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Evaluating seed blended refugia in field corn in the Southern U. S.

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in Life Sciences
in the Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology

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Helicoverpa zea (Boddie), a pest of cotton that also occurs in field corn, is commonly controlled through the use of foliar-applied insecticides or transgenic crops expressing *Bt* genes. To prevent the selection of resistant populations, refuge systems have been implemented into the agroecosystem. Historically, structured refuge compliance among growers has been low, leading to the commercialization of seed blended refugia. To test the viability of seed blended refugia in the southern U.S., field studies were conducted in Mississippi and Georgia during the 2016, 2017, and 2018 growing seasons. To quantify adult *H. zea* emergence from structured and seed blended refuge options, emergence traps were utilized. Kernel damage and moth emergence timings were recorded. Various percentages of stand loss ranging from 0% to 50% were also simulated to determine yield effects in unprotected seed blended refugia. Lastly, *H. zea* feeding and emergence in a two-gene field corn variety expressing Cry1A.105 and Cry2Ab2 were compared to non-Bt field corn. When compared to a structured refuge, *H. zea* adult moth emergence from seed blended refugia did not significantly differ. Kernel damage was not different between seed blended treatments and structured refuge treatments. Moth emergence timings were not significantly delayed between the structured refuge and seed blended refuge treatments. Significant yield losses were observed when stand loss was simulated at various

levels in field corn, suggesting that there is an opportunity to see yield losses in an unprotected seed blended refuge field corn landscape. Kernel damage did not significantly differ between field corn expressing Cry1A.105 and Cry2Ab2 and non-Bt field corn, possibly due to *H. zea* resistance to the Cry genes. However, there was a significant difference in emergence from two-gene expressing field corn and non-Bt field corn. This suggests that there may be high pupal mortality in two-gene corn plots. Based on these data, seed blended refuge could be a viable option to replace structured refuge strategies in the southern U.S., however, if left unprotected, yield loss could be observed in a case of high boring insect pressure. The significant loss of refuge plants can also compromise refuge effectiveness.

DEDICATION

For my loving grandparents, Hal and Diane Towles.

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1.1.1 Classification

Helicoverpa zea (Boddie) has multiple common names including corn earworm, bollworm, tomato fruitworm, and others depending on host (Snow and Brazzel 1965). Hardwick first established the genus, *Helicoverpa*, in 1965 which includes 18 species worldwide. *Helicoverpa zea* was first categorized in the genus *Heliothis* until it was moved into *Helicoverpa* due to morphological differences of male genitalia (Hardwick 1970). *Helicoverpa zea* is the only species of this genus that is present in North America (Hardwick 1965). Therefore, when reviewing older literature, it is not uncommon to find many varying binomial names such as *Chloridea obsoleta*, *Heliothis obsoleta*, *Noctua armigera* Hübner, and *Bombyx obsoleta*. These binomial names all refer to *H. zea* if the origin relates to the New World (Pogue 2004). *Helicoverpa zea* is closely related to another species, the tobacco budworm, *Heliothis virescens* F.; Pogue (2013) resurrected the genus *Chloridea* and *Heliothis virescens* was moved into this genus based on morphology, therefore, the tobacco budworm will be referred to as *Heliothis virescens* or *H. virescens* throughout this review to remain synonymous with the older literature.

1.1.2 Biology

Helicoverpa zea is an opportunistic, polyphagous insect pest that feeds on fruiting structures, seeds, and foliage of many important field crops including field corn, *Zea mays* L.;

cotton, *Gossypium hirsutum* L.; and soybean, *Glycine max* L. Merr. (Neunzig 1969). Although some flight activity occurs during daylight hours, most behavior takes place at night (Fitt 1989). Oviposition site preference is not well understood, but consistent patterns show that flowering hosts are preferred (Quaintance and Brues 1905; Johnson et al. 1975). Eggs are deposited in singles or groups of two or three in the vicinity of young growing points or buds, which are the preferred feeding sites of larvae (Fitt 1989). Oviposition generally begins in the early evening and can continue into early morning (Quaintance and Brues 1905), but most eggs are laid between 7:00 pm and 11:00 pm (Callahan 1958, Hardwick 1965). Eggs of *H. zea* are typically subspherical and measure a mean height of 0.42-mm and diam. of 0.50-mm (Hardwick 1965). Hardwick (1965) also described the eggs of this species to be colored greenish-yellow when deposited but will gradually change to a muddy yellow and form a pink line around the micropylar area. The eggs are flattened on the bottom where it usually rests after oviposition and the radial ribbing of the egg is commonly well defined (Hardwick 1965). A female *H. zea* can lay 500 to 3,000 eggs. Oviposition begins between two or three days after adult emergence and eggs eclose two or three days after oviposition in the warmer summer months (Quaintance and Brues 1905).

Before eclosion, the dark head capsule of the larva is visible through the chorion of the egg. The larva uses its mandibles to chew through the tough membrane of the chorion until the shell is weakened enough and an exit hole is formed. Quaintance and Brues (1905) observed this entire process and recorded two hatch times of 13 and 16 minutes. Larvae begin feeding immediately after eclosion, typically on the recently vacated chorion (Pedigo 2002). The number of larval instars varies from five to seven, but six is most common (Quaintance and Brues 1905). The larval duration of *H. zea* ranges from 12 to 16 days depending on temperature and larvae grow more rapidly as they near maturity (Quaintance and Brues 1905, Hardwick 1965). Early instar larvae can feed on vegetative material, but older instar larvae tend to migrate to reproductive material such as seeds or fruiting structures. Larvae have orange to yellow head capsules, black legs, and can measure up to 50-mm long when fully grown (Pedigo 2002). The larval coloration of *H. zea* is variable and patterns range from brown, light green, yellow, and occasionally pink. The larvae typically bear a dark band laterally above the spiracles and a light yellow or white band below the spiracles as well as four sets of abdominal prolegs. The body is covered by black protrusions, which bear setae (Drees and Jackman 1999).

The transition from one instar to the next is accomplished through molting. Quaintance and Brues (1905) presented the process of molting in *H. zea* as follows: A few hours after ecdysis occurs, the larva stops feeding and remains in an extended, slightly arched position. In this condition, the larvae become helpless, especially when nearly fully grown. At the time of molting the body contracts spasmodically, the skin splits behind the head and the head is thrust out. Three to four minutes pass before the larvae have shed the skin entirely and feeding begins after three to four hours. In midsummer, the stages leading up to molting are very short (15 to 18 hours), and in the fall longer (3 to 4 days). After the earlier molts, larvae may devour the shed skin.

Once the larvae have completed the final instar, pupation begins. The larvae begin to move down the host plant and select a suitable place to burrow beneath the soil, usually, within 0.3 to 0.6 meters of the host (Quaintance and Brues 1905). Larvae burrow anywhere from 2 to 18 cm into the soil where they will begin pupation (Quaintance and Brues 1905). *Helicoverpa zea* pupae are reddish or light brown and tend to be oblong shaped measuring 14 to 22-mm in length and 6-mm in width. Pupae are usually completely smooth and cylindrical, tapering down to a point at the lower tip of the abdomen. *Helicoverpa zea* pupae can be distinguished from *H. virescens* pupae in that the abdominal spiracles are wider and larger in *H. zea* compared to smaller and narrower in *H. virescens* (Neunzig 1960). Pupal stage duration varies from a few weeks in the warm summer months up to six months in case of the diapausing generation (Quaintance and Brues 1905). Pupal diapause can range anywhere from 187 to 243 days in the Mid-Southern United States (Stadelbacher and Pfrimmer 1972). Hogg and Calderon (1981) conducted an experiment to determine if *H. zea* that progressed through larval growth stages more quickly than normal had shorter pupal duration. The result indicated that larval and pupal development is independent of each other. There was a difference in pupal duration observed between males and females, with females developing faster. This is important with regard to modeling applications because generation time and population growth rate are functions of the development of female development only (Hogg and Calderon 1981).

Helicoverpa zea overwinters as diapausing pupae, which is a physiological feature that allows this pest to maintain local populations during periods when plant hosts are unavailable, or conditions are not conducive to reproduction and population survival (Fitt 1989). Diapause is initiated by photoperiod and nutritional quality of host plants (Stadelbacher 1981). Aestivation has been observed in *Helicoverpa armigera* (Hübner) during times of extreme heat (Liu et al. 2006) but has not in *H. zea*. Summer diapause allows the insect to slow development until the environment becomes more suitable for survival. High temperatures can also have a negative effect on survivorship of pupae, but pupae can withstand high temperatures if the humidity is high (Quaintance and Brues 1905). When pupae are exposed to dry conditions for long periods, sudden emergence can be triggered after a ground wetting or a rainfall event (Quaintance and

Brues 1905). Diapausing pupae are more tolerant of cold, dry conditions than non-diapausing pupae. Survival of overwintering pupae is greatly reduced when temperatures decline below -1.11° C (Cook and Weinzierl 2004). Schneider (2003) observed survivorship of diapausing *H. zea* was less than 5% in agricultural production fields in Mississippi. Also, for Heliothines to successfully overwinter in a field, no-tillage during the fall or winter could occur and the host cotton could not be Bt (*Bacillus thuringiensis*) before diapause (Schneider 2003). The requirement of non-Bt cotton is likely true for *H. virescens* but may not be for *H. zea*, because *H. zea* has a higher natural tolerance to Bt proteins than *H. virescens* (Luttrell et al. 1999).

Emergence of adult *H. zea* occurs when the pupal case splits along the median dorsal line of the thorax allowing the moth to escape and emerge from the burrow with wings still unexpanded (Quaintance and Brues 1905). *Helicoverpa zea* emerges from tunnels that are created by the prepupal larvae when pupation commences (Neunzig 1969). The wings expand within minutes and the moth is able to fly a few hours later (Quaintance and Brues 1905). Eclosion occurs approximately 12 days after the pupa becomes fully sclerotized (Hogg and Calderon 1981). In the mid-southern U. S., overwintering *H. zea* typically emerges from April to May. Less than 2% of overwintering pupae in an area emerge from cotton fields (Schneider 2003). This suggests that 98% of *H. zea* populations in the spring are migrating into the area or emerging from non-cotton hosts. Adult *H. zea* lifespan ranges from 5 to 17 days with a total insect lifespan of around 30 days (Quaintance and Brues 1905). The adults are approximately 25-mm in length and tan colored with a wavy band along the edge of the wing and a dark brown spot in the center of each fore wing.

1.1.3 Geographical Distribution

Helicoverpa zea can be found throughout the western hemisphere and ranges from southern Canada extending into South America (Hardwick 1965). *Helicoverpa zea* occurs in both North and South America with permanent populations existing between latitudes 40° N and 40° S (Fitt 1989). Two types of movement can occur among *H. zea* populations. The first type is common dispersal that takes place throughout an area of hosts for feeding and oviposition. The second type takes place over long distances (up to several hundred km) usually occurring with the help of prevailing winds or weather events (Hardwick 1965). *Heliothis* spp. are facultative migrants, migrating due to poor local conditions for reproduction such as a lack of host plants for oviposition or nectar shortage for adult feeding (Hardwick 1965).

1.1.4 Generations

Seasonal abundance and number of generations of *H. zea* in an area depend on temperature, the temporal sequence of plant hosts, and the suitability of those hosts (Fitt 1989). Polyphagy is important in a species such as *H. zea* in that many generations can be developing simultaneously across multiple host plants in a region (Fit 1989). The number of generations of *H. zea* per year depends on latitude and the duration of the growing season (Neunzig 1969). In tropical regions, up to 11 generations may occur per year, meaning that all life stages can be found throughout the year when adequate hosts are available. In the extreme southern U. S., some populations can continue throughout the entire year without entering diapause (Hardwick 1965). In subtropical and temperate cropping systems, three to five generations per year are more common. (Fitt 1989). The number of generations per year is often difficult to determine due to migration and dispersal of *H. zea* that results in overlapping generations (Quaintance and Brues 1905; Neunzig 1969). Within an area, *H. zea* populations tend to decline around the 5th generation due to environmental factors, parasitoid pressure, host plant senescence, adult migration, and diapausing populations (Stadelbacher et al. 1984).

1.1.5 Agroecosystem

One-third of host plants of *H. zea* are classified into the family of Leguminosae, whereas, the remaining majority of plants belong to Malvaceae, Asteraceae, and Solanaceae (Kogan et al. 1989). *Helicoverpa zea* and relatives can cause economic damage because they prefer to feed on reproductive structures and growing points, areas of the plant that are high in nitrogen (Fitt 1989; Hardwick 1965). In the crops that *H. zea* infests, reproductive structures are typically high value which leads to low damage thresholds. *Helicoverpa zea* infests high-value crops such as field corn, *Zea mays* L.; soybean, *Glycine max* Merr.; cotton, *Gossypium hirsutum* L.; grain sorghum, *Sorghum bicolor* L.; tobacco, *Nicotiana tabacum* L.; wheat, *Triticum aestivum* L.; tomato, *Solanum lycopersicum* L.; and peppers, *Capsicum* spp. (Fitt 1989). For this reason, considerable research has been conducted regarding *H. zea* to prevent economic damage. Relative to the areas grown, soybean, cotton, tobacco, and pulse crops account for the majority of economic losses due to *Heliothis spp.* (Fitt 1989). Economic losses stem not only from direct yield reduction, but also from the cost of chemical control, application, and scouting efforts (Fitt 1989). Annual estimates of the damage caused by both *H. zea* and *H. virescens* across all crops in the United States are greater than 1 billion dollars (Johnson et al. 1986). In one of the most extensive studies, Quaintance and Brues (1905) found that *H. zea* occurred on 72 different species of plants from 21 families. More recently, *H. zea* was collected on 34 species of plants covering 11 plant families from 1990 to 1991 in Tennessee (Sudbrink and Grant 1995). *Helicoverpa zea* infests high-value crops such as field corn, *Zea mays* L.; soybean, *Glycine max* Merr.; cotton, *Gossypium hirsutum* L.; grain sorghum, *Sorghum bicolor* L.; tobacco, *Nicotiana tabacum* L.;

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The pest status of *H. zea* can be maintained in unstable habitats due to four physiological, behavioral, and ecological characteristics: high mobility, high fecundity, polyphagy, and a facultative diapause (Fitt 1989). In Mississippi, spring *H. zea* development occurs mainly on crimson clover, *Trifolium incarnatum* L.; white clover, *Trifolium repens* L.; alfalfa, *Medicago sativa* L.; vetch, *Vicia sativa* L.; chickpea, *Cicer arietinum* L.; and lupine, *Lupinus* spp.; some of which are used primarily in seed blends planted by Mississippi's state highway department for erosion control (Isley 1935; Brazzel et al. 1953; Stadelbacher et al. 1986). These plant species play an important role in the buildup of *H. zea* and are present a month or more before cultivated crop hosts are available (Stadelbacher et al. 1986). Heliothines prefer corn early season and shift to other crops such as cotton and soybean later when corn has matured beyond insect preference in the growing season which is described as an "adaptive host plant shift" (Johnson et al. 1975). This insinuates that the primary preferred host suitability is decreasing, and secondary host plants and suitability are increasing (Johnson et al. 1975). The second generation of *H. zea* typically develops on corn and wild host the third generation typically develops on cotton and other hosts (Snow and Brazzel 1965). *Helicoverpa zea* has been recognized as a pest of cotton since 1820 (Quaintance and Brues 1905). *Helicoverpa zea* was the most economically destructive pest of cotton before the boll weevil, *Anthonomus grandis grandis* Boheman, was introduced (Quaintance and Brues 1905). *Helicoverpa zea* outbreaks are less likely in areas that corn is not being grown (Isley 1926). Corn is known to be the most suitable and preferred host for larval *H.*

zea. Gore et al. (2003) found that *H. zea* completed larval development more quickly when reared on corn compared to other agronomic hosts. Lukefahr and Martin (1964) found that adult female *H. zea* that were fed corn and meridic diets as larvae produced more viable eggs than *H. zea* adult females that fed on other crop diets, such as cotton, as larvae. Corn tends to be the most significant producer of adult *H. zea* across the southern United States (Jackson et al. 2008). *Helicoverpa zea* that emerges after completing a lifecycle on corn is important in resistance development to Bt cotton (Caprio et al. 2009) because larval *H. zea* that are subjected to Bt corn can be selected for resistance against Cry genes that are shared in many cotton varieties. Models show that Bt corn being absent from the landscape along with increasing refuge area for Bt cotton significantly increased the time it took for resistance development to evolve (Caprio et al. 2009). *Helicoverpa zea* larvae can attack corn from early vegetative stages until the dent stage when ripening stalks, leaves, and ears turn yellow and are no longer attractive (Quaintance and Brues 1905). Corn in the R1 growth stage (silking), is the preferential timing for *H. zea* oviposition in corn. The preference for corn tends to decrease as corn matures and other crops, such as cotton and soybeans begin to flower (Stadelbacher et al. 1986). Cotton becomes attractive to damage from *H. zea* after all other non-cotton hosts senesce (Fitt 1989). Johnson et al. (1975) found that in North Carolina, cotton was the least preferred crop compared to corn, tobacco, and soybean. Corn, cotton, and soybean preference is correlated with an increase in plant maturity that peaks at plant flowering and decreases with plant senescence (Johnson et al. 1975). *Heliothis* larvae have distinct feeding preferences for cotton flowers and small bolls that still retain the dried flower or “bloom tag” (Farrar and Bradley 1985). *Helicoverpa zea* is a yield-limiting pest of soybean, but currently can be adequately controlled using foliar insecticides (Adams et al. 2016).

