

**IMPACT OF ALTERNATE HOST PHENOLOGY AND ALTERNATE HOST-
TRANSGENIC CORN INTERACTIONS ON THE WESTERN CORN
ROOTWORM (COLEOPTERA: CHRYSOMELIDAE).**

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Doctor of Philosophy

By

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DEDICATION

To my dear family; my wife Mary, and my sons, Brian and Ian Gacii, for their loving support throughout my graduate program; and to my parents; Mary and Josephat Chege, for their great inspiration and continuous encouragement to aim higher.

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Table of Contents

ACKNOWLEDGEMENTS	II
LIST OF TABLES	VI
LIST OF FIGURES	VII
ABSTRACT	IX
CHAPTER 1	1
1.1. INTRODUCTION	1
1.2. OBJECTIVES	2
CHAPTER 2	4
2.1. REVIEW OF LITERATURE	4
2.1.1. OCCURRENCE AND DISTRIBUTION	4
2.1.2. REPRODUCTION AND LIFE CYCLE.	5
2.1.3. LARVAL FEEDING BEHAVIOR.....	6
2.1.4. DAMAGE TO CORN.....	6
2.1.5. ADULT FEEDING AND DAMAGE.....	8
2.1.6. HOST RANGE.	9
2.1.7. CONTROL.....	10
2.1.8. TRANSGENIC BT CORN FOR CORN ROOTWORM CONTROL.....	14
2.1.9. HOST PLANT PHENOLOGY.	16
2.1.10. LARVAL MOVEMENT.....	17
2.1.11. HOST SEARCHING BEHAVIOR	18
CHAPTER 3	20

ALTERNATE HOST PHENOLOGY AFFECTS SURVIVORSHIP, GROWTH AND DEVELOPMENT OF WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) LARVAE.	20
ABSTRACT	20
3.1. INTRODUCTION	21
3.2. MATERIALS AND METHODS	22
3.2.1 PLANT MATERIAL AND INSECTS.	22
3.2.2. DATA COLLECTION.....	24
3.2.3. STATISTICAL ANALYSIS.....	25
3.3 RESULTS	26
3.3.1. LARVAL SURVIVORSHIP.	26
3.3.2. LARVAL GROWTH AND DEVELOPMENT.	26
3.4. DISCUSSION AND CONCLUSION	27
CHAPTER 4	49
INITIAL FEEDING ON AN ALTERNATE HOST ENHANCES WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) BEETLE EMERGENCE ON CRY3BB1-EXPRESSING MAIZE.	49
ABSTRACT	49
4.1 INTRODUCTION	50
4.2. MATERIALS AND METHODS	53
4.2.1. EXPERIMENTAL DESIGN, PLANT MATERIAL, AND INSECTS.	53
4.2.2. INFESTATION OF ORIGINAL HOST.....	55
4.2.3. HOST-SWITCHING.....	55
4.2.4. STATISTICAL ANALYSES.....	58
4.3. RESULTS	59
4.3.1. BEETLE EMERGENCE.	59

4.3.2. SEX RATIO.....	60
4.3.3. FECUNDITY.....	60
4.3.4. EGG VIABILITY.....	61
4.4. DISCUSSION.....	61
CHAPTER 5.....	80
HOST PLANT PHENOLOGY AFFECTS WESTERN CORN ROOTWORM (COLEOPTERA: CHRYSOMELIDAE) LARVAL HOST-SEARCHING BEHAVIOR.....	80
ABSTRACT.....	80
5.1. INTRODUCTION.....	81
5.2. MATERIALS AND METHODS.....	84
5.2.1. EXPERIMENTAL DESIGN, PLANT MATERIAL, AND INSECTS.	84
5.2.2. EFFECT OF HOST PLANT PHENOLOGY ON LARVAL HOST-SEARCHING BEHAVIOR.	85
5.2.3. ETHOVISION [®] PROTOCOL.....	86
5.2.4. STATISTICAL ANALYSES.....	88
5.3. RESULTS.....	89
5.3.1. EFFECT OF HOST PLANT TYPE.....	89
5.3.2. EFFECT OF HOST PLANT PHENOLOGY.....	90
5.4. DISCUSSION.....	92
CHAPTER 6.....	115
6.1. SUMMARY AND CONCLUSIONS.....	115
REFERENCES CITED.....	118
VITA.....	136

List of Tables

Table	Page
1. Analyses of variance for percentage larval recovery, dry weight gain, and change in head capsule width.	41
2. Average weight gain (dry weight in $\mu\text{g} \pm \text{SEM}$) of larvae recovered from six host plant species in different greenhouse pots infested at one of eight weekly infestation times (I.T.) with 15 neonate western corn rootworm larvae	43
3. Analysis of variance testing differences in western corn rootworm beetle emergence, sex ratio, fecundity, and percent viability of rootworm eggs	74
4. Effect of original and final host plant treatments on the number of males and females (Mean \pm SE) of western corn rootworm beetles.	76
5. Effect of different host plant combinations on western corn rootworm females' fecundity and egg viability (Mean \pm SE).....	77
6. Analysis of variance testing differences in total distance moved by western corn rootworm neonate larvae, their velocity, total and mean turning angle, and meander within and between treatment effects and their interactions.....	106
7. Comparisons by Differences of Least Square Means for total distance traveled by neonate western corn rootworm, their velocity, total turning angle and meander.	107
8. Total distance searched, velocity, mean turning angle, and meander of neonate rootworm larvae during the 5-minute period immediately after contact with various host plant roots	108
9. The effect of host plant phenology (4, 7, 10 weeks old roots) on the total distance searched by neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.	109
10. The effect of host plant phenology (4, 7, 10 weeks old roots) on the velocity of neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.	110
11. The effect of host plant phenology (4, 7, 10 weeks old roots) on the meander of neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.	111

List of Figures

Figure	Page
1 (a, b). Experimental layout in the greenhouse.....	31
2. Thinning of plants.....	33
3. Infestation procedure.	35
4 (a, b, c). Sampling procedure.	37
5 (a, b). Phenological (root age) differences.....	39
6. Percentage larval recovery from six host plant species in different greenhouse pots infested at one of eight weekly infestation times with 15 neonate western corn rootworm larvae.....	45
7. Mean head-capsule width of larvae from six host plant species in different greenhouse pots infested at one of eight weekly infestation times with 15 neonate western corn rootworm larvae.....	47
8. Host-switching protocol. Tullgren Funnel method modified by placing recipient- /final -host box beneath the funnel to collect escaping larvae	68
9. Larva crawling towards a final host plant's (maize) roots.....	70
10. Final host plants in the greenhouse.....	72
11. Effect of larval movement between different host plant combinations on the emergence of western corn rootworm beetles.....	78
12. Experimental layout in the greenhouse. Pots were arranged in groups of 15 (5 species X 3 sample dates).	99
13. Sampling: Randomly pre-assigned samples were soaked in water to remove the plant growth media.....	100
14. Root-cleaning and infestation procedure.	102
15. The Ethovision [®] System video camera that was used to track and record the host-searching path patterns of neonate western corn rootworm.....	104

16 – 31. Neonate western corn rootworm host-searching patterns (paths) resulting from contact with roots of inbred maize, Cry3Bb1-expressing maize, large crabgrass, giant foxtail and barnyard grass at 4, 7, and 10 (weeks old) phenological (root age) stages..... 112

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ABSTRACT

The increasing adoption of transgenic maize with resistance to rootworms, combined with glyphosate tolerance, has made it necessary to evaluate the impact of host plant phenology and the potential for rootworm larvae to move between weeds and transgenic maize within the maize agro-ecosystem on biology of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and the implications of such movements for rootworm-resistant transgenic maize insect resistance management (IRM). Three studies were conducted at the University of Missouri-Columbia to address this broad objective. The first objective was to determine the impact of host plant phenology on the survivorship, growth, and development of western corn rootworm larvae. Percentage larval recovery, change in head capsule width, and weight gain were significantly impacted by infestation time and host species. Infestation in weeks 4, 5, and 6 supported western corn rootworm survivorship, growth and development better than infestation at later times. The second objective was to determine the impact of initial feeding of rootworm larvae on alternate hosts, followed by switching host to transgenic maize, on the development of western corn rootworm. Beetle emergence, the sex ratio, and fecundity were all significantly impacted by the original and the final host plant types, as well as the interaction between the two effects. The third objective was to analyze the effect of host plant phenology on western corn rootworm larval host-searching behavior using the

Ethovision® system (Noldus Information Technology Inc. Leesburg, VA). Larvae exhibited a host-searching behavior that indicated a preference for younger roots, mainly 4 weeks after planting (V5 stage). Alternate host phenology could be an important determinant for larval movement between grassy weeds and transgenic rootworm-resistant maize with important implications for rootworm-resistant transgenic maize IRM.

Key Words: Corn rootworm, *Diabrotica*, alternate host, search-behavior, Ethovision.

Chapter 1

1.1. Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is one of the most economically important insect pests of corn, *Zea mays* L. (Levine and Oloumi-Sadeghi 1991). This pest is the most economically important rootworm species because of its extensive range and abundance in North America (Krysan and Branson 1983) and its extending range in Europe (EPPO 2003, Wolfgang and Peter 1999). In the Midwestern United States, Canada, and parts of Europe, it is a serious pest of corn planted after corn (Levine and Oloumi-Sadeghi 1991).

Costs of control and yield loss associated with corn rootworms in continuous maize are annually one of the largest expenditures for insect management in the Corn Belt (Metcalf 1986, Steffey et al. 1994). Organophosphates, carbamates or pyrethroids insecticides applied to the soil at planting time or first-cultivation have been the primary means for the control of corn rootworms in continuous corn (Mayo and Peters 1978). Broadcast insecticide application has in the past been used to suppress beetles in late summer, thus reducing oviposition and subsequent larval damage the following year (Pruess et al 1974). However, Meinke et al. (1998) documented populations of adult western corn rootworms with resistance to methyl parathion and carbaryl insecticides in Nebraska, including areas where adult management (Meinke 1995), involving application of foliar insecticides to control adult populations before oviposition, has been done for years.

Transgenic corn with antibiosis to larval feeding of the western corn rootworm and northern corn rootworm *Diabrotica barberi* Smith & Lawrence has been developed by several seed companies including YieldGard® Rootworm from Monsanto, Herculex® RW from Pioneer/Dow AgroSciences, and Agrisure™ RW

from Syngenta. Increasing acreage is being planted to rootworm transgenics. For example, YieldGard rootworm surpassed 10 million acres in 2006 and Herculex rootworm surpassed 1 million acres.

The Environmental Protection Agency (EPA), under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), mandates that all registrants submit an insect resistance management (IRM) plan prior to registration of a Bt crop. There are a number of research areas for which more information is needed in order to define an optimal resistance management plan for rootworm resistant transgenic corn. Some of those areas include:

- Re-evaluate the host status of major grassy cornfield weeds and other grasses commonly found near corn; estimate the potential impact these alternate hosts may have on corn rootworm population dynamics
- Evaluate IRM options other than refuge strategy, especially if an event is not classified as high-dose.
- Examine the impacts of refuge configuration, including seed mixtures, on development of resistance and likelihood of farmer adoption.

1.2. Objectives

The objectives of my research focused on the first bulleted research need above, and were to:

1. Determine the effect of alternate host plant phenology on the survivorship, growth, and development of western corn rootworm larvae.
2. Determine the effect of larval movement from a grassy host to transgenic corn on western corn rootworm beetle emergence, sex ratio and reproductive fitness of female beetles.

3. Analyze the effect of host plant phenology on the host-searching behavior of western corn rootworm larvae.

Chapter 2

2.1. Review of Literature

2.1.1. Occurrence and distribution

Webster (1895) documented some of the earliest accounts of the origin and distribution of the genus *Diabrotica*. According to his account, the genus *Diabrotica* is almost exclusively confined to the western hemisphere, with its native habitat, seemingly, in the Neotropical Region. The western corn rootworm, *Diabrotica virgifera virgifera* LeConte and the northern corn rootworm, *Diabrotica barberi* Smith & Lawrence, are sympatric species, commonly occurring together in the Corn Belt of North America (Chiang 1973).

Recently, western corn rootworm has expanded its range beyond the Western hemisphere as it has now become established in Europe. The presence of the western corn rootworm in Europe was first reported in 1992 near Belgrade, Yugoslavia (EPPO 2003). Wolfgang and Peter (1999) documented the occurrence of the western corn rootworm in Eastern Europe. Ripka et al. (1999) reported the presence of the western corn rootworm in Hungary. The beetle was found near Venice's airport in 1998, near a loading station in Lombardy, Italy in 2000, in Paris in 2002, the Netherlands, Great Britain and in the Alsace/Basel-Mulhouse region near the borders of France, Switzerland, and Germany in 2003 (GMO Compass, 2006). As of 2005, the range of western corn rootworm activity was mainly concentrated Hungary, western Romania, southern Slovak Republic, Serbia and Montenegro, northwest Bulgaria, northeastern Bosnia Herzegovina, parts of Czech Republic and Slovenia, northern Italy, and very limited activity in Great Britain, The Netherlands, France and Ukraine (IWGO 2006). Despite previous captures of the western corn rootworm in Germany, Switzerland and

Belgium, no activity was reported in these countries and parts of France and Switzerland in 2005 (IWGO 2006).

2.1.2. Reproduction and life cycle.

The western corn rootworm is a univoltine insect with a life history closely synchronized with the phenology of corn, its primary host (Chiang 1973). Feral western corn rootworm populations overwinter as eggs in the soil in an obligatory diapause, which in temperate climates is spontaneously terminated in mid-winter (Krysan 1978). Females mate within the first few days after emergence and mating is thought to promote oocyte growth, referred to as ovarian development (Ball 1957, Hill 1975). Hill (1975) documented a mean fecundity of over 1,000 eggs for western corn rootworm females in laboratory- controlled conditions. Similar findings were documented by Boetel and Fuller (1997), who collected a maximum of 1,864 eggs per western corn rootworm female, with lot means ranging from 357 to 736. Branson and Johnson (1973) documented a mean oviposition period of 14.3 days and mean longevity of 94.8 days for western corn rootworm females. Boetel and Fuller (1997) also recorded female western corn rootworm longevity ranging from 64 to 99 days. Oviposition traditionally occurs almost exclusively in maize from July through mid-September (Shaw et al. 1978, Levine and Oloumi-Sadeghi 1991). Sherwood and Levine (1993) documented that a mating of greater than or equal to 1 hour stimulated an increase in western corn rootworm females' weight, egg-laying, and ovarian development. Female western corn rootworm call throughout the day, following emergence, and are most active during the first half of photophase on a photoperiod of 14: 10 (L: D) (Hammack 1995).

Reproduction involves an elaborate 3-4 h process of copulation, followed by migration of spermatozoa, and fertilization (Lew and Ball 1979, Sherwood and

Levine 1993). Between 0.5 and 4 h after commencement of copulation, the male deposits a complete spermatophore inside the female's bursa copulatrix, and then deposited in the spermatophore, from where the spermatozoa migrates into the spermatheca (Lew and Ball 1980). Fertilization is thought to occur just before oviposition as in most other insects (Wigglesworth 1974). Egg hatching and general western corn rootworm development varies from place to place depending on environmental as well as other factors. For example, Levine et al. (1992) documented different developmental times for western corn rootworm populations at similar temperatures in different states, suggesting significant physiological differences among populations.

2.1.3. Larval feeding behavior.

Larval host-searching behavior shifts from ranging to localized search, which is initiated by contact cues, that dominates over response to volatile cues (Strnad and Dunn 1990). Bernklau and Bjostad (1998a) documented that significantly more larvae were attracted to the side of a glass Y-tube containing a germinating corn seed than to the side containing ambient air, and significantly more larvae were attracted to the side containing cut corn roots than to the side containing an ambient air control. They concluded that the attraction of western corn rootworm larvae to corn roots is caused by CO₂ alone, and that no other volatile chemical cues are involved in attracting the larvae Bernklau and Bjostad (1998b). Neonates feed on fine roots and eventually, in the 2nd and 3rd instars, burrowing into root cores feeding on cortex tissue (Apple and Patel 1963, Riedell and Kim 1990).

2.1.4. Damage to corn.

Damage by western corn rootworm to corn is done primarily by larvae feeding on roots (Spike and Tollefson 1989). Larval damage to corn progresses from 1st and

2nd instar larvae feeding on fine roots to older larvae (2nd and 3rd instar) burrowing into the root core feeding on cortex tissue and excised root sections (Apple and Patel 1963, Riedell and Kim 1990). Such larval feeding results in reduced growth rates (Spike 1986), phenological asynchrony (attacked plants being phenologically different from plants that are not attacked by larvae), and yield reductions up to 55% (Spike and Tollefson 1989). Larval feeding on corn roots interferes with water and nutrient uptake and also reduces the plants stability such that when root pruning is extensive or when feeding damage coincides with high amounts of rainfall and strong winds, plants lodge, which hampers mechanical harvesting (Sutter et al 1990).

Low to moderate levels of injury may result in no yield loss or actually increase yield (Riedell et al. 1992). Godfrey et al. (1993a) reported that feeding by first and second instars caused detrimental reduction in maize vegetative biomass and subsequent yield reductions, except in cases where plants compensated for larval injury. Severe damage has been shown to cause yield reductions of 40–50% (Godfrey et al. 1993a, Chiang et al. 1980). Larval injury has also been shown to have significant and transient effects on maize photosynthetic rate and stomatal conductance, which subsequently leads to significant reductions in plant height (Hou et al. 1997).

Urias-Lopez et al (2000) studied photosynthetic and vegetative growth responses among genotypes of maize, documenting transient reductions in photosynthetic rate in rootworm infested maize at both low to moderate levels of root injury, and a common negative photosynthetic response to larval injury during vegetative growth stages. Hibbard et al (1999) evaluated the feeding damage of western corn rootworm to corn roots resulting from different combinations of rootworm strains, infestation times, and infestation levels in two factorial experiments

under different environments. They documented significantly more damage from an infestation of 1200 eggs/30.5 cm than from that of 600-eggs/30.5 cm and that planting-time infestation caused significantly more damage than infestation at the 2-leaf stage, and that infestation at the 2-leaf stage caused significantly more damage than infestation at the 5-leaf stage.

2.1.5. Adult feeding and damage.

Western corn rootworm adults feed on silk, pollen, and developing ears of corn and tend to remain in corn feeding on foliage or migrating to less mature fields after more suitable plant parts have senesced (Siegfried and Mullin, 1990). Feeding on corn silks can inhibit pollination and subsequent kernel development (Culy et al. 1992). McKone et al. (2001) observed *D. barberi* (but not *D. virgifera*) feeding extensively on sunflower (Asteraceae: *Helianthus spp.*) pollen, as well as on other flower parts, such as petals, occasionally. The documented feeding of western corn rootworm adults on members of plant families other than corn indicates that this species has diet flexibility as an adult (Hamilton 1965, Ludwig and Hill 1975, Hill and Mayo 1980). Recent studies have demonstrated the potential for western corn rootworm herbivory on soybean, *Glycine max* (L.) Merrill (Fabaceae), among adults resistant to annual rotation of corn, as well as rotation-susceptible adults (O'Neal et al. 2002, Mabry et al. 2004).

