Interaction of field-isolated fungi with monarch butterfly larvae and Bt maize pollen in Iowa

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION

Milkweed and Monarch Butterflies

Common milkweed (*Asclepias syriaca* L.) is a perennial, noxious weed (Bhowmik and Bandeen, 1976) and the primary host plant for monarch butterflies. The milkweed receives its name from the milky sap that is exuded from parts of the plant when severed, a substance containing alkaloids, glycosides and a resinoid (Bhowmik and Bandeen, 1976; Mitich, 1993). Milkweed plants are found in many different types of landforms and regions north of the 36th parallel through New Brunswick and as far west as South Dakota and Kansas (Woodson, 1954). Roadsides are one of the areas most populated by milkweed. In one study in Iowa, for example, milkweed plants were found in 71% of the roadside sites (Hartzler and Buhler, 2000). Agricultural land accounts for 78% of Iowa, and therefore the ecology of milkweed plants in agricultural settings is important in the life cycle of the monarch. Forty-six and 57% of the maize and soybean fields sampled, respectively, were infested by milkweed (Hartzler and Buhler, 2000). In Nebraska, Cramer and Burnside (1982) found that roadsides and soybean fields also contained more milkweed than maize fields (51%, 73%, and 36%, respectively). Yenish et al. (1997) observed that milkweed growth in maize fields may be stifled by the concurrent growth of the neighboring maize (*Zea mays* L.) plants and the competition for necessary resources for survival. In a survey of five states or provinces (Ontario, Maryland, Wisconsin, Minnesota, and Iowa), nonagricultural areas contained higher densities of milkweed plants, and monarch

productivity was four times higher in nonagricultural areas than in maize fields, although production of monarch larvae within maize fields was 45 times the amount of monarch larvae produced in nonagricultural areas (Oberhauser et al., 2001). Survival of monarchs in four of the five sites was better both within maize fields and nonagricultural areas compared to maize field edges (Oberhauser et al., 2001).

Another species, tropical milkweed, *Asclepias curassavica* L., is distributed in the southern United States and in tropical and sub-tropical regions (Woodson, 1954). *Asclepias syriaca* and *A. curassavica* differ in the structure of their leaves. *Asclepias syriaca* plants have a broader leaf containing more trichomes; whereas, *A. curassavica* leaves tend to be narrower and smoother (Zalucki et al., 2001). Tropical milkweed does not usually occur within maize fields in the United States and Canada, although it has been used in several laboratory studies (e.g., Losey et al., 1999; Jesse and Obrycki, 2000).

Monarch butterfly (*Danaus plexippus* L.) larvae use milkweed extensively throughout their development, as plants from the milkweed family (Asclepiadaceae) are their only host plants (Bhowmik and Bandeen, 1976). Adult females oviposit onto milkweed leaves, emerging larvae consume milkweed leaf material, and monarch butterflies can consume milkweed nectar. The life cycle of the monarch butterfly is spent primarily on milkweed, but a generation of adults migrates to overwinter.

Monarch butterflies are well known for their migration and the accuracy with which they migrate to and from a specific location (Wassenaar and Hobson, 1998). Two geographically separated populations of monarch butterflies migrate to two overwintering sites (Brower et al., 1995; Wassenaar and Hobson, 1998). Approximately 50,000 to several hundred thousand monarch butterflies west of the Rocky Mountains migrate to southern California, while over 100 million in the Midwest and eastern parts of the United States and

Canada migrate to approximately 13 sites within the Oyamel forests in the Mexico and Michoacan states of Mexico (Wells and Wells, 1992; Brower et al., 1995; Wassenaar and Hobson, 1998). The eastern population of monarch butterflies returns to the northern areas after mating around late March (Brower et al., 1995). As the butterflies fly north, a spring generation is started in the Gulf States, and four generations occur before the last generation of butterflies begins the migration south (Brower et al., 1995). Areas in the North or Midwest where the migratory monarch butterflies originate include many of the states of the Corn Belt, such as Iowa (Wassenaar and Hobson, 1998).

Maize Anthesis and Milkweeds

Migratory monarch butterflies may be larvae when maize anthesis occurs, and monarch and maize life cycles can overlap (Anthesis is the period when maize plants shed pollen for one to two weeks [Ritchie et al., 1992]). Monarch production within maize in Iowa and Minnesota is high (Oberhauser et al. 2001). Overlap of maize anthesis with monarch butterfly phenology was predicted to be highest (40-62%) in northern regions (Minnesota, Wisconsin, and Ontario); whereas, in Iowa and Maryland the overlap was predicted to be lower (15-20%; Oberhauser et al., 2001).

During anthesis pollen deposits onto milkweed leaf surfaces. Maize pollen grains are approximately 90 μm in size and tend to deposit rapidly (Raynor et al., 1972). Pollen densities outside maize fields are greatly reduced within a short distance (Raynor et al., 1972; Jesse and Obrycki, 2000; Sears et al., 2000; Wraight et al., 2000; Pleasants et al., 2001; Stanley-Horn et al., 2001). An estimated 40% of maize pollen can be deposited on the leaf surfaces of milkweed plants that infest maize fields (comparing pollen densities on sticky

slides and neighboring milkweed leaves through regression; Pleasants et al., 2001), while only 4% of deposited pollen was found on the underside of the leaf (Pleasants et al., 2001). Pollen tends to concentrate along the leaf midvein compared to rest of the leaf surface by a factor of 1.5 to 1.9 (Zangerl et al., 2000; Pleasants et al., 2001). Leaves at the middle nodes of milkweed plants receive the most pollen, while the upper and lower leaves collect fractions of the pollen density found on the middle leaves (Pleasants et al., 2001). For example, Pleasants et al. (2001) reported mean pollen densities of 37.3, 132.9, and 103.0 grains/cm^2 for upper, middle, and lower leaves, respectively on milkweed leaves within and up to 1 m outside of maize fields. Besides leaf position on the milkweed plant, leaf angle influences the deposition and retention of pollen (Pleasants et al., 2001). Horizontal middle leaves tend to have more pollen than upper and lower leaves, which often are more vertical (Pleasants et al., 2001). Moreover, pollen densities on leaf surfaces are reduced by rain events (e.g., Fokkema, 1971, with rye; Jesse and Obrycki, 2000; Sears et al., 2000; Zangerl et al., 2000; Stanley-Horn et al., 2001). Pleasants et al. (2001) estimated that 54-86% of deposited pollen was removed within one rain event. Pollen may be regarded as a contaminant and deterrent to female monarch butterflies, which prefer to oviposit on milkweed not surrounded by maize (Tschenn et al., 2001). The selected leaves of the milkweed plant for oviposition are typically from the upper third, which receives less pollen (Pleasants et al., 2001; Tschenn et al., 2001).

Bacillus thuringiensis, Bt Maize, and Non-target Lepidoptera

The bacterium *Bacillus thuringiensis* Berliner (Bt) produces endotoxins that are active on certain insects. These toxins have been useful in the control of pests associated

with economically important crops. Cry1Ab is a protein from *B. thuringiensis* subsp. *kurstaki*, effective against Lepidoptera. Detailed descriptions of the history of Bt, a common soil bacterium, as it was developed into an effective insecticide can be found in Beegle and Yamamoto (1992), and Milner (1994); the activity of the toxic protein Cry1Ab was reviewed in Hofte and Whiteley (1989), Van Rie et al. (1989), and Gill et al. (1992). Alkaline gut environment, proteases, and specific binding sites for the toxin on the brush border membrane of the gut epithelium are factors that determine the efficacy of the Bt toxin on insects that ingest it (Jaquet et al., 1987; Van Rie et al., 1989; Gill et al., 1992). Eventual lysis of the brush border membrane cells is caused by channels or pores formed by the toxin (through a process of reception, intercalation, and channel formation) and death later results (English and Slatin, 1992; Gill et al., 1992). The gene *Cry1Ab* that codes for production of this protein has been modified, truncated, and inserted into the maize genome. By genetically modifying maize to produce an activated form of the δ-endotoxin, some of the target insects' mechanisms to detoxify or prevent the activity of the Bt toxin are bypassed. In Bt maize the toxin affects a larva when it begins to consume host material and is most susceptible (Koziel et al., 1993). Bt events MON810 and Bt11 involve the cauliflower mosaic virus (CaMV) 35S promoter to express the Bt protein throughout the maize plant (i.e., in leaves, kernels, pith and roots) (Koziel et al., 1993; Fearing et al., 1997). The 176 Bt event, on the other hand, uses two promoters (phosphoenolpyruvate carboxylase and a maize-specific promoter) allowing for expression in green tissue and pollen (Koziel et al., 1993; Fearing et al., 1997). Cry1Ab expression in event 176 pollen can be as high as 7.1 μg/g pollen (Stanley-Horn et al., 2001); whereas expression in Bt11 and MON810 pollen is <0.09 μg Cry1Ab/g pollen (FIFRA, 2000). Event 176 maize has been phased out in the United States.

During anthesis Bt maize pollen can deposit onto the host plants of several non-target Lepidoptera. Milkweed tiger moths (*Euchaetes egle* Drury) were not affected by pollen from Cry1Ab events Bt11 and 176 (Jesse and Obrycki, 2002). Jesse and Obrycki (2002) hypothesized that the failure of the Bt to cause sublethal effects or mortality on tiger moths was due to a lack of Bt binding receptors (Jesse and Obrycki, 2002). MON810 pollen did not affect black swallowtail (*Papilio polyxenes* Fabricius) neonates at doses up to 10,000 pollen grains/cm^2, but event 176 pollen was shown to harm *P. polyxenes* larvae at this density (Wraight et al., 2000). In the field, parsnip plants near event 176 maize fields received adequate amounts of pollen deposition to cause some negative effects in black swallowtail larvae; those feeding on parsnip plants closer to the maize field had lower weights than those on more distantly located plants (0.5 m vs. 7 m; Zangerl et al., 2000). The lethal pollen density for event 176 pollen in black swallowtails was estimated to be 613 pollen grains/cm^2, although significant losses due to mortality were observed with doses of 100 pollen grains/cm^2 compared to the control (Zangerl et al., 2000).

Bt-maize pollen deposition on *A. syriaca* has been addressed in several studies. During anthesis various pollen densities (pollen grains/cm^2) within maize fields have been documented: 70-215 (Jesse and Obrycki, 2000); <900 in 99% of the fields and a maximum of 1449 (Pleasants et al., 2001); and 154-367 with similar results for six Bt and non-Bt hybrids, with the highest density after anthesis of 429 (Stanley-Horn et al., 2001). Average pollen deposition within maize fields was estimated to be 171 pollen grains/cm^2 (Pleasants et al., 2001).

Losey et al. (1999) reported in a correspondence to *Nature* that monarch larvae were negatively affected when they fed on milkweed leaves dusted with pollen from a Bt11 maize hybrid. This report incited several research projects to determine whether use of Bt-

transformed maize significantly threatens populations of monarch butterflies. Several objectives were proposed in a United States Environmental Protection Agency (EPA) 1999 Data Call-in, including testing Cry1Ab toxicity on monarch butterfly larvae and non-target insects; assessing milkweed distribution; determining the effect of pollen dispersal at varying distances from the maize field and the associated effects on the insects at these ranges; and estimating the likelihood monarch larvae and other non-target insects would encounter the Bt maize pollen on their host plants (USEPA, 1999).

The potential impact of exposure to Bt toxins by monarch larvae has been investigated (Losey et al., 1999; Jesse and Obrycki, 2000; Sears et al., 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001; and Zangerl et al., 2001). As Bt maize comprised 25% of the total amount of maize grown in Iowa, an estimated 3% of the monarch population could have been exposed to Bt maize pollen in Iowa in 2001 (Agriculture Statistics Board, 2001; Oberhauser et al., 2001). Sears et al. (2001) estimated that the risk to monarch butterfly larvae of encountering Bt pollen was very low in 2000 (2.1% and 0.8% of the Iowa population and total U.S. eastern monarch population, respectively). The risk associated with events MON810 and Bt11 was minimal as 0.7% of maize fields in 2000 shed pollen around the lowest observable effect concentration (LOEC) of 1,000 pollen grains/cm^2, and 0.1% of the maize fields had depositions of 4,000 pollen grains/cm^2 (Sears et al., 2001). In field studies, no significant differences were observed among amounts of pollen deposition for different Bt and non-Bt hybrids in 2000 (Stanley-Horn et al., 2001).

Bioassays on young (neonate/first instar) monarch larvae have helped determine toxic amounts (pollen grains/cm^2) necessary to produce sublethal effects and mortality with Bt11, MON810, and 176 events. Young monarch larvae were more susceptible to the Bt toxin after consuming Bt pollen than older instars (Jesse and Obrycki, 2000; Hellmich et al., 2001).

Cry1Ab concentration within pollen differs between Bt11/MON810 and event 176 pollen, and the effects on monarch larvae depended on the Bt pollen event and the quantity of pollen that was consumed.

Various pollen densities of Bt pollen for several Bt events have elicited sublethal effects and acute toxicity in bioassays with monarch larvae. Event 176 and Bt11 pollen were similar in toxicity to monarch larvae (in terms of survival) at low pollen densities (14 and 135 pollen grains/cm^2), but were significantly different at 1,300 pollen grains/cm^2 (Jesse and Obrycki, 2000). Non-Bt pollen, based on monarch larvae survival, was similar to Bt11 and event 176 pollen at the 14-pollen/cm^2 density, and was similar to Bt11 at 1,300 pollen grains/cm^2 (Jesse and Obrycki, 2000). Hellmich et al. (2001) found no differences in survival of *D. plexippus* larvae among non-Bt, Bt (MON810 and Bt11), and no-pollen treatments at pollen densities greater than 1,000 pollen grains/cm^2. Event 176 pollen at densities of 5-133 pollen grains/cm^2 elicited lethal and sublethal effects in *D. plexippus* larvae (Sears et al., 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001). Larvae that consumed pollen from MON810 had significantly larger larval weights at pollen densities greater than 1,000 pollen grains/cm^2 compared to the leaf discs with non-Bt pollen, but not leaf discs without pollen (Hellmich et al., 2001). There was a tendency for monarch larvae to weigh less when fed Bt11 pollen than non-Bt pollen when pollen densities exceeded 1,000 pollen grains/cm^2 (Hellmich et al., 2001). The pollen of MON810 in other studies was considered to be safe, and Bt11 was not detrimental at pollen densities observed in the field (Sears et al., 2001; Stanley-Horn et al., 2001). It is important to note that actual pollen densities over 1,400 pollen grains/cm^2 within the field setting were not common (Pleasants et al., 2001). Milkweed leaves with non-transgenic pollen compared to leaves with no pollen had significantly less feeding by monarch larvae (Losey et al., 1999) and significantly less

oviposition by monarch females (Tschenn et al., 2001). However, non-Bt pollen did not elicit such responses in feeding in other studies (Sears et al., 2000; Felke et al., 2002). The lethal density of pollen from event Bt11 or MON810 is predicted to be greater than 366 (Hellmich et al., 2001), although experimental results suggest that larger densities of Bt11 or MON810 are necessary to negatively affect monarch butterfly larvae. The EC_{50} or concentration necessary for growth inhibition with Event 176 pollen was estimated to be 5-10 pollen grains/cm^2, while 7-30 pollen grains/cm^2 is considered toxic (Hellmich et al., 2001; Sears et al., 2001). Acute effects of Cry1Ab on monarchs have been emphasized within the literature, but Stanley-Horn et al. (2001) have noted the need for long-term toxicity data.

Monarch larvae that fed on pollen mixed with anther fragments, artifacts of pollen processing, displayed sublethal effects and mortality. Jesse and Obrycki (2000) and addressed anther contamination as a means of exposing monarch larvae in bioassays to higher levels of Cry1Ab in MON810 or Bt11 pollen, and Hellmich et al. (2001) proved that tassel fragments instead of well-sifted Bt pollen caused detrimental effects (decreased weights). Anthers within maize fields on milkweed leaf surfaces remain intact; therefore, exposure of monarch larvae to Bt protein within tassel fragments in maize fields would be less likely than in laboratory experiments (Hellmich et al., 2001).

Lepidopterous Larvae and Food, Bt and Behavior

Feeding behavior of lepidopteran larvae has been investigated in relation to exposure to toxin-containing food sources. Farrar et al. (1989) noted that when administering low quality food to larvae, more material was consumed in order to compensate for the lack of adequate nutrition. Meade and Hare (1993) reported that *Spodoptera exigua* Hübner larvae consumed more Bt-containing, low quality leaf material than when administered material of

higher quality. Data on the behavioral responses of larvae can complement the understanding of physiological effects resulting from the activity of an introduced toxin to their food (Farrar et al., 1989). Armyworm larvae (*Pseudaletia unipuncta* Haworth) altered their behavior to remedy sublethal effects incurred from consuming Bt maize (i.e., consume more material in later instars) (Pilcher et al., 1997). Light brown apple moth (*Epiphyas postvittana* Walker) neonates reacted to Bt Dipel in a no-choice study by increasing their movement; ceasing the consumption of the toxin; leaving the site; or ultimately consuming the toxin-laden food (Harris et al., 1997). However, lethargy and movement to the upper side of the leaf, which is abnormal and risky, was noted with various cabbage pests that consumed event 176 Bt pollen (Felke et al., 2002).

