naturally Contaminated With Aflatoxins. *Food and Chemical Toxicology* **31**, 995-998.

Christensen, J. & Wilcoxson, R. (1966). *Stalk rot of corn.* St. Paul, MN: American Phytopathological Society.

Desjardins, A. E., Plattner, R. D. & Proctor, R. H. (1996). Linkage among genes responsible for fumonisin biosynthesis in Gibberella fujikuroi mating population A. *Applied and Environmental Microbiology* **62**, 2571-2576.

Dowden, D. (2001). Fusarium Ear Rot Resistance in Dekalb Hybrids. .

Drepper, W. J. & Renfro, B. L. (1990). Comparison of Methods For Inoculation of Ears and Stalks of Maize With Fusarium-Moniliforme. *Plant Disease* **74**, 952-956.

Fajemisin, J. (1982). International Institute of Tropical Agriculture Annual Report. , pp. 217 pp. Ibadan, NIgeria.

Gelderblom, W. C. A., Jaskiewicz, K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vleggaar, R. & Kriek, N. P. J. (1988). Fumonisins - Novel Mycotoxins With Cancer-

Promoting Activity Produced By Fusarium-Moniliforme. *Applied and Environmental Microbiology* **54**, 1806-1811.

Gulya, T. J., Martinson, C. A. & Loesch, P. J. (1980). Evaluation of Inoculation Techniques and Rating Dates For Fusarium Ear Rot of Opaque-2 Maize. *Phytopathology* **70**, 1116-1118.

Harrison, L. R., Colvin, B. M., Greene, J. T., Newman, L. E. & Cole, J. R. (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin B1, a toxic metabolite of *Fusarium moniliforme. J. Vet. Diagn. Invest* **2**, 217-221.

Headrick, J. M. & Pataky, J. K. (1991). Maternal Influence On the Resistance of Sweet Corn Lines to Kernel Infection By Fusarium-Moniliforme. *Phytopathology* **81**, 268-274.

Huss, M. J. & Leslie, J. F. (1993). Isozyme variation among six different biological species within the *Gibberella fujikuroi* species complex (*Fusarium* section *Liseola*). *Fungal Genet. Newsl* **40A**.

Jardine, D. J. & Leslie, J. F. (1992). Aggressiveness of *Gibberella fujikuroi* (*Fusarium moniliforme*) isolates to grain-sorghum under greenhouse conditions. *Plant Dis.* **76**, 897-900.

Kedera, C. J., Ochor, T. E., Ochieng, J. A. W. & Kamidi, R. E. (1994). Incidence of Maize Ear Rot in Western Kenya. *International Journal of Pest Management* **40**, 117-120.

Kellerman, T. S., Marasas, W. F. O., Thiel, P. G., Gelderblom, W. C. A., Cawood, M. & Coetzer, J. A. W. (1990). Leukoencephalomalacia in 2 Horses Induced By Oral Dosing of Fumonisin-B1. *Onderstepoort Journal of Veterinary Research* **57**, 269-275.

King, S. B. (1981). Time of Infection of Maize Kernels by *Fusarium moniliforme* and *Cephalosporium acremonium*. Phytopathology **71**, 796-799.

King, S. B. & Scott, G. E. (1981). Genotypic Differences in Maize to Kernel Infection By Fusarium- Moniliforme. *Phytopathology* **71**, 1245-1247.

Kingsland, G. & Wernham, C. (1962). Etiology of stalk rots of corn in Pennsylvania. *Phytopathology* **52**, 519-523.

Klittich, C. J. R., Leslie, J. F., Nelson, P. E. & Marasas, W. F. O. (1997). *Fusarium thapsinum* (*Gibberella thapsina*): A new species in section *Liseola* from sorghum. *Mycologia* **89**, 643-652.

Koehler, B. (1942). Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. *Journal of Agricultural Research* **64**, 421-442.

Koehler, B. (1959). Corn Ear Rots in Illinois. *Illinios Agricultural Experiment Station Bulletin* **639**, 1-87.