Western corn rootworm becomes somewhat polyphagous as an adult particularly as corn becomes phenologically unfavorable (pollen dries up and leaves begin to dry down). Western corn rootworm and other diabroticite rootworm beetles have been shown to have feeding dependence on the tetracyclic triterpenoid cucurbitacins and are thought to have coevolved with plants of the Cucurbitaceae family (Metcalf and Lampman 1990). In a recent study, Campbell and Meinke (2006)

documented that the *virgifera* group species (*D. barberi*, *D. cristata*, and *D. v. virgifera*) were closely tied to a primary habitat, corn or prairie, but moved to secondary habitat if relative attractiveness of food sources in the primary habitat decreased. They also observed that habitat type and contrasts in plant phenology affected the level of noncorn habitat use by pest species *D. v. virgifera* and *D. barberi*.

2.1.6. Host Range.

Suarez et al (2001) have documented weed communities that are common in corn and soybean fields. Among 34 weed species the grassy weeds include: Barnyardgrass, *Echinochloa crusgalli* (L.) Beav.; Large crabgrass, *Digitaria sanguinalis* (L.) Scop; Johnsongrass, *Sorghum halepensis* (L.) Pers.; Lovegrass, *Eragostis* sp; Bermudagrass, *Cynodon dactylon* (L.) Pers.; and Foxtail. *Setaria* sp.

The first successful rearing of the western corn rootworm on the roots of plants other than corn was documented by Branson and Ortman (1967a, b). In Branson and Ortman's accounts, 5 species of grasses other than corn were found to be larval hosts, 4 were potential hosts, and 13 were non-hosts. In another experiment in which they evaluated 41 grass species and 27 broadleaf species for western corn rootworm host suitability in Petri dish assays larvae survived for ten days on 18 of the 41 grass species with none surviving on the broadleaf species (Branson and Ortman 1967a, 1970).

Pavuk and Stinner (1994) examined the influence of weeds in corn plantings on abundance of adult northern and western corn rootworm. Although neither broadleaf nor grassy weeds significantly affected adult populations of western rootworm, significantly more beetles were collected from mixed weeds than from broadleaf or grassy weeds. In recent studies, Clark and Hibbard (2004), Oyediran et

al. (2004), and Wilson and Hibbard (2004) demonstrated larval development to at least 2nd instar on 50 out of the 60 species evaluated.

2.1.7. Control.

Lawson (1968) and Apple et al. (1977) have documented the use of egg sampling programs for corn rootworms to predict economically damaging densities of corn rootworm larvae, but these methods are too time-consuming and labor intensive for producers or crop consultants to utilize economically (Foster et al. 1979). A common management tactic is the prophylactic application of soil insecticides at planting time for reduction of larval rootworm root zone feeding damage (Boetel et al. 1998, Mayo and Peters 1978). An alternative management approach, which has been common in parts of Nebraska, involves aerial application of contact insecticides following beetle emergence for suppression of egg laying and resulting population reductions in the following growing season (Pruess et al. 1974). Fuller et al. (1997) investigated optimization of soil-applied granular insecticides for control of northern and western corn rootworms in eight states in the north central United States. They concluded that less-than-labeled rates of these insecticides applied in an 18-cm band can be used to manage rootworm larvae effectively with as much consistency as full rates.

Unfortunately, heavy reliance on soil insecticides for larvae control and aerial application of contact insecticides to control corn rootworm beetles, have both led to the development of insecticide resistance among western corn rootworm populations. Large-scale applications of soil insecticides were first made for western corn rootworm control in the late 1940s and 1950s, but resistance to the broadcast soil insecticides being used at the time was noted as early as 1959 (Ball and Weekman 1962, 1963). Meinke et al. (1998) reported that adult corn rootworm management

practices had led to a selection for significant levels of resistance to methyl parathion and carbaryl in Nebraska. Boetel et al. (1998) investigated the influence of soil insecticide application rates on the reproductive biologies of field-collected northern and western corn rootworms under laboratory conditions and concluded that using reduced application rates of these organo-phosphate and pyrethroid insecticides will not likely have major effects on the reproductive capacities of northern or western corn rootworm.

Attempts have been made to develop other control methods for the management of western corn rootworm larval damage to corn. For example, Journey and Ostlie (2000) documented that commercially acceptable root protection (root injury ratings below 3.0 on the 1 to 6 scale) was provided by 106 nematodes per 30.5 cm of row applied to third instars, and by 107 nematodes per 30.5 cm of row applied to second and third instars of the entomopathogenic nematode, *Steinernema carpocapsae* (Weiser) All strain. In unrelated trials, Mulock and Chandler (2000) tested the efficacy of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin against western corn rootworm, in walk-in field cages. Mortality due to *B. bassiana* was 24% when beetles were released into field cages 24 hours post-application of conidia versus 50% when beetles were present during the application. Mulock and Chandler (2001) documented overall significantly lower reproductive capacity in cohorts treated with *B. bassiana* compared with untreated cohorts, and concluded that a properly timed application of *B. bassiana* causing 75% beetle mortality could potentially reduce oviposition within adult populations western corn rootworm by approximately 70%.

Schroder et al. (1998) conducted laboratory and field-cage experiments to evaluate the efficacy of a water-soluble bait for control of adult diabroticites.

Numbers of adults were reduced by 80% in field cages with corn plants treated with the bait formulation, compared with the untreated control. This new water soluble toxic bait appeared suitable for testing on a larger scale in the field as a component of corn rootworm IPM program. The efficacy of nematodes as biological control agents for western corn rootworm has been documented. Jackson and Brooks (1995) evaluated virulence and development of the insect-parasitic nematode, *Steinernema carpocapsae* (Weiser) Mexican strain, for the immature stages of the western corn rootworm. Rootworm eggs were not susceptible, and complete life cycles were observed only in second and third instar larvae and pupae. Hibbard and Bjostad (1989) documented enhanced rate of mortality caused by larval attraction by volatile corn seedling semiochemicals to insecticides in laboratory soil bioassays.

Metcalf et al. (1987) demonstrated in small-plot field assays that baits made from dried Cucurbita fruits or corn grits and impregnated with semiochemicals and insecticides could kill corn rootworm adults. Chandler and Sutter (1997) observed that application of a semiochemical-based bait to silking corn with a high clearance sprayer effectively reduced the numbers of northern and western corn rootworm adults during studies conducted in 1994 and 1995. In addition, the semiochemical insecticide-baits had minimal effects on beneficial insects, such as predaceous adults of the family Coccinellidae. Chandler (1998) documented that semiochemical insecticide-bait applied in a broadcast fashion, where every swath was treated, as an aerial application method for corn rootworm adult management proved more effective than alternating swath treatments in reducing beetle populations

The development of rootworm-resistant maize varieties is another potential control strategy. Abel et al. (2000) argued that plant resistance is a promising control method for the two most damaging insect pests of maize: the European corn borer,

Ostrinia nubilalis (Hubner), and the western corn rootworm. However, they evaluated fifteen experimental lines of maize but no useful sources of root feeding resistance to western corn rootworm were found. Assabgui et al. (1995) documented that resistance to the western corn rootworm larvae, in the form of antibiosis, could be present in midwhorl maize inbreds and that root hydroxamic acid content may be contributing to this resistance. Chiang and French (1980) demonstrated that susceptible inbreds of maize suffered higher root damage and lodging than tolerant inbreds due to infestations of western corn rootworm. They observed that larval survival was much higher on the tolerant inbreds confirming Pathak's contention that tolerant crop varieties may be more inductive to pest population build-up than susceptible varieties. Chiang and French (1980) recommended that tolerance in host-plant resistance should be considered a short-term management tool.

For over 90 years, crop rotation has been recommended to manage western corn rootworm (Gillette 1912). However, years of annual maize and soybean rotation in some parts of the USA Corn Belt have resulted in the selection for a behavioral variant of the western corn rootworm that overcomes the maize-soybean rotation management tactic. Western corn rootworm adults were all along known to lay their eggs in maize and their larvae feed almost exclusively on maize roots (Branson and Ortman, 1967a, 1971). In 1993, however, reports of serious western corn rootworm larval injury to first-year corn in east-central Illinois and northwest Indiana began to increase and growers who had successfully used crop rotation for western corn rootworm control suffered heavy crop losses (Onstad et al. 1999).

In 1996 research and extension entomologists in Illinois and Indiana began to suspect that the cultural strategy of crop rotation had selected for a new strain of western corn rootworm capable of circumventing crop rotation by laying eggs in

soybean, *Glycine max* L., fields (Levine and Gray 1996). During the summers of 1996, 1997, and 1998, Barna et al. (1999) conducted field studies related to this behavioral shift in northwestern Indiana and found that there were higher numbers of females in soybean when compared to maize. Economic injury by western corn rootworm larvae to maize grown in rotation with soybean has been increasing in areas of the eastern Corn-Belt. The expansion of the population, into Ohio and Michigan (O'Neal et al. 2002), of this behavioral variant that circumvents soybean-corn rotation strategy has led to increased rootworm pressure on corn crops subsequently imposing a greater need for alternatives to annual crop rotation for their control.

None of the control methods described in the foregoing discussion has proved to be a silver bullet for corn rootworm management and additional strategies are therefore clearly desirable. Increased public pressure to protect the environment, including non-target organisms and ground water, and the possibility of future legal constraints on traditional pesticide use have resulted in an emphasis on the development of new, and more environmentally sound corn rootworm management technology.

2.1.8. Transgenic Bt corn for corn rootworm control.

The rapid developments in recombinant DNA technology and plant tissue culture have made it possible to engineer plants with desired genetic traits. The first transgenic plants engineered for insect resistance were released for commercial cultivation in 1996. Transgenic corn with an insecticidal protein was first developed for control of the European corn borer *Ostrinia nubilalis* (Hubner) (Koziel et al. 1993). Transgenic corn for the European corn borer was commercially available in 1996. It has been widely adopted by corn farmers in the United States, resulting in a decrease in their use of insecticides (Pilcher et al. 2002). The first transgenic cotton,

Bollgard, developed by Monsanto through research begun in the 1980s, reached the market in 1996.

Pilcher and Rice (1998) conducted a survey in late 1995 involving 799 Iowa farmers to evaluate their perception their perceptions of transgenic corn and the potential impact that transgenic *Bt* corn technology may have on insect management and farming practices. The three most important advantages were less insecticide exposure to farm workers (71.3%), potentially better yields for farmers (69.8%), and less insecticide in the environment (66.8%). The greatest advantage of transgenic corn for corn rootworms was less insecticide in the environment (40%).

Moellenbeck et al (2001) documented protection from western corn rootworm root damage offered by corn co-expressing two new delta-endotoxins from *B. thuringiensis* and that the protection exceeded that provided by chemical insecticides. Corn rootworm larvae fed on corn roots expressing the proteins showed histopathological symptoms in the midgut epithelium (Moellenbeck et al 2001). In 2003, the United States Environmental Protection Authority (EPA) approved MON863 (YieldGard Rootworm[®] developed by Monsanto Company), a genetically modified maize line expressing the Cry3Bb1 endotoxin making it resistant to the corn rootworm. Vaughn et al (2005) documented that the "Growth Stage" of different transgenic maize hybrids significant affected the expression level of Cry3Bb1 between V4 and V9 stages.

By 2004, Cry3Bb1-expressing transgenic maize was grown on >800,000 ha (Hibbard et al 2005) and as of 2005, MON863 was planted on two million hectares. In January 2006, the European Union granted authorization for MON 863 for food and feed (GMO Compass, 2006). As of 2002, transgenic corn and cotton producing *Bt* toxins for control of some key lepidopteran pests were sown to >14 million ha

worldwide (James 2003), helping to reduce reliance on insecticides (Shelton et al. 2002). Rice (2004) enumerated the potential benefits of transgenic rootworm corn (Cry3Bb1 protein), which include: increased root protection; reduced pesticide exposure to farmers, ease of use and handling, time and labor savings, better pest control and increased economic benefits to farmers (\$231 million from yield gains).

However, the demonstrated ability of corn rootworms to develop resistance to insecticides and overcome the barriers of crop rotation shows the need for a rigorous and cautious insect resistance management (IRM) program. Without such a program, the efficacy of insect-resistant transgenic plants will be cut short if pest populations evolve resistance (Gould 1998) and any benefits to farmers and/or the environment from the use of transgenic corn could be fleeting. IRM refers to practices aimed at increasing the time required for insect pests to become resistant to a pesticide. Onstad et al (2001) created a simulation model of the population dynamics and genetics of the western corn rootworm for a landscape of corn, soybean, and other crops, but the model was simulated to evaluate only resistance management plans for transgenic corn. They concluded that allele expression in the rootworm and toxin dose in the corn plant were the two most important factors affecting resistance development.

2.1.9. Host plant phenology.

Artificial habitat disturbances, food supply and quality, temperature unit accumulation, population density and other ecological factors affect insect fecundity and fertility (Boetel et al. 1998). Studies have been conducted to determine which factors affect the phenology of corn rootworms. Davis et al. (1996) for example, developed temperature-dependent models to predict regional differences in corn rootworm phenology. They theorized that rootworm phenology might be affected by genetic variation and availability of corn roots, in addition to temperature.

Darnell et al. (2000) studied how adult western corn rootworm population distribution patterns are altered over time by changes and contrasts in corn plant phenology. They documented that the length of time that a contrast in plant phenology was maintained between adjacent patches of corn strongly influenced the stability of the beetle distribution, and that as contrasts in corn phenology changed, rapid positive or negative changes in beetle densities occurred within and among fields.

2.1.10. Larval movement.

Western corn rootworm larval movement is influenced by various factors such as strong attraction by carbon dioxide released from respiring roots (Strnad et al. 1986, Massimino et al. 1980, Hibbard and Bjostad 1988). Hibbard et al (2004, 2005) documented that both neonate and later instar western corn rootworm larvae prefer nontransgenic roots to transgenic rootworm-resistant roots when a choice is possible, and that when damage to the infested, nontransgenic plant was high, western corn rootworm larvae apparently moved to neighboring transgenic rootworm-resistant plants and caused statistically significant, marginally economic damage.

According to Suttle et al. (1967) and Short and Luedtke (1970), corn rootworm larvae move through the soil as far as 100cm, in search of maize roots, from egg hatch to where adults emerged. Several studies have shown that first instar rootworm larvae locate a host by sensing CO₂ gradients in the soil given off by the root during respiration (Gustin and Schumacher 1989, MacDonald and Ellis 1990, Strnad and Bergman 1987a, b, Bernklau and Bjostad 1998b). The ability of rootworm larvae to locate and recognize host roots is therefore crucial for the survival of the larvae.

Western corn rootworm larvae could move to transgenic maize, following weed removal or phenological changes of grassy host species other than maize. Hibbard et al. (2004) demonstrated that corn rootworm larvae typically do not migrate from maize plants until significant damage has occurred. Hibbard et al. (2005) documented that when a choice is possible both neonate and later instar western corn rootworm larvae prefer nontransgenic roots to transgenic rootworm-resistant roots. Grassy weeds in corn, fencerows and erosion control areas around fields may contribute to the adult production of corn rootworms thus increasing control difficulties. This danger becomes more real in light of the fact that larvae within the *virgifera* species group (including western and northern corn rootworm) specialize on grasses (Branson and Krysan 1981, Krysan and Smith 1987).

2.1.11. Host searching Behavior

The bulk of research on host-searching behavior has focused on predators and parasites (Cade 1975, Bond 1980, Bell 1985, 1990, Chiverton 1988). Insect host-searching behavior has been defined as an active movement by which insects seek resources (Bell 1990). Host search in animals is generally categorized into ranging and local search (Jander 1975), and according to the marginal value theorem (MVT), a single animal exploiting a patch of resources should leave it and search for another patch when its rate of resource gain drops below the expected mean rate in the whole habitat (Charnov 1976). Browne (1997) categorized phytophagous insects' host selection into: host plant recognition and host plant finding. Hammack (1997) tested synthetic corn volatiles and selected analogues were tested in corn fields for attractiveness to feral adults of northern corn rootworm and western corn rootworm, and concluded that kairomonal attractants so far identified for corn rootworm adults

are unlikely to account for specificity of host-finding responses except as components of unique odorant blends.

Presence of unpleasant or toxic chemical compounds in a host could have a modifying effect on the host-searching pattern of western corn rootworm neonates. Xie et al (1992) documented that treatment of corn roots with different hydroxamic induced changes in the host searching-behavior of neonate western corn rootworm larvae, characterized by significantly reduced number of turns, significant increase in the area searched and locomotor. The responses were concentration-dependent, suggesting that hydroxamic acids act as behavior-modifying and possibly feeding-deterrent chemicals to western corn rootworm larvae. A few authors have investigated the recognition behavior of western corn rootworm. For example, Strnad and Dunn (1990) developed a novel assay for analyzing paths of larvae after initial contact of larvae with roots of potential host plants in order to determine host recognition behavior. Oyediran et al. (2004) documented significant differences in the area searched, number of turns, path crossings, and the average velocity of the northern corn rootworm neonate larvae exposed to the different plant species and the control.

It is thus necessary to document the impact of host plant phenology on the host-searching behavior, survivorship, growth and development of western corn rootworm, as well as larval movement between alternate hosts and rootworm-resistant transgenic maize. This is important with regard to the potential for the development of resistance by western corn rootworm to the *Bt* toxins in transgenic maize, and also with regard to western corn rootworm IRM programs including non-maize grass hosts as part of the refuge configuration.

Chapter 3

Alternate Host Phenology Affects Survivorship, Growth and Development of Western Corn Rootworm (Coleoptera: Chrysomelidae) Larvae.

ABSTRACT

The commercial release of transgenic maize with resistance to rootworms incorporated with glyphosate tolerance has become a reality, and questions have arisen regarding the impact of grassy weed phenology on the biology of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. A study to determine the impact of host plant phenology on the survivorship (percentage larval recovery), growth (dry-weight gain), and development (change in head capsule width) of western corn rootworm larvae was conducted in a split-split-plot randomized complete block design experiment under greenhouse conditions. Six host species were evaluated under eight weekly infestations of 15 neonate western corn rootworm larvae that were sampled for larval recovery after 7, 14, and 21 d, each with five replications. Percentage larval recovery, change in head capsule width and weight gain were affected by infestation time and host species. Other than from maize, *Zea mays* L., larval survivorship was 15% in large crabgrass, *Digitaria sanguinalis* (L.) Scop, followed by giant foxtail, *Setaria faberi* R. A. W. Herrm (7%), witchgrass (5%), *Panicum capillare* L., woolly cupgrass, *Eriochloa gracilis*(3%), and green foxtail, *Setaria viridis* (L.) P. Beauv (3%). Larval survivorship, growth and development was enhanced following infestation during weeks 4, 5, and 6 than infestation at later times. Alternate host phenology could be an important factor in larval movement between grassy weeds and transgenic rootworm-resistant maize.

KEY WORDS. *Diabrotica virgifera*, phenology, alternate host, resistance management.

3.1. Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is one of the most economically important insect pests of maize, *Zea mays* L. (Levine and Oloumi-Sadeghi 1991), with an extensive range and abundance in North America (Krysan and Branson 1983). Applications of soil insecticides and aerial application of contact insecticides in the past have led to the development of resistance to cyclodiene, organophosphate, and carbamate insecticides in the western corn rootworm (Ball and Weekman 1963, Meinke et al. 1998, Wright et al. 2000). In addition, years of annual maize and soybean rotation in some parts of the USA Corn Belt have resulted in the selection for a behavioral variant of the western corn rootworm that overcomes the maize-soybean rotation management tactic. Damage to rotated (first-year) maize by western corn rootworm has been reported in Illinois (Levine and Gray 1996), Iowa (Rice and Tollefson 1999), and Michigan, (DiFonzo 1998). These populations lay eggs in maize fields as well as outside the maize fields (Levine et al. 2002).