Fungi and the Phylloplane

Monarch larvae encounter contaminants other than maize pollen on the surfaces of milkweed leaves. Populations of microorganisms develop on the leaf surfaces (phylloplanes) of all plants and these may influence insect behavior and success. On the phylloplane microorganisms may survive in three ways or combinations of these ways: compete for available nutrients, parasitize a host, or deter the growth of other organisms by producing toxins (Belanger and Avis, 2000). The extent of variability in the structure of the microbial population on the phylloplane depends on factors associated with the plant itself (e.g., leaf age and health, structure, leaf position) and surrounding vegetation (e.g., height within canopy) (Kinkel, 1997; Zak, 2002). As the leaf ages the number of different fungi present on the leaf surface increases, but when the amount of available resources decreases and competition increases, diversity decreases (Zak, 2002). Any substance present on the leaf

surface (e.g., pollen) influences the distribution of fungal populations on the phylloplane (Kinkel, 1997).

When a nutrient source is present on the leaf surface, something similar to the growth of sooty mold on aphid honeydew can occur (e.g., Davison, 1991: Sparks and Yates, 1991). Fokkema (1968 and 1971), Warren (1972 and 1976), Garg and Sharma (1982), and Kumar and Mishra (1991) reported increased numbers of fungal populations with pollen deposition on the surface of leaves. Through field and greenhouse studies with similar pollen densities (approximately 100 pollen grains/cm^2) the growth of *Cladosporium* (usually on older leaves) could be stimulated with the pollen substrate as observed through direct microscopy and leaf washings (Fokkema, 1968). As the plant material without pollen began to senesce, colony numbers of *Cladosporium* increased and became similar to the number of *Cladosporium* colonies present with pollen (Fokkema, 1971). Fokkema (1971) suggested that during the flowering of rye there is a positive correlation between growth of *Cladosporium* and pollen densities. Warren (1972) compared pollen deposition and resulting fungal growth between two sugar beet fields (one pollinating and another without flowers, 200 m apart). Within the plot without flowers, some sugar beet pollen deposition still occurred. Fungal colony number trends of plots with or without flowers were similar, but colony numbers were comparatively lower in plots lacking flowers until the end of the sampling period (Warren, 1972).

Among different plants (barley/*Hordeum vulgare*, Triticale and eggplant/*Solanum melongena*), the predominant fungi isolated from healthy leaves were hyphomycetes, collectively termed "field fungi" (*Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Curvularia*, *Fusarium*, *Nigrospora*, *Penicillium*, and *Trichothecium*) (Garg et al., 1978). A majority of these fungi became primary colonizers upon senescence (Garg et al., 1978), and

Breeze and Dix (1981), Thomas and Shattock (1986), and Kumar and Mishra (1991) reported similar observations where the numbers of fungal populations increased on older or senescent leaves. Generally, the upper leaf surface contained more fungal spores or conidia than the lower surface (e.g., Breeze and Dix, 1981), and species diversity differed between the two surfaces (Garg et al., 1978). Some genera tended to be limited to one surface (e.g., *Epicoccum*, *Fusarium* on the upper surface), while others were present on both sides of the barley leaf (e.g., *Alternaria*, *Cladosporium*; Garg et al., 1978). Sainger et al. (1978) reported that several fungi (*Alternaria*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Cladosporium*, *Monilia*, and *Epicoccum*) colonized pollen (nasturtium/*Tropoleum majus*, carnation/*Dianthus caryophyllus*, and hollyhock/*Althaea rosea*), even after the pollen was surface sterilized.

Conidia of filamentous fungi commonly disperse through air (Andrews, 1992). Literature from all over the world can be found that identifies and quantifies fungi present in the air. Many different media and trapping apparatuses have been employed to conduct these studies. Worldwide, *Cladosporium* is likely the dominant genus (e.g., in Europe: Lacey, 1975; Larsen, 1981; Beaumont et al., 1985; Ebner et al., 1989; Larsen and Gravesen, 1991; Marchisio et al., 1992; Marchisio et al., 1997; Marchisio and Airaudi, 2001; in the Middle East: Abdel-Hafez, 1984; Shaheen, 1992; in the Far East: Takahashi, 1997; in Canada: Li and Kendrick, 1995; and in the U.S.: Lyon et al., 1984; Burge, 1986; and Katial et al., 1997). *Alternaria* species often are isolated as well, but a peak in the frequency occurs in summer and continues through early fall (Lacey, 1975; Larsen, 1981; Ebner et al., 1989; Li and Kendrick, 1995; Katial et al., 1997; Takahashi, 1997). *Fusarium* has been identified in many studies, but usually accounts for a small percentage of the total collection of genera. *Aspergillus* and *Penicillium* also comprise some of the genera commonly identified, although in some cases no distinction was made between the two due to morphological similarities in

conidia of the two genera (e.g., Larsen, 1981; Li and Kendrick, 1995; Marchisio and Airaudi, 2001). Other airborne genera include *Trichoderma*, *Aureobasidium*, *Epicoccum*, yeasts, sterile dematiaceous mycelium, *Rhizopus*/Mucorales, *Phoma*, *Scopulariopsis*, and *Drechslera*.

In the later stages of maize development, conidia of various genera (*Aspergillus*, *Penicillium*, *Alternaria*, *Cephalosporium*, *Acremonium*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Fusarium* [in particular *Fusarium verticillioides* (Sacc.) Nirenberg (synonym= *F. moniliforme* Sheldon)], *Helminthosporium*, *Mucor*, *Paecilomyces*, *Rhizopus*, *Scopulariopsis*, and *Trichoderma*) are present in the environment (Hill et al., 1984). *Penicillium* has been observed at high levels during the growing season of maize, soybeans, and wheat (Broder and Wagner, 1988). The asexual stage of *Gibberella*, *Fusarium*, is commonly isolated and present in maize fields, and several species of *Fusarium* are able to affect maize at any developmental stage (Munkvold and Desjardins, 1997). In studies of *Fusarium* in maize fields, no stalks were entirely free from fungal growth (Leslie et al., 1990), and the species of *Fusarium* associated with maize plants included *F. verticillioides*, *Fusarium proliferatum* (Matsushima) Nirenberg, and *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun, & Marasas (e.g., Leslie et al., 1990; Gillette, 1999). Soil- or debris-inhabiting *Fusarium* species associated with maize include *Fusarium oxysporum* Schlect. Emend. Snyd & Hans. and *Fusarium solani* (Mart.) Appel & Wollenw. Emend. Snyd. & Hans (Leslie et al., 1990). *Fusarium* species found in the air within maize fields of Iowa were predominantly *Fusarium semitectum* Berk. & Rav. and *Fusarium sporotrichioides* Sherb. (Gillette, 1999).

Milkweed, soybean, and maize leaf surfaces can harbor similar fungi. *Cercospora* and *Septoria* grow on *A. syriaca* in Iowa, while in other states *Alternaria*, *Cladosporium*,

Phoma, *Colletotrichum*, and *Phyllosticta* also have been present. In Florida, *Cercospora* and *Phyllosticta* have been found on *A. curassavica*. Leaves of soybeans, *Glycine max* (L.) Merr., can harbor many fungal genera including *Cercospora*, *Phomopsis*, *Alternaria*, *Cladosporium*, *Bipolaris*, *Fusarium*, *Phoma*, *Phyllosticta*, *Epicoccum*, *Septoria*, *Gliocladium*, *Aspergillus*, *Penicillium*, *Trichoderma*, and *Colletotrichum*. Fungal genera on maize leaves that also occur on either milkweed or soybeans include *Cercospora*, *Cladosporium*, *Bipolaris*, *Fusarium*, *Phoma*, *Phyllosticta*, *Epicoccum*, *Aspergillus*, *Penicillium*, and *Trichoderma*.

Insect Larvae and Fungi

Insects and fungi interact in several ways in maize agroecosystems. Some fungi produce secondary metabolites that can be toxic to insects. Insects, such as *Ostrinia nubilalis* (Hübner), can tunnel into maize tissue, carrying conidia through the tunnels, or forming entrances for fungal growth (e.g., *Fusarium*) (Christensen and Schneider, 1950). Wounding of maize tissue along with tunneling and transport of conidia by *O. nubilalis* has been found to increase incidences of maize stalk rots (Chiang and Wilcoxson, 1961; Jarvis et al., 1984) and *F. moniliforme* infection in maize kernels (Sobek, 1996). Subsequent growth of *Fusarium* on or within maize, especially in kernels, is a concern as mycotoxins (e.g., fumonisins, trichothecenes) can be produced (Munkvold et al., 1999). Insects may not only transport fungal conidia, but also may become infected and colonized by fungi. Fifteen species of *Fusarium,* nine of which are entomopathogenic, have been found to grow on insects (Claydon and Grove, 1984). Some of these species include *F. semitectum*, *F. verticillioides*, *F. oxysporum*, and *F. equiseti* (Corda) Sacc. (Claydon and Grove, 1984). Dowd et al. have conducted numerous studies on the interaction of fungi and fungal

metabolites (primarily from *Aspergillus*, *Fusarium*, and *Penicillium*) on maize pests (*Spodoptera frugiperda*, J.E. Smith and *Heliothis zea*, Boddie) or vectors (*Carpophilus hemipterus* L.; e.g., Dowd, 1988, 1989; Dowd and Middlesworth, 1989; Dowd et al., 1989, 1992). However, there is little information on the effects of fungal metabolites on non-vector or non-pest insects.

Insects that disseminate conidia (from *Fusarium*) are deterred with the active Bt present in maize, and therefore the incidence of *Fusarium* colonization (Munkvold et al., 1997) and the subsequent production of fumonisins are reduced (Munkvold et al., 1999; Dowd, 2001; Bakan et al., 2002). While the presence of Bt helps mitigate the production of mycotoxins such as fumonisins, the Bt toxin itself is not observed to be toxic to fungi (Bakan et al., 2002 from plants; and Saxena and Stotzky, 2001; Koskella and Stotzky, 2002 from purified Bt protein). Conversely, fungal colony numbers increased in the presence of Bt spores and crystals in the soil, possibly due to the use of Bt as a substrate and source of nutrients (Petras and Casida, 1985).

Bt Degradation

Bt protein is present throughout the lifespan of Bt maize. A study with event 176 tissues suggested that Bt protein levels (whole plant Cry1Ab per g fresh wt) were greater at the seedling stage than at maturity or senescence (Fearing et al., 1997). Event 176 pollen lacked protease activity that was found in leaf material and contributed to Bt degradation (Fearing et al., 1997). Half-lives of dissipation (DT_{50}) was 25.6 days without soil in insect bioassays (with *Heliothis virescens* Fabricius) with Bt maize leaf material (Sims and Holden, 1996). Measurable degradation of Cry1Ab in soil was not observed until after 21 days of incubation from which the amount of degradation was rapid through day 43 (Sims and

Holden, 1996). In one study, 176 Bt pollen retained its toxicity to *O. nubilalis*, and did not degrade significantly after exposure to ultraviolet radiation for 10 d (Ohlfest et al., 2002).

Degradation of two Bt proteins as purified protein and powder mixtures (instead of Bt protein extracted from transformed plants) was estimated by Herman et al. (2002a). Cry1F in a bioassay with *H. virescens* had a DT_{50} of 0.6 days, retaining <4% of the initial activity after 28 days. Herman et al. (2002b), using a binary insecticidal crystal protein (bICP) with southern corn rootworm larvae (*Diabrotica undecimpunctata howardi* Barber), found that the degradation was not of first-order kinetics (linear response), and the DT_{50} according to the biphasic and shift log models were 2.4 days and 1.7 days, respectively. Microorganisms were hypothesized to assist in the degradation, utilizing bICP as a nutritional source (Herman et al., 2002b). Other studies estimated degradation rates of spores and parasporal crystals within the soil matrix, and proposed that microorganisms within the soil assist in Bt protein degradation (e.g., Pruett et al., 1980; West, 1984; West et al., 1984a, 1984b; Sims and Reams, 1997).

Research Objectives

The influence of maize pollen on monarch butterfly larvae has been intensively investigated, but this relationship may be altered by the interactions among monarch butterfly larvae, maize pollen, and ubiquitous phylloplane fungi. These interactions have not been investigated previously and they form the basis of the research reported here. Three hypotheses were developed: 1) fungal populations on milkweed leaf surfaces are stimulated by maize pollen deposition (so that their effects on monarch larvae would coincide with pollen deposition effects), 2) fungal populations on milkweed leaf surfaces can influence monarch larval feeding behavior and success, and 3) fungal populations on milkweed leaf

surfaces contribute to the degradation of Bt proteins in maize pollen. Two studies were conducted for this thesis project. Objectives for the first study were (i) to identify and quantify the most frequently occurring fungi on milkweed leaves inside and outside of maize fields before, during, and after corn anthesis, and (ii) to analyze the relationship between pollen densities and numbers of fungal colonies throughout corn anthesis. Objectives for the second study were (i) to observe how fungi on milkweed leaves influenced feeding behavior of *D. plexippus* larvae and (ii) to test whether fungi assisted in the degradation of the Bt protein within the pollen on milkweed leaves and slides.

Thesis Organization

The first chapter of the thesis is an introduction and literature review. Chapter Two, "Fungi Isolated from Milkweed Leaves during Maize Anthesis," and Chapter Three, "Interaction of Fungi with Monarch Butterfly Larvae and Bt Protein in Event 176 Pollen," are to be submitted to the Canadian Journal of Botany and Environmental Entomology, respectively. Lastly, Chapter Four summarizes and concludes the research addressed in this thesis.

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CHAPTER TWO

FUNGI ISOLATED FROM MILKWEED LEAVES DURING MAIZE ANTHESIS

A paper to be submitted to Canadian Journal of Botany

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Abstract

Monarch butterfly (*Danaus plexippus* L.) larvae use milkweed plants extensively throughout their development. Milkweed plants in maize fields can be covered with maize pollen during anthesis, and pollen can influence the types and amounts of fungi present on leaf surfaces. To identify and quantify the most frequently occurring fungi on milkweed leaves inside and outside of maize fields before, during, and after corn anthesis, milkweed leaves were collected during the 2002 maize season from inside and outside three maize fields for eight sampling dates. Colony forming units (CFU) were counted, and pollen densities on milkweed leaves were measured. Correlation between means of CFU and pollen densities was considered. Pollen deposition significantly differed among fields, dates, and between positions (inside verses outside the maize fields). CFU values significantly differed among dates, but CFU values inside and outside of maize fields were similar. *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Penicillium*, *Trichoderma*, and the class Zygomycetes were the most prevalent fungi isolated. Isolates of *Fusarium* were identified to species, and *Fusarium verticillioides/proliferatum* was most frequently observed among sampling dates both inside and outside maize fields. Mean pollen densities and mean CFU were not strongly correlated, and CFU values for both inside and outside maize fields can be

influenced by phenologies of the fungi. Interaction between monarch larvae and maize pollen (transgenic and non-transgenic) has been examined extensively. However, these results indicate that exposure of larvae to leaf-surface fungi coincides with their exposure to leaf-surface pollen. Many fungi identified in this study can colonize pollen grains and produce mycotoxins. Monarch larvae that feed on milkweed leaves coated with pollen grains and fungi are involved in a complex three-way interaction that may affect larvae in ways that are not fully understood.

Introduction

Common milkweed (*Asclepias syriaca* L.) is a perennial weed (Bhowmik and Bandeen, 1976). Milkweed plants are found in many different types of landforms and regions north of the 36th parallel through New Brunswick and as far west as South Dakota and Kansas (Woodson, 1954). Roadsides are one of the areas most populated by milkweed (Cramer and Burnside, 1982; Hartzler and Buhler, 2000). Agricultural land accounts for 78% of Iowa, and 46% and 57% of the maize fields and soybean fields analyzed, respectively, were infested by milkweed (Hartzler and Buhler, 2000).

Anthesis in maize plants, the period when they shed pollen, is for one to two weeks (Ritchie et al., 1992). Maize pollen grains are approximately 90 μm in size and tend to be deposited rapidly (Raynor et al., 1972). Recorded pollen densities outside maize fields were greatly reduced within a short distance (Raynor et al., 1972; Sears et al., 2000; Jesse and Obrycki, 2000; Wraight et al., 2000; Pleasants et al., 2001; Stanley-Horn et al., 2001). During anthesis various pollen densities within maize fields have been documented, ranging from 70 to a maximum of 1449 pollen grains/cm^2 (Jesse and Obrycki, 2000; Pleasants et al., 2001; Stanley-Horn et al., 2001). The average pollen deposition within maize fields was

estimated to be 171 pollen grains/cm^2 (Pleasants et al., 2001). An estimated 40% of maize pollen can be deposited on the leaf surfaces of milkweed plants that infest maize fields (comparing pollen densities on sticky slides and neighboring milkweed leaves through regression; Pleasants et al., 2001).

Monarch butterfly (*Danaus plexippus* L.) larvae use milkweed plants exclusively throughout their development (Bhowmik and Bandeen, 1976). Adult females oviposit onto milkweed leaves, emerging larvae consume milkweed leaf material, and monarch butterflies can consume milkweed nectar. The life cycle of the monarch butterfly is spent primarily on milkweed, but a generation of adults migrates to overwinter.