Koehler, B. (1960). Cornstalk Rots of Illinois. *Illinois Agricultural Experiment Station Bulletin* **658**, 1-90.

Koehler, B., Dungan, G. & Burlison, W. (1934). Maturity of seed corn in relation to yielding ability and disease infection. *Agronomy Journal* **26**, 262-274.

Kulisek, E. & Hazebroek, J. (2000). Comparison of extraction buffers for the detection of fumonisin B1 in corn by Immunoassay and High-Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry* **48**, 65-69.

Lawrence, E. B., Nelson, P. E. & Ayers, J. E. (1981). Histopathology of Sweet Corn Seed and Plants Infected With Fusarium-Moniliforme and Fusarium-Oxysporum. *Phytopathology* **71**, 379-386.

Leslie, J. F. (1996). Introductory biology of *Fusarium moniliforme.* In *Fumonisins in Food,* pp. 153-164. Edited by J. W. D. L. S. Jackson, and L. B. Bullerman. New York: Plenum Press.

Leslie, J. F., Plattner, R. D., Desjardins, A. E. & Klittich, C. J. R. (1992). Fumonisin B1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). *Phytopathology* **82**, 341-345.

Marasas, W. F. O. (1996). Fumonisins: History, World-Wide Occurrence and Impact. In *Fumonisins in Food,* pp. 1-18. Edited by J. W. D. L. S. Jackson, and L. B. Bullerman. New York: Plenum Press.

Meadows, B. (2000). *North Carolina Agricultural Statistics 2000*. Raleigh: North Carolina Agricultural Statistics.

Merrill, A. H., Liotta, D. C. & Riley, R. T. (1996). Fumonisins: Fungal toxins that shed light on sphingolipid function. *Trends in Cell Biology* **6**, 218-223.

Merrill, A. H., Wang, E., Vales, T., Smith, E., Schroeder, J., Mendalino, D., Alexander, C., Crane, H., Xia, J., Liotta, D., Meredith, F. & Riley, R. (1996). Fumonisin Toxicity and Sphingolipid Biosynthesis. In *Fumonisins in Food,* pp. 297-306. Edited by L. Jackson, J. DeVries & L. Bullerman. New York: Plenum Press.

Miller, J. D., Young, J. C. & Trenholm, H. L. (1983). Fusarium Toxins in Field Corn .1. Time Course of Fungal Growth and Production of Deoxynivalenol and Other Mycotoxins. *Canadian Journal of Botany-Revue Canadienne De Botanique* **61**, 3080-3087.

Munkvold, G. P. & Carlton, W. M. (1997). Influence of inoculation method on systemic Fusarium moniliforme infection of maize plants grown from infected seeds. *Plant Disease* **81**, 211-216.

Nirenberg, H. (1976). Untersuchungen über die morphologische und biologische Differenzierung in der Fusarium-Sektion Liseola. In *Mitteilungen aus der Biologischen Bundesanstal für Land- und Forstwirtschaft*, pp. 1-117. Berlin-Dahlem.

Payne, G., Hagler, W. J. & Adkins, C. (1988). Aflatoxin accumulation in inoculated ears of field grown maize. *Plant Disease* **72**, 422-424.

Placinta, C. M., D'Mello, J. P. F. & Macdonald, A. M. C. (1999). A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Animal Feed Science and Technology* **78**, 21-37.

Prelusky, D., Rotter, B. & Rotter, R. (1994). Toxicology of mycotoxins. In *Mycotoxins in Grain: Compounds other than Aflatoxin*, pp. 359-403. Edited by J. Miller & H. Trenholm. St. Paul: The American Phytopathological Society.

Proctor, R. H., Desjardins, A. E. & Plattner, R. D. (1999). Biosynthetic and genetic relationships of B-series fumonisins produced by Gibberella fujikuroi mating population A. *Natural Toxins* **7**, 251-258.

Proctor, R. H., Desjardins, A. E., Plattner, R. D. & Hohn, T. M. (1999). A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in Gibberella fujikuroi slating population A. *Fungal Genetics and Biology* **27**, 100-112.