Because western corn rootworm has exhibited a plasticity to circumvent most tactics used for managing this pest, new management tactics must be developed and implemented. The latest tool is the deployment of transgenic maize expressing the Cry3Bb1 endotoxins from *Bt*, *Bacillus thuringiensis* Berliner (Vaughn et al. 2005). The demonstrated ability of corn rootworms to develop resistance to insecticides and overcome the barriers of crop rotation shows the need for a rigorous and cautious insect resistance management (IRM) program. Without such a program any benefits to farmers and/or the environment from the use of transgenic corn could be fleeting. IRM refers to practices aimed at increasing the time required for insect pests to become resistant to a pesticide.

Several interest groups have expressed concern that widespread planting of transgenic plants could hasten the development of resistance to pesticidal *Bt* endotoxins. Because of this concern, the Environmental Protection Agency (EPA 2003) mandates that all registrants submit an IRM plan prior to registration of a *Bt* crop. There are a number of research areas for which more information is needed in order to define an optimal IRM plan for rootworm-resistant transgenic corn, including the role of alternate hosts in population dynamics and biology of the western corn rootworm.

Branson and Ortman (1967a) reported the first successful rearing of the western corn rootworm on the roots of plants other than maize, and recently, Clark and Hibbard (2004), Oyediran et al. (2004), and Wilson and Hibbard (2004) demonstrated larval development to at least 2nd instar on 50 out of the 60 species evaluated. Oyediran et al. (2005) demonstrated that significantly more adults emerged from mixtures of Cry3Bb1 transgenic corn with weeds than from weeds or transgenic corn alone. These results indicate that the presence of grassy weeds potentially impacts the production of western corn rootworm adults. However, timing of weed availability and egg hatches could have a large impact. Studies conducted by Apple and Patel (1963) and Strnad and Bergman (1987) suggested that corn rootworm larvae prefer newly developed roots. The objective of this study was to document the impact of host plant phenology on the development and survivorship of western corn rootworm larvae on several host plant species.

3.2. Materials and Methods

3.2.1 Plant Material and Insects.

The study was conducted in a greenhouse on the University of Missouri-Columbia campus in 2003. The experimental design was a randomized complete

block split-split-plot in space and time with five replications. The main plot consisted of six plant species: maize, *Zea mays* L. - hybrid P3394 (Pioneer Hi-Bred Inc., Johnston, IA); large crabgrass, *Digitaria sanguinalis* (L.) Scop; giant foxtail, *Setaria faberi* R. A. W. Herrm; witchgrass, *Panicum capillare* L.; woolly cupgrass, *Eriochloa gracilis* Kunth; and green foxtail, *Setaria viridis* (L.) P. Beauv. All species except maize were purchased from Valley Seed Service, Fresno, CA. The subplot consisted of eight successive weekly infestations (4 to 11 wks after planting) and the sub-subplot was comprised of three larval recovery sampling times (7, 14, and 21 d after infestation) to examine larval development. A total of 720 pots were planted: one pot for each of the three sample times, for each of the eight infestation times, for each of the six species, and replicated five times (Figs. 1 a, and b).

The plant growth medium was comprised of soil that had been steam-sterilized for 45 minutes in an autoclave (Amsco Eagle model 2051 Lancer Medical Services Inc., Azusa, CA), and mixed with a peat-based medium (Promix, Premier Horticulture LTEE, Quebec, Canada) in a ratio of 2:1. Plant growth medium was then put into 10 liter pots (Model CL-600, Hummert International, St. Louis, MO) followed by planting. Each pot had five openings at the bottom that were fitted with a fine (114 μm openings) stainless steel mesh (TWP Inc., Berkley, CA) mesh to prevent larval escape (Clark and Hibbard 2004). Planted pots were then arranged randomly on greenhouse benches by subplots, each with columns of eight pots that corresponded to infestation time and rows that corresponded to sample date (order was randomized from subplot to subplot).

Upon germination, seedlings were watered as necessary, and fertilized every two weeks with Peter's fertilizer (20-20-20 NPK, United Industries Ltd., St Louis, MO) at a rate of 0.6 g/liter of water, and grown under 14:10 (L:D) h, with natural light

supplemented by 1000 watt sodium bulbs (GE Lighting, Cleveland, OH), and a temperature of $25 \pm 2^\circ \text{C}$ as monitored with a temperature recorder (Model SL 4350 C7C, Dickson Co., Addison, IL).

The larvae used in this study were from the non-diapausing western corn rootworm colony we maintain that was originally obtained from the Northern Grain Insects Research Laboratory, USDA-ARS laboratory, Brookings SD. Larvae from this non-diapausing colony have provided root feeding damage on corn under field conditions that was not significantly different from diapausing western corn rootworm larvae in direct field comparisons (Hibbard et al.1999). The average head capsule width and dry weight of a sub-sample of 100 neonate western corn rootworm larvae were determined before infestation and used in subsequent data analysis. From the fourth week through the 11th week after planting, randomly assigned sub-samples of each host plant species were infested weekly with fifteen neonate western corn rootworm larvae per pot. The infestations were conducted by transferring the neonates to the pots using a moistened fine camel's-hair paintbrush.

3.2.2. Data collection.

After each infestation time, larvae were allowed to feed and then recovered 7, 14, and 21 d after each respective infestation using Tullgren funnels fitted with 60W light bulbs and collecting jars half-filled with tap water. Each respective larval recovery date was comprised of five pots per alternate host species. Jars were checked after 2 and 4 d in the Tullgren funnels. All recovered larvae were transferred to 95% ethanol, counted, and measured for head capsule width. Each larva from all samples (including the initial neonate subsamples) was measured for head capsule width using an ocular micrometer (10x/21, Wild Co., Heerbrugg, Switzerland) mounted on a microscope (M3Z, Wild Co., Heerbrugg, Switzerland). After head

capsule measurements were completed, larvae were placed in scintillation vials and dried in a desiccating oven (Thelco model 16, GCA/Precision Scientific Co., Chicago, IL) at 90 °C for 24 h. The dry weight of larvae was then measured using an Electronic Balance (SR-182A, A&D Company Limited, Tokyo, Japan) with total weight for all dried larvae from individual samples (including the initial subsample) being recorded. The average dry-weight for each individual larva was then calculated by simple division.

3.2.3. Statistical Analysis.

PROC MIXED of the statistical package SAS (SAS Institute 1990) was used for data analysis. A separate analysis was done for percentage larval recovery, larval weight gain, and change in head capsule width. All data were analyzed as a 6 X 8 X 3 (plant species X infestation date X sample date) factorial randomized complete block split-split-plot in space as outlined in Steel and Torrie (1980). The linear statistical model contained the main plot of plant species, the subplot effect of infestation date, the sub-subplot effect of sample date, and all possible interactions of plant species X infestation date X sample date. Replications within plant species served as the denominator of F for testing the effects of plant species. Replications within infestation dates and sample dates were used as the denominator of F for testing treatment and the interaction of infestation dates X sample date. All other effects used the residual error for the denominator. Beyond the standard ANOVA, we compared the means for the interactions of species and infestation time that were significant ($\alpha = 0.05$). This was done with the LS MEANS output from PROC MIXED using a least significance difference (LSD) test. Although untransformed data are shown in the tables, percentage data were transformed by (arcsine square root of x) as suggested by Snedecor and Cochran (1989) for percentage data.

3.3 Results.

3.3.1. Larval survivorship.

Larval survivorship, as indicated by mean percentage larval recovery, was significantly impacted by the host plant species, infestation time, the interaction of host plant species × sample date, the interaction of infestation time × sample date (Table 1). Larval survivorship was highest in maize followed by large crabgrass (Fig. 6). The number of larvae surviving on all host plants from the first three infestations (4th, 5th, and 6th week) was relatively higher compared with those surviving following subsequent infestations except the last infestation in maize (Fig. 6). Sampling date did not have a significant impact on percentage larval recovery (Table 1).

3.3.2. Larval growth and development.

The average dry weight of neonate western corn rootworm larvae was determined to be 9.298 µg from the pooled sub-sample of 100 neonate larvae. The average dry weight-gain per larva was significantly impacted by host plant species, infestation time, sampling date, and all possible interactions (Table 1). In general, larvae gained more weight during early infestation times (four to six weeks after planting) than they did during late infestations. This difference was significant only within maize and large crabgrass (Table 2). Average weight gain was significantly greater on maize than on all other species and was significantly greater on large crabgrass than on woolly cupgrass or green foxtail (Table 2). Among the grassy weed species, larval weight gain was highest in large crabgrass followed by giant foxtail, witchgrass, woolly cupgrass and green foxtail, in descending order (Table 2).

The average head capsule width of neonate western corn rootworm larvae was determined to be 0.220 ± 0.002 mm from the subsample of 100 larvae. Increase in mean head capsule width was significantly affected by host plant species, infestation

times, sampling dates, host plant species X sampling date, and infestation time X sampling date (Table 1). The interaction between host plant species and infestation time, and the overall interaction between host plant species, infestation time and sampling date did not have a significant impact on head capsule width (Table 1). The increase in mean head capsule width generally was highest during the early stages of plant growth (the first few infestations) and then gradually declined with infestation time (Fig. 7). Increase in larval head capsule width among species was highest in maize followed by large crabgrass (Fig. 7).

3.4. Discussion and conclusion.

The commercial release of transgenic maize with resistance to rootworms incorporated with glyphosate tolerance is now a reality, and maize producers in the United States and Canada have a new tool at their disposal to combat the corn rootworm complex. Branson and Ortman (1967a, 1970), Clark and Hibbard (2004), Wilson and Hibbard (2004) showed that many grassy weed species found in or around cornfields are able to support the growth and development of western corn rootworm larvae. Larval development on grassy corn field weeds has potentially important implications regarding the development of appropriate resistance management strategies because larvae could move from weeds to transgenic maize after the weeds are removed or become phenologically less appealing. Results from this study document that host plant phenology could significantly impact survivorship, growth and development of western corn rootworm larvae and thus support the supposition that host plant phenological changes and subsequent plant nutritional status changes could encourage movement of larvae from weeds to transgenic maize. The possibility of such larval movements taking place is supported by findings in a recent study by

Hibbard et al. (2004) in which they concluded that western corn rootworm plant-to-plant movement was motivated by a search for food.

The current study documents that plant age significantly impacts survivorship, growth and development of western corn rootworm larvae as indicated by the effect of infestation time on the response variables. Weight gain was reduced and head capsule width increased more slowly when older host plants were infested compared to those that were infested four to six weeks after planting (Figs. 6 and 7). For example, it is likely that the increase in larval recovery from the last maize infestation time (Fig. 6) was due to enhanced maize root volume but reduced weight gain (Table 2) and significantly smaller head capsule widths (Fig. 7) compared to larvae recovered from the earlier maize phenologies indicates a loss in nutritional value for developing rootworm larvae as the plants aged. This shows that although older hosts do support survivorship, they are not as suitable to sustain robust larval growth (weight gain) and development (as determined by increase in head capsule width, which is indicative of development to advanced larval instars). Alternative host plants in the field might support early western corn rootworm larval survival and growth but not necessarily subsequent larval development if only older hosts are available. In related field and greenhouse studies with maize, western corn rootworm larvae could establish on late phenology maize plants, but adult emergence was greatly reduced (B.E.H., unpublished data).

Studies on the feeding behavior of many insects have demonstrated larval feeding changes with seasonal changes in plant nutrients. The nutritional quality of plants to herbivorous insects varies genetically, temporally, and spatially (Lawrence et al. 2003, Whitham 1981, Waltz and Whitham 1997). Seasonal changes in plant nutritional quality are a major source of variation on the temporal scale and have been

shown to affect the population dynamics of a broad range of phytophagous insects (Feeny 1970, Kearsley and Whitham 1989). The plant phenological age hypothesis predicts that phytophagous insects should prefer and perform better on phenologically young plants than on old plants because plant nutritional quality decreases with plant phenological age (Scheirs et al. 2002).

As a general rule, the nutrient level, i.e. nitrogen and water, decreases while the non-nutrient content and leaf toughness increase with plant phenological age (Feeny 1970). Three types of processes influence the seasonal changes in plant nutritional quality: ontogenetic processes such as maturation (Kearsley and Whitham, 1989), physiological processes such as senescence (Kearsley and Whitham 1989), and a broad range of abiotic (temperature, rainfall) or biotic (herbivores, diseases) environmental variables (Karban 1990).

Feeny (1970) reported that concentration in the spring of feeding by winter moth caterpillars, *Operophtera brumata* L., and other species of Lepidoptera on oak trees in England is believed to be related to seasonal changes in the texture and chemical composition of the leaves. Early feeding was seen to coincide with maximum leaf protein content and minimum leaf sugar content, which suggested that availability of nitrogen, rather than of carbohydrate, may be a limiting factor for spring-feeding larvae. Riipi et al. (2002) studied seasonal changes in birch leaf chemistry. They reported that co-occurring changes in physical leaf traits and concentrations of several compounds indicated a seasonal decline in foliage suitability for herbivores. Concentrations of birch leaf proteins and free amino acids declined through the growing season and individual sugars showed variable seasonal patterns. While a detailed study examining the nutritional qualities and composition of roots for western corn rootworm development has not been reported, Stavisky and Davis

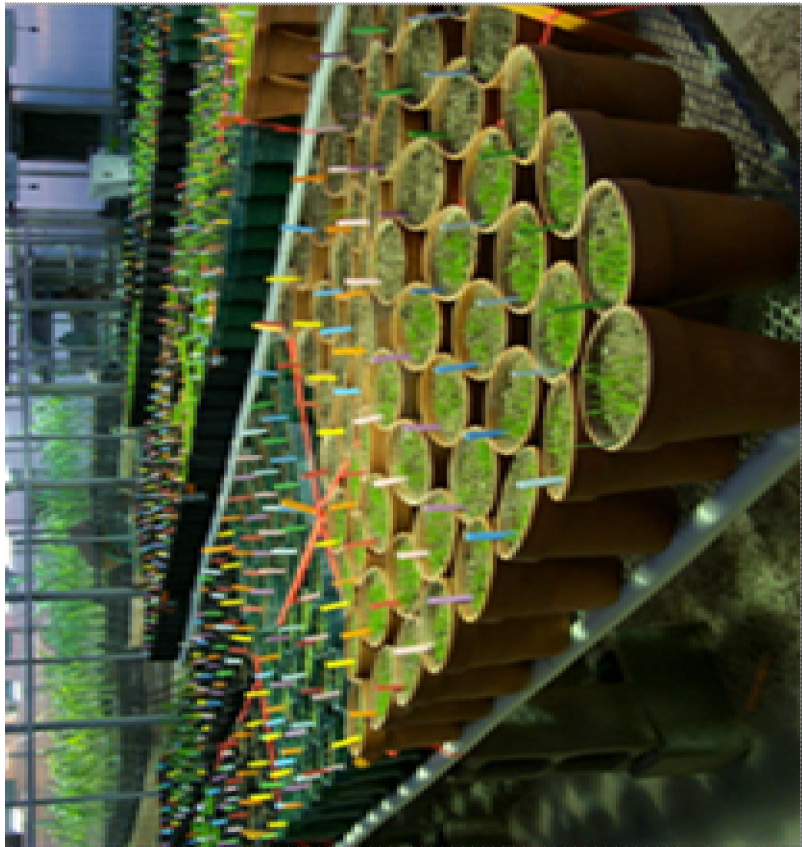
(1997) provided anecdotal evidence to this effect where they observed that western corn rootworm larvae not only prefer newly developed roots (Strnad and Bergman 1987a) but may also require younger roots for establishment. It is possible that a similar scenario occurred in the roots of the grassy weeds evaluated in this study but additional experimentation would be required to confirm this scenario.

Percentage larval recovery, head capsule width increase, and larval weight gain differed within and between the various host plant species. This indicates that plant species also significantly impacts larval survivorship, growth, and development. Certain weed species in cornfields could have more serious implications for potential insect resistance management than others. For example, on average, more larvae were recovered from large crabgrass than other grass species, except maize, and they generally gained more weight, and increased in head capsule width more as well. This implies that in a corn field, where suitable grassy weeds sometime abound, establishment and development of significant numbers of corn rootworm larvae may occur on species other than maize, and the larvae could then move on to transgenic maize, once circumstances in their micro-environment change through weed removal or phenological changes. Because larger larvae are not controlled by the Cry3Bb1 toxin as expressed in current hybrids (EPA, 2002), it is suspected that a significant number of such larvae could then withstand the *Bt* toxin in the transgenic maize. The long-term implications of such movement for transgenic events that are not high dose, such as Cry3Bb1, have yet to be determined.

Figure 1 (a, b): Experimental layout in the greenhouse. Pots were arranged by species in groups of 24 pots (8 infestation dates X 3 sample dates). Labeling stakes were color-coded according to the infestation dates. Figure 1b shows the 1000 watts sodium bulbs used to supplement natural light.



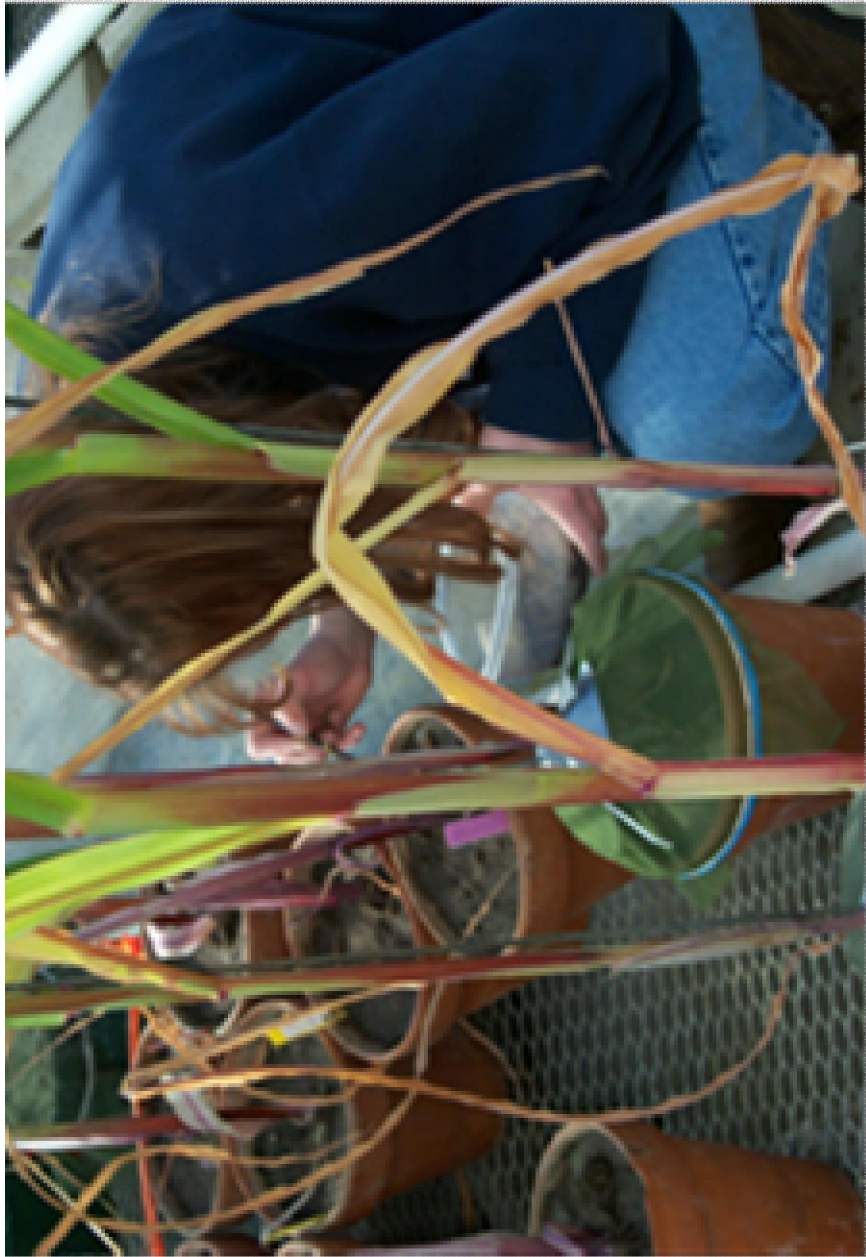
1b.