The interaction between monarch larvae and pollen (transgenic and non-transgenic) has been examined (Losey et al., 1999; Jesse and Obrycki, 2000; Sears et al., 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001; and Zangerl et al., 2001). Non-transgenic pollen on milkweed leaves has not been found to significantly affect survival or feeding behavior of monarch butterfly larvae (Sears et al., 2000; Felke et al., 2002), although one study reported significantly less feeding of leaves dusted with non-transgenic pollen than leaves without pollen in monarch larvae bioassays (Losey et al., 1999).

Monarch larvae encounter contaminants other than maize pollen on the surfaces of milkweed leaves. Fungi present on milkweed leaves, especially around anthesis within maize fields, could affect monarch larvae (feeding) behavior. Fungi, or a combination of fungi and pollen on milkweed leaves can potentially expose monarch larvae to mycotoxins.

Pollen may influence the types and amounts of fungi present on the leaf surface. The extent of variability in the structure of the microbial population on the phylloplane depends on factors associated with the plant itself (e.g., leaf age and health, structure, leaf position) and surrounding vegetation (e.g., height within canopy; Kinkel, 1997; Zak, 2002). Any

substance present on the leaf surface (e.g., pollen) influences the distribution of fungal populations on the phylloplane (Kinkel, 1997).

Stimulation of fungal growth on milkweed leaf surfaces by pollen deposition was proposed and evaluated. Objectives for this study were (i) to identify and quantify the most frequently occurring fungi on milkweed leaves inside and outside of maize fields before, during, and after maize anthesis, and (ii) to analyze the relationship between pollen densities and numbers of fungal colonies throughout maize anthesis. Maize pollen deposition on milkweed leaf surfaces has been investigated in studies where the interaction between (Bt) maize pollen and monarch butterfly larvae was tested. Maize pollen deposition and subsequent fungal colonization on milkweed leaves could influence monarch larval feeding behavior, as mycotoxins could be produced.

Materials and Methods

Milkweed leaves were collected at three locations (595/280, Shipley North, and Shipley South) in Ames, Iowa, throughout the maize-growing season of 2002 in order to isolate and identify the fungi present on the phylloplane and determine the most frequently occurring fungi. There were eight sampling dates: (1) 13 June, (2) 27 June, (3) 11 July, (4) 18 July, (5) 25 July, (6) 1 August, (7) 15 August, and (8) 29 August. The sampling regimen was organized with a 2-week interval prior to anthesis, a 1-week interval around the timeframe anthesis would occur, and a return to the 2-week interval between collection dates as pollen shed concluded. At each location, six leaves were taken both from within a maize field and from the outside of the maize field (soybean field adjacent to the maize field [at the 595/280 intersection and at a site referred to as Shipley North], or roadside [Shipley South] at approximately the same geographical location). Spatial relationships of maize fields to the

outside sampling sites differed. The 595/280 and Shipley North maize fields were located to the west and south of the soybean fields, respectively. The Shipley South maize field was situated to the east of the roadside area. Each leaf was taken from a middle node of milkweed plants and placed within a 6 x 8 2-ml plastic bag, keeping the leaf horizontal. Harvested leaves were kept cool and transferred to a refrigerator (6°C) in the laboratory.

Using a Stereo-Zoom dissecting scope (Nikon SMZ-1000) with a stage micrometer, six random 0.25-cm^2 areas on each leaf were observed, and pollen grains were counted in order to estimate average pollen shed (pollen grains/cm^2) for that date and location. Leaves were processed within three days of collection, and fungi present on the milkweed leaf surfaces were later quantified and identified through subsequent growth on media. A #9 cork borer (7 mm in diameter) was used to cut out 15 discs from each leaf. These discs were placed in individual, sterile 125-ml Erlenmeyer flasks with 100 ml of sterile phosphate buffer and 1-2 drops of Tween 20 per liter (Zaher et al., 1985). Flasks were shaken at 100 rpm (LAB-LINE Junior Orbit Shaker; LAB-LINE Instruments Inc.; Melrose Park, IL) for 20 minutes. Serial dilutions of 10^0, 10^{-1}, and 10^{-2} (at peak periods of anthesis; dates 5-8) were prepared from 1 ml of the suspension. Sterile potato dextrose agar (Difco PDA; Becton, Dickinson and Company; Sparks, MD) with antibiotics (6 ml/L of streptomycin and 3 ml/L neomycin) was poured over a 1-ml aliquot from each dilution into Petri dishes (95 x 15 mm) to further limit bacterial growth.

Individual Petri dishes were sealed with Parafilm®, and the dishes were incubated at room temperature up to 10 days. Dishes were monitored to ensure that both fast and slow growing colonies could grow and be counted. Total number of fungal colonies was recorded.

Transfers of the most frequently observed colonies (through comparison of the plates throughout the dilution series) were made and identified on carnation leaf agar (CLA; Fisher

et al., 1982). Isolates were viewed with a microscope (Olympus BH-2) and identified to genus based on colony and spore morphology (Nelson et al., 1983 and Barnett and Hunter, 1998). Numbers of colony forming units (CFU; per cm^2 milkweed leaf material) of identified specimens were recorded in order to compare genera or species (of *Fusarium*) observed from each field for each date, either inside or outside maize fields.

Statistical analyses were performed through SAS Program 8e (SAS Institute Inc., Cary, NC, 1999-2001). Pollen densities were analyzed with ANOVA (Proc mixed) to assess the effects of field, date, and milkweed plant position (inside or outside maize fields). Means for pollen deposition were generated for field, date and milkweed plant position and compared by least significant difference (Proc GLM). Field was treated as a random variable, and date and milkweed plant position were fixed variables in statistical analyses for CFU values. Means of CFU were log-transformed and analyzed with ANOVA (Proc Mixed) to assess the effects of date, position, and date*position. Correlations were tested between mean CFU values per date and pollen densities for milkweed leaves collected from inside and outside of maize fields (Proc CORR).

Results

Anthesis was first observed in the Shipley South maize field, then at the 595/280 intersection, and lastly at Shipley North with peaks in pollen deposition on the fifth, sixth, and seventh sampling, respectively (Figure 2.1). Pollen shed was at its highest level among all three maize fields on sampling date 5, although date 6 was statistically similar. Pollen density was statistically different among dates, fields, and positions ($P<0.0001$; $P = 0.0224$; and $P<0.0001$, respectively; data summarized in Table 2.1). Interaction effects (i.e.,

field*date, date*position, field*position, and field*date*position) were all significant ($P<0.0001$) for pollen deposition.

Total mean CFU values from leaves collected from the inside or outside maize fields did not differ significantly ($P = 0.0823$; Figure 2.2). However, total mean CFU values significantly differed among sampling dates ($P<0.0001$). There was not a significant interaction between date and position ($P = 0.36$). Collection date 6 had the most CFU reported, which was significantly greater than all other dates (based on differences of least squares means, $P<0.05$). Mean CFU values on dates 5, 7, 4, and 1 were not significantly different ($P>0.05$). CFU Means on dates 8 and 1 were similar ($P = 0.12$). Lowest CFU values were recorded on dates 3 and 2, which were significantly different from date 8 ($P = 0.0038$ and $P = 0.0007$, respectively). Order of highest to lowest mean CFU values for the overall effect of date for maize fields is 6>5>7>4>1>8>3>2.

Alternaria, *Aspergillus*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Penicillium*, *Trichoderma*, and Zygomycetes were the most frequently isolated genera or class of fungi (Tables 2.2A and B). Mean *Alternaria* CFU significantly differed among dates ($P = 0.0118$), where a peak at date 4 was observed and differed significantly from other dates (based on differences of least squares means, $P<0.05$). Mean *Aspergillus* CFU differed significantly among dates ($P<0.0001$) with a statistically significant peak of mean CFU on date 5 compared to other dates ($P<0.05$). Values of *Cladosporium* mean CFU significantly differed among sampling dates ($P=0.0299$), but without distinct peaks. *Fusarium* mean CFU values significantly differed ($P<0.0001$) among sampling dates and tended to have higher frequencies during dates 4-7 than date 8 and earlier dates. *Gliocladium* and *Penicillium* mean CFU did not significantly differ among sampling dates ($P=0.089$ and $P=0.19$, respectively). *Trichoderma* CFU values were nearly statistically significant ($P=0.0506$)

among sampling dates and tended to be higher on dates 7 and 1. Zygomycete mean CFU values were not significantly different among sampling dates (P=0.08). Mean CFU values for all genera did not significantly differ (P>0.05) inside and outside maize fields; while mean CFU values of class Zygomycetes inside the maize fields were significantly greater than those isolated on leaves outside the maize fields (P=0.0422). Interaction between date and position was tested and was not significant for all isolated fungi (P>0.05).

Correlations between mean total CFU and mean pollen densities on milkweed leaves inside and outside maize fields were tested. Inside maize fields the correlation coefficient (R) was 0.61 (P <0.0001; Figure 2.3). Mean pollen density and mean CFU outside of maize fields were weakly correlated (P = 0.31), and the R value was very small and not representative of the overall comparison, as more emphasis was placed on outliers for the test (when pollen density was not zero; Figure 2.4). Pollen densities and CFU amounts on milkweed leaves outside of the maize field were analyzed by sampling from milkweed plants in heterogeneous locations (two soybean fields and a roadside), which were exposed compared to the milkweed located within the confines of the maize canopy. However, total CFU did not significantly differ with or without pollen (inside or outside maize fields; Figure 2.5). Trends of CFU for the most frequently isolated fungi throughout the sampling period are compared to mean pollen deposition in Figure 2.6.

Isolates were identified to the species level for the genus *Fusarium* (Figure 2.7). *Fusarium graminearum* was the most frequently identified species on date 1 inside maize fields. On date 2 and dates 4-8, CFU of *Fusarium verticillioides/proliferatum* were the most prevalent inside maize fields. *Fusarium verticillioides/proliferatum* CFU values were also the highest *Fusarium* species or species grouping outside of maize fields on dates 4 through 8. *Fusarium sporotrichioides* was the most frequently identified species on date 3 inside

maize fields and on date 1 outside maize fields. *Fusarium subglutinans* and *Fusarium acuminatum* were the most prevalent species on dates 2 and 3 outside maize fields, respectively. The order of the most to the least frequently isolated species over all sampling dates was: *F. verticillioides/proliferatum*, *Fusarium semitectum*, *F. sporotrichioides*, *F. graminearum*, *Fusarium equiseti*, *Fusarium subglutinans*, *Fusarium acuminatum*, and *Fusarium poae*. Five percent of *Fusarium* CFU was not identified to species.

Discussion

Total area of milkweed leaf material used in statistical analyses included upper and lower surfaces of leaf discs, which resulted in conservative mean CFU values per cm^2 milkweed leaf material. Leaf discs were immersed in the buffer solution and shaken, which permitted both lower and upper surfaces to be rinsed. Breeze and Dix (1981) reported that the upper side contained more fungal spores or conidia than the underside of leaf. Excluding the lower surface area would produce exaggerated results for mean CFU values assumed to represent only fungi located on the upper surface of milkweed leaves. Garg et al. (1978) found that species diversity differed between the two surfaces. Some genera tended to be limited to one surface (e.g., *Epicoccum*, *Fusarium* on the upper surface), while others were present on both surfaces of barley leaves (e.g., *Alternaria*, *Cladosporium*; Garg et al., 1978). Therefore, some genera may be better represented because both surfaces were considered, while others may be underestimated.

Pollen on surfaces of leaves provides an adequate substrate for fungal growth. Previous studies (Fokkema [1968 and 1971], Warren [1972 and 1976], Garg and Sharma [1982], and Kumar and Mishra [1991]) indicated that fungal populations on leaf surfaces tend to increase with pollen deposition. Therefore, fungal CFU on milkweed leaves located

inside and outside of the maize field were expected to differ. However, fungal population trends were similar between two sugar beet fields (one pollinating and another without flowers, 200 m apart; Warren, 1972). In the sugar beet study, some pollen was deposited on leaves of flowerless plants, and fungal colony numbers were lower on leaves of flowerless plants until the end of the sampling period (Warren, 1972). Results with milkweed plants inside and outside of maize fields also demonstrated this trend. Fungal populations within maize fields may increase with pollen shed, alter the composition of aeromycoflora, and be present in areas surrounding maize fields (i.e., milkweeds outside of maize fields). Fungi reported as being on the surface of leaves are not necessarily colonizing leaf surfaces, but may be colonizing pollen. Conidia transported through the air may be present on leaf surfaces and removed by leaf washing (Fokkema, 1968; Garg et al., 1978; Dickinson, 1971; Warren, 1972).

The decrease of CFU values on date 2 is puzzling. Rainfall from the first to the second collection date was less than 1 cm; whereas, total rainfall between dates 2 and 3 was about 8 cm, which could explain the low number of CFU recorded for date 3. Rainfall can wash off pollen (Jesse and Obrycki, 2000; Sears et al., 2000; Zangerl et al., 2000; Pleasants et al., 2001; Stanley-Horn et al., 2001) and associated fungal material from milkweed leaf surfaces. However, humidity and leaf wetness stimulate fungal growth offsetting the removal.

Fungi isolated from milkweed leaf surfaces in this study are similar to other reports of fungi harbored on milkweed. *Cercospora* and *Septoria* grow on *A. syriaca* in Iowa, while in other states *Alternaria*, *Cladosporium*, *Phoma*, *Colletotrichum*, and *Phyllosticta* also have been present. *Nigrospora*, pycnidial fungi, and other hyphomycetes were also present in this study, but were not as prevalent or frequently observed as the other fungi discussed. Yeasts

commonly inhabit the phylloplane (e.g., Last and Deighton, 1965), but were not identified. This could be due to methods used to grow, isolate, and identify fungi.

Fusarium verticillioides, *F. proliferatum*, and *F. subglutinans* are commonly found in maize fields (e.g., Leslie et al., 1990; Munkvold and Desjardins, 1997; Gillette, 1999). Gillette (1999) found *F. semitectum* and *F. sporotrichioides* to be the most frequently observed *Fusarium* species in ambient air of maize fields. Results from the present study show that the *F. verticillioides/proliferatum* grouping was the most prevalent *Fusarium* species followed by *F. semitectum* and *F. sporotrichioides*.

Various fungal CFU peaked as pollen deposition increased throughout anthesis. *Alternaria* CFU, recorded from milkweed leaf surfaces inside and outside maize fields, peaked on date 4 when pollen deposition inside maize fields was beginning to increase. *Aspergillus* CFU outside of maize fields on date 5 were observed to increase considerably; CFU values recorded from inside maize fields did not raise as abruptly on date 5. CFU values for *Cladosporium* and *Fusarium* recorded from milkweed leaves inside and outside maize fields appeared to increase when mean pollen deposition values were escalating (dates 4-6). CFU values for *Aspergillus* and *Fusarium* from inside and outside maize fields seemed to have pronounced increases throughout the period of anthesis compared to the values associated with other identified fungi. CFU values of *Gliocladium*, *Penicillium, Trichoderma* and Zygomycetes from inside maize fields tended to be low, although some increases in CFU were observed as mean pollen density was decreasing (e.g., on date 7). Outside of maize fields, small peaks in *Gliocladium*, *Trichoderma* and Zygomycetes CFU were observed around the peak period of anthesis.

Some fungi produce secondary metabolites that can be toxic to insects. Dowd et al. have conducted numerous studies on the interaction of fungi and fungal metabolites

(primarily from *Aspergillus*, *Fusarium*, and *Penicillium*) on maize pests (*Spodoptera frugiperda*, J.E. Smith and *Heliothis zea*, Boddie) or vectors (*Carpophilus hemipterus* L.) (e.g., Dowd, 1988, 1989;; Dowd and Middlesworth, 1989; Dowd et al., 1989, 1992). Most of the fungi identified in this study are capable of being toxigenic. Mycotoxins can be produced by *Alternaria* (e.g., cyclopiazonic acid), *Cladosporium* (e.g., epicladosporic acid), *Trichoderma* (e.g., glioviridin; Klein and Eveleigh, 1998), and *Gliocladium* (e.g., gliotoxin; Samuels et al., 1984). *Fusarium* can produce mycotoxins, such as butenolides, trichothecenes, and a polyketide (Marasas et al., 1984; Samuels et al., 1984). *Fusarium verticillioides* and *F. proliferatum*, the most frequently observed species grouping in this study, for example, can produce fumonisins (Nelson et al., 1992; Bacon and Nelson, 1994). However, there is little information on the effects of fungal metabolites on non-vector or non-pest insects.

Pollen deposition on milkweed leaf surfaces was not found to stimulate fungal growth significantly in this study. Mean pollen densities were correlated with mean total CFU values inside maize fields, although CFU values did not significantly differ inside and outside maize fields. Monarch butterfly larvae consuming milkweed leaf tissue in conditions similar to those analyzed in this study could be adversely affected. Larvae consuming milkweed material coated with pollen during anthesis or accumulated from anthesis (i.e., inside maize fields) could be exposed to peak amounts of fungal CFU. Results also indicate that monarch larvae feeding on milkweed leaves outside of maize fields may be exposed to smaller amounts of pollen, but these larvae could be exposed to large amounts of fungal CFU. The most frequently isolated fungi were capable of producing mycotoxins, which could affect monarch feeding behavior and survival.