1.1.6 Control Methods

Arthropod management should incorporate multiple control methods that will decrease negative ecological, economic, and sociological consequences. These methods may include pest population monitoring, judicious use of pesticides, or communication that no pest control method is necessary (Luckmann and Metcalf 1975). Protecting and promoting beneficial insects and microbes is also extremely important when decisions are being made to help decrease reliance on insecticides and promote insecticide resistance management (IRM) (Bottrell and Adkisson 1977).

There are many natural predators of *H. zea* including 142 species from 2 arachnid and 8 insect orders (King and Coleman 1989). The soil bacterium *Bacillus thuringiensis* (*Bt*) var. *kurstaki* and the baculovirus *H. zea* nuclear polyhedrosis virus (NPV) are currently two largely marketed biological control agents for *H. zea* management in agricultural settings. Another issue with relying on biological control is that sizable populations of *H. zea* need to be present and established for biological agents to be effective. In most cases. *Helicoverpa zea* thresholds on high-value crops do not allow for this to occur (King and Coleman 1989). In general, biological control agents of *Heliothis* spp. present in the environment alone are not sufficient to prevent economic losses in high-value crops (Goodenough 1986; Knipling and Stadelbacher 1983). For this reason, chemical control tends to be warranted in pest management decisions regarding *H. zea*.

Before the widespread usage of insecticides for the control of *H. zea*, growers implemented many cultural practices to reduce the establishment of the pest such as adjusting planting dates, increasing crop density, eliminating early-season non-crop hosts, and field cultivation to reduce overwintering pupae (Fitt 1989). In Georgia and Virginia, spring plowing controlled 92% of overwintering pupae and fall plowing controlled 98% of overwintering pupae (Barber and Dicke 1937). Cultural control methods alone do not provide adequate control of *H. zea*, however, when implemented with other control tactics, they can help decrease the insect's impact.

Proper timing of chemical control is important for the successful management of *H. zea* due to the insect's behavior of boring into fruiting structures. This behavior can provide refuge from chemical applications. Research conducted by Bibb et al. (2018) showed that *H. zea* did not produce enough damage to ear stage field corn to cause yield loss. *Helicoverpa zea* is not considered a yield-limiting pest in field corn and insecticide applications are not economically beneficial for control in field corn. In cotton, tolerance for *H. zea* infestations is reduced due to the traditionally high crop value (Isley 1935). When making insecticide control selections, insecticides that are selective to the pest while leaving beneficial insects relatively unharmed is preferred. However, this has not always been common practice. For example, in the 1950s, broad-spectrum insecticides, such as organophosphates and organochlorines, were commonly used for *H. zea* control. Those insecticides worked well in controlling *H. zea*, however, they were non-selective and detrimental to populations of beneficial insects as well (Lincoln and Phillips 1970). DDT (Dichlorodiphenyltrichloroethane) became the first effective synthetic insecticide to control *H. zea* and was being used across the landscape for several other pests. This led to resistance and the ultimate decrease in efficacy against *H. zea* (Lincoln and Phillips 1970). In the 1940's, excellent control of *H. zea* was accomplished using DDT with 560 g per hectare, by the 1950's control was merely acceptable at a rate of 1,120 g per hectare (Lincoln and Phillips 1970). *Helicoverpa zea* resistance to the pyrethroid class of insecticides was observed in the early 1990's, however, this class of insecticides was used in efforts to control *H. zea* until the early 2010's (Reisig et al. 2019). Currently, the diamide class of insecticides is the most effective class against *H. zea*.

1.2 *Bacillus thuringiensis*

1.2.1 Agroecosystem

Bt cotton, specifically BollGard® cotton (Monsanto, St. Louis, MO), was introduced and approved for commercial use by the Environmental Protection Agency in 1995. Bt cotton is a transgenic crop that contains a gene from the soil bacterium *Bacillus thuringiensis.* This bacterium produces proteins that have insecticidal properties upon being ingested by certain lepidopteran species. Over 200 crystalline proteins or prototoxins that express biological activity on insects have been discovered from *B. thuringiensis*. Specifically, BollGard cotton was modified using a gene from *B. thuringiensis* Berliner that coded for the production of the Cry1Ac protein. Cry1Ac is crystalline in shape with good to excellent insecticidal activity against *H. zea* and *H. virescens*, among other species (Shelton et al. 2002). Lepidopteran insects are the targets of >99% of all hectares that are planted in Bt technology (James 2002). Cry1Ac expression in Bt cotton tends to be lower in the flowers, squares, and bolls of cotton compared to terminal and leaf tissue (Greenplate et al. 1998). Gene expression also tends to decrease below effective levels after 80 days after planting (Greenplate et al. 1998). BollGard provided nearly complete control of *H. virescens* but, *H. zea* was only partially controlled due to a higher natural tolerance to the Cry1Ac toxin produced by BollGard (Luttrell et al. 1999). It was not uncommon to find *H. zea* infesting first generation varieties expressing the Cry1Ac protein (Mahaffey et al.1995). *H. virescens* populations rapidly decreased in areas where a large percentage of the cotton was planted with Bt varieties during the 1996 growing season (Schneider 2003).

1.2.2 Insecticide Resistance Management

In efforts to prolong the durability of the Bt technology and decrease the rate of resistance development, a high dose refuge strategy was implemented. The high-dose, refuge strategy relies on the assumptions that; resistance allele frequency is low (<0.0001) and that 50% of fields are planted to refuge, mating is completely random, and resistance alleles are inherited recessively (Carrière and Tabashnik 2001). This form of insecticide resistance management (IRM) assumes that the Bt crops express a high level of toxins to kill all but the homozygous resistant individuals and planting a refuge to produce healthy susceptible individuals. For the toxin to be considered high-dose, the dose must kill ≥95% of the heterozygotes, which prevents heterozygous insects from conveying resistance alleles to the next generation (Huang et al. 2011). Before the initial exposure to the toxin, resistance alleles are assumed to be rare (Gould 1998).

A refuge is an area planted to non-Bt crop that produces Bt susceptible insects. In theory, the rare resistant individuals will mate with susceptible individuals emerging from the refuge to produce heterozygous offspring (Hurley et al. 2001). In theory, resistance is conferred by recessive alleles, which is when the high-dose refuge strategy is expected to delay resistance development most effectively (Tabashnik et al. 2003). Heterozygous individuals carry both alleles for susceptibility and resistance, meaning that it is important for the Bt crops to control the heterozygous individuals. If resistance inheritance is recessive, the progeny of susceptible-resistant crosses would die upon feeding on Bt crops (Gould 1998). Fitness costs and incomplete resistance also play a role in resistance development. A fitness cost occurs when an individual that contains alleles conferring resistance to Bt toxins occurs in an environment lacking Bt and has lower fitness than an individual not conferring the same resistance alleles (Gassmann et al.

2009). Incomplete resistance is a form of impaired performance in Bt-resistant insects that develop on Bt expressing plants that can negatively affect things such as developmental rate, fecundity, and pupal weight (Tabashnik et al. 2003).

Dual-gene Bt cottons were introduced in 2003 and 2005 to improve control of *H. zea* and to strengthen resistance management. BollGard II® (Monsanto, St. Louis, MO) is a pyramided gene cotton expressing both Cry1Ac and Cry2Ab that became commercially available in 2003. WideStrike™ (Dow AgroSciences, Indianapolis, IN) is also a pyramided gene cotton expressing Cry1Ac and Cry1F that was released in 2005. TwinLink® (BASF, Florham Park, NJ) is another pyramided gene cotton expressing Cry1Ab and Cry2Ae that was released in 2003. The introduction of dual-gene cottons offered improved control of *H. zea* compared to single gene cotton and continued to provide excellent control of *H. virescens*. Dual-gene cotton also allowed for the elimination of the structured cotton refuge strategy. This was justified based on the fact that there is no cross-resistance between the Bt proteins being expressed in the plant and that non-cotton hosts provided a significant contribution of Bt susceptible adult *H. zea* into the landscape across the southern U. S. (Jackson et al. 2008). The natural refuge strategy was implemented in 2006, however, this strategy was only allowed for dual-gene cottons.

Pyramided cotton varieties incorporating three different Bt proteins were released in 2017. WideStrike 3™ was released by Dow AgroSciences (Indianapolis, IN) which incorporated Vip3A along with Cry1F and Cry1Ac in the original WideStrike cotton. BollGard 3® was introduced by Monsanto (St. Louis, MO) which incorporated Vip3A along with Cry1Ac and Cry2Ab in BollGard II cotton. TwinLink Plus® (BASF, Florham Park, NJ) was also introduced which incorporates Vip3A along with Cry1Ab and Cry2Ae. Vip3A is a vegetative insecticidal protein with insecticidal activity against lepidopterans, including *H. zea*. Epithelial cells of susceptible lepidopterans are the site of action for Vip3A and cell lysis is the mechanism of action (Yu et al. 1997).

YieldGard® was the first Bt incorporated field corn hybrid developed by Monsanto (St. Louis, MO). YieldGard expressed a single Cry1Ab protein for the control of lepidopterous pests. The introduction of Bt in field corn was primarily for the control of the European corn borer, *Ostrinia nubilalis* Hübner, and the southwestern corn borer, *Diatrea grandiosella* Dyar because control of these species is difficult with foliar insecticides. In cotton-growing areas, the required structured refuge size for single-gene Bt corn was 50 hectares of non-Bt corn refuge for every 50 hectares of Bt corn planted.

When pyramided gene corn became commercially available, the refuge requirement changed. In cotton-growing areas, the dual-gene corn refuge requirement is 20% while non-cotton growing areas were 5%. This was due to corn's role in pre-selecting for resistance in insects that infest both corn and cotton, such as *H. zea*. In areas where cotton is not normally grown, the structured refuge requirement is 5 hectares of non-Bt corn for every 95 hectares of dual-gene Bt corn planted. In some regions of the U.S., this is commonly implemented through the usage of seed blended refuges (RIB) which constitutes Bt seed corn blended with the correct percentage of required non-Bt seed. Upon planting, this gives a random dispersal of refuge plants throughout a field. The blended refuge strategy leads to both easier planting and ensures refuge compliance. However, computer simulations indicate that the structured refuge tends to be more durable than seed blended refuges, even when 75% of the structured refuge was not planted (Caprio et al. 2019). Cross-pollination from Bt plants to non-*Bt* refuge plants may play an important role in IRM for pests that feed on kernels (Pan et al. 2015). For example, Burkness et al. (2011) conducted several experiments with sweet corn expressing Cry1Ab showing 63% survival of naturally infested *H. zea* in refuge ears that had been cross-pollinated by adjacent Bt plants compared to 100% survival in non-Bt ears that were not cross-pollinated. Horner et al. (2003) conducted an experiment comparing survival on both non-Bt and Bt corn ears that showed that *H. zea* sample kernels more frequently when feeding on Bt ears than that of non-Bt ears. This suggests that larval *H. zea* may detect Bt toxins and attempt to avoid feeding on Bt expressing kernels.

Bt expressing plants provided season-long protection against select susceptible pests. However, selection pressure for resistance with Bt plants is greater than that for foliar applications of *B. thuringiensis* that had short residual and rapid decay (Luttrell et al. 1999). When single gene cotton was commonly planted, three refuge options were available in the United States. These options were a 5% unsprayed refuge, a 5% embedded refuge, and a 20% sprayed refuge. To comply with a 5% unsprayed refuge, at least 5 acres of non-Bt cotton needed to be planted for every 95 acres of Bt cotton and no insecticides with lepidopteran activity could be used on the refuge. The 5% embedded refuge was similar to the other 5% unsprayed option when it came to acreage, however, the refuge had to be planted within the same field as the Bt cotton and could be sprayed as long as the entire field was sprayed (Jackson et al. 2008). Lastly, the 20% sprayed option required at least 20 acres of non-*Bt* refuge for every 80 acres of *Bt* cotton planted. This option allowed for the use of lepidopteran active foliar insecticides except for foliar Bt applications. Currently, 100% of the cotton in Mississippi is planted to Bt cotton varieties (Cook and Cutts 2018). Since commercialization and adoption, Bt crops continue to be successful in controlling target pests, however, supplemental foliar insecticide applications are often needed to control *H. zea*, especially in dual-gene Bt cottons. Currently, there is documented evidence of widespread *H. zea* resistance to the Cry genes in Bt cotton and Bt corn in the southern U. S. (Yang et al. 2017; Reisig et al. 2018).

1.2.3 Cry Protein Mode of Action

Plant material expressing a Cry protein is broken down by the foregut and passes into the midgut where further digestion takes place. The midgut is lined with the peritrophic membrane which protects epithelium cells along midgut walls. The midgut is separated into two parts by the peritrophic membrane, the ectoperitrophic membrane which maintains the food bolus and the endoperitrophic space which is located between the peritrophic membrane and cell membrane of the midgut (Lehane and Billingsley 1996). Food will begin to be digested by enzymes in the ectoperitrophic space and digested molecules pass through the peritrophic membrane where they are absorbed by epithelial cell microvilli. Insects in both lepidopteran and dipteran orders have an alkaline midgut pH of up to 11, which is important in the breakdown of plant material and detoxification of compounds produced by the host (Dow 1986). Upon entering the midgut, midgut paralysis begins, the insect feeding stops within minutes, and death follows in several days (Murray et al. 1991). The high pH is important, as it leads to prototoxin activation and proteolysis and the solubilized inactivated protoxins are cleaved by midgut proteases which yield 60-70 kDa protease-resistant proteins (Whalon and Wingerd 2003; Bravo et al. 2005). The toxin becomes activated and then binds to highly specific receptors on the brush border membrane of the epithelium columnar cells of the midgut just before inserting into the cell membrane (Bravo et al. 2005). The columnar cell microvilli begin to swell and protrusions form in the lumen followed by cell lysis (Murray et al. 1991). Disruption to the midgut epithelium and cell lysis results in the leaking of cell contents which gives spores a medium to germinate, leading to septicemia and ultimately, insect death (de Maagd et al. 2001). Chemical imbalances occur inside the insect upon pore formation that leads to mortality. The creation of pores allows for midgut content diffusion into the hemocoel causing an increase in hemolymph pH and leakage of the midgut contents causes a decrease in midgut pH (Whalon and Wingerd 2003). Lastly, as previously stated, the insect eventually dies due to either septicemia or starvation, not from a direct poisoning of the Bt itself. Cry gene resistance is most commonly observed through an altered binding site in the insect's epithelial cells due to exposure to a high dosage (Lee et al. 1995).