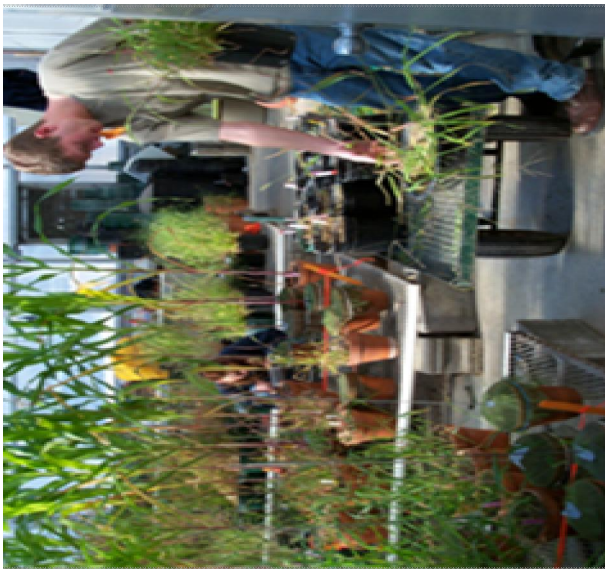
1a.



Figure 3. Infestation procedure. Each host plant pot was infested with 15 neonate western corn rootworm larvae using a wet camel's-hair paintbrush.



37 Figures 4 (a, b, c). Sampling procedure. The foliage was trimmed close to the soil (a) and the contents of the pots (b) were transferred to Tullgren funnels (c).



4a:



4b:



4c:

Figure 5 (a, b). Phenological (root age) differences.



5a. Young roots (5 weeks old)



5b. Old roots (11 weeks old)

Table 1. Analyses of variance for percentage larval recovery, dry weight gain, and change in head capsule width.

Analysis	Effect	df ₁	df ₂	F	P
Larval recovery					
	Species	5	20	46.09	<0.0001
	Infestation time	7	162	9.03	<0.0001
	Species × Infest. time	35	162	1.38	0.0940
	Sampling date	2	372	2.09	0.1254
	Species × Sampling date	10	372	4.51	<0.0001
	Infest. time × Sampling date	14	372	2.42	0.0029
	Species × Infest. time × Date	70	372	1.16	0.1999
Dry wt. gain					
	Species	5	20	16.54	<0.0001
	Infestation time	7	162	8.63	<0.0001
	Species × Infest. time	35	162	2.76	<0.0001

Sampling date	2	372	16.85	<0.0001
Species × Sampling date	10	372	14.98	<0.0001
Infest. time × Sampling date	14	372	2.28	0.0054
Species × Infest. time × Date	70	372	1.61	0.0030
Change in head capsule width				
Species	5	20	60.98	<0.0001
Infestation time	7	162	23.84	<0.0001
Species × Infest. time	35	162	1.26	0.1668
Sampling date	2	372	68.24	<0.0001
Species × Sampling date	10	372	13.59	<0.0001
Infest. time × Sampling date	14	372	1.73	0.0472
Species × Infest. time × Date	70	372	0.84	0.8191

df₁: numerator degrees of freedom

df₂: denominator degrees of freedom

Table 2. Average weight gain (dry weight in $\mu\text{g} \pm \text{SEM}$) of larvae recovered from six host plant species in different greenhouse pots infested at one of eight weekly infestation times (I.T.) with 15 neonate western corn rootworm larvae*

I.T.	Green foxtail	Witchgrass	Cupgrass	Giant foxtail	Large crabgrass	Maize	I.T. main effects
4 th	49.00 \pm 24.09aC	56.49 \pm 24.54aBC	25.01 \pm 6.89aC	58.63 \pm 20.12aBC	129.64 \pm 49.36aB	479.48 \pm 263.27aA	133.04 \pm 28.07a
5 th	13.78 \pm 1.00aC	57.55 \pm 24.93aBC	26.37 \pm 13.31aBC	27.68 \pm 10.69aBC	96.07 \pm 39.40abB	372.94 \pm 207.99bA	99.07 \pm 20.43b
6 th	5.09 \pm 3.25aC	46.68 \pm 36.80aBC	12.09 \pm 9.20aC	197.10 \pm 181.89aB	37.18 \pm 15.04bBC	273.49 \pm 158.17cA	95.27 \pm 34.25b
7 th	0.00 \pm 0.00aB	46.68 \pm 36.80aBC	13.97 \pm 8.82aB	15.23 \pm 6.53aB	32.32 \pm 16.85bB	145.21 \pm 66.00deA	34.67 \pm 10.23c
8 th	0.00 \pm 0.00aB	1.38 \pm 1.38aB	7.19 \pm 3.90aB	4.25 \pm 4.25aB	55.32 \pm 32.60bB	168.78 \pm 99.05dA	39.61 \pm 14.00c
9 th	7.17 \pm 7.67aB	11.81 \pm 11.41aB	14.76 \pm 0.78aB	2.44 \pm 5.37aB	40.89 \pm 16.70bAB	111.87 \pm 49.35deA	34.07 \pm 8.12c
10 th	6.85 \pm 1.36aB	8.19 \pm 1.57aB	7.52 \pm 4.79aB	5.52 \pm 1.38aB	49.33 \pm 30.86bAB	82.19 \pm 50.13eA	26.60 \pm 6.05c
11 th	3.43 \pm 1.80aB	3.21 \pm 1.65aB	8.19 \pm 2.35aB	5.08 \pm 5.08aB	19.67 \pm 7.38bB	103.09 \pm 49.48deA	23.78 \pm 5.65c
SME ^a	10.83 \pm 2.60C	23.78 \pm 6.65BC	14.34 \pm 2.76C	40.89 \pm 23.44BC	58.09 \pm 8.48B	220.77 \pm 27.76A	

*Data from three sample dates (7, 14, and 21 d) for each infestation time were combined and averaged. Means in the same row followed by the same uppercase letter and means in the same column followed by the same lowercase letter are not significantly different according to Fisher's

Protected LSD test ($\alpha = 0.05$).^a Species Main Effects

Figure 6: Percentage larval recovery from six host plant species in different greenhouse pots infested at one of eight weekly infestation times with 15 neonate western corn rootworm larvae. LSD1 is the least significant difference in percentage larval recovery between host plant species within an infestation date. LSD2 is the least significant difference in percentage larval recovery between infestation times within a plant species. The three sampling dates for each infestation time were pooled and averaged because there was no significance among them.

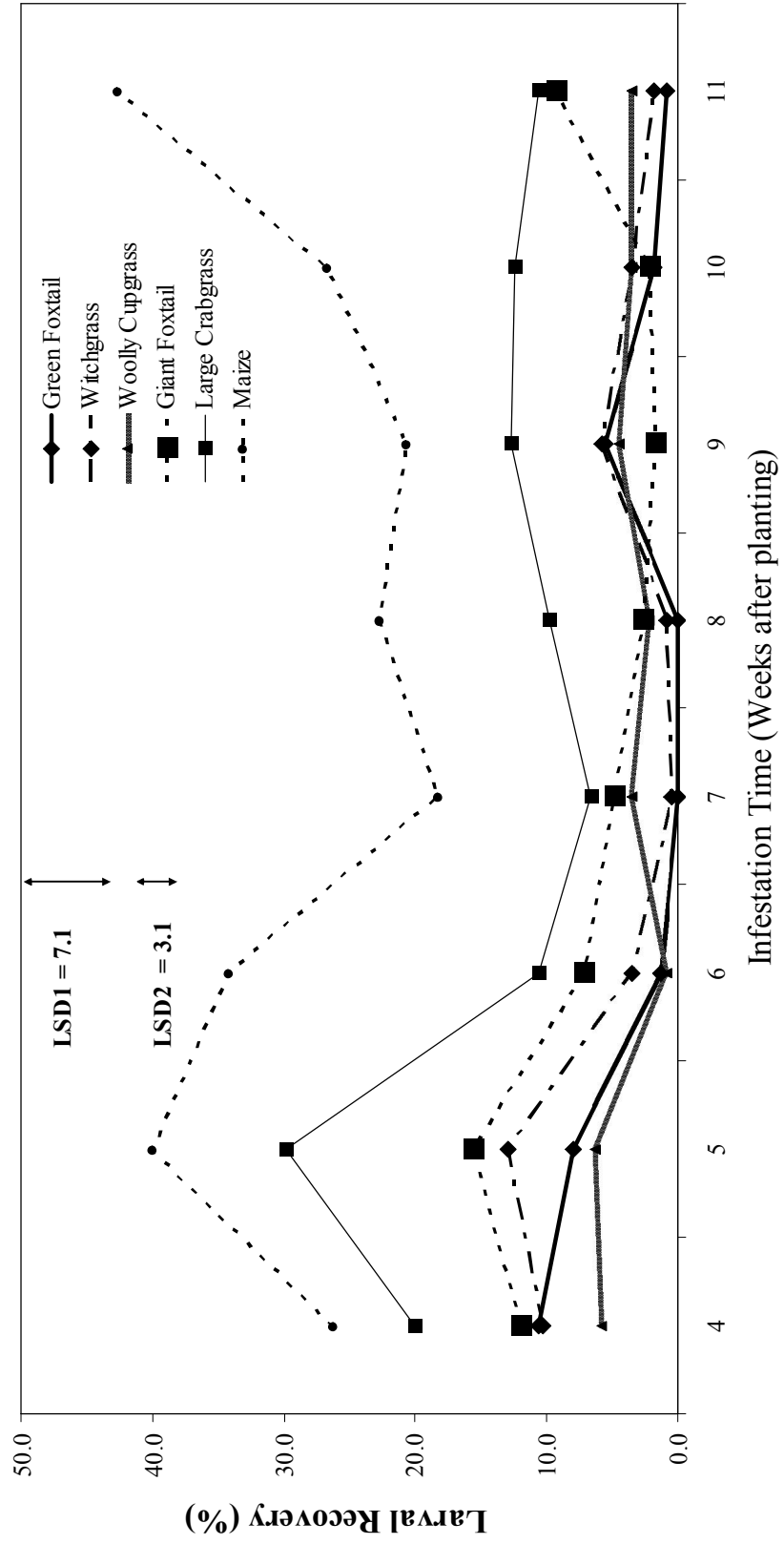
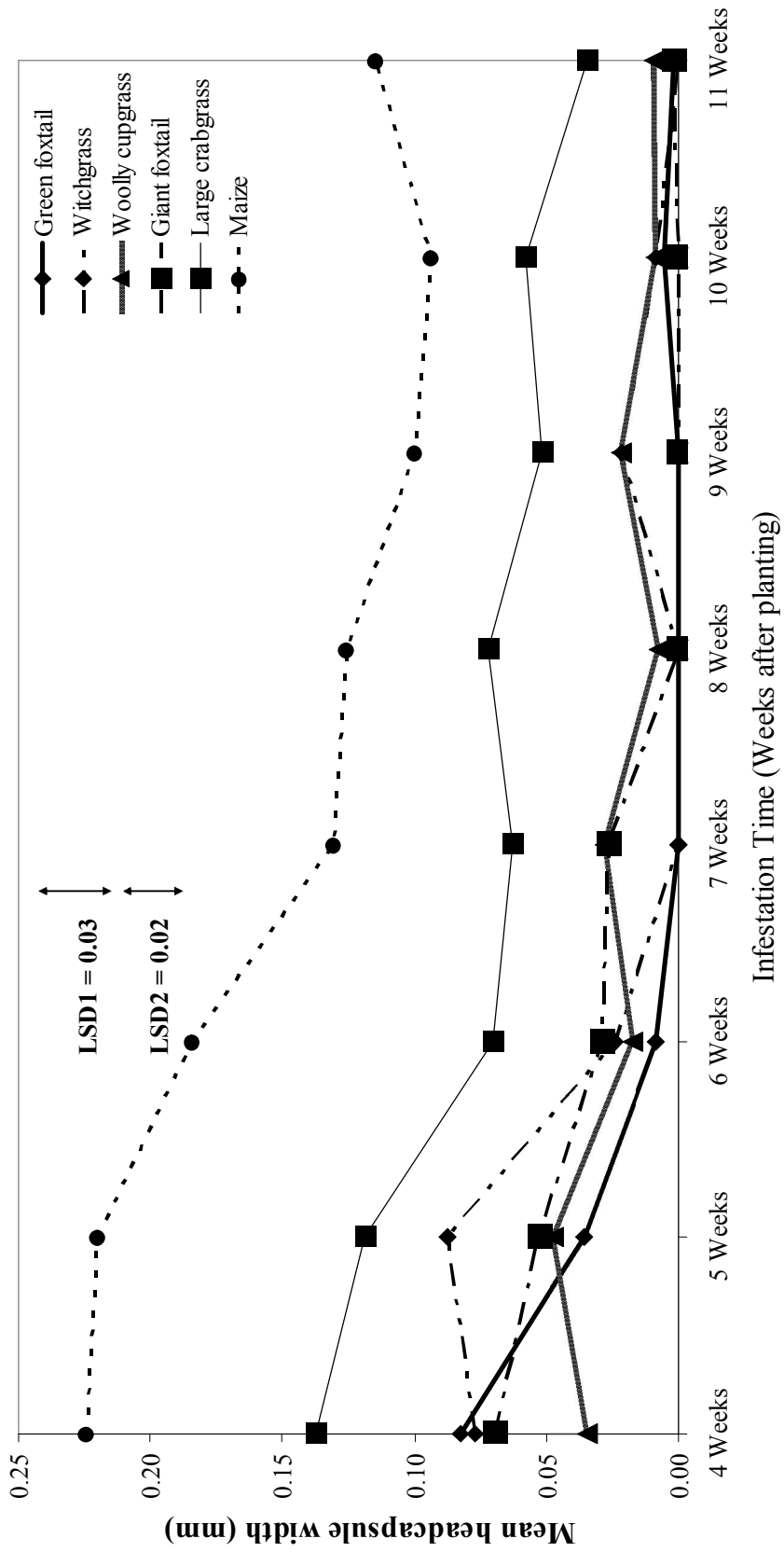


Figure 7: Mean head-capsule width of larvae from six host plant species in different greenhouse pots infested at one of eight weekly infestation times with 15 neonate western corn rootworm larvae. LSD1 is the least significant difference in head capsule width between host plant species within an infestation date. LSD2 is the least significant difference in head capsule width between infestation times within a plant species. The three sampling dates for each infestation time were pooled and averaged.



Chapter 4

Initial Feeding on an Alternate Host Enhances Western Corn Rootworm (Coleoptera: Chrysomelidae) Beetle Emergence on Cry3Bb1-Expressing Maize.

ABSTRACT

The potential for rootworm larvae to move between weeds and transgenic maize may be important in resistance management of transgenic-rootworm maize. To determine the impact of initial feeding of rootworm larvae on alternate hosts, followed by switching host to transgenic maize, on the development of western corn rootworm (*Diabrotica virgifera virgifera* LeConte), a split-split-plot randomized complete block design experiment was conducted under growth chamber and greenhouse conditions. Two weed species, large crabgrass [*Digitaria sanguinalis* (L.) Scop], giant foxtail (*Setaria faberi* R. A. W. Herrm), rootworm-resistant Cry3Bb1 transgenic maize (MON863, YieldGard[®] Rootworm) and its 2003 isoline, were infested with 50 neonate larvae per container' four weeks after planting. On the 5th, 10th, and 15th d after infestation, a modification of the Tullgren funnel technique was used to facilitate larval movement (host-switching) from the original host plants to the final hosts (MON863 or its isoline). Beetle emergence, and fecundity were significantly impacted by the original, the final host, and the interaction between the two effects. Only the original and the final host plant effects impacted the sex ratio significantly. The host-switching date did not impact any of those three variables. Only the original host plant effect had a significant impact on egg viability. Significantly more reproductively fit beetles were produced from larvae that fed on an alternate host before moving to transgenic maize than from larvae fed exclusively on

transgenic maize. Interactions with alternate hosts may influence transgenic maize resistance management models

KEY WORDS. *Diabrotica virgifera virgifera*, rootworm, host-switching, transgenic maize.

4.1 Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is the most destructive insect pest of continuous maize in the United States. Damage to maize is mainly caused by larvae, with neonates feeding on fine roots and eventually, in the 2nd and 3rd instars, burrowing into root cores feeding on cortex tissue (Apple and Patel 1963, Riedell and Kim 1990). Low to moderate levels of injury may result in no yield loss or actually increase yield (Riedell et al. 1992). Godfrey et al. (1993a) reported that feeding by first and second instars caused detrimental reduction in maize vegetative biomass and subsequent yield reductions, except in cases where plants compensated for larval injury. Severe damage has been shown to cause yield reductions of 40–50% (Godfrey et al. 1993a, Chiang et al. 1980). Larval injury has also been shown to have significant and transient effects on maize photosynthetic rate and stomatal conductance, which subsequently leads to significant reductions in plant height (Hou et al. 1997, Urias-Lopez et al. 2000).

A common management tactic is the application of soil insecticides at planting time for reduction of larval rootworm root zone feeding damage (Mayo and Peters 1978). An alternative management approach, which has been common in parts of Nebraska, involves aerial application of contact insecticides following beetle emergence for suppression of egg laying and resulting population reductions in the following growing

season (Pruess et al. 1974). Unfortunately, heavy reliance on soil insecticides for larvae control and aerial application of contact insecticides to control corn rootworm beetles, have both led to the development of insecticide resistance among western corn rootworm populations. Large-scale applications of soil insecticides were first made for western corn rootworm control in the late 1940s and 1950s, but resistance to the broadcast soil insecticides being used at the time was noted as early as 1959 (Ball and Weekman 1962, 1963).

Meinke et al. (1998) reported that adult corn rootworm management practices had led to a selection for significant levels of resistance to methyl parathion and carbaryl in Nebraska. Crop rotation is a popular and successful strategy for managing maize rootworms in many areas (Vidal et al. 2005). In 1993, however, reports of serious western corn rootworm larval injury to first-year maize in east-central Illinois and northwest Indiana began to increase and growers who had successfully used crop rotation for western corn rootworm control suffered heavy crop losses (Levine et al. 2002).