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Figure 2.1. Trend of pollen deposition on milkweed leaves within and outside three maize fields throughout 8 dates in the summer of 2002.

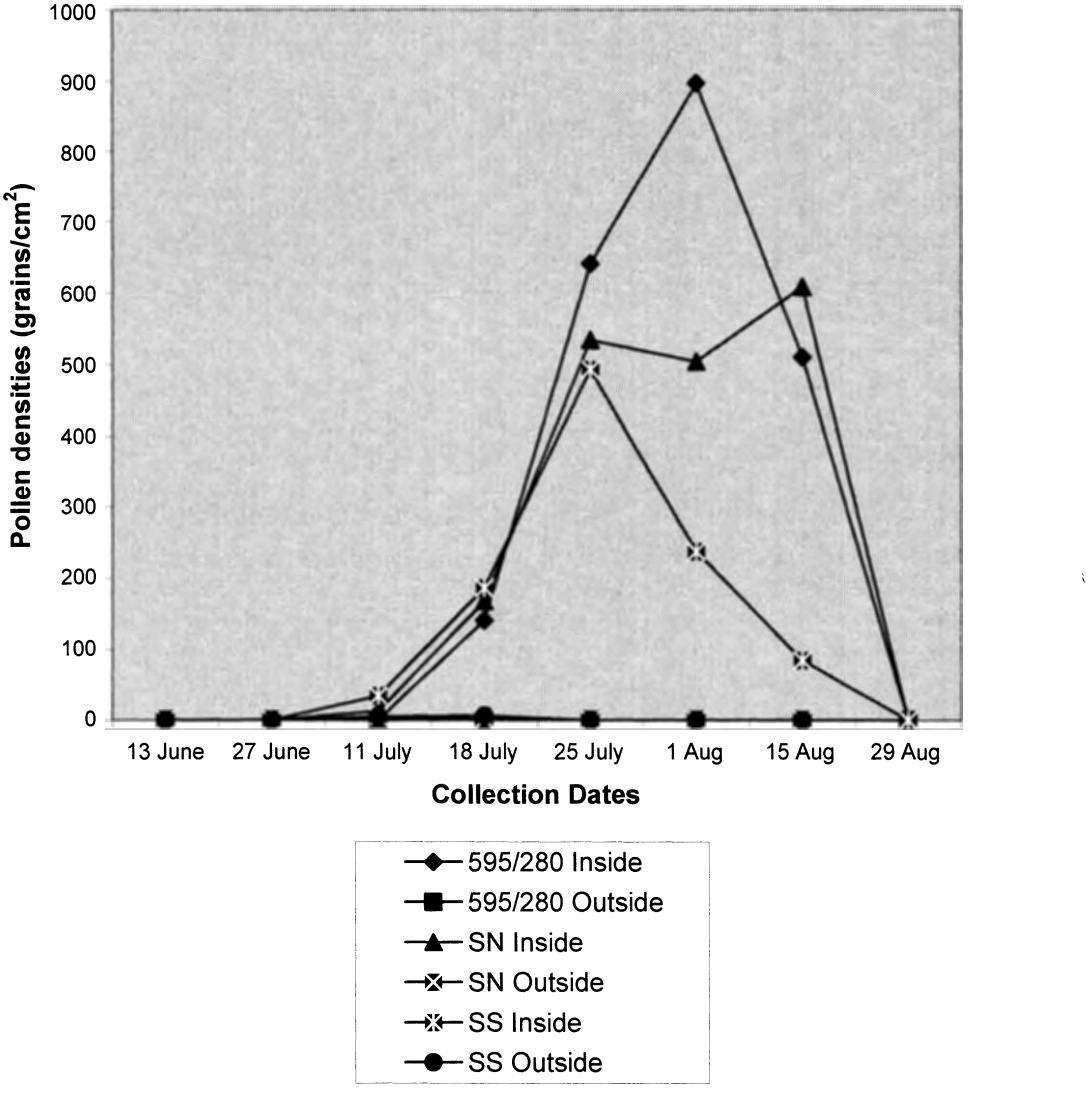


Table 2.1. Mean pollen deposition (grains/cm^2) on randomly collected milkweed leaves inside and outside of three maize fields on eight dates during the summer of 2002.

	595/280		Shipley North		Shipley South	
Date	Inside	Outside	Inside	Outside	Inside	Outside
13 June	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
27 June	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
11 July	1 +/- 1	1 +/- 1	12 +/- 3	5 +/- 4	0 +/- 9	0 +/- 2
18 July	140 +/- 60	0 +/- 0	170 +/- 60	1 +/- 1	34 +/- 81	4 +/- 3
25 July	640 +/- 70	0 +/- 0	530 +/- 75	1 +/- 1	236 +/- 93	7 +/- 0
1 August	900 +/- 140	0 +/- 0	500 +/- 88	0 +/- 0	493 +/- 71	0 +/- 0
15 August	500 +/- 97	0 +/- 0	610 +/- 62	0 +/- 0	84 +/- 28	0 +/- 0
29 August	0 +/- 0	0 +/- 0	0 +/-0	0 +/- 0	0 +/- 0	0 +/- 0

(Means +/- standard error)

Figure 2.2. Trend of fungal growth on collected milkweed leaves from within and outside three maize fields throughout 8 dates in the summer of 2002.

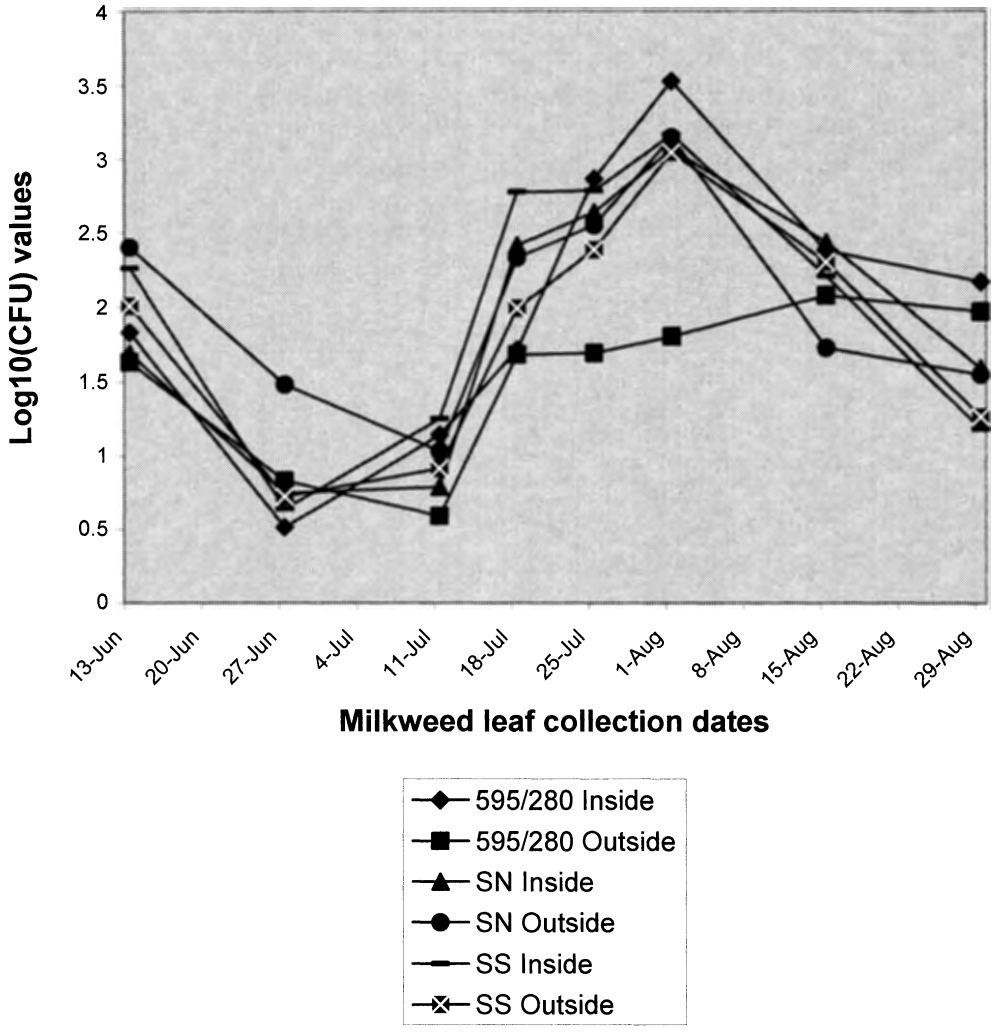


Table 2.2A. Means and standard deviations of colony-forming units/cm^2 of leaf surface frequently isolated genera and class of fungi from the surface of milkweed leaves inside maize fields.

Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD
1	*Fusarium*	0.32	0.48	2	*Fusarium*	0.42	0.11	3	*Aspergillus*	0.40	0.69	4	*Alternaria*	0.40	0.63
	Cladosporium	0.18	0.43		*Aspergillus*	0.21	0.45		*Alternaria*	0.11	0.29		*Fusarium*	0.38	0.55
	Trichoderma	0.15	0.31		*Alternaria*	0.07	0.15		*Fusarium*	0.08	0.26		*Cladosporium*	0.14	0.42
	Gliocladium	0.06	0.23		*Penicillium*	0.06	0.23		*Penicillium*	0.05	0.23		Zygomycete	0.09	0.31
	Penicillium	0.04	0.22		*Cladosporium*	0.01	0.05		*Trichoderma*	0.03	0.08		*Gliocladium*	0.04	0.22
	Alternaria	0.04	0.22		*Gliocladium*	0	0		*Gliocladium*	0.01	0.05		*Trichoderma*	0.03	0.10
	Aspergillus	0.04	0.22		*Trichoderma*	0	0		*Cladosporium*	0	0		*Penicillium*	0.02	0.07
	Zygomycete	0.01	0.05		Zygomycete	0	0		Zygomycete	0	0		*Aspergillus*	0.01	0.05
5	*Fusarium*	0.71	0.84	6	*Fusarium*	0.45	0.78	7	*Fusarium*	0.54	0.69	8	Zygomycete	0.18	0.45
	Aspergillus	0.46	0.91		*Cladosporium*	0.19	0.67		*Trichoderma*	0.35	0.68		*Fusarium*	0.15	0.48
	Cladosporium	0.29	0.81		Zygomycete	0.17	0.48		Zygomycete	0.23	0.56		*Gliocladium*	0.14	0.43
	Zygomycete	0.18	0.46		*Alternaria*	0.17	0.53		*Alternaria*	0.23	0.59		*Aspergillus*	0.06	0.30
	Alternaria	0.14	0.46		*Gliocladium*	0.12	0.43		*Penicillium*	0.22	0.59		*Alternaria*	0.05	0.32
	Penicillium	0.08	0.36		*Trichoderma*	0.09	0.30		*Gliocladium*	0.15	0.44		*Penicillium*	0.02	0.10
	Trichoderma	0.08	0.36		*Aspergillus*	0.02	0.18		*Cladosporium*	0.05	0.25		*Trichoderma*	0.01	0.06
	Gliocladium	0.03	0.18		*Penicillium*	0	0		*Aspergillus*	0.03	0.18		*Cladosporium*	0	0

Table 2.2B. Means and standard deviations of colony-forming units/cm^2 of leaf surface frequently isolated genera and class of fungi from the surface of milkweed leaves outside maize fields.

Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD	Date	Fungus	Mean[a]	SD
1	*Trichoderma*	0.29	0.48	2	*Aspergillus*	0.12	0.37	3	*Aspergillus*	0.16	0.48	4	*Fusarium*	0.47	0.57
	Fusarium	0.18	0.42		*Alternaria*	0.08	0.15		*Alternaria*	0.13	0.37		*Alternaria*	0.34	0.55
	Cladosporium	0.16	0.40		*Penicillium*	0.05	0.23		*Fusarium*	0.10	0.32		*Trichoderma*	0.14	0.37
	Aspergillus	0.07	0.30		*Fusarium*	0.02	0.07		*Cladosporium*	0.09	0.45		Zygomycete	0.13	0.37
	Alternaria	0.07	0.25		*Cladosporium*	0	0		*Penicillium*	0.09	0.31		*Gliocladium*	0.07	0.30
	Gliocladium	0.04	0.22		*Gliocladium*	0	0		*Trichoderma*	0.03	0.10		*Cladosporium*	0.04	0.22
	Penicillium	0.04	0.22		*Trichoderma*	0	0		Zygomycete	0.01	0.05		*Aspergillus*	0.02	0.10
	Zygomycete	0	0		Zygomycete	0	0		*Gliocladium*	0	0		*Penicillium*	0.01	0.05
5	*Aspergillus*	1.04	1.12	6	*Fusarium*	0.29	0.58	7	*Fusarium*	0.37	0.63	8	*Gliocladium*	0.27	0.66
	Fusarium	0.42	0.72		*Gliocladium*	0.26	0.64		*Alternaria*	0.16	0.51		*Aspergillus*	0.12	0.44
	Alternaria	0.18	0.56		*Aspergillus*	0.13	0.50		*Trichoderma*	0.12	0.40		*Alternaria*	0.09	0.38
	Trichoderma	0.17	0.46		*Cladosporium*	0.13	0.50		*Penicillium*	0.04	0.19		*Trichoderma*	0.08	0.30
	Zygomycete	0.14	0.49		*Alternaria*	0.11	0.36		*Aspergillus*	0.01	0.06		*Penicillium*	0.03	0.18
	Cladosporium	0.11	0.47		Zygomycete	0.05	0.32		*Gliocladium*	0.01	0.06		Zygomycete	0.01	0.06
	Penicillium	0.10	0.40		*Penicillium*	0.04	0.31		Zygomycete	0.01	0.04		*Fusarium*	0.01	0.04
	Gliocladium	0.09	0.44		*Trichoderma*	0.04	0.19		*Cladosporium*	0	0		*Cladosporium*	0	0

a Means are based on log10 values of CFU

Figure 2.3. Similar trends for pollen shed and CFU on the surface of milkweed leaves throughout the 8 dates for the mean of three maize fields in the summer of 2002.

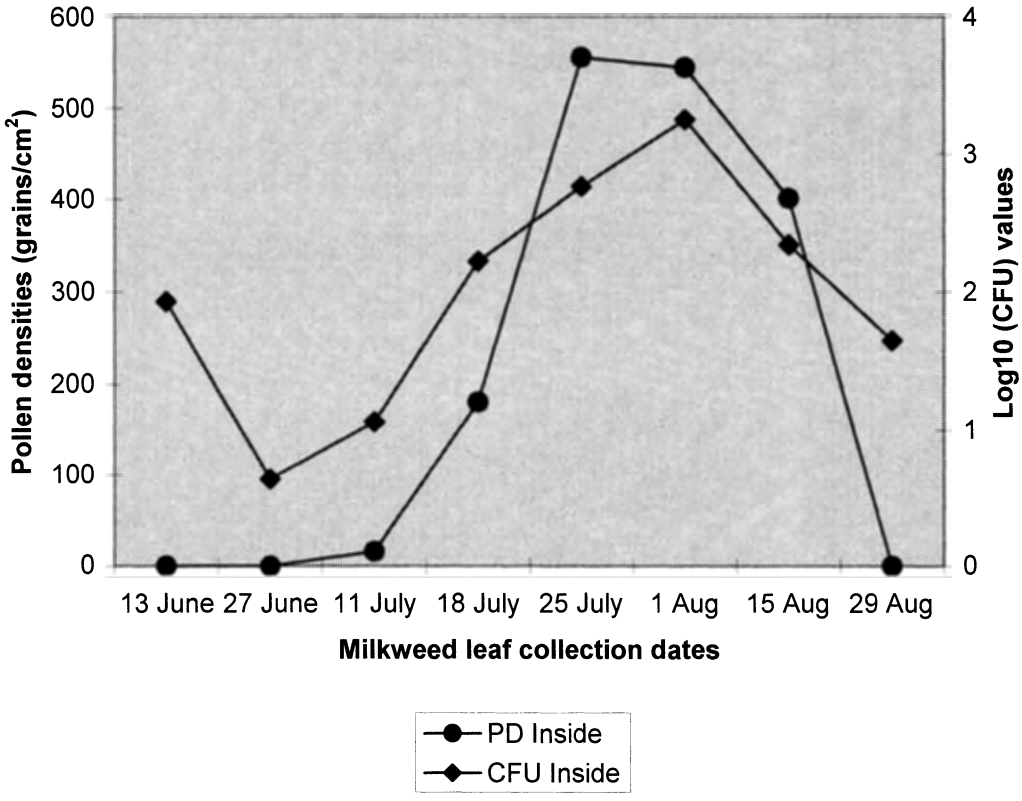


Figure 2.4. Contrast of CFU with pollen shed on milkweed leaves for the mean of three areas outside of maize fields throughout 8 dates in the summer of 2002.