1.3 Introduction and Justification of Further Research

As previously stated, Bt crops play a major role in the control of *H. zea* across multiple crops across the U. S. With the possibility of development of Bt resistance in target insects, we incorporate refugia across the landscape to serve as a source of Bt susceptible insects. A major issue with the high-dose refuge strategy in the mid-southern U. S. is that grower compliance and refuge planting is believed to be lower than satisfactory. In the Midwest U. S., seed-blended refuge (RIB) is an option that incorporates certain percentages of non-Bt seed with Bt seed in a standard commercial seed bag. This would ensure compliance if made an option in cotton-growing areas. This project was implemented to determine if RIB produces proportionate amounts of *H. zea* adults compared to structured refuge plantings in field corn. Another objective of this experiment was to quantify the amount of adult *H. zea* production from several different refuge percentages.

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CHAPTER II

QUANTIFYING THE CONTRIBUTION OF SEED BLENDED REFUGE IN FIELD CORN TO CORN EARWORM (LEPIDOPTERA: NOCTUIDAE) POPULATIONS

2.1 Abstract

Helicoverpa zea (Boddie), a pest of cotton that also occurs in field corn, is commonly controlled through the use of foliar-applied insecticides or transgenic crops expressing *Bt* genes. To prevent the selection of resistant populations, refuge systems have been implemented into the agroecosystem. Historically, structured refuge compliance among growers has been low, leading to the commercialization of seed blended refugia. To test the viability of seed blended refugia in the southern U.S., field studies were conducted in Mississippi and Georgia during the 2016, 2017, and 2018 growing seasons. To quantify adult *H. zea* emergence from structured and seed blended refuge options, emergence traps were utilized. Kernel damage among seed blended refuge and structured refuge corn ears were recorded and compared. Moth emergence timings were recorded. When compared to a structured refuge, *H. zea* adult moth emergence from seed blended refugia did not significantly differ. Kernel damage was not different between seed blended treatments and structured refuge treatments. Moth emergence timings were not significantly delayed between the structured refuge and seed blended refuge treatments.

2.2 Introduction

The corn earworm, *Helicoverpa zea* (Boddie), is a highly polyphagous pest that has the potential to be yield-limiting in cotton, *Gossypium hirsutum* (L.); soybean, *Glycine max* (L.)

Merr.; and grain sorghum, *Sorghum bicolor* (L.) Moench; and other crops in the mid-southern United States. However, *H. zea* is not considered a yield-limiting pest of field corn, *Zea mays* L., in the mid-southern U. S. and management is generally not considered to be economically beneficial (Bibb et al. 2018; Olivi et al. 2019). Field corn is one of the most abundant agronomic crops grown in the U. S. (USDA NASS 2018), and it plays an important role in the population dynamics of *H. zea* in the agroecosystem (Jackson et al. 2008, Head et al. 2010). Corn tends to be the most significant producer of *H. zea* adults during the early and mid-season across the landscape in the southern U. S. (Jackson et al. 2008). Experiments using pheromone trapping were conducted, stable carbon isotope analyses and gossypol analyses on adult *H. zea* to determine percentages of moths arising from C_3 and C_4 plants (Head et al. 2010). During the early season, *H. zea* develop mainly on C3 hosts and mid to late season *H. zea* develop mainly on C4 crops such as corn and grain sorghum. A minimum of 10% - 41% of moths had developed on C_4 hosts when production is expected to be mainly from cotton (Head et al. 2010). This suggests that C4 crops are driving the population dynamics of *H. zea* in the Southeastern U.S. and that *H. zea* populations are influenced more by C_4 crops rather than cotton (Head et al. 2010). Corn is the preferred and most beneficial host for *H. zea* (Barber 1936), and larvae generally progress through stadia more rapidly when reared on corn compared to other hosts (Gore et al. 2003).

Bt is a soil bacterium that produces crystalline (Cry) proteins during sporulation that have insecticidal properties (Macintosh et al. 1990). In 1996, Bt expressing corn hybrids expressing the Cry1Ab toxin were introduced into corn to aid in the control of European corn borer, *Ostrinia nubilalis* (Hübner), and southwestern corn borer, *Diatraea grandiosella* (Dyar), (Andow and Hutchison 1998). The Cry1Ac Bt toxin was also genetically incorporated into cotton for control of tobacco budworm, *Heliothis virescens* (F.), and pink bollworm, *Pectinophora gossypiella* (Saunders), (Umbeck et al. 1987; Perlak et al. 1990; Stewart et al. 2001). Bt crops can potentially place greater selection pressure for resistance on an insect population than foliar insecticides because the toxins are expressed throughout the plant during the entire growing season (Wearing and Hokkanen 1995).

An important condition of the registration of Bt crops was the implementation of a resistance management plan (Gould and Tabashnik 1998). The high-dose refuge strategy was implemented as a method of insecticide resistance management (IRM). For this strategy to be considered most effective, the Bt must kill >99.9% of the wild-type individuals, the resistance allele must be rare, resistance should be mostly recessive, and random mating must occur between moths emerging from the Bt crop and refuge crop (Onstad and Knolhoff 2014). *Helicoverpa zea* is inherently more tolerant of Bt Cry proteins and damaging infestations can occur in Bt incorporated cotton (Mahaffey et al. 1995). As a result, the toxins expressed in Bt corn or Bt cotton events do not meet the high dose criteria for *H. zea* (Huang et al. 2011).

A refuge can be deployed in multiple ways including blocks separate from the Bt crop, strips within the field, natural areas (for polyphagous pests), or by randomly dispersed refuge plants in a blended mixture of both refuge and Bt seed (Onstad et al. 2017). Seed blended refuge was not approved by the U. S. Environmental Protection Agency (EPA) for single-gene Bt corn hybrids based on the movement of *O. nubilalis* between Bt and non-Bt plants and the potential exposure to sub-lethal doses of the toxin when this occurs (US EPA 1998). Structured refuge requirements were initially mandated at 50 hectares of non-Bt refuge for every 50 hectares of Bt corn planted in cotton-growing regions. Pyramided Bt crops produce two or more Bt proteins, either Cry or Vip, that control the same pest species and further delay the development of resistance (Carrière et al. 2015). When two-gene pyramided corn was released, the Environmental Protection Agency reduced the structured refuge to 20 hectares of non-Bt corn for every 80 hectares of Bt corn planted in cotton-growing regions.

A major issue with the high-dose refuge strategy is low compliance among growers who are responsible for deployment. In 2014 and 2016, North Carolina corn growers were surveyed to determine if they planned to plant a corn refuge. Only 38.3 to 44.3% of corn growers responded that they planned to plant the required refuge and 22 to 29.4% of growers were uncertain (Reisig 2017). Another report showed that approximately 25% of corn growers in the U.S. that planted Bt corn were not in full compliance with current refuge requirements (Jaffe 2009). Bt corn refuge compliance is most likely similar to or lower than this across most of the cotton-growing states. Refuge compliance is not always an all or none scenario, meaning that refugia can be too small or too far away from the Bt field to be effective (Onstad et al. 2017). Refugia also need to be as attractive to the target insect as the insecticidal crop in terms of management and growth stages (Onstad 2011). Low refuge compliance is more detrimental in cotton-growing areas because corn and cotton varieties that contain similar Bt toxins may be planted in the same area (Von Kanel et al. 2016). *Helicoverpa zea* commonly feeds in succession on corn and cotton for multiple generations meaning that exposure to closely related proteins can last for the entire growing season (Caprio et al. 2019).

The benefits of planting seed blended corn refuge are that it maximizes adult mixing in a field setting as refuge plants would be distributed uniformly across Bt fields, and the burden of refuge deployment is placed on the seed distributor instead of the grower (Carroll et al. 2012; Onstad et al. 2017). Models have shown that a seed blend mixture would more effectively delay resistance than a structured refuge with a single gene corn hybrid in an area where non-compliance of the structured refuge is ≥50% (Carroll et al. 2012). Seed blends can have negative impacts on resistance development as well. One factor to consider is cross-pollination with neighboring Bt plants. Kernels on a non-Bt plant fertilized by pollen from a Bt plant producing two unlinked Bt toxins will express anywhere from 0 to 2 toxins, therefore, ear feeding *H. zea* would be exposed to a mosaic of toxins (Caprio et al. 2015). Corn is a cross-pollinated crop, and rates of self-pollination in corn are as low as 5% in a field setting (Waller 1917). This means that as much as 95% of kernels on an ear are fertilized by pollination can play a negative role in the development of resistance through sublethal doses of Bt proteins in individual kernels. One study found that up to 75% of non-Bt corn ears that were within four rows of Bt corn were affected by cross-pollination (Burkness and Hutchison 2012). *Helicoverpa zea* survival rates were as low as 63% on ears of maternal non-Bt plants that were cross-pollinated from a Bt plant producing Cry1Ab compared to 100% survival of *H. zea* feeding on ears of non-Bt plants pollinated by non-Bt pollen (Burkness et al. 2011). Gene flow through pollen dispersal can cause low to moderate levels of Bt toxin expression in ears of non-Bt refuge corn plants from Bt corn plants up to 31-m away from Bt corn plants (Chilcutt and Tabashnik 2004). In one study, 94.4% of refuge ear kernels expressed at least one Bt protein in a seed blended refuge field setting (Yang et al. 2014). Bt pollen contamination of refuge ears (cross-pollination) did not significantly affect early larval survival, but larval growth was delayed by one instar (Yang et al. 2014). A seed blended refuge may not produce as many non-selected moths as a structured refuge because of cross-pollination, leading to less overall refuge area (Chilcutt and Tabashnik 2004). It is also possible for foliage feeding larvae to encounter and ingest Bt expressing pollen during peak pollen shed which can promote the selection of Bt-resistant alleles (Burkness and Hutchison 2012).

The objective of the current study was to quantify adult *H. zea* emergence in corn seed blended refuge scenarios compared to structured refuge scenarios. This information will provide insight into the proportion of adults that are produced among both refuge strategies. These findings will help determine if seed blended refuge can play a role in cotton-growing areas.

2.3 Materials and Methods

Seven field experiments were conducted across four locations from 2016 to 2018 to determine adult *H. zea* production from corn hybrids expressing the Vip3A protein blend with varying percentages of non-Bt corn seed (blended refuge). Locations included the Monsanto Learning Center in Scott, Mississippi (2016), the Delta Research and Extension Center (DREC) in Stoneville, Mississippi (2017 & 2018), the R. R. Foil Plant Science Research Center in Starkville, Mississippi (2017 & 2018), and the Bledsoe Research Farm in Williamson, Georgia (2017 & 2018). Field corn hybrids with pyramided traits that included the Vip3Aa protein were planted with and without refuge (non-Bt) blends at a rate of 79,040 seed Ha^{-1}. The experimental design was a randomized complete block with five treatments and four replications. Treatments consisted of (1) 100% non-Bt corn; (2) 90% Vip3Aa hybrid with 10% non-Bt seed blend; (3) 80% Vip3Aa hybrid with 20% non-Bt seed blend; (4) 70% Vip3Aa hybrid with 30% non-Bt seed blend; (5) 100% Vip3A hybrid. Corn hybrids and Bt traits varied across locations (Table 2.1). The Williamson, GA (2018) location utilized five replications. Corn was planted on 101.6-cm, 96.5-cm, and 91.4-cm row beds in Stoneville, MS; Starkville and Scott, MS; and Williamson GA, respectively. Seed were planted using an Almaco plot research specific cone-planter (Almaco, Nevada, IA) at a depth of 3.81-cm below the soil surface. Corn seed were treated with clothianidin at a rate of 0.5 mg seed^{-1} before planting to protect seedlings from early-season insect pests. Plot size consisted of 8 rows wide by 13.7-m in length at all locations except, the

Williamson, GA location (2018) which utilized 10 rows of 15.8-m in length. For eight-row plots, the center four rows were used for emergence traps and ear sampling. For ten row plots, rows 2-9 were used for emergence traps and ear sampling. *H. zea* oviposition was monitored during the R1 growth stage (silking). After *H. zea* eggs hatched, larvae could move into the corn husk, and feed until pupation. Before pupation, corn ears were examined, and kernel damage and larval presence were recorded. In the Williamson, GA locations (2017 & 2018), larval presence counts were recorded at the R3 (milk) growth stage and damaged kernel counts were recorded at the R6 (Physiological maturity) growth stage. At the Starkville, MS location (2017 & 2018), kernel damage and larval presence were recorded at the R6 growth stage. At the Stoneville, MS location (2017 & 2018), kernel damage counts were recorded at the R6 growth stage. At the Scott, MS location (2016), kernel damage counts and larval presence counts were not recorded. After larvae left plants to pupate in the soil, plants were removed by cutting stalks at the soil level with a sickle bar mower (Model BSB-284, BEFCO, Inc., Rocky Mount, NC). Twenty-five pyramid emergence traps were placed in each plot to collect adult *H. zea* as they emerged from the soil. Emergence traps measured 132.1 cm in length by 106.7 cm in width by 76.2 cm in height in Mississippi (covering 15.2 sq. ft.) and 122 cm in length by 76.2 cm in width in Georgia (covering 10 sq. ft) (Fig. 2.1). Trap frames were custom-built using 3.2mm diameter solid steel rods. Frames were covered using 6.4mm mesh galvanized hardware cloth. The hole used for moth exit was made using a steel tube with an outside diameter of 19.1mm and measured 38.1mm in length. Corn plants removed from the plot area were placed around the base of the emergence traps to prevent insect movement into or out of the traps. Openings (19.1 mm diameter) were cut in the bottom 10.9 cm diameter plastic serving cups with matching lids and positioned at the peak of the traps. *Helicoverpa zea* adult emergence was recorded weekly at the

Scott, MS (2016) and Stoneville, MS (2017 & 2018) locations. In the Starkville, MS (2017 & 2018) locations, *H. zea* emergence was monitored every 2 to 3 days until emergence ceased. At the Williamson, GA location in 2017, *H. zea* emergence was monitored daily for a total of 27 days followed by every 2 to 3 days until emergence ceased. At the Williamson, GA location in 2018, *H. zea* emergence was monitored daily for a total of 49 days followed by every 2 to 3 days until emergence ceased. *H zea* emergence was then calculated to the number of moths emerging per hectare. The plot areas were maintained weed and disease-free throughout the growing seasons. Damaged kernel counts were recorded from refuge plants in each plot and converted to damage per cm^2. Plants were verified as refuge by subjecting each plant in the plot to glufosinate (plant marking) ((*RS*)-2-Amino-4-(hydroxy(methyl)phosphonoyl)butanoic acid). Bt expressing plants were glufosinate tolerant, but non-Bt plants were not, therefore, displaying glufosinate injury. Corn plants with glufosinate injury were assumed to be refuge plants. Additionally, all moth collection dates were recorded in attempts to understand how cross-pollination to seed blended refuge plants affects *H. zea* adult emergence. Emergence timings were analyzed to show when 50, 75, and 100% of *H. zea* moths had emerged from the plots.

For analyses, cumulative total *H. zea* emergence was calculated for each plot across all sample dates. For the initial analysis, cumulative *H. zea* emergence was compared across all treatments. A second analysis was conducted to compare adult emergence from each seed blended refuge treatment to the same percentage emerging from the 100% non-Bt hybrid treatment which acted as a structured refuge. For the 90:10, 80:20, and 70:30 seed blends, the total number of moths that emerged from the non-Bt hybrid was multiplied by 0.10, 0.20, and 0.30, respectively, to determine if the seed blend refuge produced a similar number of moths as the structured refuge (non-Bt isoline). In addition to total moth emergence, the days to 50%, 75%, and 100% emergence were calculated for each plot to determine if a delay in emergence occurred. Over the 7 site-years, only 2 *H. zea* adults emerged from the 100% Vip3A hybrid so it was excluded from this analysis. *Helicoverpa zea* emergence data and damaged kernel data were analyzed with a mixed model analysis of variance (PROC GLIMMIX, SAS Institute 2019). Treatment was considered a fixed effect in the model. Location and replication nested in location were considered a random effect. Degrees of freedom were estimated with the Kenward-Roger method. PROC MEANS was used to determine means and standard errors. Means were separated using Tukey's HSD test ($\alpha = 0.05$).