Increased public pressure to protect the environment, including non-target organisms and ground water, and the possibility of future legal constraints on traditional pesticide use have resulted in an emphasis on the development of new, and more environmentally sound corn rootworm management technology. Recent attempts have been made to develop other control methods, like the recent deployment of rootworm-resistant transgenic maize expressing the *Bacillus thuringiensis* Berliner (Bt) Cry3Bb1 endotoxin, known as YieldGard Rootworm[®] and YieldGard Plus[®] (Monsanto). Because pest *Diabrotica* have exhibited potential to develop resistance to most management practices, companies that sell transgenic products are required by the Environmental

Protection Agency (EPA) to develop and implement insect resistance management (IRM) plans to safeguard any benefits to farmers and/or the environment from the use of these products.

It is well documented that hosts beyond maize and larval movement are important factors in western corn rootworm biology. For example, several studies have documented that many grassy weeds can support at least partial development of western corn rootworm larvae (Branson and Ortman 1967a, 1970, Clark and Hibbard 2004, Wilson and Hibbard 2004). Oyediran et al. (2005) demonstrated that the presence of grassy weeds enhances the production of western corn rootworm beetles from mixtures of Cry3Bb1-expressing maize and weeds but noted that timing of weed availability and egg hatch could impact results. These findings are supported by our (Chege et al. 2005) recent study in which we documented that host plant phenology significantly impact survivorship, growth and development of western corn rootworm larvae (Chege et al. 2005).

As IRM efforts are developed and refined, there are additional needs to gather comprehensive data on the implications on resistance management, including western corn rootworm larval movement between grassy weeds and rootworm resistant transgenic maize. For example, initial feeding by western corn rootworm larvae on alternate hosts followed by movement to transgenic plants could increase the rate of resistance development if the larger insects tolerate the toxin of the transgenic plants and insects with a heritable resistance to the transgenic plants survive at a greater rate or are more fecund. Alternatively, if appropriately located within the rootworm resistant transgenic

maize agroecosystem alternate hosts could serve as a refuge, effectively delaying the rate of resistance development by supporting the production of susceptible beetles.

Hibbard et al. (2004) demonstrated that corn rootworm larvae typically do not migrate from maize plants until significant damage has occurred. Hibbard et al. (2005) documented that when a choice is possible both neonate and later instar western corn rootworm larvae prefer nontransgenic roots to transgenic rootworm-resistant roots. They also reported that when damage to the infested, nontransgenic plant was high, western corn rootworm larvae apparently moved to neighboring transgenic rootworm-resistant plants and caused statistically significant, albeit marginally economic damage. However, studies have not been done to determine the impact of one-way movement between alternate hosts and transgenic maize. The objective of this study was to determine the effect of larval movement from a grassy host to transgenic maize on western corn rootworm beetle emergence, sex ratio and reproductive fitness of female beetles.

4.2. Materials and Methods

4.2.1. Experimental Design, Plant material, and Insects.

The experiment was conducted in two phases in summer and early fall of 2004. The original host plant phase was conducted in a growth chamber (37002-GROWC-13, Percival Boone, Iowa) under controlled conditions ($\approx 25^{\circ}\text{C}$, 14:10 (L:D) photoperiod), and then the larvae were switched to their final host plants and maintained in a greenhouse on the University of Missouri-Columbia campus, until beetle emergence. The experimental design was a randomized complete block split-split plot in space and time with four replications. The main plot consisted of four different original host plant treatments: large crabgrass (*Digitaria sanguinalis* (L.) Scop) and giant foxtail (*Setaria*

faberi R. A. W. Herrm) purchased from Valley Seed Service, Fresno, CA, and rootworm resistant maize YieldGard[®] Rootworm hybrid CS 304YGRW/RR and its near isoline hybrid CS304RR (Monsanto Co. St. Louis MO). The subplot consisted of three host switch/transfer times: 5, 10, and 15 days after infestation that were randomly assigned to boxes containing the original host plants. The sub sub-plot consisted of two final host plant types; the transgenic YieldGard[®] Rootworm maize and its isoline hybrids.

The original host plant seeds were sown into 2.25 liter translucent plastic shoe boxes (Ziploc[®], Wal-Mart Company), which were covered with aluminum foil on the bottom and sides to minimize potential effects of white light on root growth (Pilet and Ney, 1978). Four sets of boxes, each containing 24 randomly arranged boxes (corresponding to 4 plant species × 3 switch dates × 2 final hosts), were placed in a growth chamber (37002-GROWC-13, Percival Boone, Iowa). The plant growth medium comprised of soil that had been steam-sterilized for 45 minutes in an autoclave (Amsco Eagle model 2051 Lancer Medical Services Inc., Azusa, CA), and mixed with a peat-based medium (Promix, Premier Horticulture LTEE, Quebec, Canada) in the ratio of 2:1. Two weeks following the sowing of the original host plants, an additional set of rootworm resistant YieldGard[®] and its isoline maize were sown as final hosts in bigger boxes, 11.4 liters (Sterilite[®] Model 1854N5-C, 2002 Sterilite Corporation, Townsend, MA), and maintained in a greenhouse. These containers were also covered with aluminum foil on the bottom and sides to minimize potential effects of white light on root growth (Pilet and Ney, 1978). The larvae used in this study were hatched from diapausing western corn rootworm eggs preserved in the laboratory.

4.2.2. Infestation of original host

Four weeks after planting (V5 stage), each original host plant was infested with 50 neonate western corn rootworm larvae. A pair of wooden labeling stakes were pushed between the inner edge of a pot and its contents and gently lifted to expose the roots. The larvae were then gently placed on the roots using moistened camel's-hair paintbrush. The infested plants were maintained in a growth chamber (37002-GROWC-13, Percival Boone, Iowa) under controlled conditions ($\approx 25^{\circ}\text{C}$, 14:10 (L: D) photoperiod).

4.2.3. Host-switching

At 5, 10, and 15 d, the foliage of each randomly predetermined original host plant was excised and the remaining roots and soil were then visually examined for exposed larvae. Exposed larvae were transferred directly to predetermined final host plant boxes using moistened camel's-hair paintbrush. To transfer the remaining larvae, contents (soil mixture and infested rootmats) were individually placed in Tullgren funnels fitted with 60W Soft white light bulbs (Great value, Wal-Mart Corp. Bentonville, AR). Below each Tullgren funnel containing the original host plant, a box with the predetermined final host plant seedlings was placed to facilitate direct host switch with minimum handling (Figs. 8 and 9). The final host plant boxes were removed from the Tullgren funnels after 72 h, covered on the sides and bottom with aluminum foil, and transferred back to the greenhouse (Fig. 10). Collecting jars containing moist soil were then fitted onto the Tullgren funnels for an additional 48 hours to collect any remaining larvae, which were then transferred to the appropriate container. Some larvae were collected and transferred to the appropriate final host treatments.

The infested final host plants were maintained in the greenhouse under 14:10 (L:D), with natural light supplemented by 1000 watt sodium bulbs (GE Lighting, Cleveland, OH) and a temperature of $25 \pm 2^\circ \text{C}$ as monitored with a temperature recorder (Model SL 4350 C7C, Dickson Co., Addison, IL). Twenty-eight days post infestation (original host), the final host boxes were covered with a beetle-proof insect mesh (0.60 by 0.60 mm opening, ECONET L, LS Americas, Co., Charlotte, NC) and monitored every 2d for teneral beetles. Beetles from each box were collected using a mechanical aspirator (GAST Model 0211-V45F-G8CX, MFG Corp., Benton Harbor Michigan), counted, and placed in labeled rearing boxes. After three days of feeding, the beetles were sexed and the sex ratios recorded. Female beetles were removed from the rearing cages after 3d (Boetel et al. 1998) and sequestered individually in transparent oviposition boxes, 7.6 by 5.7 by 5.7-cm clear H/H, Omniline Plastic Boxes with Flush Lid (Gary Plastic Packaging, Bronx, NY). The beetles were reared after the methods and materials of Boetel and Fuller (1997) with few modifications that included an artificial diet and others described below.

To promote successful insemination, two males from the same treatment as the female were added to each oviposition box. Where there were no sufficient males to match females from a given treatment, males were supplemented from a non-diapausing colony maintained temporarily during the study period in the laboratory. The beetles were provided with an artificial diet (Branson et al. 1975, Jackson 1986), watering wicks and a pinch of maize pollen to promote maximum fecundity. The diet comprised of 290 g Beekeeper's pollen substitute, 54 g wheat germ, 63 g casein, 48 g sucrose, 9 g cellulose powder, 18 g salt mix, 18 g Vanderzant vitamin mix, 0.125g cholesterol moistened with

honey to peanut butter-like consistency (all the ingredients were obtained from Bio-Serve, Frenchtown, NJ). The food (diet, pollen, and water) was replaced with fresh supplies every two days. A layer, approximately 1.5 cm thick, of autoclaved silt soil, that had been sieved through a 70-mesh USA Standard Sieve, Series ASTM Designation E11 (Newark Wire Cloth Co, Newark NJ 07104), was provided at the bottom of each box to facilitate oviposition.

After all females had died, the oviposition boxes, along with the eggs and the oviposition substrate, were placed in a refrigerator at 4° C for a minimum of 150 d to terminate diapause (Fisher et al. 1994). After removal from the cold storage the eggs were incubated in a growth chamber at 25 ± 2°C in order to enable viable eggs to hatch. This was a modification of Boetel et al (1998) since the eggs were allowed to hatch instead of being recovered from the soil first. The oviposition boxes were checked three times weekly for larval hatch. By the 15th day of incubation, hatched larvae could be seen crawling inside the oviposition boxes. After the 21st when no more hatched larvae could be seen crawling about, a shower of cold water was used to separate eggs from soil using a 60-mesh sieve. The eggs were then transferred to a moistened filter paper in a Petri dish (14 by 2 cm).

All eggs recovered were counted under a microscope (M3Z, Wild Co., Heerbrugg, Switzerland). The eggs from each oviposition box were also microscopically examined for hatching and quality – that is healthy, cream colored and robust; or unhealthy, dark brown, empty, misshapen or moldy (Fisher et al. 1994), and also counted. Eggs were classified into two categories: healthy – viable eggs (including hatched eggs) and unhealthy – unviable eggs that were dark brown, empty, misshapen or moldy.

Hatched eggs were represented by empty shells, each with a distinct larval exit opening. The egg quality (viability) and quantity (fecundity) data for all females placed in oviposition chambers were recorded.

The following variable (“Viability”) was derived, as a modification of Boetel and Fuller (1997), and taken as a reflection of a predicted total number of viable offspring that would have been produced by an individual offspring. Healthy eggs include hatched eggs.

$$\text{Viability} = (\text{healthy eggs}/\text{total eggs oviposited}) \times 100\%$$

4.2.4. Statistical analyses

PROC MIXED of the statistical package SAS (SAS Institute 1990) was used to analyze the beetle emergence, sex ratio, and percentage egg viability. The data were analyzed as a randomized complete block split-split plot design in which the main plot contained the effect of original host plant type, the sub-plot contained the effect of host-switching time, and the interaction of original host plant species \times host-switching time, while the sub-sub-plot contained the effects of the final host plant variety, and all possible interactions with the main and sub-plot effects, as outlined in Steel and Torrie (1980). Beyond the standard ANOVA, we compared beetle emergence means for the interactions of original host plant type and final host plant variety that were significant ($\alpha = 0.05$). Fisher’s protected least significant difference (LSD, $\alpha = 0.05$) test was done using LS MEANS and the t-value at the appropriate degrees of freedom from the PROC MIXED output. A pre-planned comparison of the percentage viability of eggs for the different host plant combinations was also conducted. Although untransformed data are

presented, sex ratio and percentage viability data were transformed by $(\arcsin \sqrt{x})$ prior to analysis as suggested by Snedecor and Cochran (1989) for percentage data.

4.3. Results

4.3.1. Beetle emergence.

Beetle emergence, that is the mean number of larvae that developed into beetles, was significantly affected by the original host plant, the final host, and the interaction of the two effects (Table 3). The highest number of beetles emerged from a combination of giant foxtail as the initial host and isoline maize as the final host (Fig. 11). The host combination of crabgrass as the initial host followed by isoline maize also produced significantly more beetles than the remaining treatments (Fig. 11). Significantly more beetles emerged from larvae that first fed on giant foxtail as an initial host and then were switched to rootworm-resistant transgenic maize than from larvae raised exclusively on the rootworm-resistant transgenic maize (Fig. 11).

Although the first beetles to emerge were from isoline maize, the overall number of beetle emerged from larvae reared exclusively on isoline maize was significantly lower than the number of beetles emerged from larvae reared initially on giant foxtail or large crabgrass and then switched to isoline maize (Fig. 11). There was no significant difference in beetle emergence within or between the 5 d, 10 d, and 15 d host-switching (F 0.50; $df = 2, 24$; $P = 0.6144$). The overall interaction between the original host, the host-switching date, and the final host plant variety also did not have a significant effect on the number of beetles that emerged.

4.3.2. Sex ratio.

The sex ratio was significantly affected by the original host plant type and the final host plant type, but not the interaction between the two effects (Table 3). The sex ratio was not significantly affected by the 5th, 10th, or 15th d host-switching, larval movement date. The interactions between the original host plant type, the host-switching date, and the final host plant variety also did not have a significant effect on the sex ratio (Table 3). More males than females emerged from all treatments (Table 4). The overall sex ratio was two males to one female. In host plant combinations where beetle emergence was generally low, only male beetles were mostly recorded. Male beetles also generally emerged earlier than female beetles, and continued to emerge late into the study season.

4.3.3. Fecundity.

Fecundity was significantly affected by the original and final host plant types, and the interaction between the two effects (Table 3). Females that emerged from larvae that were raised exclusively on rootworm-resistant transgenic maize had the lowest mean fecundity (3.33 ± 1.2). A mean fecundity (65.67 ± 20.7) was recorded for females that developed from larvae originally raised on giant foxtail or large crabgrass and then switched to inbred maize (Table 5). The host-switching date and all its interactions did not have a significant effect on the fecundity of the females. Although fecundity did not differ significantly between the three host-switching dates, a higher mean fecundity was recorded for females that emerged from larvae that switched hosts late, that is during the third (15 d) host-switching date.

4.3.4. Egg viability.

Only the original host plant affected egg viability significantly (F 4.75; df = 3, 9; P = 0.0298). Eggs oviposited by females from larvae originally reared on giant foxtail and then switched to isoline maize had the highest viability ($60.60 \pm 13.6\%$), whereas eggs oviposited by females from larvae reared on rootworm-resistant transgenic maize as an original host plant had the lowest viability. For example, for eggs oviposited by females from larvae reared on rootworm-resistant transgenic maize alone viability was $8.26 \pm 2.3\%$, whereas for eggs for females from the rootworm-resistant transgenic maize – isoline maize host switch viability was $8.10 \pm 3.1\%$. Eggs oviposited by females that emerged from larvae that initially fed on the weed species followed by host-switching to rootworm-resistant transgenic maize had a significantly higher viability than the eggs from beetles from larvae raised on an exclusive rootworm-resistant transgenic maize host-switch. For females from the giant foxtail – rootworm-resistant transgenic maize host-switch egg viability was $48.94 \pm 17.1\%$, whereas for large crabgrass – rootworm-resistant transgenic maize host-switch egg viability was $36.28 \pm 10.4\%$.

4.4. Discussion

The results from the present study showed that significantly more beetles emerged from larvae that were initially reared on giant foxtail then switched to rootworm-resistant transgenic maize than from larvae maintained exclusively on the rootworm-resistant transgenic maize. Numerically more beetles emerged from larvae that were initially reared on large crabgrass then switched to rootworm-resistant transgenic maize than from larvae maintained exclusively on the rootworm-resistant transgenic maize. Beetle emergence, sex ratio, fecundity or egg viability did not differ significantly among the

three host-switching dates. This suggests that even a short period (5d) of larval feeding on an alternate host before feeding on transgenic maize, enhances the ability of the larvae to withstand the deleterious effects of the Cry3Bb1 toxin.

The possibility of rootworm larvae to move between weeds and transgenic maize plants may have the potential to impact the outcome of resistance management strategies for rootworm-resistant transgenic maize, although the long-term implications of rootworm larval movement between weeds and a low-dose transgenic maize event have not been fully studied. Movement of rootworm larvae between weeds and transgenic maize potentially occur when the initial host weeds become less nutritious to the larvae due to phenological changes (Chege et al. 2005), or are removed mechanically or using herbicides. Maize root, in the soil, quality decreases with time (Kieselbach, 1949) and larvae must leave older roots and infest younger, higher quality roots, to survive (Apple and Patel, 1963, Strnad and Bergman, 1987b).

The critical period for weed control is the period in the crop growth cycle during which weeds must be controlled to prevent unacceptable yield losses. This is dependent on site-specific factors and its onset could be delayed, for example, by the addition of nitrogen (Evans et al. 2003). Weed control in maize fields could be delayed due to weather, which in turn could lead to scenarios evaluated in the current experiment. With the increasing adoption of rootworm-resistant transgenic maize with high levels of antibiosis stacked with genes conferring resistance to post-emergence herbicides such as glyphosate, grassy weeds in and adjacent to maize fields could play an important role in the western corn rootworm life cycle.

The emergence of more beetles from larvae initially maintained on weeds then switched to rootworm-resistant transgenic maize (significantly higher in the giant foxtail – to MON863 host switch) than beetle emergence from larvae reared on rootworm-resistant transgenic maize alone corroborates the findings of Oyediran et al. (2005). Oyediran et al. (2005) documented significantly higher beetle emergence on a combination of rootworm-resistant transgenic maize and grassy weeds versus rootworm-resistant transgenic maize alone. The current study also supports speculation by Oyediran et al. (2005) that enhancement in beetle emergence from rootworm-resistant transgenic maize plus weeds was likely due to larvae that initially survived on weeds prior to their removal. The emergence of a higher number of beetles, that was recorded for the 15d host-switching date as compared to earlier host switching dates, indicate that the longer the larvae stayed on a suitable original host before being switched to rootworm-resistant transgenic maize, the higher the chances of their survival to adulthood became.

Sex ratio was skewed in favor of males with a ratio of two males to one female. Generally, there was a very close relationship between beetle counts, male to female ratios and fecundity, with the highest records for each of these parameters being documented for giant foxtail – isoline maize host plant combination, and rootworm-resistant transgenic maize alone generally ranking the lowest. In host plant combinations where beetle emergence was generally low, only male beetles were recorded. Male beetles also generally emerged earlier than female beetles. Unfavorable changes in an insect's microenvironment, such as exposure to insecticides could lead to sex ratio shifts in favor of males and reduced female fecundity (Rosenheim and Hoy 1998, Parkman and

Pienkoski 1990). Findings in a study by Rosenheim and Hoy (1998) indicated that exposure to chlorpyrifos elicited a sex-ratio shift in *Aphytis melinus* DeBach offspring toward that of more males. The findings documented in this study suggest that host-switch manipulations, such as larval extraction using Tullgren Funnels that could be harsh to the larvae, could have prompted a sex-ratio shift toward more males. The predominant late emergence of females partly explains the correspondingly low fecundity recorded for some of the host plant combinations. This supposition is supported by Boetel and Fuller (1997) who documented better health and reproductive fitness in early-emerging beetles, with an increase in fecundity in early-emerging females as compared to late-emerging females of both western corn rootworm and northern corn rootworm, *Diabrotica barberi*, Smith & Lawrence.

