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Effects of infection with *Nosema pyrausta* on survival and development of offspring of laboratory selected Bt-resistant and Bt-susceptible European corn borers

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ABSTRACT

Infection with *Nosema pyrausta* Paillot lengthens developmental period of Bt-susceptible *Ostrinia nubilalis* (Hübner) to a similar extent as feeding on Cry1Ab-incorporated diet in Cry1Ab-resistant *O. nubilalis*, and these two factors combined lengthen developmental period further than either alone. Resistant *O. nubilalis* mating with infected susceptible, or infected resistant partners would produce partially- and fully-resistant offspring, respectively, infected with *N. pyrausta*. To investigate the impacts on the progeny of such matings, test crosses were set up to produce partially- and fully Cry1Ab-resistant *O. nubilalis* offspring transovarially infected and not infected with *N. pyrausta,* which were exposed to Cry1Ab toxin at doses of 0, 3, or 30 ng/cm^2 for 7 days. Transovarial infection with *N. pyrausta* significantly decreased 7 day survival of partially and fully-resistant *O. nubilalis* feeding on 30 ng/cm^2 Cry1Ab. In addition, *N. pyrausta* infection delayed larval development (as measured by weight) of partially- and fully-resistant *O. nubilalis* feeding on 3 and 30 ng/cm^2 Cry1Ab. Impacts of natural enemies on target pests may have the potential to impact evolution of resistance. *N pyrausta*-infected *O. nubilalis* are more strongly affected by feeding on Bt, and would be less likely to survive to adulthood to pass on resistance to the next generation. This indigenous microsporidium may work to delay evolution of resistance in *O. nubilalis* by lowering their ability to survive on Bt.

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1. Introduction

Feeding by the European corn borer, *Ostrinia nubilalis* (Hübner), injures maize plants resulting in yield loss (Mason et al., 1996). For more than a decade, populations of *O. nubilalis* have been effectively controlled by Bt transgenes isolated from the bacterium, *Bacillus thuringiensis* Berliner. The insertion of these transgenes into the maize genome caused great concern regarding the effective lifespan of Bt technology because of the possibility of pest insects evolving resistance. All events of Bt maize have a required resistance management plan to delay resistance evolution, the most common being the high dose/refuge strategy (US EPA, 2001). The defined purpose of the refuge is to produce sufficient numbers of genetically-susceptible adults to mate with resistant adults. Because matings between resistant and susceptible adults will never produce homozygous-resistant offspring, this strategy should slow or prevent the evolution of resistance to Bt maize. One assumption of the high dose/refuge strategy is that resistant adults emerging from Bt maize will mate randomly with susceptible adults from the refuge. Susceptible and resistant adults would need to be present at the same time in order for random mating to occur.

Delays in larval development and subsequent adult emergence could lead to non-random mating, especially if larvae of resistant and susceptible genotypes develop at different rates. Feeding on Bt proteins has been shown to delay larval development in resistant pink bollworm, *Pectinophora gossypiella* (Saunders) (Liu et al., 1999, 2001). Resistant *P. gossypiella* exhibited slower larval development when feeding on Cry1Ac-incorporated diet and Cry1Ac-expressing cotton compared with susceptible larvae feeding on non-Cry1Ac diet and cotton (Liu et al., 1999, 2001). Lopez et al. (2010) also found that feeding on Cry1Ab diet lengthened time to adult emergence of resistant *O. nubilalis* compared to susceptible *O. nubilalis* feeding on non-Cry1Ab diet.

Other abiotic and biotic factors may also impact larval development and timing of adult emergence. Peck et al. (1999) demonstrated via evolution models that ecological variables affecting the timing of diapause and adult emergence will impact the rate of resistance evolution. *Nosema pyrausta* Paillot is a widespread obligate intracellular parasite of *O. nubilalis*. Effects of *N. pyrausta*

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on *O. nubilalis* vary with intensity of infection and include increased mortality, decreased adult longevity, and reduced fecundity (Zimmack and Brindley, 1957; Windels et al., 1976; Siegel et al., 1986; Sajap and Lewis, 1992). In addition, infection with *N. pyrausta* has been shown to delay larval and pupal development (Solter et al., 1990b; Lopez et al., 2010). Lopez et al. (2010) also found that both Cry1Ab-resistant and susceptible *O. nubilalis* infected with *N. pyrausta* feeding on non-Cry1Ab diet were delayed to a similar degree as Cry1Ab-resistant *O. nubilalis* feeding on Cry1Ab diet. Cry1Ab-resistant *O. nubilalis* infected with *N. pyrausta* that were fed Cry1Ab diet were delayed to an even greater extent than caused by infection alone or feeding on Cry1Ab diet alone (Lopez et al., 2010). The authors concluded that if similar developmental delays were present in a field situation, resistant *O. nubilalis* feeding on Cry1Ab maize would emerge later than uninfected, susceptible *O. nubilalis* from the refuge, leading to assortative mating. However, susceptible *O. nubilalis* infected with *N. pyrausta* would also experience delayed emergence relative to uninfected *O. nubilalis*. Because they experience a delay in adult emergence when feeding on Cry1Ab, resistant moths may be more likely to encounter and mate with similarly delayed, *N. pyrausta*-infected moths from the refuge than uninfected refuge moths. If a Bt-resistant male mates with a refuge female infected with *N. pyrausta* the infection will be transmitted transovarially, thereby impacting their heterozygous-resistant offspring. Additionally, assortative mating among resistant *N. pyrausta*-infected moths, because of prolonged developmental delays, would produce fully-resistant offspring which would also be transovarially infected.