2.4 Results and Discussion

There were no differences observed between kernel damage in ears of the non-Bt structured refuge plants and ears of non-Bt refuge plants in the seed blended refuges (F = 2.59; df = 3, 60; *P* = 0.06). Mean (± standard error) damaged kernels ranged from approximately 25.8 ± 5.1 to 40.7 ± 5.3 cm^2 per 10 refuge ears (Fig. 2.2). These data are similar to what has been observed in previous research regarding the number of kernels damaged for non-Bt corn (Buntin et al. 2004, Bowen et al. 2014). As expected, differences in cumulative total emergence were observed among treatments (F = 54.28; df = 4, 130.1; *P* <0.01). Averaged over the seven site years, 14,175; 3,963; 2,474; 1,713; and 56 adult *H. zea* emerged per hectare from the 100% non-Bt, 70:30, 80:20 and 90:10 (Bt:non-Bt) seed blend refuges, and 100% Vip3Aa hybrid, respectively (Fig. 2.3). Less *H. zea* emergence from Vip3Aa corn was expected because the Vip3Aa trait currently provides adequate control of *H. zea* (Tabashnik and Carrière 2017) and there were proportionally fewer non-Bt ears in the blended refuge treatments than the 100% non-Bt treatment. These observations are similar to what has been seen in previous research. A study in Texas measuring *H. zea* emergence in non-Bt field corn observed 15,500 adult moths produced per hectare (Beerwinkle et al. 1993), which is similar to non-Bt corn in the current study.

Emergence was adjusted and compared to the percentages of the seed blend refuge treatments (Fig. 2.4). Emergence from the structured refuge plots was not significantly different than the 90:10 (F = 0.34; df = 1, 56; *P* = 0.56), 80:20 (F = 0.30; df = 1, 56; *P* = 0.59), and 70:30 (F = 0.09; df = 1, 52; *P* = 0.77) seed blended refuge plots. This suggests that a blended refuge strategy can produce similar numbers of moths as a structured refuge of equal size. Yang et al. (2014) suggested that a 5% seed blended refuge would not produce proportionate amounts of adults as a 5% structured refuge. The data presented in this paper indicate that a seed blended refuge as low as 10% can produce as many or more *H. zea* moths when compared to a structured refuge.

Differences in adult *H. zea* emergence were not observed among treatments for days to 50% emergence (Fig. 2.5) (F = 2.00; df = 3, 63.93; *P* = 0.12). Differences were observed among treatments for days to 75% emergence (F = 3.69, df = 3, 78.04; *P* = 0.02) and days to 100% emergence (F = 5.23; df = 3, 78.06; *P* = <0.01). Moths in the 90:10 (Bt: Non-Bt) seed blend plots emerged sooner than moths from the structured refuge plots and the 80:20 seed blend plots, but not the 70:30 seed blend plots for days to 75% emergence. For the number of days to 100% emergence, emergence in the 90:10 seed blend plots occurred sooner than all other plots (Fig. 2.6 & 2.7). This is most likely a result of fewer total moths emerging from the 90:10 seed blend and the tail of the emergence curve could not be detected. These data suggest that larval *H. zea* are either not ingesting enough Bt toxin from cross-pollination in kernels of refuge ears to delay development, or that they are resistant to the Bt toxin and growth and development was similar to that of larvae in the structured refuge plots. It is also possible that the insects are selecting kernels expressing lower levels or no Bt toxins. Multiple studies have documented that exposure of larval *H. zea* to Cry1Ab Bt proteins resulted in a 5-7-day delay in developmental times to reach the sixth instar compared to non-Bt controls (Horner et al. 2003; Storer et al. 2001). *H. zea* can detect and avoid cotton plant structures, such as terminal tissue, that express high levels of Bt and move to areas of lower Bt expression such as flowers (Greenplate 1999; Gore et al. 2002). Also, the development and survival of *H. zea* tend to be influenced by the variability of Bt expression in cotton plants (Bommireddy and Leonard 2008). It could be assumed that this shift in behavior and development in the presence of low Bt expression would translate to corn. Larval *H. zea* produced scattered patches of partial feeding in-ears expressing Cry1Ab compared to compact, localized feeding in non-Bt expressing ears (Horner et al. 2003). Avoidance of Bt toxins has also been observed when incorporated into the diet (Gould et al. 1991). Based on these observations, larval *H. zea* may feed on many kernels in corn ears avoiding those expressing Bt and consuming kernels with no expression. Recent data modeling the impact of seed blended refugia suggest that the durability could be dramatically reduced for kernel feeding insects because the refuge is compromised due to cross-pollination and that block refugia was more durable due to lack of gene flow (Caprio et al. 2016). However, in areas where block refugia are not planted, such as across most of the midsouthern U.S., seed blended refugia were found to provide an effective alternative IRM method for delaying the evolution of resistance (Carroll et al. 2012). Based on emergence timing data, it is possible that *H. zea* larvae displayed avoidance of possible cross-pollinated kernels in refuge ears, were already resistant to the Bt proteins being expressed, or simply did not ingest enough Bt toxin to affect developmental times compared to those in structured refuge plots. If the previous statement is true, this could be problematic for this method and IRM.

Based on data in this study, it appears that implementing a seed blended refuge strategy in the mid-southern U.S. could be viable in terms of *H. zea* adult production across a landscape, however, it is undetermined how possible selection pressure affected these adults. Introducing seed blended refugia would ensure that grower compliance would return to a desirable level which could be argued is equally an important factor as other negative implications such as cross-pollination and high larval movement. To better grasp seed blended refugia productivity, efficacy, and possible implications across a region, more landscape-level emergence research should be conducted to improve resistance models. More research should also be conducted to determine how much of a role cross-pollination plays in selecting for resistance alleles. Prior to conducting these experiments, landscape-level data on the emergence of *H. zea* from seed blended corn refuge in the mid-southern U.S. were lacking. These data provide a look at the comparison of *H. zea* production from seed blended corn refuge compared to the current requirement of a structured corn refuge in areas that cotton is grown. Field trials were conducted in multiple locations in Mississippi and Georgia in attempts to capture a better understanding of seed blended corn refuge performance across the southern U.S.

Table 2.1 Corn hybrids and Bt protein incorporations to quantify *H. zea* emergence from seed blended refuges at each location.

Location	**Bt Variety / Bt Incorporation**	**Non-Bt Variety**
Scott, MS 2016	DKC 67-99[1] (Cry1A.105, Cry2ab, & Vip3Aa)	DKC 67-70[1]
Stoneville, MS 2017	DKC 67-99[1] (Cry1A.105, Cry2ab, & Vip3Aa)	DKC 67-70[1]
Stoneville, MS 2018	DKC 67-99[1] (Cry1A.105, Cry2ab, & Vip3Aa)	DKC 67-70[1]
Starkville, MS 2017	DKC 67-99[1] (Cry1A.105, Cry2ab, & Vip3Aa)	Pioneer 2088R[2]
Starkville, MS 2018	DKC 67-99[1] (Cry1A.105, Cry2ab, & Vip3Aa)	Pioneer 2088R[2]
Williamson, GA 2017	Pioneer 2088VYHR[2] (Cry1Ab, Cry1F, & Vip3Aa)	Pioneer 2088R[2]
Williamson, GA 2018	Pioneer 2088VYHR[2] (Cry1Ab, Cry1F, & Vip3Aa)	Pioneer 2088R[2]

[1] Bayer CropScience, St. Louis, MO

[2] Pioneer, Johnston, IA

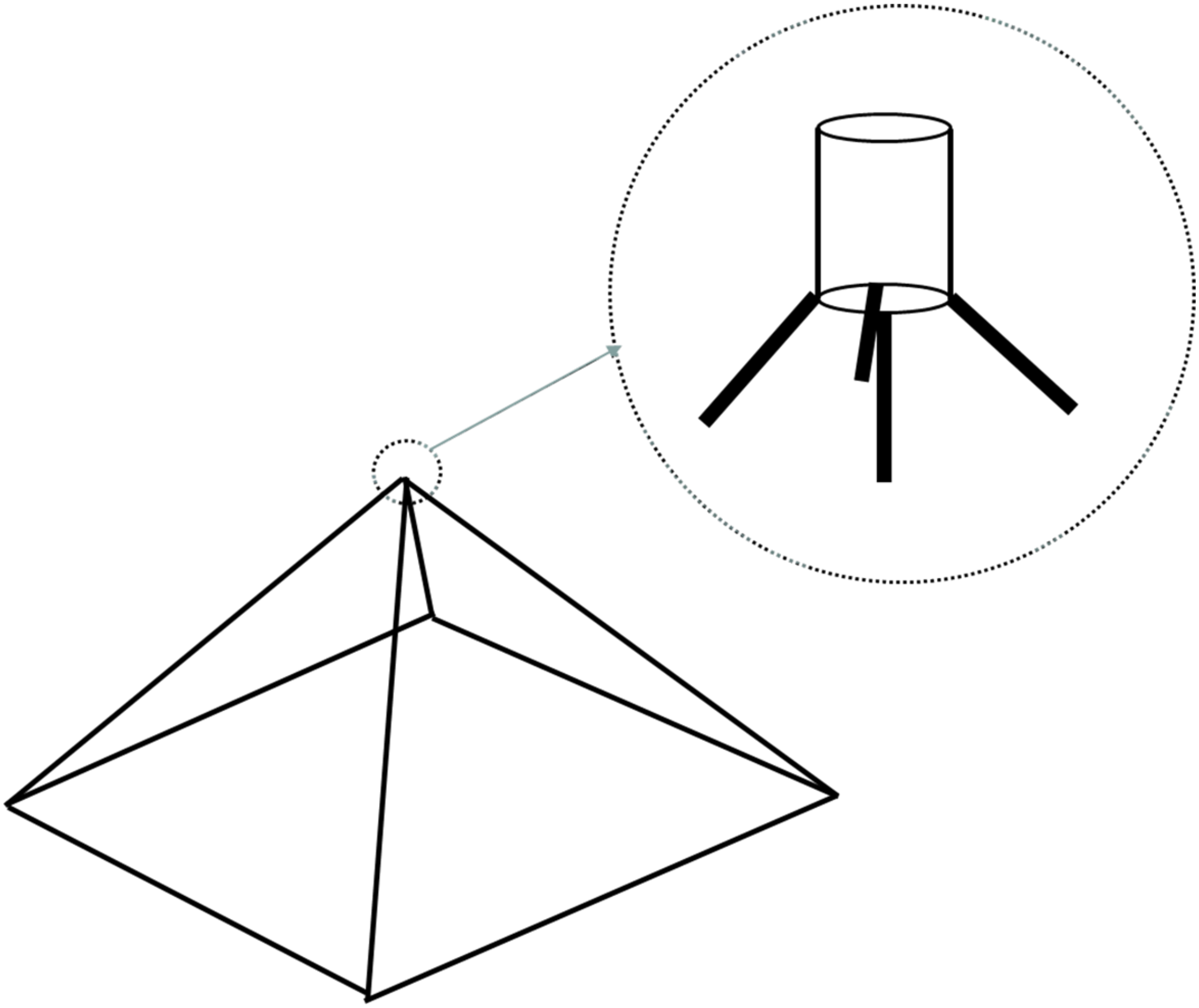

Figure 2.1 Diagram of emergence trap design used for the collection of adult *H. zea* in studies to evaluate moth emergence from seed blended corn refuges in Mississippi and Georgia from 2016 to 2018.

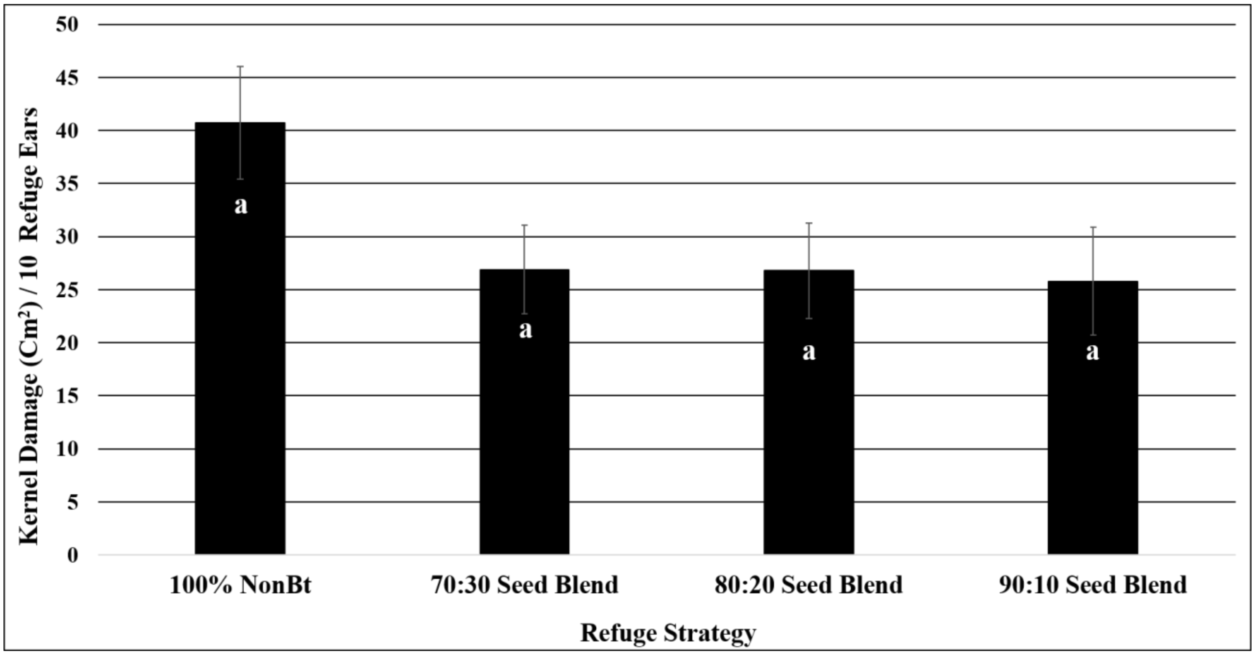

Figure 2.2 Mean (SE) area of damaged kernels (cm2) per 10 refuge ears in seed blended corn refuge treatments compared to the 100% structured refuge treatment for studies conducted in Mississippi and Georgia from 2016 to 2018. (*P* = 0.06)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