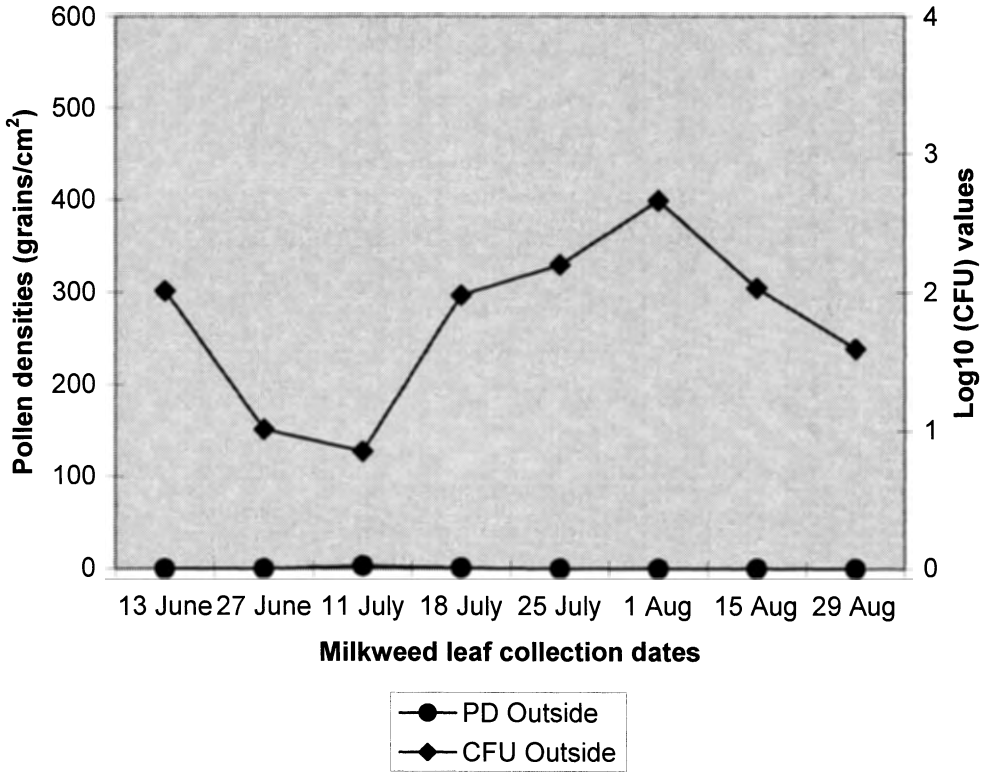


Figure 2.5. Similar trends for pollen shed and CFU on the surface of milkweed leaves throughout the 8 dates for means inside and outside of three maize fields in the summer of 2002.

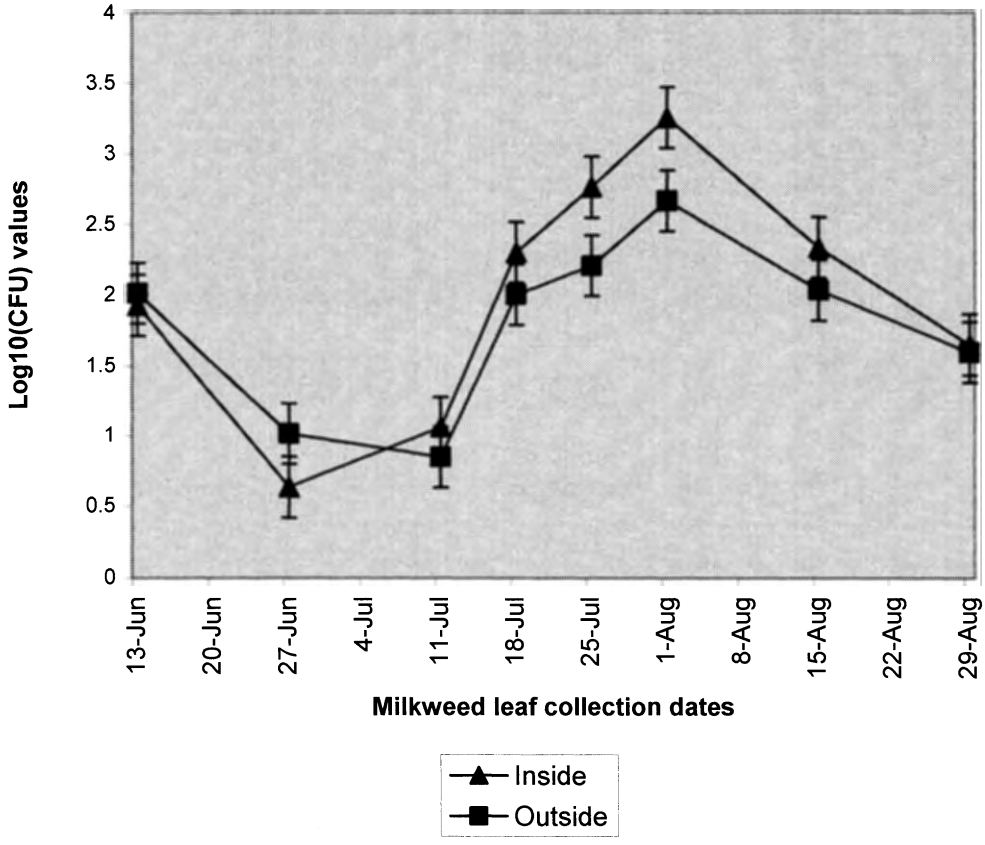
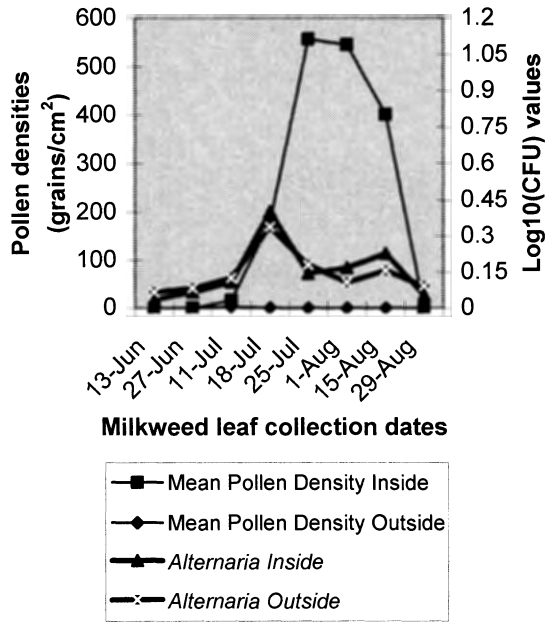
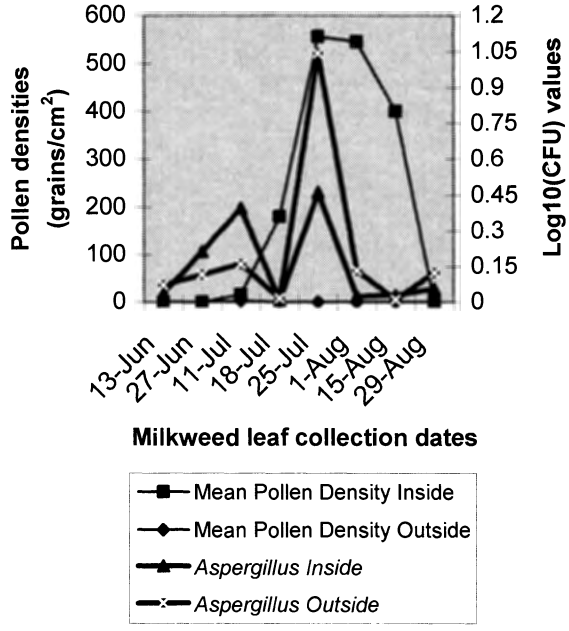


Figure 2.6. Trends for pollen shed and fungal CFU on the surface of milkweed leaves throughout the 8 dates for means inside and outside of three maize fields in the summer of 2002 for fungi including A) *Alternaria*, B) *Aspergillus*, C) *Cladosporium*, D) *Fusarium*, E) *Gliocladium*, F) *Penicillium*, G) *Trichoderma*, and H) Zygomycetes.

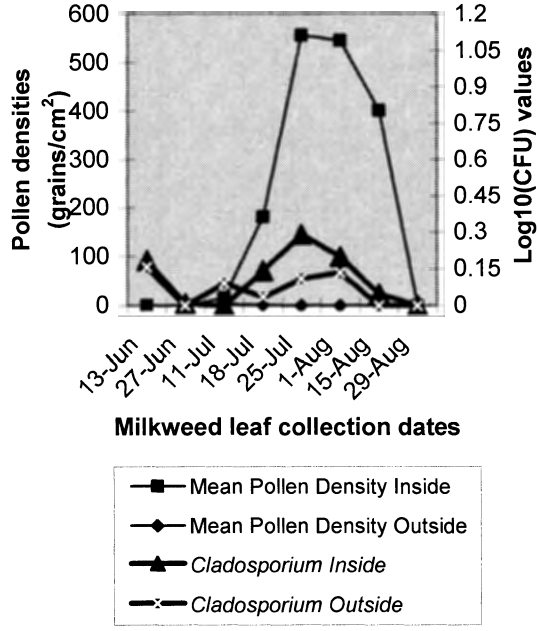
A.



B.



C.



D.

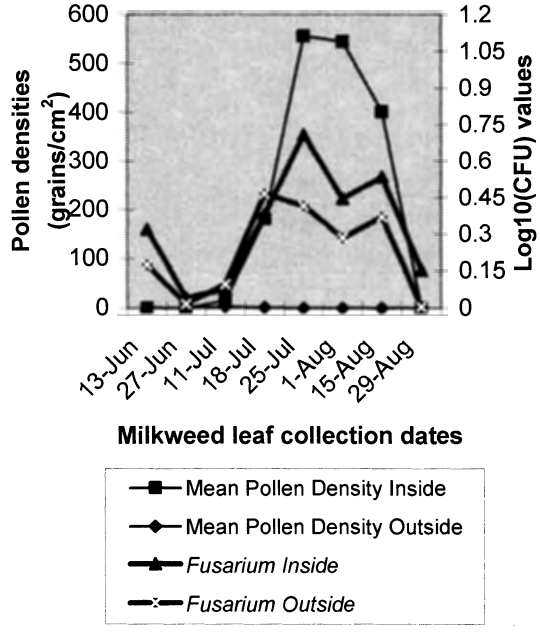
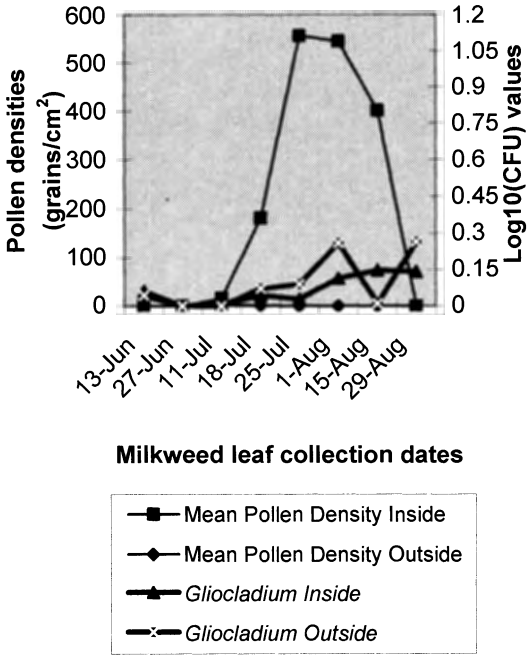
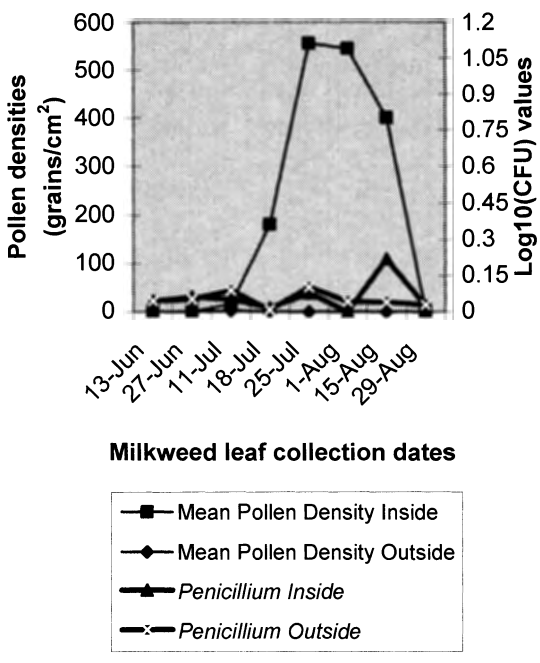


Figure 2.6. (continued)

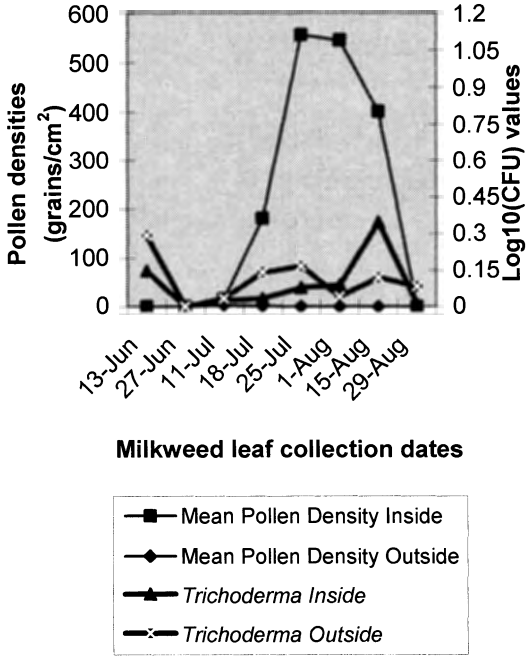
E.



F.



G.



H.

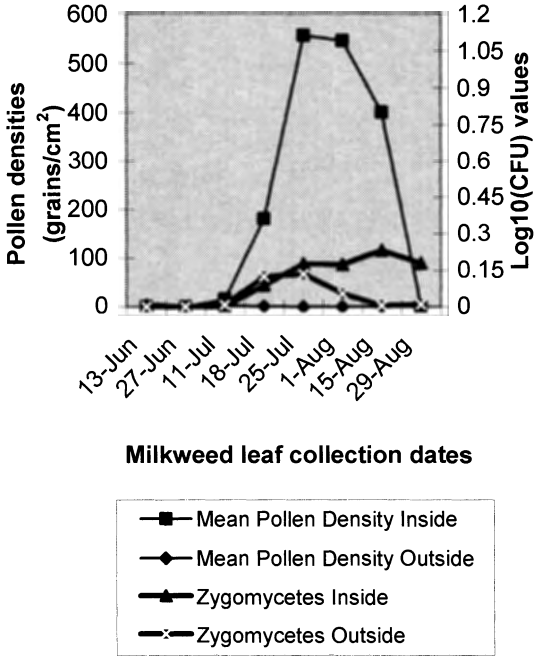
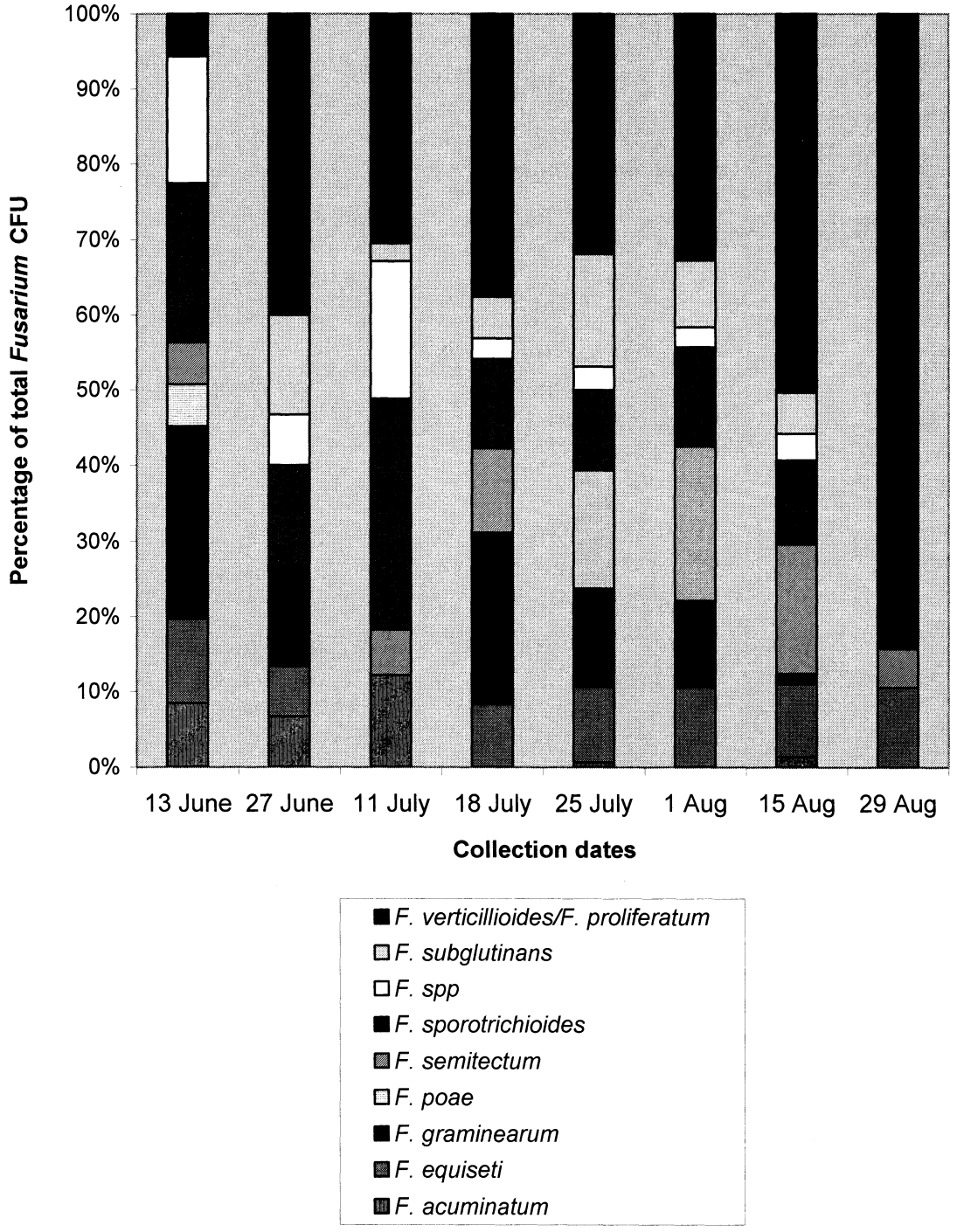


Figure 2.7. An overall comparison of the *Fusarium* species present on the milkweed leaves throughout the 8 dates in the summer of 2002.