Previous studies have shown increased detrimental effects on Bt-susceptible *O. nubilalis* infected with *N. pyrausta* when challenged with Bt diet compared with uninfected *O. nubilalis* (Pierce et al., 2001; Reardon et al., 2004). Little work has been done to elucidate how infection with *N. pyrausta* may influence evolution of resistance to Bt maize. The present study was designed to assess effects on the offspring of matings that may result from delayed adult emergence in susceptible and resistant populations of *O. nubilalis* infected and not infected with *N. pyrausta*. Of particular interest was the effect of transovarial infection with *N. pyrausta* by itself and in combination with Cry1Ab on the offspring of both resistant-susceptible matings, and resistant-resistant matings. These offspring represent the generation produced by mating of delayed resistant and susceptible adults. The objective of the study was to determine the impacts on offspring of the following matings: (1) resistant × uninfected susceptible, (2) resistant × *N. pyrausta*-infected susceptible, (3) resistant × uninfected resistant, and (4) resistant × *N. pyrausta*-infected resistant.

2. Materials and methods

2.1. Insect preparation

Four populations of *O. nubilalis* were established in the laboratory and later mass-mated to produce offspring having the desired resistance and infection types: (1) resistant, uninfected, (2) resistant, *N. pyrausta*-infected, (3) susceptible, uninfected, and (4) susceptible, *N. pyrausta*-infected. A population of *O. nubilalis* resistant ("RES") to high levels of the Bt insecticidal crystal protein Cry1Ab was selected for and maintained in the laboratory for >50 generations (Sumerford, personal observation). This population is greater than 2500× resistant and can feed and survive on reproductive stage maize. Eggs from the RES population were used to create infected and uninfected populations as follows: approximately half of the eggs were placed into rearing containers (25 cm diameter) (Pioneer Plastics, Inc., Dixon, KY) containing 930 g meridic diet (Guthrie et al., 1985), which were inoculated with *N. pyrausta* spores 3–4 days post egg hatch. The spores were harvested from highly infected frozen *O. nubilalis* larvae; cadavers were homogenized in a blender with water, the resulting solution was filtered through several layers of cheesecloth and then diluted to a final concentration to equal 1000 spores/mm^2 of diet surface. The remaining eggs were placed into containers of meridic diet without *N. pyrausta* spores to establish the uninfected resistant population. A susceptible population free from infection with *N. pyrausta* was continuously maintained in the laboratory, and eggs from this population were used to establish infected and uninfected populations as described above. These four populations were specifically mated to produce transovarially infected and uninfected F_1 offspring. Test populations resulting from mass matings using the four populations described above were established to produce larvae partially- (F_1 populations #1 and #2, Table 1, 50% of their genetic material from a susceptible female) or fully-resistant to Cry1Ab (#3 and #4, Table 1). The Cry1Ab resistance in this colony is not sex-linked (Sumerford, personal observation). Reciprocal crosses using resistant females crossed to susceptible males would have been redundant with respect to the resistance phenotype and would not have produced infected offspring.

N. pyrausta can be transmitted to the offspring of an infected female via transovarial transmission (Sajap and Lewis, 1988, 1992). Male *O. nubilalis* are either incapable of transmitting *N. pyrausta* infection to offspring, or, if it does occur, is very infrequent and unlikely to contribute to spread of the disease in the field (Solter et al., 1990a; Lopez, 2008). Therefore, in matings designed to produce a *N. pyrausta*-infected F_1 population, the female was always the infected partner. Before placement into mating arenas, infection status for all emerged females was verified by examining the meconium for *N. pyrausta* spores (Inglis et al., 2003). All females that came from inoculated rearing containers were positive, and all that came from control containers were negative. Between 20 and 30 adults of each sex were placed into mating arenas in the combinations shown in Table 1. Eggs from the four mating combinations were collected daily and maintained at 27 °C, 70% relative humidity (Rh), and continuous light until hatch.

2.2. Diet and experimental arenas

The surface overlay method developed by Siegfried et al. (2001) was used to bio-assay Cry1Ab resistance. Meridic diet was prepared, metered into opaque 128 cell Bio-Assay trays (Bio-serv, Frenchtown, NJ) using a repetitive pipetter, 1 ml per cell, taking great care to prevent any surface imperfections such as bubbles or depressions, and allowed to solidify overnight. Cry1Ab toxin (trypsin-digested Cry1Ab; obtained from Dr. M. Carey, Dept. of Biochemistry, Case Western Reserve University, Clevelend, OH), was mixed in 0.1% Triton-X 100 non-ionic detergent (used to ensure even spread across the diet surface), and pipetted onto the diet surface. Larvae were exposed to two doses of Cry1Ab; a low, sublethal dose (3 ng/cm^2) and a high dose (30 ng/cm^2). The current diagnostic dose of Cry1Ab used to monitor resistance in field populations of *O. nubilalis* is 10 ng/cm^2 (Marçon et al., 2000). These doses along with a control of 0.1% Triton-X 100 were overlaid onto the diet cells using a repetitive pipetter (30 μl), and allowed to dry (see (Siegfried et al., 2001). Neonatal *O. nubilalis* were placed one per cell onto the control diet or diet overlaid with one of two doses of Cry1Ab toxin. Trays were sealed with 16-cell self-adhesive Bio-Assay tray lids (Bio-serv) that allowed for air exchange and prevented larval movement between cells. Trays were maintained at ideal larval conditions (27 °C, 70% Rh, and continuous light) for 7 days. Data were recorded from all larvae at 7 days, and included weight and survival.