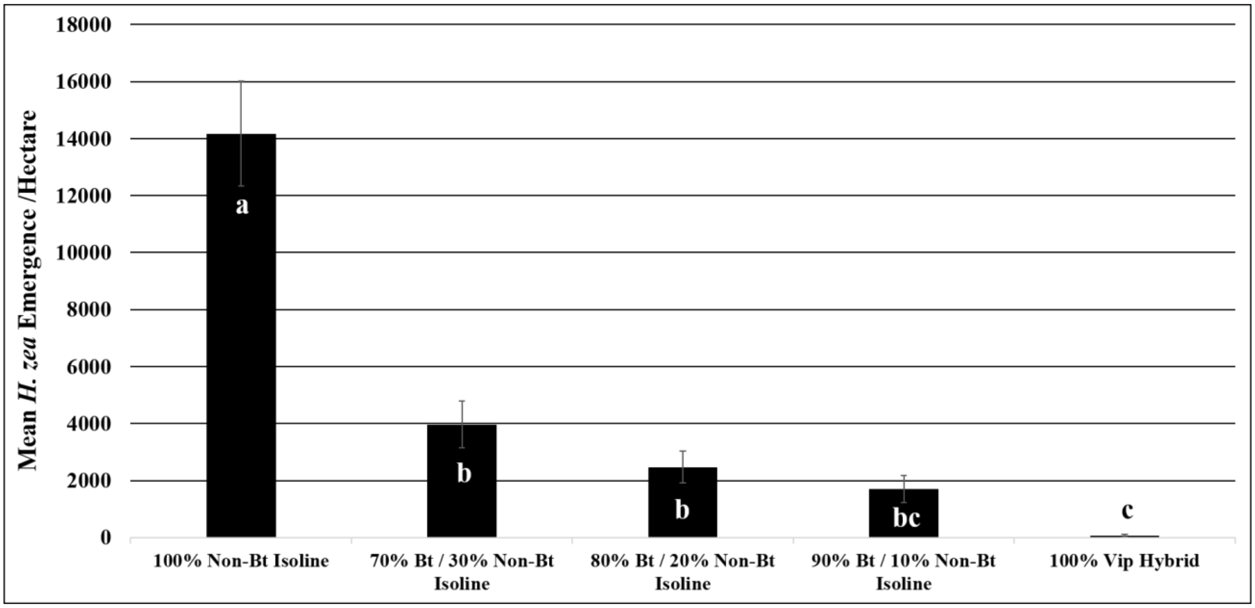

Figure 2.3 Mean (SE) *Helicoverpa zea* production per hectare from seed blended corn refuges across 7 site years in Mississippi and Georgia in 2016, 2017, and 2018. (P = <0.01)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

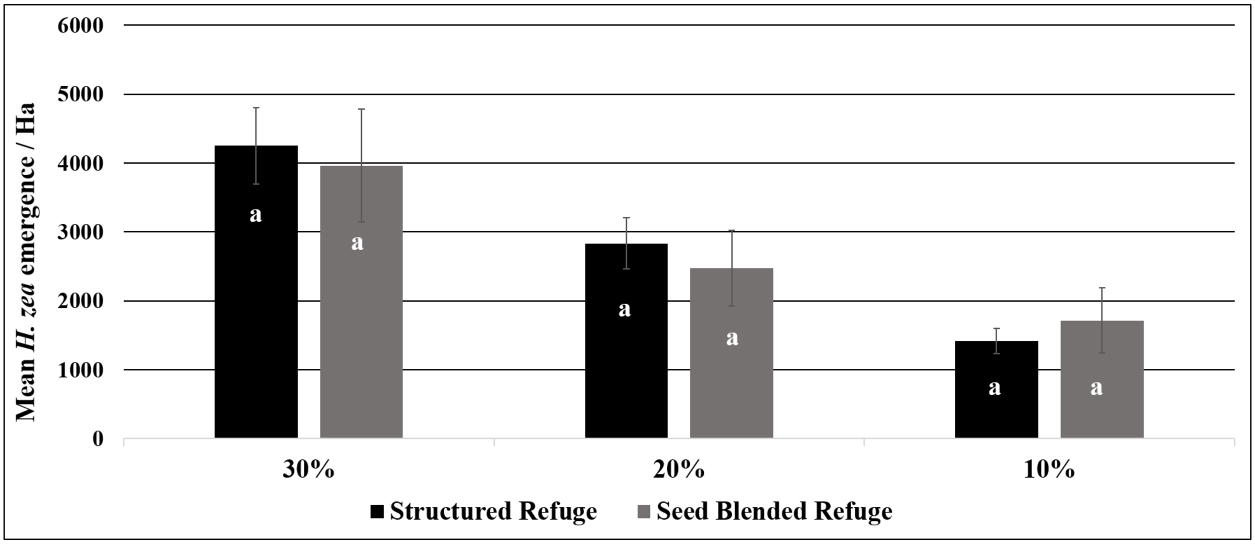

Figure 2.4 Mean (SE) *Helicoverpa zea* emergence from seed blended corn refuge treatments compared to equal percentages of structured refuge across 7 site years in Mississippi and Georgia from 2016 to 2018. (*P* = 0.77, *P* = 0.59, *P* = 0.56)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

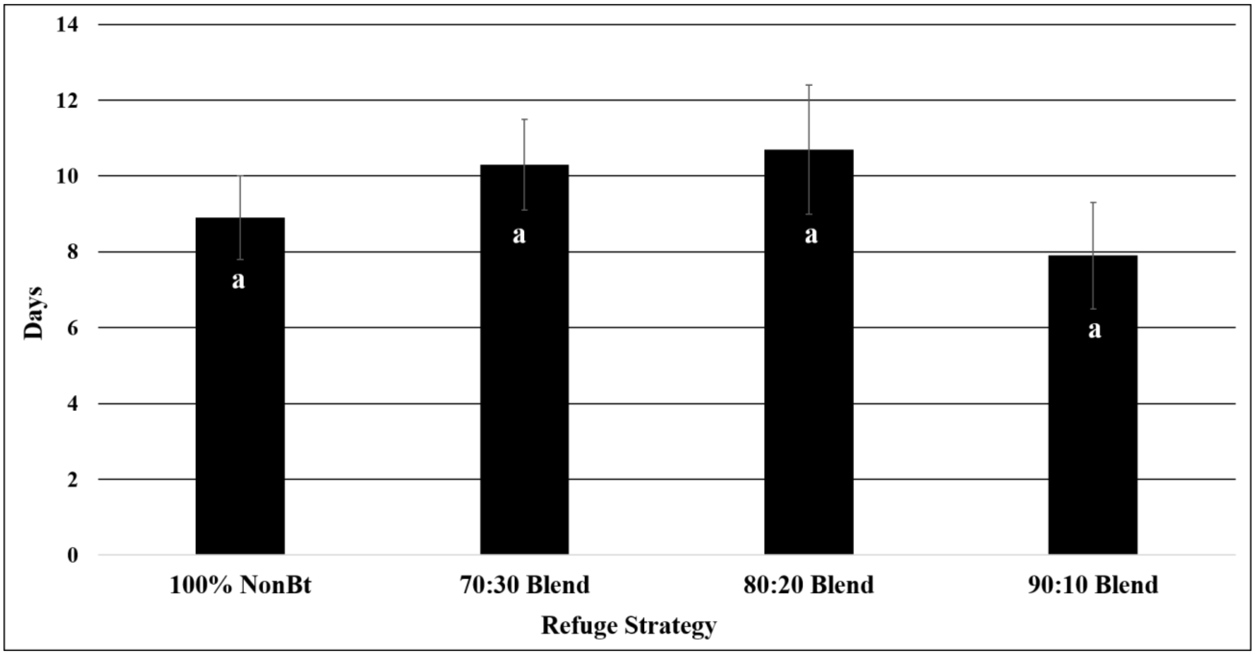

Figure 2.5 Mean (SE) number of days to reach 50% production of adult *H. zea* in all corn refuge treatments across 6 site years in Mississippi and Georgia in 2017 and 2018. (P = 0.12)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

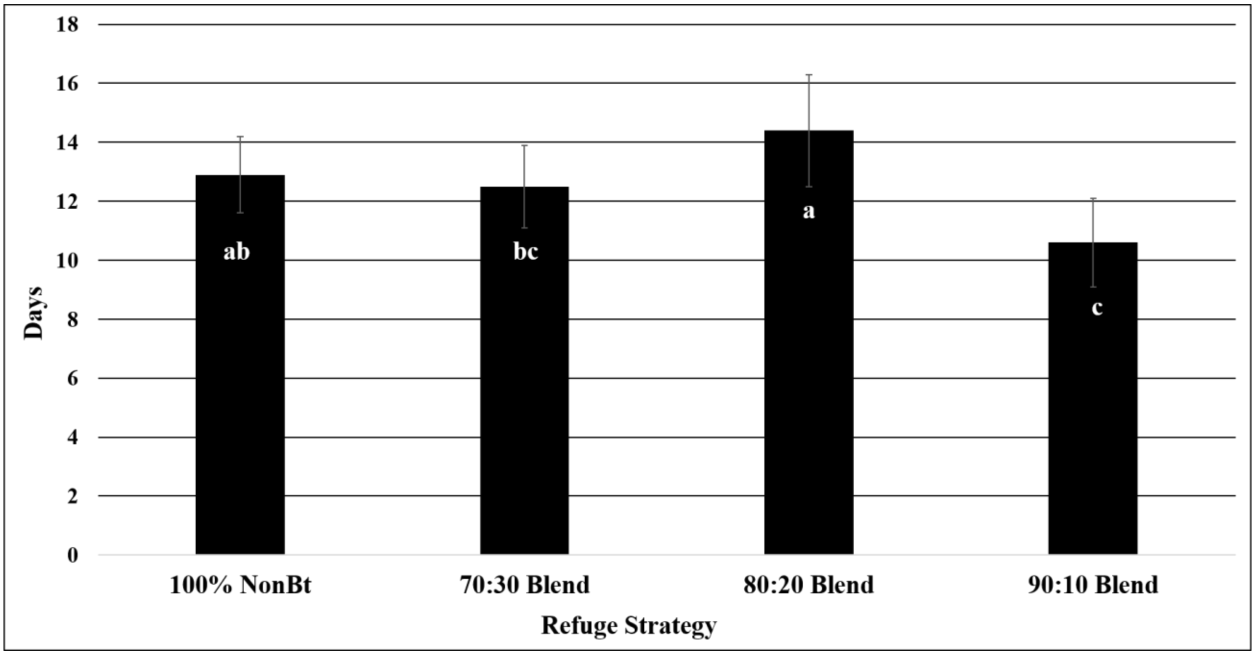

Figure 2.6 Mean (SE) number of days to reach 75% production of adult *H. zea* in all corn refuge treatments across 6 site years in Mississippi and Georgia in 2017 and 2018. (P = 0.02)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

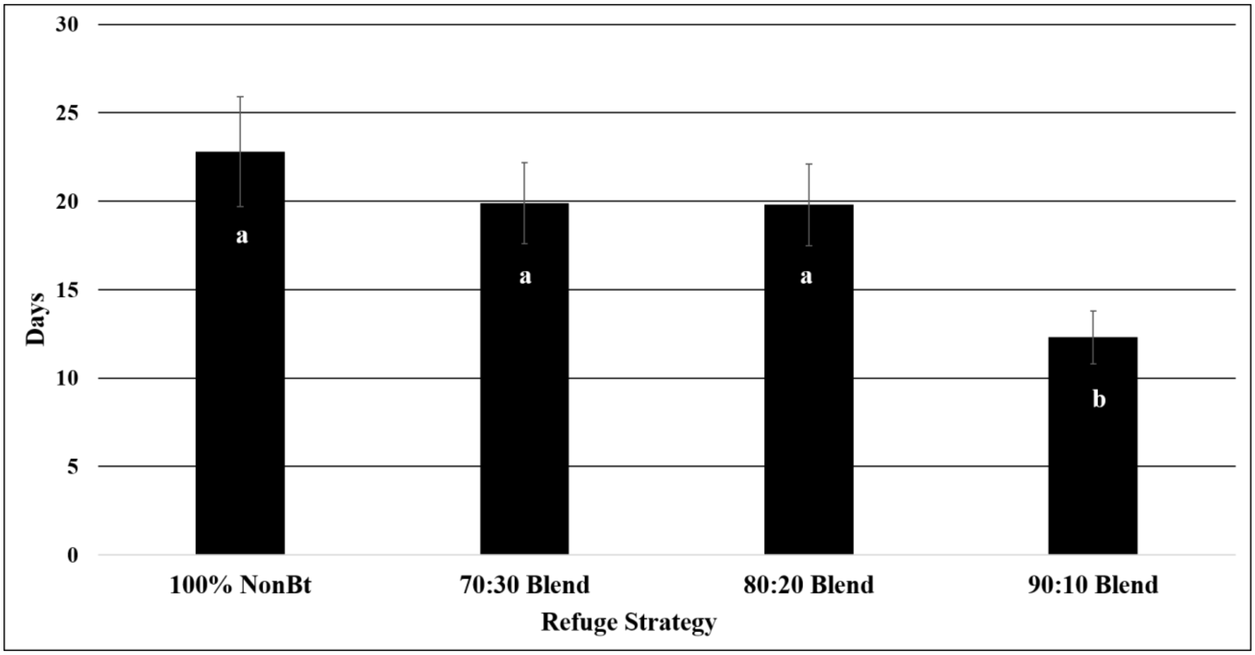

Figure 2.7 Mean (SE) number of days to reach 100% production of adult *H. zea* in all corn refuge treatments across 6 site years in Mississippi and Georgia in 2017 and 2018. (*P* <0.01)

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

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CHAPTER III

DETERMINING HOW VARIOUS LEVELS OF SIMULATED STAND LOSS AFFECT FIELD CORN YIELDS

3.1 Abstract

The borer complex, consisting of *Diatraea grandiosella* (Dyar), *Ostrinia nubilalis* (Hübner), and *Diatraea saccharalis* (F.), poses a risk to field corn that is not protected through the use of foliar-applied insecticides or Bt incorporation. In the southern U.S., corn borers have been controlled extremely well through the widespread usage of Bt corn hybrids. To prevent the selection of Bt resistant populations, refuge systems have been implemented into the agroecosystem. Historically, structured refuge compliance among growers has been low, leading to the commercialization of seed blended refugia in areas of the U.S. in which cotton is not grown. It could be assumed that if seed blended refugia was approved for use in the southern U.S., growers would not manage corn borers leading to the possible loss of refuge plants. To determine how this loss of refuge plants would affect yield, insect related stand loss was simulated at levels of 0, 10, 20, 30, 40, and 50%. Seed was counted into percentages and hand blended to simulate a seed blended refuge. Termination percentages were simulated at both the V5 and V10 growth stages. In low yielding environments, every one percent loss in plant population resulted in a 26.6 Kg Ha^{-1} reduction in corn yield. Subsequently, in high yielding environments, every one percent loss in plant population resulted in a 78.86 Kg Ha^{-1} reduction in corn yield.

3.2 Introduction

The borer complex of corn, *Zea mays* (L.), consists of the European corn borer, *Ostrinia nubilalis* (Hübner); the southwestern corn borer, *Diatraea grandiosella* (Dyar); and the sugarcane borer, *Diatraea saccharalis* (F.). *O. nubilalis* and *D. saccharalis* tend to be uncommon pests in the midsouthern U.S., and *D. grandiosella* populations are more commonly observed in the region (Baldwin et al. 2006). Both *O. nubilalis* and *D. saccharalis* have become more common across the southernmost Gulf States which may be due to reduced tillage practices and increasing corn hectares across the region (Castro et al. 2004, Huang et al. 2006). *D. grandiosella* is periodic in occurrence and yield losses may be greater than realized due to damage being hidden in the stalk (Davis et al. 1933). *O. nubilalis* is considered the most damaging insect pest of corn in the U.S. and Canada with losses exceeding one billion dollars each year (Ostlie et al. 1997). The feeding behaviors and damage to corn among species in the borer complex are relatively similar. Adults lay eggs on leaves of corn and grain sorghum, *Sorghum bicolor* (L.) Moench. Larvae hatch and feed on leaves for a short time before boring into the stalk (Baldwin et al. 2005). Larvae tunnel up and down the stalk as they mature, ultimately weakening the stalk (Baldwin et al. 2005). Corn borers feed within the vascular tissue of corn and disrupt the movement of water and nutrients within the plant (Culy 2000). This feeding can result in stunting, plant deformation, deadheart, and sometimes plant death (Culy 2000). Tissue injury caused by this complex can also lead to stalk lodging and ear drop contributing to yield reductions (Edwards et al. 1992). Later planted corn typically experiences more *D. grandiosella* damage than early planted corn suggesting that earlier planted corn will mature before damaging infestation levels can occur (Starks et al. 1982; Baldwin et al. 2005).