CHAPTER THREE

INTERACTION OF FUNGI WITH MONARCH BUTTERFLY LARVAE AND BT PROTEIN IN EVENT 176 POLLEN

A paper to be submitted to Environmental Entomology

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Abstract

Milkweed leaves are the primary food source for larvae of *Danaus plexippus*. Maize pollen present on the milkweed leaf surface can affect organisms that reside on or consume milkweed. Pollen on leaf surfaces can influence distributions of fungal populations on milkweed leaves, and fungal colonization on pollen can present a hazard to *D. plexippus* larvae in addition to pollen from Bt (event 176) maize. *Danaus plexippus* larvae administered milkweed leaf discs sprayed with *Fusarium sporotrichioides* and *Cladosporium* consumed less leaf tissue and weighed significantly less after 14 or 15 d compared to water-treated leaves. Fungi present with event 176 pollen on milkweed leaf discs fed to *D. plexippus* larvae did not significantly reduce the toxicity of Bt pollen. Event 176 pollen sprayed with conidial suspensions of *Alternaria*, *Cladosporium*, *Fusarium proliferatum*, and *F. sporotrichioides* did not significantly differ in detected concentrations of Cry1Ab from event 176 pollen treated with water. Cry1Ab concentration per pollen grain differed significantly among incubation periods as degradation occurred. Fungi did not significantly

degrade Cry1Ab with event 176 pollen in *D. plexippus* bioassays, and ELISA results did not demonstrate that fungal colonization contributed to lower Cry1Ab concentrations.

Introduction

Monarch butterfly (*Danaus plexippus* L.) larvae use milkweed extensively throughout their development, as plants from the milkweed family (Asclepiadaceae) are their only hosts (Bhowmik and Bandeen, 1976). Common milkweed (*Asclepias syriaca* L.) is a perennial weed (Bhowmik and Bandeen, 1976). Milkweed plants are widespread north of the 36th parallel through New Brunswick and as far west as South Dakota and Kansas (Woodson, 1954); roadsides are one of the areas most populated by milkweed. Agricultural land accounts for 78% of Iowa, and 46% and 57% of the maize fields and soybean fields analyzed, respectively, were infested by milkweed (Hartzler and Buhler, 2000). In a survey of five states or provinces (Ontario, Maryland, Wisconsin, Minnesota, and Iowa), nonagricultural areas contained higher densities of milkweed plants, and monarch productivity was four times higher in nonagricultural areas than in maize fields, although production of monarch larvae within maize fields was 45 times the amount of monarch larvae produced in nonagricultural areas (Oberhauser et al., 2001). Survival of monarchs in four of the five sites was better both within maize fields and nonagricultural areas compared to maize field edges (Oberhauser et al., 2001).

Cry1Ab, an insecticidal protein, is produced by the bacterium *Bacillus thuringiensis* subsp. *kurstaki* (Bt). The gene *Cry1Ab* that codes for the production of this protein (a δ-endotoxin) has been modified, truncated, and inserted into the maize genome. Bt toxin on host plant material has influenced feeding behavior of insects (e.g., consume more host plant material when their food was substandard, or compensate for any sublethal effects incurred

as younger instars; Farrar et al., 1989; Meade and Hare, 1993; Pilcher et al., 1997). Several events of genetically modified maize exist (e.g., event 176), and the location and concentration of Bt toxin differ among events. Two promoters (phosphoenolpyruvate carboxylase and a maize-specific promoter) in event 176 maize plants are used to produce Cry1Ab, which is expressed in green tissue and pollen (Koziel et al., 1993; Fearing et al., 1997). Cry1Ab expression in event 176 pollen can be as high as 7.1 μg/g pollen (Stanley-Horn et al., 2001).

Bt (event 176) maize pollen present on the milkweed leaf surface can affect organisms that reside on or consume milkweed. Bt maize pollen shed and deposition was studied in response to a 1999 Data Call-in by the Environmental Protection Agency (EPA) to evaluate whether pollen from Bt maize posed a threat to *D. plexippus* larvae (Jesse and Obrycki, 2000; Sears et al., 2000; Wraight et al., 2000; Pleasants et al., 2001; Stanley-Horn et al., 2001) following the correspondence by Losey et al. (1999). Maize pollen grains are approximately 90 μm in size and are prone to deposit rapidly (Raynor et al., 1972). An estimated 40% of maize pollen can be deposited on the leaf surfaces of milkweed plants that infest maize fields (comparing pollen densities on sticky slides and neighboring milkweed leaves through regression; Pleasants et al., 2001). Mean pollen deposition within maize fields was estimated to be 171 pollen grains/cm^2, and pollen densities over 1,400 pollen grains/cm^2 within fields were not common (Pleasants et al., 2001). Sears et al. (2001) estimated that the risk to monarch butterfly larvae of encountering Bt pollen was very low in 2000 (2.1% and 0.8% of the Iowa population and total eastern monarch population, respectively). Nonetheless, event 176 pollen at pollen densities of 5-133 pollen grains/cm^2 can elicit lethal and sublethal effects in *D. plexippus* larvae (Sears et al., 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001).

Danaus plexippus larvae may encounter other contaminants on milkweed leaf surfaces as pollen can influence the distribution of fungal populations on leaves (Kinkel, 1997). Pollen acts as a nutrient source on the leaf surface, stimulating fungal growth similar to the growth of sooty mold on aphid honeydew (e.g., Davison, 1991; Sparks and Yates, 1991). The effect pollen deposition on fungal populations on the surface of leaves was reported in Fokkema (1968 and 1971), Warren (1972 and 1976), Garg and Sharma (1982), and Kumar and Mishra (1991). Pollen grains served as substrates for fungi, and numbers of fungal colonies differed between leaves with and without pollen.

Milkweed leaves, when coated with pollen and colonized by fungi associated with maize, may present another hazard to *D. plexippus* larvae in addition to (event 176) Bt maize pollen. Some fungi produce secondary metabolites that can be toxic to insects. Dowd et al. have conducted numerous studies on the interaction of fungi and fungal metabolites (primarily from *Aspergillus*, *Fusarium*, and *Penicillium*) on maize pests (*Spodoptera frugiperda*, J.E. Smith and *Heliothis zea*, Boddie) or vectors (*Carpophilus hemipterus* L.) (e.g., Dowd, 1988, 1989; Dowd and Middlesworth, 1989; Dowd et al., 1989, 1992). However, there is little information on effects of fungal metabolites on non-vector or non-pest insects.

Fungal colonization also may affect Bt toxin concentration within event 176 maize pollen. Soil microorganisms likely assist in degrading Bt in maize tissue (e.g., Sims and Holden, 1996) or purified Bt protein (e.g., Herman et al., 2002) in soil. However, studies examining Bt protein degradation in Bt maize pollen are not common.

Fungal growth on milkweed leaves has been observed in the field. While many studies have assessed the effect Bt maize pollen has on *D. plexippus* larvae, feeding behavior and success may be influenced by fungal populations on milkweed leaf surfaces. Fungi have

been assumed to assist in Bt degradation of purified protein and in plant tissues in soil. Likewise, fungal colonization following Bt maize pollen deposition on milkweed leaf surfaces may contribute to the degradation of Bt proteins in maize pollen. Objectives for this study were (i) to observe how fungi on milkweed leaves compared to water- and event 176-treated milkweed leaves influenced feeding behavior, weights, and mortality of *D. plexippus* larvae after two time periods and (ii) to test whether fungi assisted in degrading Bt protein within pollen on milkweed leaves and slides.

Materials and Methods

Insect bioassays with *D. plexippus* larvae were conducted to observe and compare effects on mortality, consumption and weight for different incubation periods (duration from the spraying of milkweed leaves until bioassays are initiated). Pollen and fungal material on glass slides were analyzed for Cry1Ab concentrations by enzyme-linked immunosorbent assays (ELISA) to determine how the presence of fungi affected the rate of Bt degradation.

Bioassay *Insects and pollen. D. plexippus* eggs and larvae were collected around Ames, Iowa, during the summers of 2002 and 2003 in order to establish the monarch colony. Monarch butterflies oviposited on *Asclepias curassavica* L. plants in cages, and eggs were removed from milkweed leaves, surface sterilized and incubated in environmental chambers at 26.7°C. Neonates <24 h old were introduced into arenas in order to conduct bioassays. Pollen was from Mycogen hybrid 2249 IMI, which contains Bt event 176 (Mycogen Seeds, Altoona, IA). Pollen had been collected July 29- August 1, 2001 and stored at –70°C.

Greenhouse Study A. Within the ISU Plant Pathology Greenhouse, leaves of *A. curassavica* were sprayed with (i) water, (ii) water and event 176 pollen, (iii) *F. sporotrichioides* conidial suspension, and (iv) event 176 pollen and *F. sporotrichioides*

conidial suspension. Conidia of *F. sporotrichioides* (10^5 conidia per ml) were suspended in sterile distilled water and were obtained from fungal growth on potato dextrose agar (isolates from milkweed leaf surfaces in 2001).

Milkweed leaves were sprayed with a thin-layer chromatography (TLC) sprayer (model 422550; Kimble-Kontes, Vineland, NJ) in which 4 ml of sterile distilled water that served as the control and vehicle for pollen, and 4 ml of the conidial suspension were used for spraying. Approximately 3 ml of the suspension was sprayed onto milkweed leaf surfaces. The progression of spraying began with treatment (i) followed by treatment (ii) and the pollen portion of the event 176 pollen + fungus treatment. The conidial suspension was sprayed last. Separate vials were designated per treatment (i.e., a water/pollen vial, and a vial for the fungus), and the TLC sprayer was thoroughly rinsed between each spraying. Plastic dividers were constructed to prevent contamination among treatments.

Milkweed leaves were collected separately by treatment and transported to the laboratory after incubation periods ended, laying flat in separate coolers on ice packs wrapped in paper towels. Treated leaves were transported back to the lab within individual plastic bags (4 x 6 2-ml or 6 x 8 2-ml) in order to estimate pollen densities with a dissection scope and stage micrometer (Olympus SZ40). Arenas were prepared by forming a double layer of solidified agar (2.5% wt/vol.; approximately 2 mm thick/layer) and cutting two holes in the agar with a #12 cork borer (16 mm in diameter) in small Petri dishes (60 x 15 mm). Milkweed leaf discs for each treatment were cut from treated *A. curassavica* leaves with a flamed # 14 cork borer (20 mm in diameter) and sandwiched between the layers of agar within the arenas labeled for each treatment. Neonate *D. plexippus* were introduced into arenas with a camelhair paintbrush. Arenas were arranged in a randomized complete block design on trays and incubated for four days at 26.7°C with 18:6h (L:D) and 50% relative

humidity. Milkweed leaves were collected and processed 7 and 14 days after spraying the treatments. Each treatment had 25 replicates.

Greenhouse Study B. This experiment followed the same procedures as Greenhouse Study A, except the fungus *Cladosporium* was used at a conidial suspension concentration of 10^6 conidia/ml sterile distilled water. Each treatment had 25 replicates.

Data Collection. Observations (leaf consumption and larval mortality) were made at 48 and 96 h. Consumption (mm^2) was measured, using a dissecting microscope (Stereo-Zoom; Nikon SMZ-1000) fitted with a stage micrometer. Live larvae on the fourth day were weighed (mg).

Degradation. *Slides.* Similar to the spraying of the milkweed leaves, slides (plain, 25 x 75 x 1 mm) were sprayed with a pollen from event 176 hybrid (Mycogen) suspended in water, followed by either water or four different conidial suspensions. *Alternaria*, *Cladosporium*, *F. proliferatum*, and *F. sporotrichioides* were sprayed at concentrations of 10^5 conidia per ml.

Sixteen slides were placed on each tray, and four trays per treatment were sprayed with a TLC sprayer under a fume hood. Each tray was lined with paper towels, which were moistened with sterile distilled water and arranged to completely cover the tray. Event 176 maize pollen in 4 ml sterile distilled water was sprayed onto the slides for each tray. Treatments included (i) sterile distilled water, (ii) *Alternaria*, (iii) *Cladosporium*, (iv) *F. proliferatum*, and (v) *F. sporotrichioides*. Water was first sprayed on top of the pollen suspension before each conidial suspension was sprayed. Beginning with the water treatment, sprayed slides were removed and placed into crispers before proceeding to the next treatment to prevent contamination. Slides were allowed to dry and were placed on top of moistened paper towels within a designated crisper for each tray. Four crispers per

treatment were then stacked and wrapped in garbage bags to incubate. Crispers were randomly placed on a bench top and rerandomized after each sampling period.

Periods of incubation were 0, 1, 2, 3, 4, 7, 14, and 21 days. The percentage of fungal colonization and mean pollen density per slide were estimated and recorded before processing. Areas were considered to be colonized if hyphae were present (6 random areas/slide; within grid of 0.25 cm^2). A sterile razor was used to scrape the material off of the slide onto a piece of filter paper (90 mm in diameter, Whatman®) to funnel the material into a microcentrifuge tube in order to remove the pollen and associated fungal growth effectively. Two slides were sampled per collection date per treatment, and the tubes were immediately placed on ice until stored in a freezer (-13°C). All samples were lyophilized for 24 h.

A Mini Beadbeater (BioSpec Products Inc.; Bartlesville, OK) was utilized to pulverize the lyophilized pollen and fungal material gathered from the slides. A consistent volume of 0.5-mm glass beads was poured into 1.5-ml beadmill tubes. To the pollen/fungal material of each sample 750 µl of 1X PBS (pH 8.0) was added, and each sample was transferred to beadbeater tubes. The beadbeater was set at 50 x 10 RPM and 4, 3, and 2 x 10 seconds for the first, second, and third beatings, respectively.

Using the bead-beaten samples, a double antibody sandwich (DAS) ELISA was used to determine the amount of Cry1Ab present within the pollen and fungal material at each interval. Chemicals and antibodies were purchased through Sigma: St. Louis, MO unless noted otherwise. Rabbit anti-Cry1Ab antibody (polyclonal; purified through protein A column chromatography; USDA-ARS, Corn Insects and Crop Genetics Unit), purified Cry1Ab protein (Case Western Reserve University; Cleveland, OH), mouse monoclonal anti-Cry1Ab (Abraxis Kits; Warminster, PA), and sheep anti-mouse IgG alkaline phosphatase

conjugate were used. This procedure was performed as described in Clark et al., 1986, with some modifications. Controls and samples were loaded and analyzed in triplicate. Whenever rinsing was necessary, 1X PBST (0.5% Tween) was used three times, except before loading the enzyme-labeled conjugate when the plate was rinsed seven times. The plate was blocked a second time after the incubation of the mouse anti-Cry1Ab antibody. The reaction was not stopped after the incubation of the alkaline phosphatase substrate p-nitrophenylphosphate. Cry1Ab amounts were read through a SPECTRAMax Plus plate reader (Molecular Devices Corp.; Sunnyvale, CA) at a wavelength of 405 with the SoftMax Pro software (2.2.1; 1998; Molecular Devices Corp.).

Analyses. Data were analyzed for normal distribution, and logarithmic transformations were applied as necessary. Treatments were analyzed as fixed effects and replicates were considered random effects. Analysis of variance (Proc Mixed) was used for bioassay data analyses to test for differences among treatments for consumption at 48 h and 96 h and larval weights, whereas, Chi Square (Proc Freq) and Fisher tests were used to compare mortality among treatments (SAS Institute, 2001). Means were separated through least significant differences and contrasts. ELISA-derived Cry1Ab concentrations (ng Cry1Ab/pollen grain) were compared and analyzed for the effects of the treatments, days, and fungal colonization through analysis of variance (Proc Mixed).