The predominant emergence of male beetles as compared to females could be attributed to the larval-host plant density relationship that in turn determines the quality and the abundance of food whereby males emerge earlier before food quality and amount declines in the confined host plant boxes. Similarly, the unexpectedly emergence of small numbers of beetles from larvae raised exclusively on isoline maize suggests density-dependent rapid depletion of food by the numerous larvae in a confined space. The size of the containers we used in the study held enough soil to support only one or two maize plants.

In the present study, the poor emergence of beetles from susceptible isoline maize could have been due to good initial establishment of larvae followed by subsequent starvation and death of the growing larvae as available food resources within each box became limiting. Comparable observations were documented by Hibbard et al. (2004).

After evaluating different rootworm egg densities, they reported that post-establishment larval movement and plant damage were density-dependent and that availability of food resources was an important factor in larval movement. On the other hand, a smaller initial establishment of larvae on the grassy weeds and the availability of more numerous albeit small grassy weeds roots could have been responsible for the fairly high beetle emergence recorded for host plant combinations in which the grassy weeds were the original larval hosts.

Fecundity was significantly impacted by the interaction of the original and final host plant types. Fecundity and egg viability for females from beetles reared on isoline maize was comparatively lower than that of females reared on the grassy weeds before being switched to a final host. Branson and Ortman (1967a, b, 1970) documented that although larvae may have higher survival on maize roots, western corn rootworm females reared on some of the alternate hosts oviposited a higher number of viable eggs than those reared on maize. Fecundity was highest from beetles first reared on giant foxtail or large crabgrass and then switched to isoline maize. There was no significant difference in the fecundity of beetles from larvae reared on the grassy weeds or isoline maize and then switched to rootworm-resistant transgenic maize. Fewer eggs were oviposited by beetles from larvae reared on rootworm-resistant transgenic maize alone. Egg viability was generally also lowest for beetles from larvae reared on rootworm-resistant transgenic maize. The reproductive fitness of emerged females seems to be strongly influenced by the host plant combination, especially the host on which the larvae fed during the early stages of development. This is supported by the observation that only the original host plant type impacted egg viability significantly.

The establishment of grassy weeds in substantial areas within and around the maize agroecosystem and erosion control areas may contribute to beetle production of maize rootworms. It is also possible that in transgenic fields selection will now favor development on non-maize hosts. Although the difference was not significant, females that emerge from larvae reared on the grassy weeds or isoline maize and then switched to rootworm-resistant transgenic maize were more fecund than females from larvae reared on rootworm-resistant transgenic maize alone. However, the viability of eggs from females that emerged from larvae reared on the grassy weeds or isoline maize and then switched to rootworm-resistant transgenic maize was significantly higher than the viability of eggs oviposited by females from larvae reared on rootworm-resistant transgenic maize alone. My results provide some support to the supposition that grassy weed in or around maize fields may contribute to the production of reproductively-capable beetles in rootworm-resistant transgenic maize fields. On one hand, this may act as a built in refuge, which could play a role in the slowing down or prevention of development of rootworm resistance to the transgenic maize. On the other hand, it could lead to significant damage to transgenic maize, with consequent reduction in yield. This latter possibility is accentuated by the fact that host plant phenological changes and subsequent plant nutritional status changes or weed removal by herbicides could encourage movement of larvae from weeds to transgenic maize.

In summary, initial larval feeding, for even relatively short periods of time, on an alternate host followed by movement to rootworm-resistant transgenic maize enhances the emergence beetles. In transgenic maize agroecosystems where the control of especially grassy weeds is delayed for any reasons, it may be possible to have enhanced

emergence compared to fields where grassy weeds were controlled prior to egg hatch. This, coupled with other factors, could play a role in the development of rootworm resistance to the transgenic maize. The positive possibility is that the presence of the weeds in the maize agroecosystem could act as a built in refuge, which could play a role in the slowing down or prevention of development of rootworm resistance to the transgenic maize. Therefore, the findings in the current study and previous works (Clark and Hibbard 2004, Oyediran et al. 2004, Chege et al. 2005) have generated information that could be useful in designing appropriate refuge configurations and resistance management models as grassy weeds can play a significant role in western corn rootworm biology.

Figure 8. Host-switching protocol. Tullgren Funnel method modified by placing recipient-/final -host box beneath the funnel to collect escaping larvae



Figure 9. Larva crawling towards a final host plant's (maize) roots.



Figure 10. Final host plants in the greenhouse.



Table 3. Analysis of variance testing differences in western corn rootworm beetle emergence, sex ratio, fecundity, and percentage viability of eggs within and between treatment effects and their interactions. Significance based on $P < 0.05$.

Analyses	Effect	df ₁	df ₂	F	P
Beetle emergence	Original host	3	9	8.35	0.0058*
	Switch date	2	24	0.50	0.6144
	Original host × Switch date	6	24	1.57	0.1985
	Final host	1	36	24.08	<0.0001*
	Orig. host × Final host	3	36	8.95	0.0001*
	Switch date × Final host	2	36	0.51	0.6036
	Orig. host × S. date × Final host	6	36	1.03	0.4248
Sex ratio	Original host	3	9	4.50	0.0343*
	Switch date	2	24	0.08	0.9270
	Original host × Switch date	6	24	0.14	0.9887
	Final host	1	36	4.20	0.0478*
	Orig. host × Final host	3	36	2.20	0.1051
	Switch date × Final host	2	36	0.15	0.8631
	Orig. host × S. date × Final host	6	36	0.63	0.7041
Fecundity	Original host	3	9	5.66	0.0185*
	Switch date	2	24	0.52	0.6019
	Original host × Switch date	6	24	1.66	0.1742
	Final host	1	36	8.90	0.0051*
	Orig. host × Final host	3	36	5.40	0.0036*
	Switch date × Final host	2	36	0.74	0.4829
	Orig. host × S. date × Final host	6	36	1.19	0.3348
Egg viability	Original host	3	9	4.75	0.0298*
	Switch date	2	24	0.40	0.6742
	Original host × Switch date	6	24	0.98	0.4572

Final host	1	36	0.40	0.5304
Orig. host × Final host	3	36	1.52	0.2259
Switch date × Final host	2	36	0.36	0.6992
Orig. host × S. date × Final host	6	36	0.68	0.6645

df₁: numerator degrees of freedom. df₂: denominator degrees of freedom.

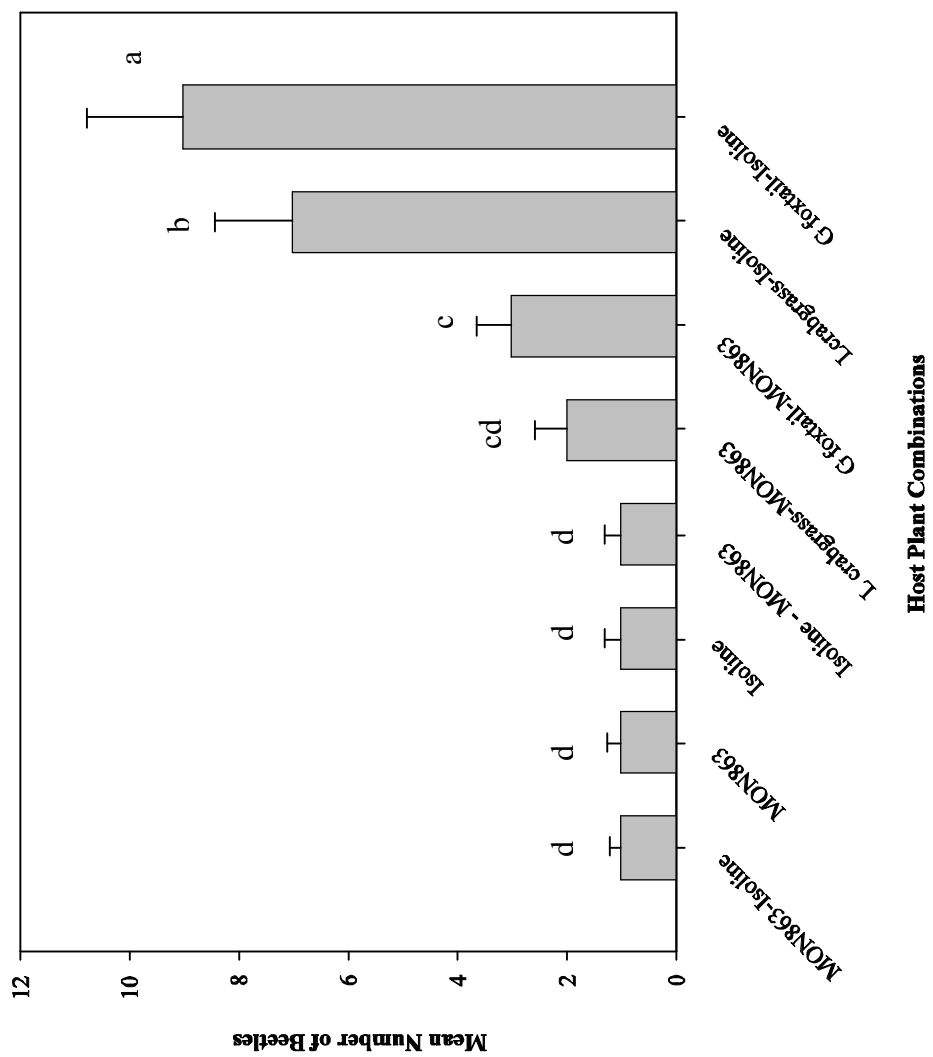
Table 4. Effect of original and final host plant treatments on the number of males and females (Mean \pm SE) of western corn rootworm beetles. Data from three host-switching (larval movement) dates 5, 10, and 15 d for each host plant type were combined and averaged. Within column means followed by the same letter are not significantly different [Fisher's Protected LSD test ($P < 0.05$)].

Treatment	Males (Mean \pm SE)	Females (Mean \pm SE)
<u>Original host</u>		
MON863 maize	0.29 \pm 0.04c	0.08 \pm 0.04b
Isoline maize	0.54 \pm 0.08c	0.29 \pm 0.11b
Giant foxtail	4.13 \pm 0.75a	1.33 \pm 0.40a
Large crabgrass	2.83 \pm 0.41b	1.04 \pm 0.18a
<u>Final host</u>		
MON863 maize	3.50 \pm 0.04a	0.40 \pm 0.02b
Isoline maize	2.99 \pm 0.34a	0.98 \pm 0.19a

Table 5. Effect of different host plant combinations on western corn rootworm females' fecundity and egg viability (Mean \pm SE). Data from three host-switching (larval movement) dates 5, 10, and 15 d for each host plant combination were combined and averaged. Within column means followed by the same letter are not significantly different [Fisher's Protected LSD test ($P < 0.05$)].

Host plant combination (Treatment)	Fecundity	Egg viability (Percentage)
MON 863 maize	3.33 \pm 1.2b	8.26 \pm 2.3bc
MON863 – Isoline maize	3.50 \pm 0.9b	8.10 \pm 3.1bc
Giant foxtail – MON863	16.00 \pm 4.7b	48.94 \pm 17.1a
Giant foxtail – Isoline maize	65.67 \pm 20.7a	68.23 \pm 14.8a
Isoline – MON863	17.67 \pm 4.2b	36.01 \pm 9.3ab
Isoline maize	5.67 \pm 1.7b	14.10 \pm 4.2b
Large crabgrass – MON863	15.17 \pm 2.2b	36.28 \pm 10.4ab
Large crabgrass – Isoline maize	53.17 \pm 17.4a	60.60 \pm 13.6a
LSD	26.0	22.6

Figure 11: Effect of larval movement between different host plant combinations on the emergence of western corn rootworm beetles. Bars indicate mean + SE. Data from three host-switching dates (5, 10, and 15 d) for each host plant combination were combined and averaged. Bars bearing the same letter are not significantly different [Fisher's Protected LSD test ($P < 0.05$)]. NB: On the graph, MON863 refers to MON 863-to-MON 863 host-switch and Iso line refers to Iso line-to-Iso line host switch.



Chapter 5

Host Plant Phenology affects Western Corn Rootworm (Coleoptera:

Chrysomelidae) Larval Host-Searching Behavior

ABSTRACT

A study to determine the impact of host plant phenology on the host-searching behavior of western corn rootworm neonate larvae was conducted in a split-plot randomized complete block design experiment under greenhouse conditions. Five host plant types, 3 grassy weed species large crabgrass [*Digitaria sanguinalis* (L.) Scop], giant foxtail (*Setaria faberi* R. A. W. Herrm) and barnyard grass (*Echinochloa muricata* (Beauv.) Fernald)], and 2 varieties of maize [rootworm-resistant Cry3Bb1 transgenic maize (YieldGard[®] Rootworm hybrid CS 304YGRW/RR) and MON863 isoline hybrid CS304RR (Monsanto Co. St. Louis MO)], were evaluated at three different phenological stages (root age); 4, 7, and 10 weeks after planting, and replicated four times. The behavioral movement of neonate western corn rootworm larvae after contact with root samples from each of the host plant species was tracked and recorded for 5 minutes in an arena, using the Ethovision[®] system, and the paths were analyzed to determine host-finding behavior. Plant phenology (sampling time) significantly affected the total distance moved (movelength) by larvae, the velocity at which the larvae moved, and the total turning angle of larvae. The interaction of plant phenology (sampling time) and host plant species had a significant effect on the mean turning angle and the meander of neonates.

KEY WORDS. Grassy weeds, alternate hosts, Ethovision, host-search behavior, phenology, corn rootworm.

5.1. Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a major pest of maize (*Zea mays* L.), which costs maize producers in North America, millions of dollars in yield losses and control expenses. This pest is widely distributed in the corn belt of North America (Chiang 1973). Damage is primarily caused by larvae burrowing, tunneling and feeding on maize roots, which results in decreased plant growth and reduced yield by interfering with photosynthetic rates, limiting the uptake of water and nutrients, and by making plants to become more susceptibility to lodging (Spike and Tollefson 1989, Apple and Patel 1963, Godfrey et al. 1993, Urias-Lopez et al. 2000, Riedell and Kim 1990).

The western corn rootworm is a univoltine insect whose life history is closely synchronized with the phenology of corn, its primary host (Chiang 1973). According to Suttle et al. (1967) and Short and Luedtke (1970), corn rootworm larvae move through the soil as far as 100 cm, in search of maize roots, from egg hatch to where beetles emerge. Several studies have shown that first instar rootworm larvae locate a host by sensing CO₂ gradients in the soil given off by the root during respiration (Gustin and Schumacher 1989, MacDonald and Ellis 1990, Strnad and Bergman 1987a, b, Bernklau and Bjostad 1998b). The ability of rootworm larvae to locate and recognize host roots is therefore crucial for the survival of the larvae.

Bell (1990) described searching behavior is "an active movement by which insects seek resources." According to Jander (1975), search behavior in animals, generally, occurs in two phases described as: ranging and local search. Ranging search is involves relatively straight locomotion in which the animal has limited resource

information whereas local search involves increased turning rate and decreased locomotor rate when animals perceive information of suitable resources (Bell 1985).

Browne (1997) categorized phytophagous insects' host selection as choice behavior with two extremes that coincide with host plant recognition and host plant finding respectively. The first extreme is distinguished by the fact that insects choose solely after contact with a potential host plant and the frequency of visits to a host and nonhost plant is dependent on the relative abundance of the plant in the environment. The other extreme is that insects perceive host plant characteristics, such as volatile chemicals, color, and shape, at a distance and choose to visit the host because of these host-specific signals. Calkins et al. (1967) reported that many phytophagous subterranean insects locate their hosts by host-oriented responses as opposed to random movements.

Bell (1990) described four decision rules that an insect may use to remain within a patch. Strnad and Dunn (1990) developed a novel assay in which they analyzed paths of larvae after initial contact of larvae with roots of potential host plants in order to determine host recognition behavior. They observed that larvae shifted from long distance ranging behavior to localized search behavior after 5 min of contact with the roots of maize and wheat, but not contact with oats, giant foxtail, or soybean. They argued that this shift from ranging to localized search is initiated by contact cues, and that it dominated over response to volatile cues because immediately after contact with maize roots, larvae were no longer attracted to carbon dioxide.

According to Strnad and Dunn (1990), factors in host plant roots trigger a localized search behavior when larvae are removed from the host and this localized

search behavior is not triggered by non-host roots. Larvae that perceive a root as a suitable host exhibit increased turning, reduced velocity, and increased crossovers in their range of movement (meander), whereas larvae that do not recognize a root as a host travel at a higher velocity and display few or no sharp turning angles or meanders.

Oyediran et al. (2004) used an assay similar to that of Strnad and Dunn (1990) to analyze the host search behavior of the north corn rootworm neonates. They recorded a significant difference in the area searched, number of turns, crossings and the velocity the behavior of the northern corn rootworm neonates exposed to the different plant species and the control (moistened filter paper). The control had the highest ranging behavior while maize and barnyard had the mostly localized search behavior. Recently, the use of novel technology in the study of the behavior of insect herbivores is becoming increasingly popular.

In a recent study, we demonstrated that host plant species and phenology affects western corn rootworm survivorship, growth, and development (Chege et al. 2005). Evidence also suggests that larvae prefer to feed on younger, more succulent roots, often migrating to, and tunneling into the root apices as they penetrate the soil (Chiang 1973, Strnad and Bergman 1987a, b). Understanding of the impact of corn phenology on rootworm larval host-search behavior and movement could help us explain the propensity of larval movement between alternate hosts and transgenic rootworm-resistant maize.

The increasing adoption of transgenic maize with resistance to rootworms and the potential for rootworm larvae to move (Hibbard et al. 2004) between weeds and transgenic maize within the maize agroecosystem makes it important to evaluate the impact of host plant phenology on the feeding behavior of the western corn rootworm.

This experiment was a follow-up to two earlier studies. In the first one (Chege et al. 2005) we documented that host plant phenology affects western corn rootworm survivorship, growth, and development, and in the second one we reported that initial feeding on an alternate host enhances western corn rootworm beetle emergence on Cry3Bb1-expressing maize. In the second study, Chege et al. (200X) we documented that initial feeding on an alternate host enhances western corn rootworm beetle emergence on Cry3Bb1-expressing maize. In this study, the objective was to analyze the effect of host plant phenology on western corn rootworm larval host-searching behavior.

5.2. Materials and Methods

5.2.1. Experimental Design, Plant material, and Insects.

The study was conducted in a greenhouse on the University of Missouri-Columbia campus from fall 2005 to winter 2006. The experimental design was a randomized complete block split-plot with four replications. The main plot consisted of five different original host plant treatments: three weed species (purchased from Valley Seed Service, Fresno, CA) and two corn hybrids. The weed species were: large crabgrass (*Digitaria sanguinalis* (L.) Scop), giant foxtail (*Setaria faberi* R. A. W. Herrm), and barnyard grass [*Echinochloa muricata* (Beauv.) Fernald]. The corn hybrids were: a rootworm resistant Cry3Bb1-expressing hybrid, CS 304YGRW/RR and its rootworm susceptible near-isoline hybrid, CS304/RR (Monsanto Co. St. Louis MO) as a positive control. All the non-maize hosts had been identified as viable alternative hosts for corn rootworm in previous studies (Oyediran et al. 2004, Clark and Hibbard 2004). The subplot consisted of three sampling times (4, 7, and 10 weeks after planting), to examine the effect of host plant phenology on the feeding behavior of western corn rootworm

larvae. A total of 60 pots were planted: one pot for each of the three sample times, for each of the five host plant varieties (two varieties of maize and three grassy weed species) and replicated four times.