Corn hybrids that express insecticidal proteins from the soil bacterium, *Bacillus thuringiensis* (Bt) var. *kurstaki* (Bt), have provided excellent control of the corn borer complex and other lepidopteran pests (Ostrý et al. 2015). To prevent the selection of resistant alleles in target insects, a high-dose refuge strategy was implemented as a method of insecticide resistance management (IRM) (US EPA FIFRA SAP 1998). Several assumptions must be met for structured refuges to effectively delay resistance in target species. The assumptions are that the Bt must kill >99.9% of the wild-type individuals, the resistance allele is rare, resistance is mostly recessive, and random mating occurs between moths emerging from the Bt crop and refuge crop (Onstad and Knolhoff 2014). Initially, refuge deployment involved planting non-Bt hybrids in structured blocks separate from the Bt crop or as strips of non-Bt hybrids within the Bt field (Onstad et al. 2017). Recently, seed blended refugia were approved in some regions of the U.S. for dual-gene Bt corn hybrids, this consist of refuge seed mixed with Bt seed.

Structured refuge compliance has been low among growers leading to lower Bt susceptible insect populations and the selection for Bt resistance alleles in *H. zea* (Reisig 2017). The benefits of planting a seed blended corn refuge are that it maximizes adult mixing in a field setting as refuge plants would be distributed randomly across Bt fields. Blended seed refuge also places the burden of refuge deployment on the seed distributor instead of the grower (Carroll et al. 2012; Onstad et al. 2017). Low refuge compliance is especially an issue in cotton-growing areas because both corn and cotton express the same or similar Bt proteins and *Helicoverpa zea* (Boddie) feeds on both crops in succession (Von Kanel et al. 2016). For this reason, corn seed blended refugia are being considered as an option in cotton-growing areas to slow the development of resistance. Despite the potential benefits of a seed blended refuge, several implications such as cross-pollination could limit the effectiveness of this strategy. With the potential introduction of seed blends as a refuge option in the midsouthern U.S., this would change the way growers protect corn. Structured refugia can be easily treated with insecticides as they are typically planted in blocks or strips. Block planting refugia is typical due to timeliness and ease of planting and growers will likely choose the most economical compliance method (Hyde et al. 1999). However, because seed blended refugia would be randomly dispersed across a field of majority Bt protected corn, it is likely that growers would not try to protect these refuge plants. Untreated refuge plants are left vulnerable to attack from corn borers as well as other pests. In a worst-case scenario infestation of corn borers, it is possible to have negative effects such as decreasing overall crop yield and decreasing susceptible insect production from the refuge. This experiment examines potential yield loss in a seed blended field corn refuge when deployed at various non-Bt percentages. Additionally, loss of refuge plants can decrease the overall size and effectiveness of the refuge regarding Bt susceptible adult production. For every one percent loss in plant population in high yielding environments, there was a 26.6 Kg Ha^{-1} reduction in corn yield. For every one percent loss in plant population in high yielding environments, there was a 78.86 Kg Ha^{-1} reduction in corn yield.

3.3 Materials and Methods

A field study was conducted from 2017 to 2019 to determine how various percentages of simulated insect damage could affect yield in field corn. During 2017, this study was conducted at the R. R. Foil Plant Science Research Center in Starkville, Mississippi and at the Delta Research and Extension Center in Stoneville, Mississippi. In 2018, this study was conducted at the R. R. Foil Plant Science Research Center in Starkville, Mississippi. During 2019 the same study was conducted in two separate fields at the R. R. Foil Plant Science Research Center in Starkville, Mississippi. Planting dates for each trial in Starkville were 3 May in 2017, 12 April and 9 May in 2018, 29 May and 16 June in 2019. The planting date for the trial in Stoneville was 9 May in 2017. Field experiments were arranged as a randomized complete block with a 2 x 6 factorial arrangement of treatments and four replications. This study was repeated for a total of five site years. The factors included plant population loss timing and percent stand loss. Stand loss timings were imposed at the V5 and V10 growth stages. The timings were selected to determine if corn populations compensate for stand loss in early and mid-vegetative growth stages. Percent plant population loss treatments were 0%, 10%, 20%, 30%, 40% and 50%. Stand loss occurred by mixing the appropriate percentage of non-Roundup Ready corn seed with glyphosate [N-(phosphomethyl) glycine] (Roundup®, Monsanto Company, St. Louis, MO) resistant corn seed (DEKALB® DKC67-72, Monsanto Company, St. Louis, MO). The two cultivars were mixed thoroughly for random in-plot trait distribution. This occurred for every planting row within each plot. Two packages of equal amounts of blended seed (one package per row) were prepared for each plot. Seed were planted using an Almaco plot research specific cone-planter (Almaco, Nevada, IA). Corn was planted at the Starkville, MS location in two-row plots at a rate of 79,040 seeds per hectare on 96.5-cm row beds at a depth of 3.81-cm below soil level. At the Stoneville, MS location, corn was planted on two-row plots on 101.6-cm row beds. Plot lengths were 12.2 meters in length. Corn seed were treated with clothianidin at a rate of 0.5 mg ai/seed to protect plants from early-season underground insect pests. Plant population counts were recorded at the V3 growth stage prior to termination events to determine initial plant populations. Plant population counts were conducted by counting every live plant in each plot.

Glyphosate (Roundup®, Monsanto Company, St. Louis, MO) was applied at the V5 and V10 growth stages at a rate of 1.54 kg ai ha^{-1} to terminate glyphosate susceptible plants and to achieve the desired plant population loss percentage. Plant population counts were recorded following termination events to determine termination success. Plots were maintained weed-free across all locations through hand weeding and the application of pre-emergence and post-emergence herbicides. Fertilizer applications were based on soil test recommendations across locations. Furrow irrigation was utilized in experiments that were conducted in Stoneville, MS, but not in Starkville, MS. Plots were then allowed to develop to full maturity. At maturity, the entire plot was harvested, yields and percent moisture were recorded. Prior to analysis, corn grain yields were standardized to 15% moisture for all plots. Trials were harvested using a research scale combines with a weight system and moisture meter.

In the initial analysis, yield data were analyzed using a mixed model analysis of variance (PROC GLIMMIX, SAS 9.4, SAS Institute; Cary, NC) to determine how stand loss events affect corn yields. Test (site year), plant loss timing, plant loss percentage, and all interactions were considered fixed effects in the model. Replication, replication nested in test, and replication by plant loss timing nested in test were considered random effects in the model. In this analysis, there was a significant test by percent plant loss interaction (Table 3.1). The effect of test was then analyzed using LSMEANS and mean yields among tests were separated based on Tukey’s HSD ($\alpha = 0.05$). Starkville 2017 and Starkville 2018 tests were grouped into high yielding environments while the location in Stoneville 2017 and the two Starkville locations in 2019 were grouped into low yielding environments (Fig. 3.1). Corn yields in each environment were analyzed with regression analysis (PROC GLM, SAS 9.4, SAS Institute; Cary, NC). For each plant loss timing within an environment, plant population loss percentage was the independent variable and corn grain yield was the dependent variable. Analysis of covariance was used to test the slopes of the regression equations between the two plant loss timings within each environment. The slopes of the regression equations were not different between plant loss at V5 and V10 as indicated by a non-significant plant loss timing by plant loss percentage interaction in the low yielding (F = 1.29; df = 1, 140; *P* = 0.26) and high yielding (F = 0.15; df = 1, 92; *P* = 0.70) environments. As a result, data within each environment were combined across plant loss timings for corn yields. Analysis of covariance was used for the final analysis to test the slopes of the regression equations across the low yielding and high yielding environments. For all regression analyses, both linear and quadratic terms were tested for each model.

3.4 Results

There were no significant differences in plant populations among treatments prior to glyphosate applications being made (F = 0.58; df = 5, 200.9; *P* = 0.71). Based on plant population counts, the method used to blend the glyphosate-resistant and conventional seed was an effective means of simulating stand loss in a field setting (Fig. 3.2 & 3.3). After the V5 terminations, percent plant population loss had an effect on plant population as would be expected (F = 41.01; df = 5, 80.27; *P* <0.01). Plant populations in the different percent plant loss treatments were different from each other, except that plant populations in the 40% and 50% plant loss treatments were similar to each other (Fig. 3.2). The 10, 20, 30, 40, and 50% plant loss treatments resulted in plant populations that were 91, 82, 71, 60, and 52% of the zero plant loss treatment, respectively. After the V10 terminations, percent plant population loss had an effect on plant populations as would be expected (F = 37.33; df = 5, 90; *P* <0.01). Plant populations in the different percent plant loss treatments were different from each other except the plant populations in the 20% and 30% and the 30% and 40% plant loss treatments were similar to each other (Fig. 3.3). The 10, 20, 30, 40, and 50% plant loss treatments resulted in plant populations that were 91, 78, 70, 64, and 52% of the zero plant loss treatment, respectively.

In the low yielding environment, there was a linear relationship between plant loss percentage and corn grain yield when averaged across plant loss timings (F = 9.98; df = 3, 140; *P* <0.01). For every one percent loss in plant population, there was a 26.6 Kg Ha^{-1} reduction in corn yield (Fig. 3.4). Subsequently, in high yielding environments, there was a significant linear relationship between plant loss and corn grain yield when averaged across plant loss timings (F = 75.74; df = 3, 92; *P* <0.01). (Fig. 3.4). For every one percent loss in plant population, there was a 78.86 Kg Ha^{-1} reduction in corn yield (Fig. 3.4). The percent loss by test interaction was significant (F = 43.98; df = 1, 236; *P* <0.01) suggesting that the response of corn to plant loss was different between the low yielding and high yielding environments (Fig. 3.4).

3.5 Discussion

The introduction of Bt incorporated crops has provided near-complete control of *O. nubilalis* and *D. grandiosella* while reducing insecticide applications (Ostlie et al. 1997). Surveys conducted by Rice and Ostlie (2013) concluded that growers typically did not manage *O. nubilalis* because yield losses were not always obvious, they are unwilling to scout for the pest, history suggested no problems, and failure to recognize the cause of yield loss among many other reasons. The corn borer complex can be a serious pest in both sweet corn and field corn due to stalk and shank tunneling causing plant lodging or ear drop (Capinera 2000; Bessin N.D.). Unlike *H. zea*, corn borers can be gregarious feeders leading to multiple individuals per plant resulting in greater yield loss potential (Chiang et al. 1960). Small plants are more susceptible to corn borer injury, so early season cultural practices are encouraged to promote large healthy plants before corn borer establishment (Arbuthnot et al. 1958). Moving to a seed blended refuge could potentially put growers at risk of yield losses from the corn borer complex. Seed blended refugia would essentially ensure that refuge compliance is met, however, it could lead to unprotected corn plants and possible significant yield losses if large enough populations become established. In the current study, yield losses from severe simulated corn borer injury resulted in 26.6 Kg Ha^{-1} and 78.9 Kg Ha^{-1} yield losses for every one percent loss in plant populations in low yield and high yield environments, respectively. Previous research showed that *D. grandiosella* could cause 8% to 100% yield losses in dent stage field corn (Walton and Bieberdorf 1948). Another study conducted in Iowa indicated that *O. nubilalis* caused a 127- and 304.8-kilogram yield losses when treated and not treated with an insecticide, respectively (Bergman et al. 1985). Although the current study investigated a worst-case scenario for corn borer injury, the data suggest that some risk of yield loss may be realized from planting a seed blended refuge. Scouting and the implementation of a comprehensive trapping program would be needed to monitor populations. Future research looking at actual infestations of corn borer spp. in seed blended refugia incorporated fields would be beneficial to supplement this study.

Table 3.1 Results of the analysis of variance evaluating yield effects of stand loss in field corn across 5 site years in Mississippi in 2017, 2018, and 2019.

Effect	**F**	**df**	***P***
Test	16.75	12	<0.01
Timing	0.15	15	0.70
Timing*Test	0.98	15	0.45
Percent Loss	11.29	190	0.01
Percent Loss*Test	2.82	190	0.03
Percent Loss*Timing	1.62	190	0.20
Timing*Percent Loss*Test	0.61	190	0.66

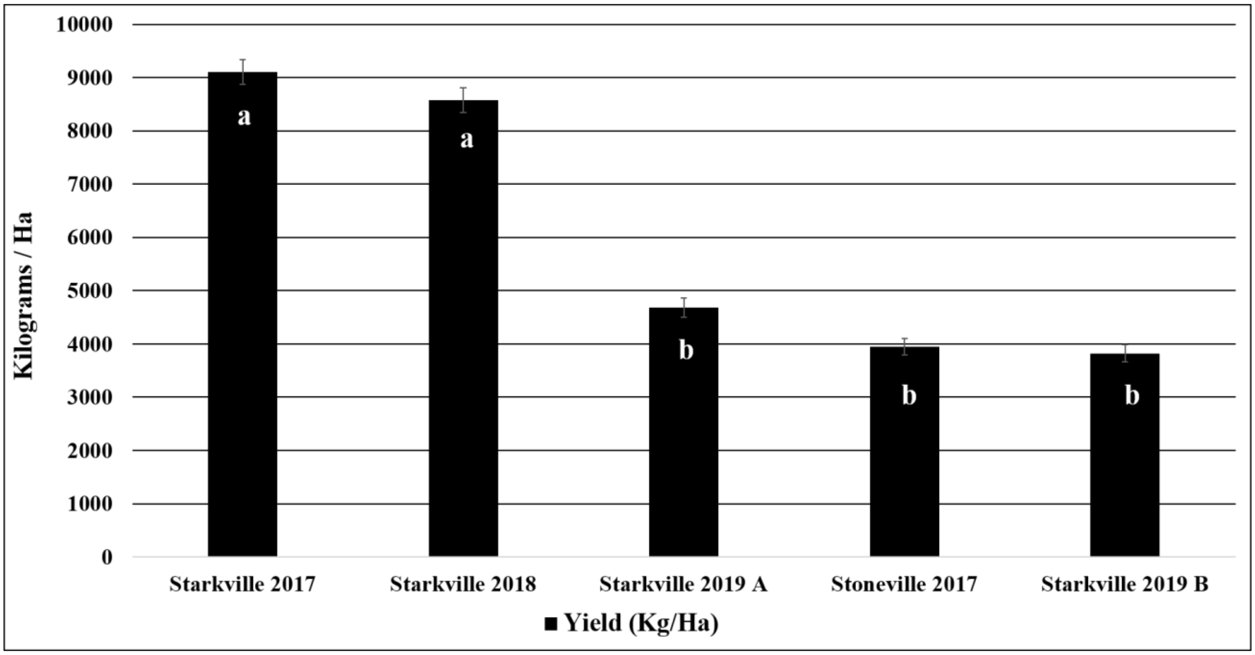

Figure 3.1 Mean yields across all locations from experiments evaluating stand loss in field corn.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

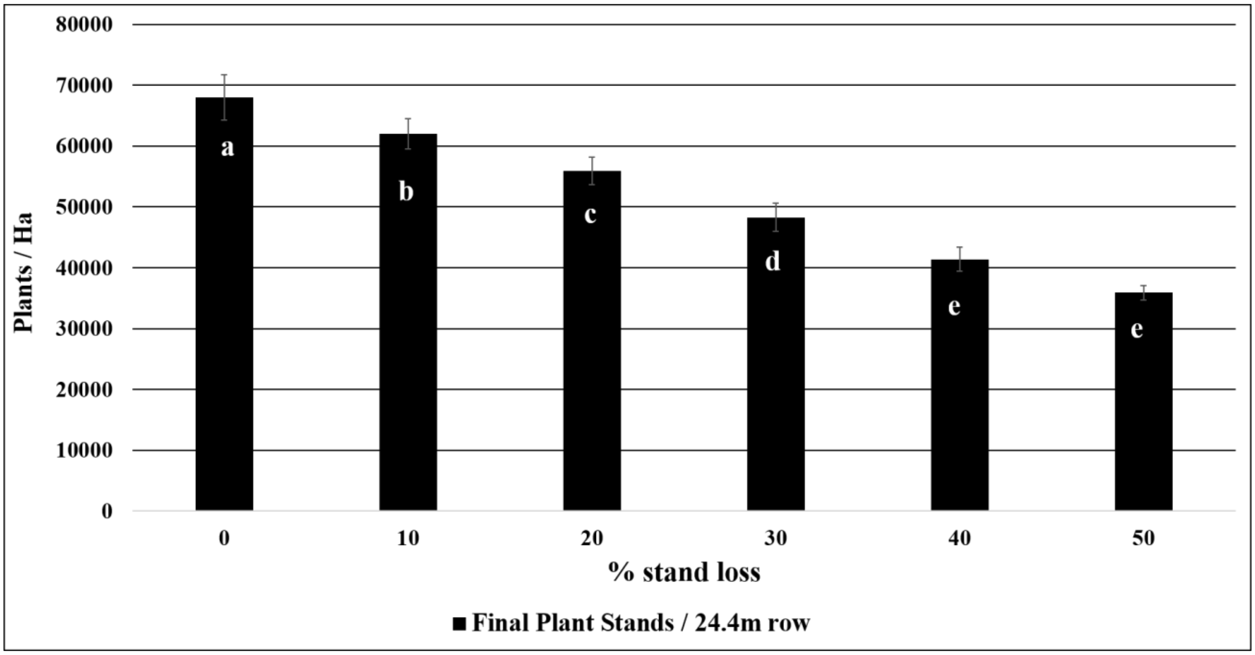

Figure 3.2 Mean plant population from stand loss treatments at the V5 stage of corn plants, averaged across experiments conducted in Starkville and Stoneville, Mississippi from 2017 and 2018.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