Results

Bioassays. *Greenhouse Study A.* Mean pollen densities for the pollen treatment and the pollen/fungus treatment were 174 and 93, respectively for greenhouse study A after spraying. *Fusarium sporotrichioides* was identified on sprayed milkweed leaves and on

milkweed leaf discs within arenas of the *D. plexippus* larvae, although the fungus was frequently overgrown by populations of *Cladosporium*, *Epicoccum* and *Alternaria.*

Significantly different results were recorded for the consumption of milkweed leaf material 7 days after being sprayed among treatments 48 h and 96 h after the beginning of the bioassay, as well as the weights of *D. plexippus* larvae recorded after 96 h in Greenhouse Study A ($F = 40.37$, df = 3, 88 for F48h; $F = 44.31$, df = 3, 58.8 for F96h; and $F = 17.66$, df = 3, 55 for weight; all $P<0.0001$; Table 3.1). Two distinct groupings were demonstrated by treatment means. Milkweed leaf disc consumption did not differ significantly between water and *F. sporotrichioides* treatments ($F = 0.09$, df = 1, 88, $P = 0.77$). *Danaus plexippus* larvae fed upon event 176 pollen- and event 176 pollen/*F. sporotrichioides*-treated milkweed leaf discs in similar amounts, also ($F = 0.00$, df = 1, 88, $P = 0.96$). Consumption of milkweed leaf material after 48 h significantly differed between the water and event 176 pollen treatments and between *F. sporotrichioides* and event 176 pollen/*F. sporotrichioides* ($F = 62.45$, df =1, 88, and $F = 58.6$, df = 1, 88, respectively; both $P<0.0001$). *Danaus plexippus* larvae consumed significantly less milkweed leaf material treated with event 176 pollen. Mortality among treatments did not significantly differ after 48 h (percentages of mortality for water, event 176 pollen, *F. sporotrichioides*, and event 176 pollen/*F. sporotrichioides* treatments were 9%, 23%, 4%, and 22%, respectively; $P > 0.05$). After 96 h *D. plexippus* larvae continued to feed more on water-treated milkweed leaf discs compared to event 176 pollen-treated milkweed leaf discs ($F = 76.13$, df = 1, 59.7, $P<0.0001$). Consumption of milkweed leaf discs between water and *F. sporotrichoides* treatments and between event 176 pollen and event 176 pollen/*F. sporotrichioides* did not significantly differ after 96 h ($F = 0.02$, df = 1, 57, $P = 0.89$; and $F = 0.59$, df = 1, 59.2, $P = 0.44$, respectively). Significant differences existed between the amount of milkweed leaf material consumed by *D. plexippus*

larvae given *F. sporotrichoides*- and event 176 pollen/*F. sporotrichioides*-treated milkweed leaf material (F = 61.52, df = 1, 59.8, P<0.0001). *Danaus plexippus* weights significantly differed between water and event 176 pollen treatments and between *F. sporotrichioides* and event 176 pollen/*F. sporotrichioides* treatments (F = 21.19, and F = 31.79, respectively; for both df = 1, 55 and P<0.0001). Similar weights of *D. plexippus* larvae were recorded for treatment comparisons between water and *F. sporotrichioides* treatments and between event 176 pollen and event 176 pollen/*F. sporotrichioides* treatments (F = 0.12, df = 1, 55, P = 0.73; and F = 0.38, df = 1, 55, P = 0.54, respectively). However, after 96 h the frequency of mortality differed significantly between water- and pollen-treated milkweed leaf discs and water- and pollen/*F. sporotrichioides*-sprayed milkweed leaf discs (percentages of mortality for water treatment, event 176 pollen treatment, *F. sporotrichioides* treatment, and event 176 pollen/*F. sporotrichioides* treatments were 20%, 58%, 20%, and 60%, respectively; $P < 0.01$ for both comparisons).

Significantly different values for consumption of milkweed leaf material 14 days after being sprayed at 48 and 96 h and weights were observed (F = 19.27, df = 3, 71.1 for F48h; F = 16.52, df = 3, 70.7 for F96h; and F = 22.62, df = 3, 61 for weight; all P<0.0001; Table 3.1). Milkweed leaf consumption after 48 and 96 h showed that the trend observed with milkweed leaf material 7 d after being sprayed changed with an additional week. *Danaus plexippus* larvae consumed significantly more milkweed leaf material treated with water than with other treatments after 14 d (water verses event 176 pollen at 48 h: F = 49.37, df = 1, 71.7, P <0.0001; at 96 h: F = 41.71, df = 1, 71, P<0.0001). Consumption of milkweed leaf material after 48 and 96 h significantly differed between *F. sporotrichioides* and water treatments (F = 29.57, df = 1, 70.6, and F = 16.08, df = 1, 70.3, respectively; all P<0.0001). Consumption of milkweed leaf discs after 48 h between event 176 pollen and event 176 pollen/*F.*

sporotrichioides treatments and between *F. sporotrichioides* and event 176 pollen/*F. sporotrichioides* treatments did not significantly differ ($F = 0.14$, df = 1, 71.7, $P = 0.71$, and $F = 1.88$, df = 1, 70.6, $P = 0.17$, respectively). After 96 h these comparisons did not change ($F = 0.49$, df = 1, 71, $P = 0.48$ for event 176 pollen verses event 176 pollen/*F. sporotrichioides*, and $F = 3.28$, df = 1, 70.3, $P = 0.07$ for *F. sporotrichioides* verses event 176 pollen/*F. sporotrichioides*). Weights also did not follow the trend of the previous bioassay, although again *D. plexippus* larvae weighed significantly more when administered milkweed leaf discs treated with water than other treatments (than event 176 pollen: $F = 55.71$, df = 1, 61.2, $P<0.0001$; than *F. sporotrichioides*: $F = 22.84$, df = 1, 59.4, $P<0.0001$). Weights of *D. plexippus* larvae fed event 176 pollen/*F. sporotrichioides*-treated milkweed leaf discs were not significantly different from weights associated with event 176 pollen and *F. sporotrichioides* treatments (with event 176 pollen: $F = 2.06$, df = 1, 62.5, $P = 0.16$, and with *F. sporotrichioides*: $F = 2.42$, df = 1, 61.9, $P = 0.12$). Mortality after 48 and 96 h did not significantly differ among the treatments (for 48 h: percentages of mortality for water, event 176 pollen, *F. sporotrichioides*, and event 176 pollen/*F. sporotrichioides* treatments were 0%, 4%, 0%, and 0%, respectively; $P>0.05$) with the exception of the water- vs. pollen-sprayed treatments after 96 h (percentages of mortality for water, event 176 pollen, *F. sporotrichioides*, and event 176 pollen/*F. sporotrichioides* treatments were 4%, 30%, 17%, and 16%, respectively; $P< 0.02$).

Greenhouse Study B. Milkweed leaves sprayed with event 176 pollen alone and *Cladosporium* and event 176 pollen had mean pollen densities of 80 and 62, respectively in the greenhouse study B. Mean pollen densities for the second bioassay of greenhouse study B were 47 pollen grains/cm^2 for event 176 pollen alone and 34 pollen grains/cm^2 for event 176 pollen and *Cladosporium*. *Cladosporium* was identified on sprayed milkweed leaves in

concert with *Alternaria*, and both were later confirmed to be present in arenas with *Epicoccum*, *Penicillium* and *Fusarium*.

Consumption of milkweed leaf material 7 days after spraying at 48 and 96 hours in the bioassay and larval weights were significantly different among treatments for the second greenhouse study as in Greenhouse Study A results ($F = 26.57$, df = 3, 88 for F48h; $F = 35.16$, df = 3, 92 for F96h; and $F = 29.12$, df = 3, 84 for weight; all $P<0.0001$; Table 3.2). Two groupings of treatment means were observed as larvae fed leaf material treated with pollen consumed less milkweed leaf material and weighed less than larvae not feeding on pollen after 48 h and 96 h. *Danaus plexippus* larvae fed water-treated and *Cladosporium*-treated leaf discs had similar consumptions after 48 and 96 h ($F = 0.74$, df = 1, 88, $P = 0.39$, and $F = 0.04$, df = 1, 92, $P = 0.84$, respectively). Significant differences were observed between the consumption of water- and event 176 pollen-treated and between *Cladosporium*- and event 176 pollen/*Cladosporium*- treated milkweed leaf discs by *D. plexippus larvae* after 48 h ($F = 40.91$, df = 1, 88, $P<0.0001$, and $F = 36.68$, df = 1, 88, $P<0.0001$, respectively). These significant differences were observed after 96 h, also (between water and event 176 pollen: $F = 68.31$, df = 1, 92, and between *Cladosporium* and event 176 pollen/*Cladosporium*: $F = 33.53$, df = 1, 92; both $P<0.0001$). Consumption by *D. plexippus* of event 176 pollen and event 176 pollen/*Cladosporium* were similar after 48 h ($F = 1.44$, df = 1, 88, $P = 0.23$), but were significantly different after 96 h ($F = 6.28$, df = 1, 92, $P = 0.014$). Weights of *D.plexippus* larvae did not significantly differ between water and *Cladosporium* treatments and between event 176 pollen and event 176 pollen/*Cladosporium* ($F = 0.02$, df = 1, 84, $P = 0.88$, and $F = 0.00$, df = 1, 84, and $P = 0.95$, respectively). Significant differences of weights for *D. plexippus* larvae were observed between water and event 176 pollen treatments and between *Cladosporium* and event 176 pollen/*Cladosporium*

treatments (F = 41.98, df = 1, 84, P<0.0001, and F = 44.81, df = 1, 84, P<0.0001, respectively). Survival of *D. plexippus* larvae after 48 h did not differ significantly among treatments (percentages of mortality for water, event 176 pollen, *Cladosporium*, and event 176 pollen/*Cladosporium* treatments were 0%, 4%, 4%, and 9%, respectively; P >0.24). However, mortality of larvae exposed to water- vs. event 176 pollen-sprayed treatments and event 176 pollen- vs. event 176 pollen/*Cladosporium*-sprayed treatments significantly differed after 96 h (P = 0.0016 and P < 0.02, respectively; percentages of mortality for water, event 176 pollen, *Cladosporium*, and event 176 pollen/*Cladosporium* treatments were 0%, 33%, 4%, and 4%, respectively).

Treatment means for consumption and weight measurements were not grouped together (i.e., water and *Cladosporium*; event 176 pollen and event 176 pollen/*Cladosporium*) 15 days after spraying milkweed leaves like treatment means from milkweed leaves sprayed 7 d earlier. Amounts of milkweed material consumed by *D. plexippus* larvae after 48 hours were not significantly different among all treatments (F = 1.75, df = 3, 88, P = 0.16). Means associated with consumption and weights after 96 hours were significantly different among treatments (F = 4.41, df = 3, 91, P = 0.0061, and F = 6.93, df = 3, 78, P = 0.0003, respectively). Significantly different consumptions by *D. plexippus* larvae between water- and event 176 pollen-treated milkweed leaf discs and between water- and *Cladosporium*-treated milkweed leaf material were observed after 96 h (F = 9.79, df = 1, 91, P = 0.0024 and F = 9.46, df = 1, 91, P = 0.0028, respectively). Similarities existed between the milkweed leaf material consumed by *D. plexippus* larvae for event 176 pollen and event 176 pollen/*Cladosporium*, as well as between *Cladosporium* and event 176 pollen/*Cladosporium* (F = 0.29, df = 1, 91, P = 0.59, and F = 0.21, df = 1, 91, P = 0.65, respectively). Weights significantly differed for the same comparisons as consumption

measured after 96 h (water verses event 176 pollen: $F = 13.02$, df = 1, 78, $P = 0.0005$, and water verses *Cladosporium*: $F = 14.21$, df = 1, 78, P = 0.0003). Similar weights were observed between event 176 pollen and event 176 pollen/*Cladosporium* and between *Cladosporium* and event 176 pollen/*Cladosporium* ($F = 0.01$, df = 1, 78 for both comparisons, and $P = 0.94$ and 0.92, respectively). Means for leaves treated with water were significantly higher than means of other treatments for consumption and weight after 96 h (Table 3.2). Mortality of larvae among treatments after 48 and 96 h did not significantly differ (for 48h: percentages of mortality for water, event 176 pollen, *Cladosporium*, and event 176 pollen/*Cladosporium* treatments were 9%, 4%, 0%, and 0%, respectively; for 96h: percentages of mortality for water, event 176 pollen, *Cladosporium*, and event 176 pollen/*Cladosporium* treatments were 13%, 25%, 17%, and 12%, respectively; both $P>0.05$).

Degradation. Pollen densities for the water-, *Alternaria*-, *Cladosporium*-, *F. proliferatum*-, and *F. sporotrichioides*-sprayed slides were 236 (+/-31, SD), 241 (+/- 9), 256 (+/-38), 236 (+/-9), and 227 (+/-25) pollen grains/cm^2, respectively. Fungal colonization was rapid. Hyphae of *Alternaria*, *F. proliferatum*, and *F. sporotrichioides* had colonized nearly 100% of observed areas on slides within one day, while *Cladosporium* colonization took approximately 2-3 d to cover nearly 100% of observed areas (Figure 3.1). Pollen densities could not be accurately enumerated for slides treated with water at 21 d, and at 14 and 21 d for slides treated with a fungus due to dense hyphal growth.

Cry1Ab concentrations did not differ significantly among treatments comparing water and conidial suspensions ($F = 0.56$, df = 4, 176, $P = 0.69$; Table 3.3 and Figure 3.1). Percentage of fungal colonization also was not a significant factor affecting Cry1Ab concentrations ($F = 0.76$, df = 23, 176, $P = 0.77$; Figure 3.2). Nevertheless, Cry1Ab concentrations for incubation times significantly differed ($F = 7.72$, df = 6, 176, $P< 0.0001$).

About 1.1-7.1 μg Cry1Ab /g pollen is present in event 176 pollen, and approximately 1.5 million pollen grains are in 1 g of pollen (Hellmich et al., 2001; Stanley-Horn et al., 2001). Thus, approximately 0.000733-0.004733 ng Cry1Ab/pollen grain is estimated for event 176 pollen, assuming that pollen grains express equal amounts of Cry1Ab. Results from ELISAs are larger than this estimate, even when Cry1Ab concentration is reduced after 14 and 21 d. Cry1Ab concentrations after 14 and 21 d of incubation were not significantly different (F = 0.01, df = 1, 176, P = 0.93), but both were significantly different from earlier dates (F = 10.69 to 24.37, df = 1, 176, $P < 0.0013$ for all contrasts of d0, d1, d2, d3, d4, and d7 with both d14 and d21).

Discussion

Results from greenhouse studies showed that feeding behavior and weights after 96 h of *D. plexippus* larvae administered milkweed leaves treated with fungi changed with an additional week of time after spraying milkweed leaves. Larvae administered milkweed leaf material treated with water and fungi consumed similar amounts of milkweed material and weighed similarly 7 d after spraying milkweed leaves. However, 14 d or 15 d after spraying milkweed leaves, larvae fed milkweed material with only fungi present consumed less milkweed leaf material and weighed less than larvae that consumed water-treated milkweed leaf discs.

Results from larvae feeding on event 176 pollen demonstrated Cry1Ab toxicity in greenhouse studies. *Danaus plexippus* larvae were exposed to larger pollen densities of Cry1Ab than necessary to elicit sublethal and lethal effects (5-133; Sears et al., 2000; Hellmich et al., 2001; Stanley-Horn et al., 2001) and the average pollen deposition of 171 pollen grains/cm^2 in maize fields (Pleasants et al., 2001). Treatments with pollen and a

fungus had approximately half of the pollen deposition of the pollen treatment, perhaps resulting from inconsistency in the spraying the leaves, or selecting non-representative leaves to estimate pollen densities for treatments. Mortality 7 days after spraying milkweed leaves differed significantly between event 176 pollen alone and event 176 pollen with *Cladosporium*. Perhaps *Cladosporium* assisted in degrading Bt toxin within the bioassay (96 h compared to 48 h), although this result was not repeated in the other bioassay (15 days after spraying milkweed leaves).

Larvae consuming fungus-treated milkweed leaf discs tended to consume less milkweed leaf material and weigh less when administered milkweed leaf material 14 or 15 d after being sprayed compared to milkweed leaf material 7 d after being sprayed. As milkweed leaf discs are cut, severed leaf material does not contain as much latex or sap and quality decreases. Fungi were sprayed onto milkweed leaves at high concentrations, and once leaf material was optimal for colonization, the sprayed fungi, as well as any other saprophytic fungi in air, or fungi associated with pollen or *D. plexippus* larvae, could begin to grow. *Danaus plexippus* larvae may have encountered increasing amounts of fungal hyphae as milkweed leaf discs were consumed and leaf disc quality declined. Arenas provided a suitable environment for fungal growth with water agar and milkweed leaf material. *Danaus plexippus* larvae, consuming milkweed leaf material in the field, trench and sever veins in order to reduce the flow of latex (Zalucki et al., 2001). However, leaves generally remain attached to the milkweed plant as *D. plexippus* larvae feed. Arenas may simulate the latex or sap reduction, although the age and quality of the leaf material are inferior to those observed in the field during the period the bioassays were conducted.