The plant growth medium comprised of soil that had been steam-sterilized for 45 minutes in an autoclave (Amsco Eagle model 2051 Lancer Medical Services Inc., Azusa, CA), and mixed with a peat-based medium (Promix, Premier Horticulture LTEE, Quebec, Canada) in a ratio of 2:1. The seedlings were planted the growth medium 10 liter pots (Model CL-600, Hummert International, St. Louis, MO). Planted pots were then arranged randomly on greenhouse benches by subplots (Fig. 12), each subplot having columns of three pots that corresponded to sampling time and rows of five pots that corresponded to host plant variety (order was randomized from subplot to subplot).

Upon germination, seedlings were watered as necessary and grown under 14:10 (L:D) h, with natural light supplemented by 1000 watt sodium bulbs (GE Lighting, Cleveland, OH), and a temperature of $25 \pm 2^\circ$ C. We used neonate (>12 h old) larvae from the non-diapausing western corn rootworm colony we maintain that was originally obtained from the Northern Grain Insects Research Laboratory, USDA-ARS laboratory, Brookings SD.

5.2.2. Effect of host plant phenology on larval host-searching behavior.

To determine the impact of host plant phenology on the host-search behavior of neonate western corn rootworm larvae, host plant samples were taken during each sampling time, 4, 7, and 10 weeks after planting. On each sampling occasion, the foliage of the randomly assigned original host plant was cut close to the soil and the rootmats and soil mixture transferred into a plastic pan and soaked in water to remove the plant growth

media. After removing the bulk of the growth media, the roots were washed using a gentle shower of tap water first and finally rinsed with distilled water (Figs. 13 and 14). A clean sub-sample of roots from each of the host plant varieties was then transferred to a 2 liter translucent plastic shoe boxes (Ziploc[®] Wal-Mart Company) and infested with 20 neonate western corn rootworm larvae, by gently placing them on the roots using a moistened fine camel's-hair paintbrush. After allowing the larvae to feed on the roots for five minutes, one larva, out of the 20 originally placed on the roots each host plant variety, was randomly picked and its host-searching behavior recorded using the Ethovision[®] system (Noldus Information Technology Inc. Leesburg, VA) video camera and software (Fig. 15). This was replicated four times for each host plant variety.

To record the host-searching behavior of larvae on each sampling occasion, the randomly selected larvae were individually placed on the arena and their behavioral movement tracked and recorded for 5 minutes using the Ethovision[®] system video camera and software. The arena comprised of a moist 185 mm Whatman[®] filter paper circle (Whatman International Ltd., Maidstone England) mounted on a wooden platform under the Ethovision[®] system video camera. The moist filter paper was replaced prior to each sampling. Therefore, on each of three (4, 7, and 10 weeks after planting) sampling occasions, a total of 20 track-files were recorded, corresponding to five larvae, one from each of the five host plant varieties, replicated four times. A total of 60 track files were recorded for the entire study.

5.2.3. Ethovision[®] Protocol.

For optimum viewing of larvae, we defined the arena and calibrated the “tracking” settings to the following ideal specifications: Detection method, subtraction;

processing settings, only detect objects that are darker than background; image enhancement, contrast, 86 and -10 brightness; search window, 20 pixels; minimum object size, one pixel; maximum object size, 20 pixels; sample rate, two seconds per sample. Under the “trial protocol,” tracking time was set at five minutes, and auto-settings for background noise removal engaged.

For each larval tracking, we selected “acquire data” option under the “experiment” menu. The appropriate user defined variables (host species/variety, sampling date, and replication) were then specified and the larval gently introduced into the arena using a moist camel’s-hair paintbrush. After pressing the start button, the behavior of the larva was tracked and recorded for five minutes. Before putting a new larva on the arena, the blotting paper was replaced with a fresh moist one. To mitigate for any changes in the settings, the detection variables were updated as follows: under “tracking” menu, we chose “update detection variable,” then chose “measure,” and then clicked “update.”

The recorded larval host-searching behavior, primarily movement, was defined by the following dependent variables or parameters measured during the five minutes of tracking:

1. Total distance moved (cm). The distance traveled by the ‘center of gravity’ of the object between two samples.
2. Mean velocity (cm/s).
3. Total turn angle (degrees).
4. Mean turn angle (degrees), and

5. Mean meander (degrees/cm). The change in direction of movement of an object relative to the distance it moves. It is measured in degrees per unit of distance (the unit one calibrated ones arena in). Meander = Relative turn angle/distance moved. Because meander is corrected for distance, it is also corrected for speeds (unlike angular velocity and turn angle). Therefore you can use meander to compare the amount of turning of objects moving at different speeds (Bell, 1991).

5.2.4. Statistical Analyses.

PROC MIXED of the statistical package SAS (SAS Institute 1990) was used to analyze the total distance, maximum data, total turning angle, mean meander, as well as the mean turning angle data. The data was analyzed as a RCBD split plot in which the main plot contained the effect of original host plant species (or variety) the sub-plot contained the effect of the different plant phonologies (root age) represented by the three sampling times, and the interaction of original host plant species and sampling time, and all possible interactions with the main and sub-plot effects, as outlined in Steel and Torrie (1980). Replications within plant species served as the denominator of F for testing the effects of plant species. Replications within sampling dates and sample dates were used as the denominator of F for testing treatment and the interaction of sampling dates. Beyond the standard ANOVA, we did pre-planned comparisons of means for the host plant species main effects. We also compared the interactions of original host plant species and sampling time ($\alpha = 0.05$). Fisher's protected least significant difference (LSD, $\alpha = 0.05$) test was done using LS MEANS and the t-value at the appropriate degrees of freedom from the PROC MIXED output.

5.3. Results.

5.3.1. *Effect of host plant type.*

Neonates exposed to roots from the various host plant types displayed one of two general host-searching patterns to varying degrees: Either a ranging searching pattern following a relatively less convoluted path, and characterized by long total distance movement (long movelength), high velocity, low turning angles and a low meander; or a localized searching pattern along a convoluted path, restricted within a relatively short total distance (movelength) at a low velocity, and displaying high turning angles and a high meander (Figs. 16 - 20). Host plant type did not have a significant effect on the total distance searched, larval velocity, total and mean turning angle, or meander (Table 6), but these variables were significantly affected by host plant phenology (Tables 6 and 7). Larvae exposed to the roots of isoline maize on average displayed a more localized search, with a shorter movelength (8.64 ± 2.73 mm), compared to the movelength searched by larvae exposed to roots of Cry3Bb1-expressing maize (9.51 ± 3.32 mm), large crabgrass, and giant foxtail (Table 8). Only larvae exposed to the roots of barnyard grass searched a shorter total distance than isoline maize (7.12 ± 2.21 mm). A corresponding pattern was recorded for larval velocity in all host plant treatments (Table 8). The order was reversed in the pattern of meanders, with the meander being relatively higher for larvae that searched the shortest total distance and moved at the lowest velocity and vice versa (Table 8). Larvae exposed to large crabgrass searched the longest total distance (11.40 ± 1.88 mm), moved at the fastest velocity (0.04 ± 0.01 mm/sec), and displayed the lowest meander (2593.23 ± 66.42 degrees/cm).

Least square mean differences between host plants showed that there was unprotected significant difference between the total distance searched by larvae exposed to roots of barnyard grass and large crabgrass ($P = 0.0201$). All other comparisons of total distance between different host plants did not show statistically significant differences.

5.3.2. Effect of host plant phenology.

Plant phenology (sampling time) significantly ($F = 19.57$ df 2, 30 $P < 0.0001$) affected the total distance moved by larvae, the velocity at which the larvae moved, and the total turning angle of larvae however, the interaction of host phenology and host plant species significantly affected larval meander (Table 6). In general, the total distance searched, larval velocity, total and mean turning angle differed significant between week 4 (V5) and week 10 (V10), but not between week 7 (V8) and week 10 (Table 7).

The interaction of plant phenology (sampling time) and host plant species had a highly significant effect on the mean turning angle of larvae ($F = 4.11$ df 8, 30 $P = 0.0021$) and slightly significant effects on: the total distance searched by larvae ($F = 2.06$ df 8, 30 $P = 0.0730$), the velocity of the larvae ($F = 2.04$ df 8, 30 $P = 0.0749$), and the meander (df 8, 30 $F = 2.10$ $P = 0.0678$). In all the host plant treatments, the shortest total distance searched was recorded for larvae exposed to roots of 4 week old (V5) plants (Table 9). The total distance searched by larvae exposed to roots of Cry3Bb1-expressing maize and giant foxtail increased with root age, whereas it fluctuated for larvae exposed to roots of isoline maize, large crabgrass and barnyard grass (Table 9). A similar pattern was recorded for velocity (Table 10).

Larval meander fluctuated depending on the interaction of host plant phenology (sampling dates) and the host plant treatments (Table 11). For example, the path patterns for some neonates that contacted 10 week old isoline maize roots indicated a more localized search characterized by a high mean meander (2920.39 ± 128.83 degrees/cm), compared to the search displayed by neonates that contacted 4 weeks (V5) old or 7 weeks old (V8) isoline maize roots, which had mean meanders of 2859.45 ± 238.65 degrees/cm and 2728.21 ± 96.71 degrees/cm respectively. For Cry3Bb1-expressing maize, on the other hand, neonates that contacted 7 week old (V8) roots displayed the highest meander (3175.45 ± 297.83 degrees/cm) compared to larvae exposed to 4 week old (V5) roots (2556.89 ± 0.00 degrees/cm) or 10 weeks old (V10) roots (2219.93 ± 83.25 degrees/cm).

Some of the neonates that contacted Cry3Bb1-expressing maize became moribund and did not move significantly after being transferred to the arena (Fig. 19). In a few cases, neonates that contacted V5 and V8 Cry3Bb1-expressing maize roots displayed more ranging-type search behavior (Figs. 20 and 21) whereas some neonates that contacted 10 week old Cry3Bb1-expressing maize roots displayed a more localized search pattern (Fig. 22). Generally, however, neonates that contacted younger, 4 weeks and 7 weeks old (V5 and V8) Cry3Bb1-expressing maize roots searched a shorter movelength and moved slower than neonates that contacted older, 10 weeks (V10) Cry3Bb1-expressing maize roots.

The search pattern (meander), the total distance searched and the velocity of neonates that contacted large crabgrass roots fluctuated with phenological changes of the roots. Generally, the mean total distance searched was shortest (8.50 ± 2.13 mm) among the larvae that contacted 4 week old large crabgrass roots, longest for the 7 week old

phenology (14.91 ± 2.38 mm), and in between the two extremes for neonates that contacted 10 week old roots (9.66 ± 3.21 mm). The trend was exactly the same for the velocities, and reversed in the pattern meander (Figs. 23, 24, & 25); the lowest being for neonates that contacted 7 week old roots (2501.46 ± 158.96 degrees/cm). Neonates that contacted barnyard grass roots searched the within the least mean total distance, moved at the lowest mean velocity, and had relatively higher meanders compared to neonates that contacted roots from other plant species at each sampling time. The mean total distance searched by neonates after contacting 4, 7, and 10 week old barnyard grass roots was; 3.08 ± 0.82 mm, 10.66 ± 0.76 mm, and 7.58 ± 3.03 mm respectively. The same pattern was recorded for the neonates' velocities, and reversed for the meander (Figs. 26, 27, & 28). A very similar trend was recorded for neonates that contacted roots of isoline maize (Figs. 29, 30, & 31).

5.4. Discussion

The findings in this study demonstrate that host plant phenology affects the host-searching and feeding behavior of western corn rootworm larvae. Neonates exposed to roots from the various host plant types exhibited either a localized or ranging search. Neonate western corn rootworm have to find a suitable host within their first 24 hours in order to enhance their survivor, and the shift in their host-searching behavior between a localized and ranging search enhances their chances of locating a viable host (Strnad and Bergman 1987b). Bell (1990) described four possible rules that an insect may follow to locate food. The first is the decision to restrict searching to the current patch after resource utilization (e.g. feeding), whereby the insect adopts a turning bias, turning sharply either left or right, but over time, if resources are not encountered the search path

becomes straighter. Secondly, an insect may decrease movelength or move only short distances before stopping to scan, and if resources are not encountered the movelength gradually increases. The third decision rule directs the insect to leave the resource patch in a different direction from the arrival direction, whereas the fourth decision rule directs the insect to turn back when the edge of the patch is detected (Bell 1990). The observations documented in the current study show that western corn rootworm neonates seem to follow these rules in the process of searching for a suitable host.

The search pattern exhibited by larvae after contact with host plant roots indicates whether the larvae perceive the roots as a suitable host or not. Strnad and Dunn (1989) observed that larvae searched the smallest area and moved the slowest after contact with a maize root. The results demonstrated that sampling time, which was a measure of plant phenology, significantly affected the larval movelength, the velocity at which the larvae moved, and the total turning angle of larvae in the process of host-searching. Larval meander fluctuated with phenological changes, but contact of neonates with 4 week old roots (V 5 stage) from each of the host plant species generally elicited a more localized search behavior, with a shorter movelength, low velocity and relatively high meander (more convoluted path). This indicates that generally the V5 stage of maize and the grassy weeds was phenologically more appealing to neonates than subsequent phenological stages.

The plant phenological age hypothesis predicts that phytophagous insects should prefer and perform better on phenologically young plants than on old plants because plant nutritional quality decreases with plant phenological age (Scheirs et al. 2002). However, there were some exceptions to this general rule in the current study, possibly due to the

interaction effect of host plant species and phenology. For example, the path patterns for neonates that made contact with V10 (10 week old) isoline maize roots indicated a more localized search compared to the search pattern of neonates that contacted V5 or V8 (4 or 7 weeks old) isoline maize roots. A similar observation was made for V10 MON863 maize. Recent field and greenhouse studies with maize have also demonstrated that western corn rootworm larvae could establish on late phenology maize plants, but adult emergence was greatly reduced (B.E.H., unpublished data).

The unexpected localized searching behavior by neonates that contacted V10 isoline maize roots corresponds to a similar unexpected increase in larval recovery from the last maize infestation time (V10 stage) that we documented in a previous study (Chege et al 2005), and which was due to enhanced maize root volume. However, although the localized search behavior may in this case indicate that the neonates had located a suitable food patch, feeding on a V10 isoline maize root could subsequently be nutritionally less rewarding than a V5 or V8 isoline maize root. As documented in Chege et al (2005), larvae that fed on V10 maize roots had reduced weight gain and significantly smaller head capsule widths compared to larvae recovered from the earlier maize phenologies indicates a loss in nutritional value for developing rootworm larvae as the plants aged.

The same reasoning could explain the host-searching behavior pattern for western corn rootworm neonates that contacted some of the Cry3Bb1-expressing maize root samples at different phenological stages. In addition the apparent departure in the host-searching behavioral pattern of neonates that contacted different phenological stages of Cry3Bb1-expressing maize roots could also be as a result of balancing the tradeoffs of

younger nutritious roots that express high concentrations of Cry3BB1 toxin against older less nutritious roots with weaker toxin expression. Vaughn et al (2005) documented that the "Growth Stage" of different transgenic maize hybrids significantly affected the expression level of Cry3Bb1 between V4 and V9 stages. They reported a general tendency of a decrease in root expression as the plant matured from V4 to V9. According to Nakamuta (1985), the intensity of the feeding stimulus partly determines the duration of localized search. The documented varying search patterns of neonates after contacting Cry3Bb1-expressing maize in the current study could thus be an indication of how nutritiously rewarding or toxically repelling the Cry3Bb1-expressing maize roots were perceived by the neonates in each case.

Changes in larval feeding with seasonal changes in plant nutrients have been documented in past studies of the feeding behavior of many insects. For example, Whitham (1981), Waltz and Whitham (1997), and Lawrence et al. (2003), have documented that the nutritional quality of plants to herbivorous insects varies genetically, temporally, and spatially. Awmack and Leather (2002) reported that the responses of insect herbivores to changes in host plant quality vary within and between feeding guilds. According to Feeny (1970) and Kearsley and Whitham (1989) seasonal changes in plant nutritional quality are a major source of temporal variation that also impacts the population dynamics of a broad range of phytophagous insects.

In the current study, grassy weeds, notably barnyard grass, elicited a more localized search pattern in neonates, characterized by a shorter movelength, low velocity, and a high meander compared to Cry3Bb1-expressing maize or isoline maize or any of the other grassy weeds. The behavioral pattern elicited by barnyard grass, therefore,

indicates that the contact of neonates with barnyard could be energetically rewarding. On the other hand, the behavioral patterns elicited by contact of neonates with large crabgrass or giant foxtail may also indicate that these grassy weeds are also nutritionally attractive to the neonates. In the absence of a more attractive choice, neonates could remain within a “patch” (roots) of these grassy weeds. Past studies have documented that many grassy weed species found in or around cornfields are able to support the growth, development and fecundity of western corn rootworm larvae (Branson and Ortman, 1967a, 1970, Clark and Hibbard, 2004, Wilson and Hibbard, 2004), and that host plant phenology affects western corn rootworm survivorship, growth and development (Chege et al. 2005).

Western corn rootworm larvae prefer newly developed roots (Strnad and Bergman 1987). As the results in this study show, host plant phenology affects the host-searching behavior of neonate western corn rootworm. These findings support the supposition that growth and development, and subsequent movement of rootworm larvae between weeds and transgenic maize could potentially occur when the initial host weeds become less nutritious to the larvae due to phenological changes. Recent studies have also supported the possibility of plant-to-plant western corn rootworm larval movement motivated by a search for food (Hibbard et al. 2004). Therefore, larval development on grassy corn field weeds and subsequent movement to rootworm-resistant transgenic maize has potentially important implications for the development of appropriate resistance management strategies. This is important with regard to the potential for resistance development and refuge configuration in insect resistance management (IRM) programs involving the inclusion of western corn rootworm non-maize grass hosts as part of the refuge. Western

corn rootworm larvae could move to rootworm-resistant transgenic maize, following changes in their micro-environment such as weed removal or phenological changes of grassy host species other than maize after initial establishment and development of significant numbers of the larvae may occur on those non maize hosts.

Figure 12: Experimental layout in the greenhouse. Pots were arranged in groups of 15 (5 species X 3 sample dates).



Figure 13: Sampling: Randomly pre-assigned samples were soaked in water to remove the plant growth media.



Figure 14: Root-cleaning and infestation procedure. The host plant roots were rinsed with distilled water and then neonate western corn rootworms were placed the roots. After 5 minutes, randomly selected neonates were individually transferred to the arena and their host-searching path patterns tracked and recorded using the Ethovision[®] System video camera and software.



Figure 15: The Ethovision® System video camera that was used to track and record the host-searching path patterns of neonate western corn rootworm. The camera was connected to a computer equipped with the Ethovision® software.