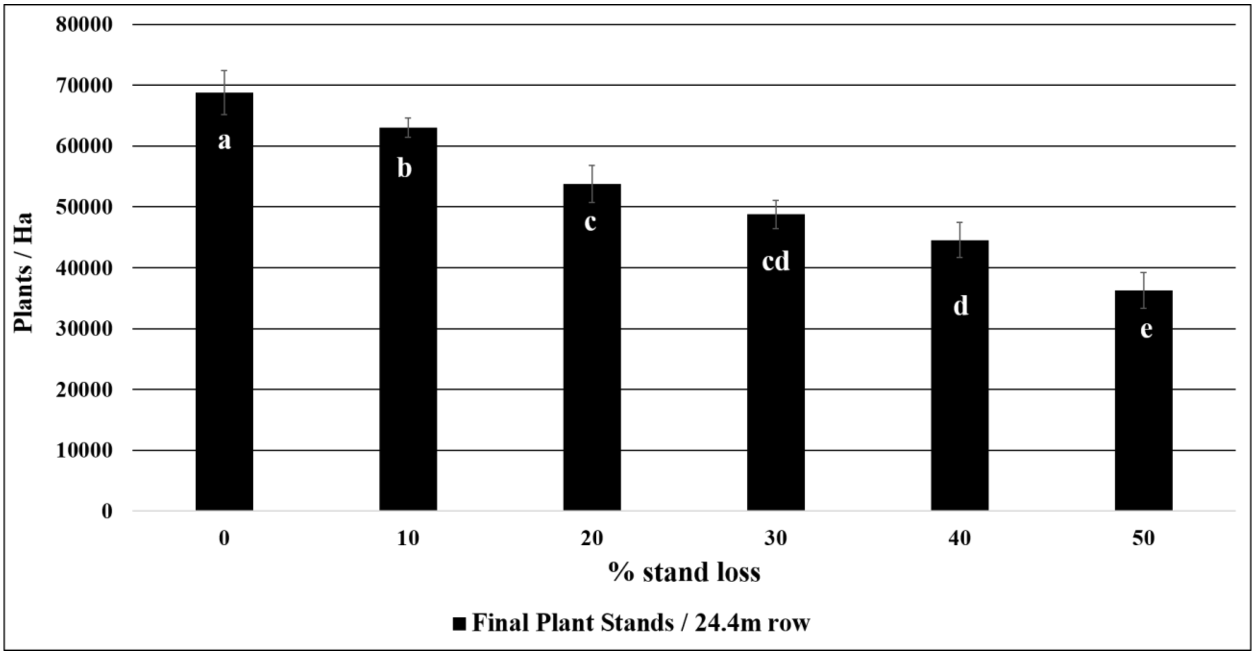

Figure 3.3 Mean plant population from stand loss treatments at the V10 stage of corn plants, averaged across experiments conducted in Starkville and Stoneville, Mississippi from 2017 and 2018.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

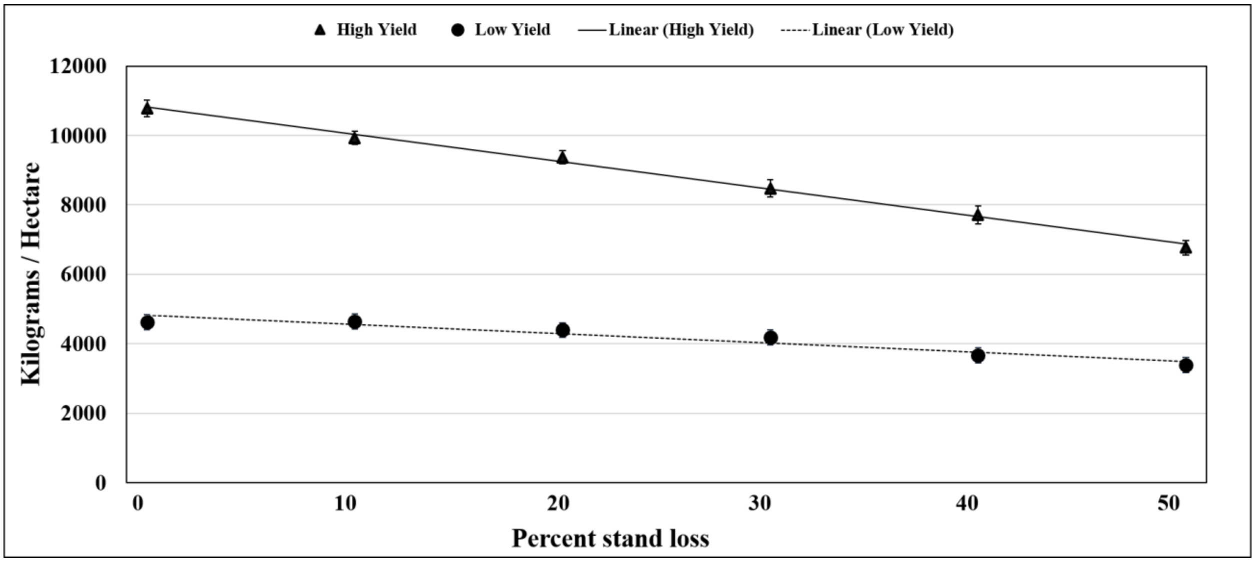

Figure 3.4 Impacts of varying levels of stand loss on corn yield in high and low yielding scenarios in environments in Mississippi from 2016 to 2019.

(▲) $y = -78.86x + 10809$; $P < 0.01$
(●) $y = -26.57x + 4814$; $P < 0.01$

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CHAPTER IV

SURVIVAL AND EMERGENCE OF CORN EARWORM (LEPIDOPTERA: NOCTUIDAE) FROM CORN HYBRIDS EXPRESSING THE TWO PYRAMIDED BT PROTEINS CRY1A.105 AND CRY2AB2

4.1 Abstract

Corn hybrids expressing Bt proteins have been utilized by growers in the U.S. to control insect pests of corn for over two decades. This technology has been instrumental in controlling corn pests such as *Ostrinia nubilalis* and *Diatrea grandiosella*. Refugia is implemented into the agroecosystem to prevent the selection of Bt resistance alleles and prolong the durability of Bt corn. Due to the lack of grower refuge compliance, field evolved resistance has been documented in *Helicoverpa zea* to Cry proteins in field corn and cotton. To test the efficacy of Cry genes on *H. zea* in field corn, field studies were conducted once in Scott, Mississippi in 2016 and once in Stoneville Mississippi in 2017 and 2018. Treatments consisted of a corn hybrid expressing both Cry1A.105 and Cry2Ab2 and a non-Bt expressing corn hybrid. Kernel damage among two-gene and non-Bt expressing corn plants were recorded and compared. To quantify adult *H. zea* emergence from two-gene pyramided corn and non-Bt expressing corn, emergence traps were utilized. *H. zea* survivorship and emergence was recorded from two-gene pyramided corn and compared to emergence from non-Bt expressing corn. Moth emergence timings were recorded and analyzed to 50, 75, and 100% emergence. Kernel damage was not different between the non-Bt expressing treatment and the two-gene pyramided treatment. When compared to non-Bt

expressing corn, *H. zea* adult moth emergence from two-gene pyramided corn significantly differed. Moth emergence timings were not significantly delayed between the non-Bt expressing treatment and two-gene pyramided treatment.

4.2 Introduction

Since the introduction of corn hybrids expressing insecticidal proteins from *Bacillus thuringiensis* (Bt), growers have been utilizing the technology to aid in the control of European corn borer, *Ostrinia nubilalis* Hübner, and southwestern corn borer, *Diatrea grandiosella* Dyar (Peferoen 1997). Bt cotton was genetically engineered to protect against *Heliothis virescens* (F.), and *Pectinophora gossypiella* Saunders (Huang et al. 2011). Approximately, 92% of cotton and 83% of corn planted in the U.S. express one or more Bt proteins (USDA ERS 2019). To prevent the development of resistant strains of some species, refugia are incorporated into the Bt containing landscape to provide a source of unexposed, Bt susceptible insects. The theory is that if a rare, naturally occurring resistant individual survives and emerges from Bt expressing crops, it will mate with one of the many susceptible individuals produced by the refuge (Hurley et al. 2001). If resistance is inherited recessively, mating between a Bt-resistant individual and Bt susceptible individual produces heterozygous offspring that will be killed by Bt (Gould 1998). Surveys have shown that corn refuge compliance is low among growers in the U.S. (Jaffe 2009; Reisig 2017). Low refuge compliance is especially detrimental in cotton-growing areas due to the same or closely related Bt proteins present in both corn and cotton. Currently, Bt pyramided corn and cotton varieties expressing two to three different Bt proteins are being marketed. In corn and cotton, some commonly incorporated gene combinations for lepidopteran control are Cry1Ab, Cry1F, Cry1A.105, Cry2Ab2, Cry2Ae, Cry1Ac, and Vip3Aa20 (Reisig and Kurtz 2018). Vip3Aa20 is a vegetative insecticidal protein (VIP) that has recently been commercialized for lepidopteran control in Bt maize and cotton. Vip3Aa20 is currently being engineered into some corn hybrid pyramids along with Cry1A.105 and Cry2Ab2 in an attempt to further delay insecticidal resistance from occurring. The method of pyramiding Bt proteins aids in prolonging the development of resistance by combining proteins that target separate receptors in the pest (Carrière et al. 2015). However, it is documented that resistance has already occurred to gene pyramids such as Cry1Ac and Cry1F, Cry1Ac and Cry2Ab2, Cry1Ab and Cry1F, and Cry1A.105 and Cry2Ab2 (Dively et al. 2016; Reisig et al. 2018). If resistance is developed against one Bt protein in a two or three-protein pyramided corn variety, the effectiveness of the pyramided Bt proteins to delay resistance is reduced (Carrière et al. 2016).

The bollworm, *Helicoverpa zea* (Boddie), feeds on both corn and cotton in succession (Quaintance and Brues 1905). Second generation *H. zea* emerging from non-cultivated hosts typically infest corn followed by the third generation infesting to other hosts such as cotton (Snow and Brazzel 1965). Corn hybrids expressing Bt proteins are selecting *H. zea* which will infest cotton in subsequent generations. This results in those populations that subsequently infest cotton having a higher tolerance to Bt due to selection in corn during previous generations. *H. zea* is naturally more tolerant of Bt proteins in corn and cotton compared to the target pests, such as *H. virescens* and *O. nubilalis,* resulting in the possibility of yield-limiting populations becoming established in Bt cotton. Early resistance studies suggested that *H. virescens* and *H. zea* are biologically able to develop resistance to Bt endotoxins, however, there is only evidence of field evolved resistance in *H. zea* (Gould et al. 1996; Luttrell et al. 1999). Ear damage and *H. zea* larval survival have increased in two gene pyramided Bt corn hybrids expressing the Bt proteins Cry1A.105 and Cry2Ab across the mid-southern U.S. since first planting in 2010 (Kaur et al. 2019). These results are likely due to field-evolved resistance to the proteins expressed by

Bt corn hybrids. However, susceptibility levels of larvae varied among populations across the region (Kaur et al. 2019). The objective of the current study was to determine how field evolved resistance has affected the efficacy of Cry1A.105 and Cry2Ab2 and to quantify *H. zea* emergence from dual-protein corn in relation to non-Bt corn.

4.3 Materials and Methods

A total of three field experiments were conducted during the 2016, 2017, and 2018 growing seasons. the effectiveness of a popular two gene pyramided Bt expressing corn hybrid compared to non-Bt field corn to determine *H. zea* adult production and developmental timing. Locations included the Monsanto Learning Center in Scott, Mississippi (2016) and the Delta Research and Extension Center (DREC) in Stoneville, Mississippi (2017 and 2018). The experimental design was a randomized complete block with two treatments and four replications. Treatments consisted of DKC 67-70 (Bayer CropScience, St. Louis, MO) non-Bt and DKC 67-72 (VT DoublePro Bayer CropScience, St. Louis, MO) expressing the Cry1A.105 and Cry2Ab2 Bt proteins. Corn was planted at a rate of 79,040 seed Ha^{-1}. Corn was planted on 101.6-cm row beds in Stoneville, MS locations and 96.5-cm row beds in Scott, MS. Seed were planted using an Almaco plot research specific cone-planter (Almaco, Nevada, IA) at a depth of 3.81-cm below the soil surface. Corn seed were treated with clothianidin at a rate of 0.5 mg seed^{-1} prior to planting to protect seedlings from early-season soil insect pests. Plot size consisted of 8 rows wide by 13.7-m in length at all locations. *H. zea* oviposition was monitored during the R1 growth stage (silking). After *H. zea* eggs hatched, larvae could move into the corn husk, and feed until pupation. Kernel damage from *H. zea* feeding was determined at the R6 (physiological maturity) growth stage during 2017 and 2018. Kernel damage was not determined during the 2016 growing season (Scott, MS). The number of damaged kernels per ear was converted to cm^2 of damage. It was assumed that 1 cm^2 was equal to 4 kernels. After larvae left plants to pupate in the soil, plants were removed by cutting stalks at the soil level with a sickle bar mower (Model BSB-284, BEFCO, Inc., Rocky Mount, NC). Twenty-five pyramid emergence traps were placed in each plot to collect adult *H. zea* as they emerged from the soil. Emergence traps measured 132.1 cm in length by 106.7 cm in width by 76.2 cm in height covering 15.2 sq. ft. each. Trap frames were custom-built using 3.175mm diameter solid steel rods. Frames were covered using 6.35mm mesh galvanized hardware cloth. The hole used for moth exit was made using a steel tube with an outside diameter of 19.05mm and measured 38.1mm in length. Plant material removed from the plots was placed around the base of the emergence traps to prevent insect movement into or out of the traps. Entry holes were cut in 10.92 cm diameter plastic serving cups and positioned at the peak of the traps to collect and hold *H. zea* adults. *H. zea* adult emergence was recorded for 15 consecutive weeks during all three years. *H zea* emergence per hectare was calculated. The plots were maintained weed and disease-free throughout the growing seasons. Twenty-five ears per plot were examined for *H. zea* feeding.

For analyses, cumulative total *H. zea* emergence was calculated for each plot across all sample dates. For the initial analysis, cumulative *H. zea* emergence was compared across both corn types. In addition to total moth emergence, the days to 50%, 75%, and 100% moth emergence were calculated for each plot to determine if a delay in emergence occurred. *H. zea* emergence data and damaged kernel data were analyzed with a mixed model analysis of variance (PROC GLIMMIX, SAS Institute 2019). Corn type was considered a fixed effect in the model. Location and replication nested in location were considered a random effect. Degrees of freedom were estimated with the Kenward-Roger method. Proc means was used to determine means and standard errors. Data regarding the number of damaged kernels were subjected to analysis of variance procedures using PROC GLIMMIX (SAS Institute 2019).