Reid, L. M., Nicol, R. W., Ouellet, T., Savard, M., Miller, J. D., Young, J. C., Stewart, D. W. & Schaafsma, A. W. (1999). Interaction of Fusarium graminearum and F-moniliforme in maize ears: Disease progress, fungal biomass, and mycotoxin accumulation. *Phytopathology* **89**, 1028-1037.

Salazar, F. & Vargas, E. (1977). Methods of evaluation and levels of resistance in maize to the rotting of ears caused by Fusarium moniliforme. *Agronomia Costarricense* **1**, 93-99.

Scott, G. E. & King, S. B. (1984). Site of Action of Factors For Resistance to Fusarium moniliforme in Maize. *Plant Disease* **68**, 804-806.

Shelby, R. A., White, D. G. & Bauske, E. M. (1994). Differential Fumonisin Production in Maize Hybrids. *Plant Disease* **78**, 582-584.

Shim, W. B. & Woloshuk, C. P. (1999). Nitrogen repression of fumonisin B1 biosynthesis in Gibberella fujikuroi. *Fems Microbiology Letters* **177**, 109-116.

Shim, W. B. & Woloshuk, C. P. (2001). Regulation of fumonisin B-1 biosynthesis and conidiation in Fusarium verticillioides by a cyclin-like (C-type) gene, FCC1. *Applied and Environmental Microbiology* **67**, 1607-1612.

Smith, F. L. & Madsen, C. B. (1949). Susceptibility of Inbred Lines of Corn to Fusarium Ear Rot. *Agronomy Journal* **41**, 347-348.

Toussoun, T. A. (1981). Present concept of Fusarium classification. In *Fusarium: disease, biology and taxonomy*, pp. 413-427. Edited by T. A. T. P. E. Nelson, and R. J. Cook. University Park: The Pennsylvania State University Press.

Vainio, H., Heseltine, E. & Wilbourn, J. (1993). Report on an IARC working group meeting on some naturally- occurring substances. *Int. J. Cancer* **53**, 535-537.

Valleau, W. (1920). Seed corn infection with Fusarium moniliforme and its relation to the root and stalk rots. *Kentucky Agricultural Experiment Station Bulletin* **226**, 27-51.

Wang, E., Merrill, A. H., Norred, W. P., Bacon, C. & Riley, R. T. (1991). Inhibition of Sphingosine Biosynthesis By Fumonisins, Mycotoxins Produced By Fusarium Moniliforme. *Faseb Journal* **5**, A1605-A1605.

Warfield, C. Y. & Gilchrist, D. G. (1999). Influence of kernel age on fumonisin B-1 production in maize by Fusarium moniliforme. *Applied and Environmental Microbiology* **65**, 2853-2856.

Warren, H. L. (1978). Comparison of Normal and High-Lysine Maize Inbreds For Resistance to Kernel Rot Caused By Fusarium moniliforme. *Phytopathology* **68**, 1331-1335.

Whitaker, T. B., Trucksess, M. W., Johansson, A. S., Giesbrecht, F. G., Hagler, W. M. & Bowman, D. T. (1998). Variability associated with testing shelled corn for fumonisin. *Journal of Aoac International* **81**, 1162-1168.

White, D. (1999). Fusarium Kernel or Ear Rot. In *Compendium of Corn Diseases*. Edited by D. White. St. Paul: American Phytopathological Society.

Xu, J. R., Yan, K. Y., Dickman, M. B. & Leslie, J. F. (1995). Electrophoretic karyotypes distinguish the biological species of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). *Mol. Plant-Microbe Interact* **8**, 74-84.

Yoshizawa, T., Yamashita, A. & Luo, Y. (1994). Fumonisin Occurrence in Corn From High-Risk and Low-Risk Areas For Human Esophageal Cancer in China. *Applied and Environmental Microbiology* **60**, 1626-1629.

Zummo, N. & Scott, G. E. (1992). Interaction of Fusarium-Moniliforme and Aspergillus-Flavus On Kernel Infection and Aflatoxin Contamination in Maize Ears. *Plant Disease* **76**, 771-773.