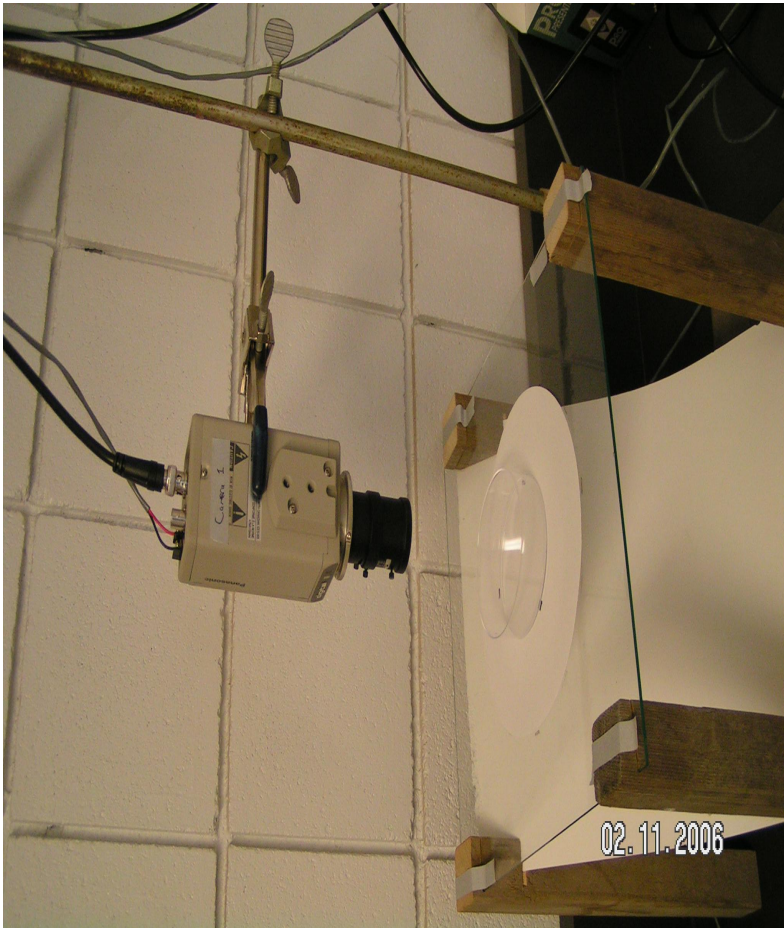


Table 6. Analysis of variance testing differences in total distance moved by western corn rootworm neonate larvae, their velocity, total and mean turning angle, and meander within and between treatment effects and their interactions. Significance based on $P < 0.05$.

Analyses	Effect	df ₁	df ₂	F	P
Distance moved	Host plant	4	12	2.06	0.1498
	Sampling date (phenology)	2	30	19.57	<0.0001*
	Host plant × Sampling date	8	30	2.06	0.0730
Velocity	Host plant	4	12	2.03	0.1538
	Sampling date (phenology)	2	30	19.12	<0.0001*
	Host plant × Sampling date	8	30	2.04	0.0749
Total turning angle	Host plant	4	12	2.15	0.1364
	Sampling date (phenology)	2	30	15.96	<0.0001*
	Host plant × Sampling date	8	30	1.64	0.1554
Mean turning angle	Host plant	4	12	2.42	0.1364
	Sampling date (phenology)	2	30	1.16	0.3283
	Host plant × Sampling date	8	30	4.11	0.0021*
Meander	Host plant	4	12	1.19	0.3629
	Sampling date (phenology)	2	30	0.19	0.8310
	Host plant × Sampling date	8	30	2.10	0.0678*

df₁: numerator degrees of freedom, df₂: denominator degrees of freedom

Table 7. Comparisons by Differences of Least Square Means for total distance traveled by neonate western corn rootworm, their velocity, total turning angle and meander.

Dependent Variable	Sampling date (Phenology, i.e. root age in weeks)	DF	t	$P > t $
Total Distance	4	30	5.56	<.0001*
	4	30	5.27	<.0001*
	7	30	0.29	0.7738
Velocity	4	30	5.49	<.0001*
	4	30	5.20	<.0001*
	7	30	0.29	0.7740
Total Turning Angle	4	30	5.38	<.0001*
	4	30	4.19	0.0002*
	7	30	1.19	0.2430
Meander	4	30	0.61	0.5494
	4	30	0.37	0.7141
	7	30	0.24	0.8153

“*” indicates significant differences.

Table 8. Total distance searched (larval movelength), velocity, mean turning angle, and meander of neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots. Data from three sampling dates 4, 7, and 10 weeks for each host plant type were combined and averaged. Within column means followed by the same letter are not significantly different [Fisher's Protected LSD test ($P < 0.05$)].

Treatment	Total Distance (mm) (Mean \pm SE)	Velocity (mm/sec) (Mean \pm SE)	Mean Turning Angle (Degrees) (Mean \pm SE)	Meander (degrees/cm). (Mean \pm SE)
<u>Host Plant</u>				
MON863 maize	9.51 \pm 3.32a	0.03 \pm 0.01b	119.02 \pm 9.94ab	2650.76 \pm 279.80a
Isoline maize	8.64 \pm 2.73a	0.03 \pm 0.01b	129.03 \pm 2.36a	2836.02 \pm 56.70a
Giant foxtail	10.23 \pm 2.40a	0.03 \pm 0.01b	120.95 \pm 7.25a	2653.90 \pm 203.91a
Large crabgrass	11.40 \pm 1.88a	0.04 \pm 0.01a	122.19 \pm 3.00a	2593.23 \pm 66.42a
Barnyard grass	7.12 \pm 2.21b	0.02 \pm 0.01c	135.02 \pm 12.47a	2985.47 \pm 245.78a
LSD	4.02	0.001	15.11	524.87

Table 9. The effect of host plant phenology (4, 7, 10 weeks old roots) on the total distance searched by neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.

Total Distance (mm)				
<u>Host Plant</u>	Week 4	Week 7	Week 10	
MON863 maize	3.83 ± 0.15	9.38 ± 2.01	15.33 ± 1.60	
Isoline maize	3.615 ± 0.16	13.34 ± 1.60	8.71 ± 2.90	
Giant foxtail	6.59 ± 2.28	10.11 ± 2.15	14.2 ± 2.39	
Large crabgrass	8.50 ± 2.13	14.91 ± 2.38	9.66 ± 3.21	
Barnyard grass	3.08 ± 0.82	10.66 ± 0.76	7.58 ± 3.03	

Table 10. The effect of host plant phenology (4, 7, 10 weeks old roots) on the velocity of neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.

<u>Host Plant</u>	Velocity (mm/sec)		
	Week 4	Week 7	Week 10
MON863 maize	0.013 ± 0.00	0.0313 ± 0.01	0.051 ± 0.01
Isoline maize	0.014 ± 0.00	0.0445 ± 0.01	0.029 ± 0.01
Giant foxtail	0.028 ± 0.01	0.0337 ± 0.01	0.048 ± 0.01
Large crabgrass	0.028 ± 2.13	0.0497 ± 0.01	0.036 ± 0.01
Barnyard grass	0.010 ± 0.00	0.0357 ± 0.00	0.025 ± 0.01

Table 11. The effect of host plant phenology (4, 7, 10 weeks old roots) on the meander of neonate rootworm larvae during the 5-minute period immediately after contact with host plant roots.

<u>Host Plant</u>	Meander (degrees/cm).		
	Week 4	Week 7	Week 10
MON863 maize	2556.89 ± 198.23	3175.45 ± 297.83	2219.93 ± 83.25
Isoline maize	2859.45 ± 238.65	2728.21 ± 96.71	2920.39 ± 128.83
Giant foxtail	3056.16 ± 23.21	2510.87 ± 89.19	2394.65 ± 262.96
Large crabgrass	2555.93 ± 691.41	2501.46 ± 158.96	2722.29 ± 114.12
Barnyard grass	2954.48 ± 210.68	2576.11 ± 176.50	3425.82 ± 378.15

Figures 16 – 31: Neonate western corn rootworm host-searching patterns (paths) resulting from contact with roots of isoline maize, Cry3Bb1-expressing maize, large crabgrass, giant foxtail and barnyard grass at 4, 7, and 10 (weeks old) phenological (root age) stages.

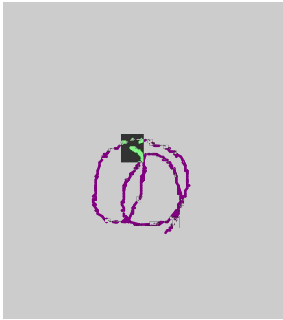


Figure 16.
Giant foxtail Week 10

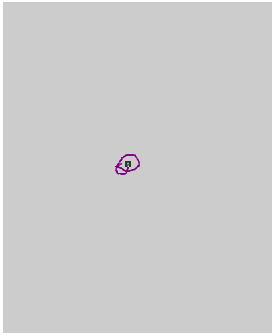


Figure 17
Giant foxtail week 10

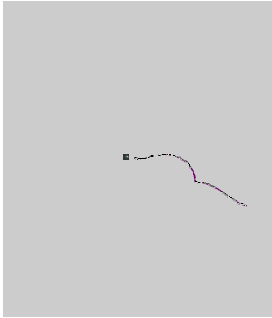


Figure 18
Giant foxtail week 7

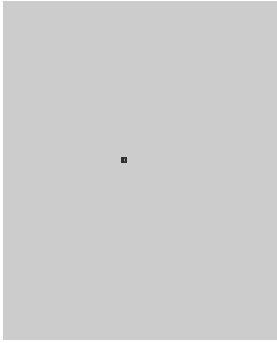


Figure 19
MON863 week 7

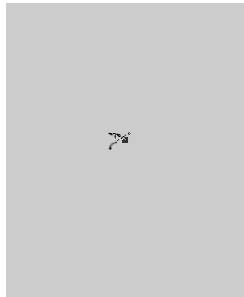


Figure 20
MON863 maize week 4

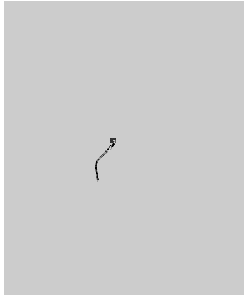


Figure 21
MON863 maize week 7

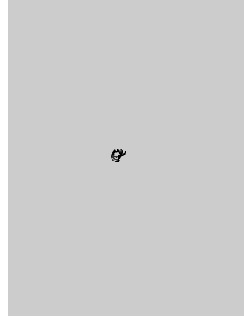


Figure 22
MON863 maize week 10

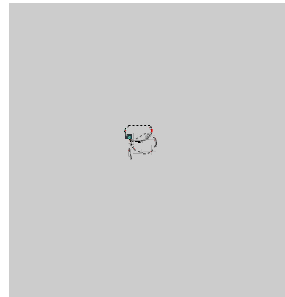


Figure 23
Large crabgrass week 4

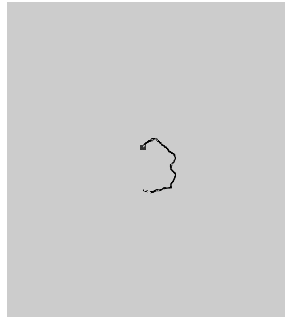


Figure 24
Large crabgrass week 7

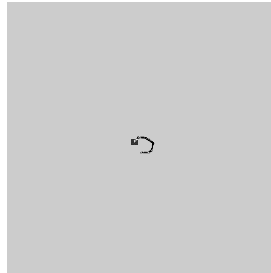


Figure 25
Large crabgrass week 10

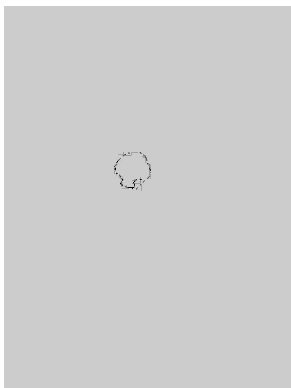


Figure 26
Barnyard week 4

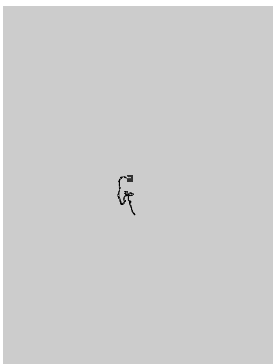


Figure 27
Barnyard week 7

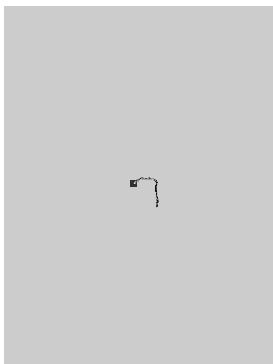


Figure 28
Barnyard week 10

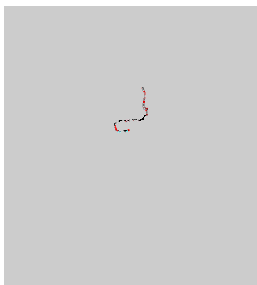


Figure 29
Isoline maize week 4

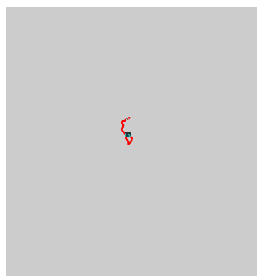


Figure 30
Isoline maize week 4

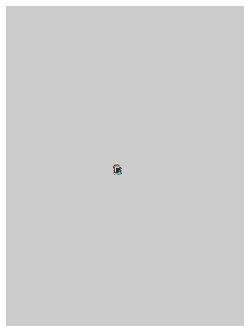


Figure 31
Isoline maize week 10

Chapter 6

6.1. Summary and Conclusions.

Host plant phenology affects western corn rootworm larval host-searching behavior, survivorship, growth, and development. Young 4-6 weeks old (V5) host plants favor survivorship, growth and development of western corn rootworm larvae. Infestations occurring during the 4-6 weeks window after planting resulted in better larval survivorship, growth, and development than infestations at later phenologies. Host-searching path patterns of western corn rootworm neonates also showed that searching V5 plant growth stage roots was generally more nutritionally rewarding than searching older roots. Initial larval feeding on an alternate host followed by movement to rootworm-resistant transgenic maize enhances the emergence of western corn rootworm beetles.

With the increasing usage of Bt corn, there is concern about the development of tolerance to Cry3Bb1 (Bt) toxin within corn rootworm populations. Over time, some individuals of the western corn rootworm populations that can tolerate high doses of Bt could increase, making rootworm-resistant Bt corn less effective. Roundup Ready rootworm-resistant corn with stacked traits has been developed. In 2005, Monsanto Company introduced the first triple-trait technology product in the agriculture industry, YieldGard Plus with Roundup Ready Corn 2, providing farmers with in-seed insect protection against the corn rootworm, corn borer, and herbicide tolerance to Roundup and other glyphosate-based herbicides (Monsanto Company, 2005). This technology affords farmers excellent flexibility due to a wide window of glyphosate-based herbicide application.

Herbicide timing is critical in corn when using one-pass postemergence programs (Gower et al. 2003) and delaying the herbicide application could lead to

early season weed competition and reduce yields in conventional or Roundup Ready corn. Delayed glyphosate applications may increase the risk of yield loss in corn due to weed competition (Carey and Kells 1995, and Gower et al. 2002) and also allow initial establishment of corn rootworm larvae on grassy weeds in corn fields. Initial larval establishment on alternate hosts (weeds) and subsequent movement to transgenic maize could occur following delayed weed removal. This is especially likely to occur due to late glyphosate-based herbicides application in Bt-glyphosate-Ready corn systems, or when the nutritional resources of the weeds drop due to phenological changes of weeds.

Movement due to decline in nutritional resources could occur according to the marginal value theorem (MVT), which states that a single animal exploiting a patch of resources should leave it and search for another patch when its rate of resource gain drops below the expected mean rate in the whole habitat (Charnov 1976). Grassy weeds in the transgenic maize agro-ecosystem could thus become a recipe for resistance development if initial establishment of larvae on the grassy weeds is followed by movement to transgenic maize. On the other hand, the presence of weeds in transgenic maize fields could be advantageous if deliberately configured within the refugia in order to delay development of rootworm resistance to the Cry3Bb1 (Bt) toxin.

IRM strategies should be refined and implemented to prevent or delay development of tolerance to Cry3Bb1 (Bt) toxin in corn rootworm populations. These strategies should include maintaining corn rootworm populations that are genetically susceptible to Bt toxin by adopting measures similar to those that have been used in IRM programs for European Corn Borer. These measures include: having refugia, that is areas planted to non-Bt maize or grassy weeds around or within the transgenic corn

agro-ecosystem where the Bt genes are not used; introducing more than one kind of Bt gene into corn lines; incorporating another effective insecticidal component into transgenic corn other than Bt, and; combining Bt insecticidal qualities with other insecticidal traits in transgenic corn.

In transgenic maize agroecosystems where the control of especially grassy weeds is delayed for any reasons, large numbers of beetles are likely to eventually emerge. This, coupled with other factors, could play a role in the development of rootworm resistance to the Bt toxin in transgenic maize. The positive possibility is that the presence of the weeds in the maize agroecosystem could act as a built-in refuge, which could play a role in the slowing down or prevention of development of rootworm resistance to the transgenic maize. If movement of rootworm larvae from alternate hosts or susceptible maize to complete development on Cry3Bb1-expressing transgenic corn produces susceptible adults, this could prolong the lifespan of transgenic maize tactic through production of large numbers of susceptible beetles from within the transgenic field (Hibbard et al. 2005). The findings in these studies suggest that where no pre-emergence herbicides are used, the timing of postemergence herbicide application, especially glyphosate-based herbicides in Roundup Ready rootworm-resistant transgenic corn systems, should be scheduled to ensure early removal of weeds, particularly around the 4-6 weeks window after planting. Prompt removal of weeds in rootworm-resistant transgenic corn agroecosystems would deny rootworm larvae the opportunity of initial establishment on the alternate hosts that could enable them to eventually withstand the Bt toxin once they move to the Bt corn. The full implications of these findings for IRM are dependent on other factors, such as the scale of implementation of refugia, selection intensity of transgenic plants on rootworm neonates, soils, and growing conditions.

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VITA

Peter Gacii Chege was born and attended primary school in Murang'a (formerly called Fort Hall) in Central Province, Kenya. He then joined Nakuru High School (formerly European School Nakuru), in the Rift Valley Province, for grades 9 through 12. He proceeded to Njiiri School in Murang'a District for Advanced Level high school education (equivalent to grades 13 and 14), and studied Math, Chemistry and Biology (MCB). After completing A-Levels in 1988, he taught biology at Kibutha Girls High School for six months and then went for four months of the basic pre-university paramilitary training at the Gilgil National Youth Service Center.

In fall 1989, he joined Kenyatta University in Nairobi Kenya and graduated in fall 1993 with a Bachelor's of Science, Zoology major. He taught high school biology in Nairobi, and in 1998 he won a DAAD (German) scholarship to study for a master's degree in Entomology at Addis Ababa University in Ethiopia under the African Regional Post-graduate Program in Insect Science (ARPPIS). His thesis research was in IPM-Horticulture. After graduating in summer 2000, he taught freshman Biology and Environmental studies at the Kisama Africa University College in Addis Ababa. In fall 2001, he joined the University of Missouri to pursue a PhD in Entomology under the supervision of Dr. Thomas Clark. He is married to Mary Mwihaki and they have two sons: Brian Chege and Ian Jakes. His interests are in working with clients in the horticulture and biotechnology industries dealing with integrated pest management (IPM) and transgenic insect-resistant plants.