4.4 Results and Discussion

Mean number of damaged kernels was not different among corn types and ranged from approximately 94.1 ± 1.4 to 89.7 ± 5.4 cm^2 per 25 ears (Fig. 4.1). In the past, it was common to observe significant reductions in *H. zea* damaged kernels for pyramided Bt corn compared to non-Bt corn (Storer et al. 2001; Buntin et al. 2004; Burkness et al. 2010; Yang et al. 2014). However, with the combination of widespread adoption of pyramided Bt corn hybrids and low refuge compliance, the reduction of kernel damage between Bt and non-Bt is no longer consistently observed, possibly due to the development of resistance (Dively et al. 2016; Reisig and Kurtz 2018; Kaur et al. 2019;). However, differences among cumulative totals of *H. zea* adult emergence were observed between the non-Bt and Bt corn hybrids (F = 13.52; df = 1, 17; *P* < 0.01). This indicates that Cry1A.105 and Cry2Ab2 may still be causing mortality to *H. zea* during the pupal stage despite resistance being well documented (Reisig and Reay-Jones 2015; Dively et al. 2016; Reisig et al. 2018). The observance of similar kernel damage between Bt and non-Bt corn ears and differences in survival and emergence would need to be further investigated to understand this relationship. Over the three site years, mean *H. zea* emergence was 7,874 and 5,391 from non-Bt and Bt respectively (Fig. 4.2). Even though differences in total number of moths emerged were observed between non-Bt and Bt corn expressing Cry1A.105 and Cry2Ab2, there were no differences observed between corn types for number of days to 50% (F = 2.02; df = 1,11; *P* = 0.18), 75% (F = 0.07; df = 1,20; *P* = 0.79), or 100% moth emergence (F = 3.22; df = 1,20; *P* = 0.08) (Fig.4.3-4.5). This suggests that there is no longer a significant delay in *H. zea* developmental time in non-Bt and Bt corn expressing Cry1A.105 and Cry2Ab2 (Fig. 4.3-4.5).

These data suggest that durability has been lost in two-gene corn pyramids expressing Cry1A.105 and Cry2Ab2 in-ear feeding *H. zea* due to field evolved resistance. However, since *H. zea* is not a yield-limiting pest in field corn, this technology still has great value in controlling key target pests such as *D. grandiosella* and others.

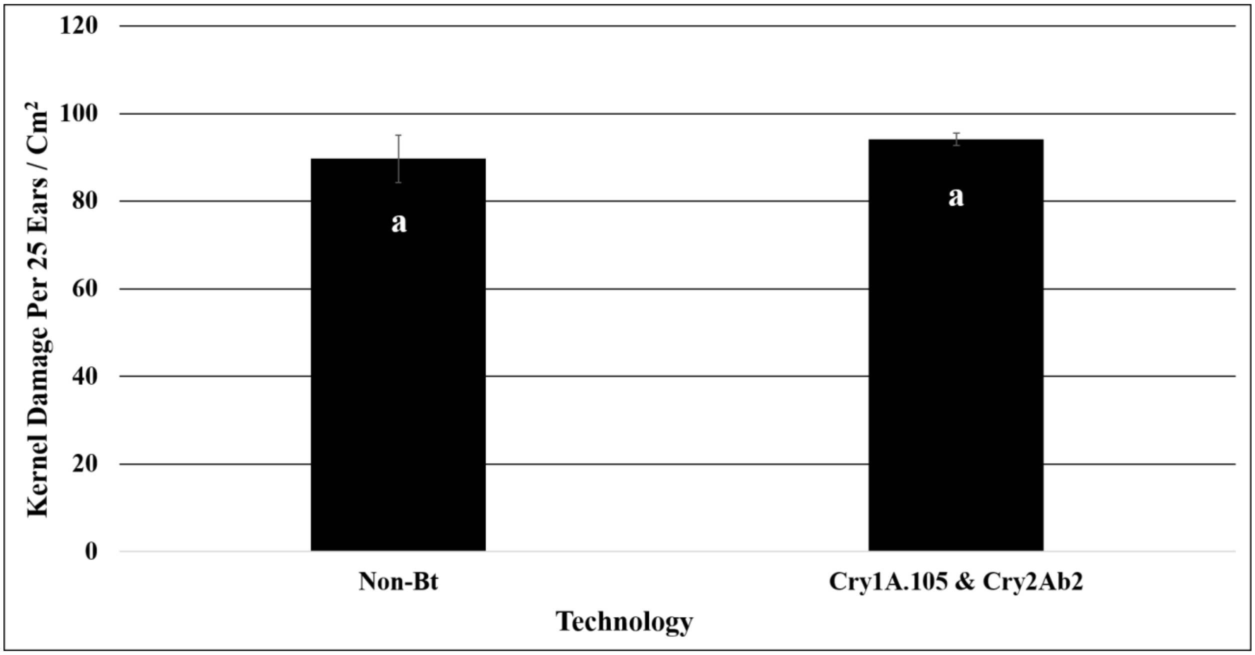

Figure 4.1 Mean (SE) kernel damage per 25 ears in cm^2 among non-Bt and Bt technologies.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

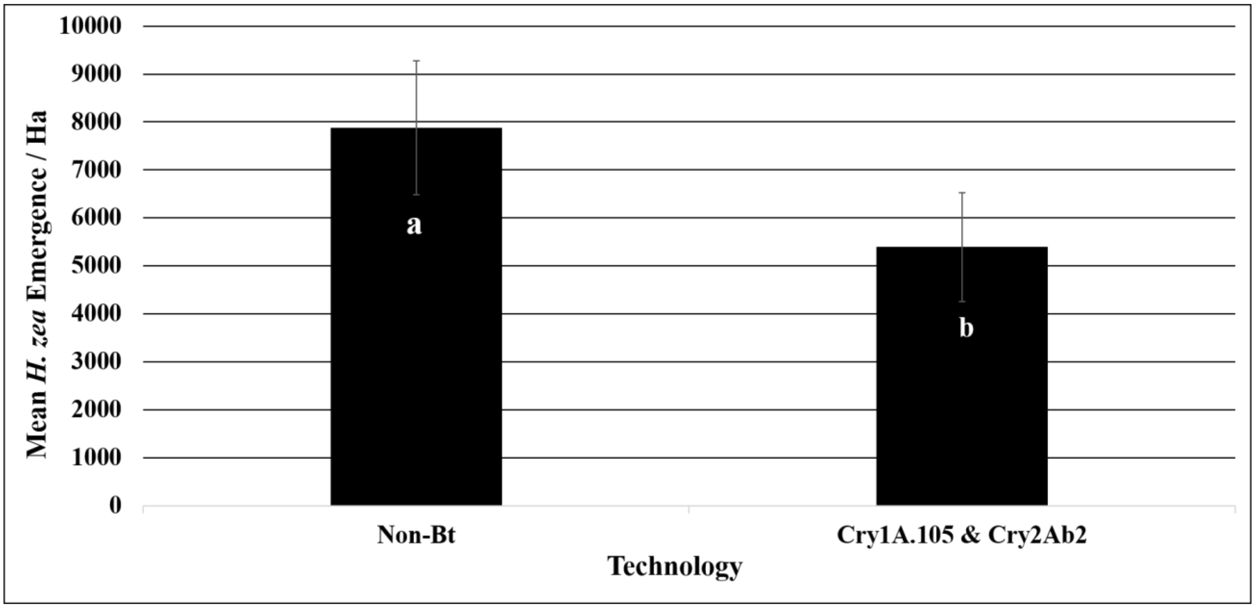

Figure 4.2 Mean (SE) *H. zea* adult survivorship and emergence from non-Bt and Bt corn plots.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

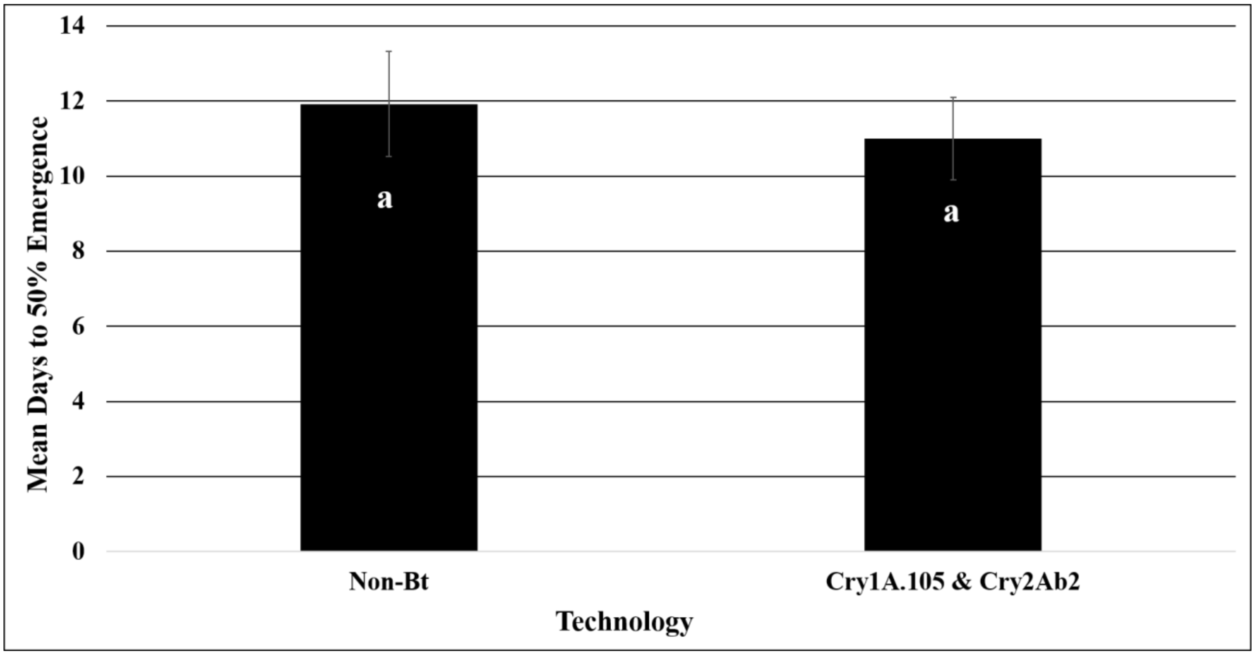

Figure 4.3 Mean (SE) days to 50% of *H. zea* emergence among treatments.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

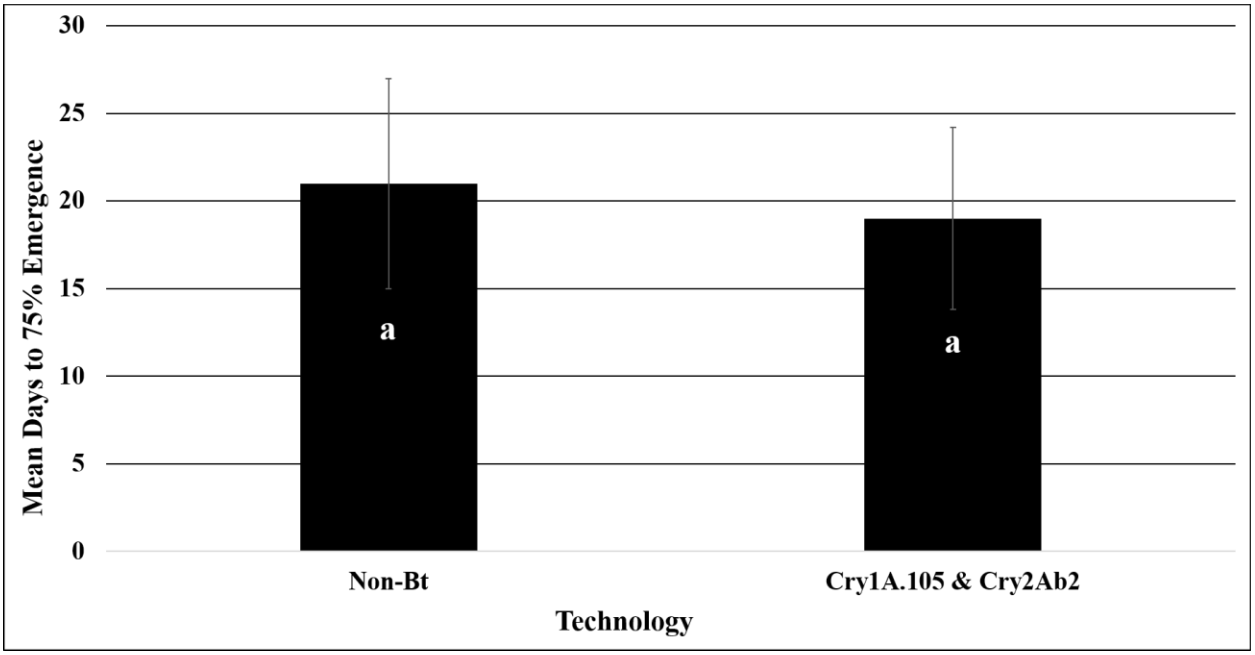

Figure 4.4 Mean (SE) days to 75% of *H. zea* emergence among treatments.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

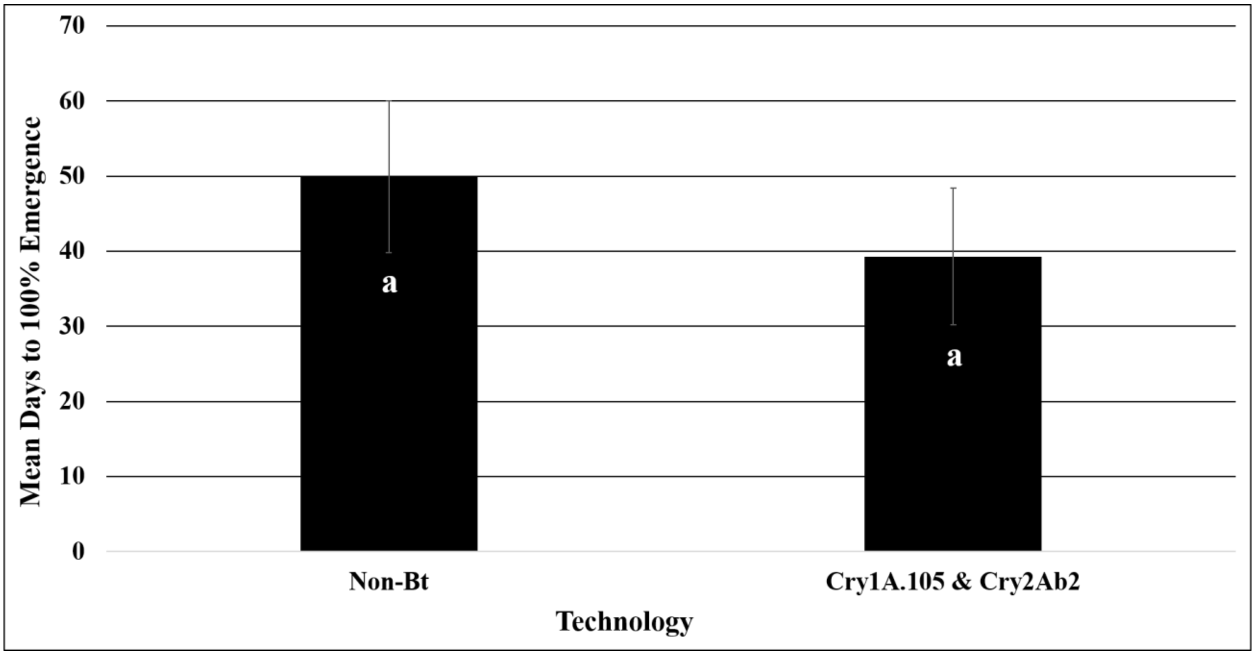

Figure 4.5 Mean (SE) days to 100% *H. zea* emergence among treatments.

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

4.5 References

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