Fungi used within larval bioassays were capable of producing mycotoxins when colonizing substrates, although mycotoxins were not tested for or identified in this work.

Numerous mycotoxins, such as deoxynivalenol, diacetoxyscirpenol, T-2, and zearalenone, are produced by *F. sporotrichioides* (Marasas et al., 1984). *Cladosporium*, a ubiquitous fungus, has been reported to produce toxins, such as epicladosporic and fagicladosporic acids (Samuels, 1984). Larvae administered treatments of *F. sporotrichioides* or *Cladosporium* consumed significantly less milkweed leaf material and weighed significantly less than the water treatment 14 or 15 days after spraying the milkweed leaves. The presence of fungi on milkweed leaf surfaces and/or mycotoxin production may account for these reductions.

Fungal colonization on Cry1Ab-containing pollen presents complications in measuring Cry1Ab concentration by ELISA. The percentage of pollen to fungus decreases as fungi grow and form hyphal mats on pollen. Actual Cry1Ab concentration within pollen-fungal substances could be masked or diluted with fungi present. Slides were used in order to simplify colonization, and the bead-beater was used to destroy fungal cells as well as pollen grains. ELISA results for Cry1Ab concentration were higher than estimates of 1.1-7.1 μg/g Cry1Ab in event 176 pollen. Nearly 55% of all samples were included in data analyses. Perhaps the large amounts of Cry1Ab are attributable to the presence of the fungus, which may have created false positive results. However, samples after 14 and 21 d of incubation show decreased Cry1Ab concentration/pollen grain when fungal growth is at its highest value. Pollen density for latter incubation dates (14 and 21) were diluted less for use in ELISAs, and therefore more pollen grains were present per well for these dates.

All treatments were eventually colonized by fungi. Fungal colonization was slower on slides treated with water, while pollen grains on slides were quickly colonized when treated with fungi. Pollen grains sprayed with water were colonized by *Cladosporium*, which could have grown from within the pollen grains. Sainger et al. (1978) reported that several fungi (*Alternaria*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Cladosporium*, *Monilia*, and

Epicoccum) colonized pollen (nasturtium/*Tropoleum majus*, carnation/*Dianthus caryophyllus*, and hollyhock/*Althaea rosea*), even after the pollen was surface sterilized.

Cry1Ab concentrations were significantly different among incubation periods, and similar Cry1Ab concentrations can be observed throughout the early sampling dates (days 0-7). Increases in Cry1Ab concentrations were observed within days 0 through 4 in treatments except *Alternaria*. These peaks may result from detection of Cry1Ab fragments as fungi colonized event 176 pollen. However, results at d 0 were also large when compared to standard estimates of ng Cry1Ab/pollen grain.

The two hypotheses for this research included (i) fungal populations on milkweed leaf surfaces can influence monarch larval feeding behavior and success and (ii) fungal populations on milkweed leaf surfaces contribute to the degradation of Bt proteins in maize pollen. Bioassay results demonstrated that *D. plexippus* larvae fed milkweed leaves sprayed with *Cladosporium* and *F. sporotrichioides* consumed significantly less milkweed leaf material and weighed significantly less than the water-sprayed milkweed leaves 14 or 15 days after spraying the milkweed leaves. These results supported the first hypothesis. Degradation of Cry1Ab was not detected in the results from *D. plexippus* bioassays (consumption and weight) and ELISAs. The second hypothesis was not supported by these results.

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Table 3.1. Comparison of the means for the results of the monarch larvae bioassays of greenhouse study A 7 and 14 days after spraying milkweed leaves with event 176 pollen and the fungus *F. sporotrichioides*.

Treatment	Feeding (mm^2) 48h		Feeding (mm^2) 96h[a]		Weight (mg)	
	7 d	14 d	7d	14 d	7 d	14 d[b]
Water (i)	49.9a	49.9a	177a	253a	13.2a	16.5a
Event 176 pollen (ii)	11.1b	16.3b	18.2b	36.4b	3.8b	2.3c
F. sporotrichioides (iii)	48.5a	24.5b	142a	79.1b	13.8a	6.6b
176+*F. sporotrichioides* (iv)	10.9b	18.1b	16.4b	59.3b	2.4b	4.6bc

a F96h values were log transformed for statistical analyses.
b Weights were log transformed for statistical analyses for the bioassay 14 d after spraying the milkweed leaves.

Table 3.2. Comparison of the means for the results of the monarch larvae bioassays of greenhouse study B 7 and 15 days after milkweed leaves with event 176 pollen and the fungus *Cladosporium*.

Treatment	Feeding (mm^2) 48 h		Feeding (mm^2) 96 h[a]		Weight (mg)	
	7 d	15 d	7d	15 d	7 d	15 d[b]
Water (i)	48.9a	39.7a	239a	161a	20.2a	13.9a
Event 176 pollen (ii)	15.4b	28.0a	47.8c	58.7b	4.4b	5.3b
Cladosporium (iii)	53.5a	29.8a	245a	60.7b	19.9a	5.4b
176+*Cladosporium* (iv)	21.7b	29.4a	60.3b	70.1b	4.6b	5.2b

a F96h values were log transformed for statistical analyses.
b Weights were log transformed for statistical analyses for the bioassay 15 d after spraying the milkweed leaves.

Table 3.3 Cry1Ab/pollen grain values for slides treated with water, *Alternaria*, *Cladosporium*, *F. proliferatum*, and *F. sporotrichioides* at different incubation periods.

	Treatments									
Day	Water		*Alternaria*		*Cladosporium*		*F. proliferatum*		*F. sporotrichioides*	
	Mean[a]	SE	Mean[a]	SE	Mean[a]	SE	Mean[a]	SE	Mean[a]	SE
0	0.47	0.11	0.96	0.33	0.34	0.10	0.35	0.07	0.44	0.14
1	0.51	0.06	0.59	0.09	0.20	0.05	0.51	0.13	0.38	0.17
2	0.49	0.14	0.43	0.12	0.27	0.05	0.70	0.43	0.51	0.15
3	0.36	0.08	0.93	0.15	1.23	0.58	0.53	0.21	0.28	0.04
4	0.36	0.16	0.58	0.13	0.24	0.04	0.46	0.10	0.52	0.15
7	0.50	0.26	0.49	0.15	0.59	0.25	0.53	0.19	0.51	0.23
14	0.11	0.06	0.13	0.02	0.15	0.04	0.16	0.04	0.12	0.03
21	0.10	0.04	0.13	0.03	0.19	0.05	0.12	0.04	0.20	0. 10

a Approximately 0.000733-0.004733 ng Cry1Ab/pollen grain is estimated for event 176 pollen.

Figure 3.1. Comparisons of Cry1Ab degradation over 21 d for event 176 pollen sprayed with water, *Alternaria*, *Cladosporium*, *F. proliferatum*, and *F. sporotrichioides* on slides.

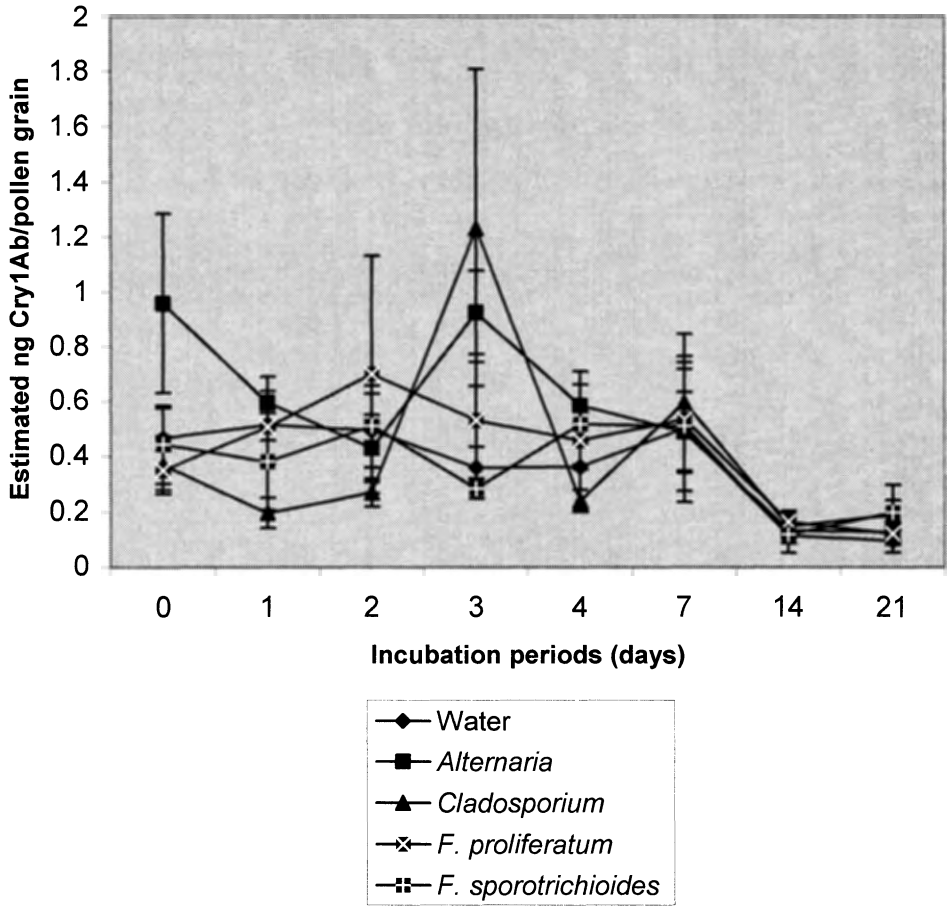
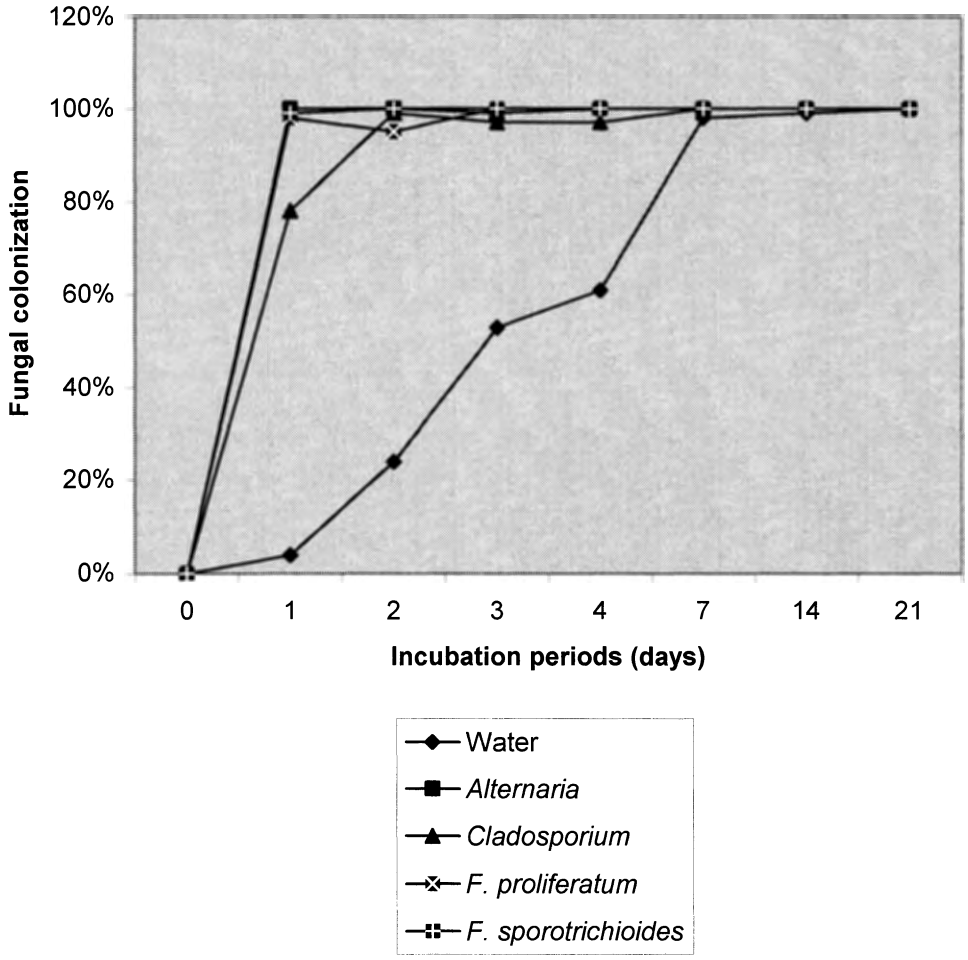


Figure 3.2. Percentage of fungal colonization for each incubation date on event 176 pollen sprayed onto glass slides.



CHAPTER FOUR

GENERAL CONCLUSIONS

The effect of Bt maize pollen (particularly event 176 pollen) on monarch butterfly (*Danaus plexippus* L.) larvae that consume milkweed leaves coated with Bt pollen has been explored, although milkweed phylloplane fungi involvement with the Bt maize pollen-*D. plexippus* larvae interaction has not been tested. Three hypotheses for this thesis project were developed: 1) fungal populations on milkweed leaf surfaces are stimulated by maize pollen deposition (so that their effects on *D. plexippus* would coincide with pollen deposition effects), 2) fungal populations on milkweed leaf surfaces can influence monarch larval feeding behavior and success, and 3) fungal populations on milkweed leaf surfaces contribute to the degradation of Bt proteins in maize pollen. Two main studies, each with two objectives were conducted in order to identify commonly occurring fungi around maize fields and observe how these fungi affect *D. plexippus* feeding behavior and degradation of Cry1Ab in event 176 pollen. Milkweed leaves were collected from three maize fields (inside and outside) on eight sampling dates in the first study in order to (i) identify and quantify the most frequently occurring fungi on milkweed leaves inside and outside of maize fields before, during, and after maize anthesis, and (ii) analyze the relationship between pollen densities and numbers of fungal colonies throughout maize anthesis. Two experiments were conducted for the second study. First, *D. plexippus* larvae were fed milkweed leaf discs sprayed with (i) water, (ii) event 176 pollen in water, (iii) a conidial suspension, or (iv) event 176 pollen in water and a conidial suspension. Secondly, event 176 pollen in water was sprayed onto glass slides followed by water or a conidial suspension and incubated for 0, 1, 2, 3, 4, 7, 14, and 21 days. Objectives for this study were (i) to observe how fungi on

milkweed leaves influenced feeding behavior of *D. plexippus* larvae and (ii) to test whether fungi assisted in degrading Bt protein within pollen on milkweed leaves and slides.

In Chapter Two, fungi were isolated from milkweed leaves and identified. Common field fungi were prevalent inside and outside of maize fields when anthesis was at its peak, which may be influenced by phenologies of these fungi, and the environment or surrounding vegetation. Observations of the fungal ecology associated with milkweed phylloplanes, inside or outside of maize fields, were confined to the patterns for seven genera and one class of fungi. Many of these fungi are common airborne fungi, although some strains of these fungi have been found to be toxigenic. In general, CFU both inside and outside maize fields seemed to follow the trend of mean pollen density (inside maize fields) throughout the sampling period. Pollen deposition was not necessary on milkweed leaf surfaces outside of maize fields to have CFU levels similar to those on milkweed leaves inside maize fields. However, as milkweed leaf discs were washed in the phosphate buffer-Tween solution, conidia, as well as fungal mycelium and pollen, were included in the isolations.

In Chapter Three fungi were sprayed with event 176 pollen on milkweed leaves and glass slides. Two fungi, *Cladosporium* sp. and *F. sporotrichioides*, affected monarch larval feeding behavior as fungal-treated milkweed leaf material (14 or 15 days after spraying milkweed leaves) was consumed less and these larvae weighed less than the larvae fed water-treated milkweed leaf discs. Significant differences between the water- and fungal-treated milkweed leaves were not observed in the bioassay seven days after spraying milkweed. Fungal colonization and mycotoxin production (if present at all) may not have been effective until 14 days after spraying milkweed leaves. Mycotoxins may have been produced, although no testing was conducted in order to confirm their presence. *Fusarium*

sporotrichioides could have produced trichothecenes, and *Cladosporium* could have produced epicladosporic/fagicladosporic acid. While the consumption of milkweed leaves sprayed with fungi elicited altered feeding behavior by monarch larvae and smaller end weights, fungi were not found to significantly affect the degradation of Cry1Ab protein in event 176 pollen.

In conclusion, results support one of the three hypotheses. Pollen deposition did not contribute to higher levels of total colony forming units present on milkweed phylloplanes inside maize fields compared to those outside of maize fields (Hypothesis 1). *Danaus plexippus* larvae consumed and weighed less when fed milkweed leaf discs sprayed with a conidial suspension 14 or 15 days earlier compared to leaves sprayed 7 days before bioassays commenced (Hypothesis 2). Fungal involvement with Cry1Ab degradation in event 176 pollen was not proven in *D. plexippus* bioassays (comparing effects on consumption and weight between event 176 pollen alone and event 176 pollen and a fungus), and ELISA results could not prove that different fungi cause increased degradation compared to the water treatment (Hypothesis 3).

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