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Do pigeon pea refuges in *Bt* cotton pull their weight as "genetic diluters" to counter *Bt* resistance in *Helicoverpa* moths?

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ABSTRACT

Helicoverpa armigera (Hübner) and *H. punctigera* (Wallengren) are destructive pests that could develop resistance to *Bt*-cotton (*Gossypium hirsutum* L.). In Australia resistance is countered within a season by diluting resistance genes with susceptible genes ("Genetic Dilution"); or limiting the gene flow of resistance genes between seasons ("Season Quarantining"). Planting non-Bt host plants ("refuges") like pigeon pea (*Cajanus cajan* (L.)) to produce sufficient susceptible genes to dilute resistance genes from *Bt*-cotton, is part of the Genetic Dilution strategy. The current resistance management plan for *Bt*-cotton mandates that pigeon pea refuges are half the size of non-*Bt*-cotton refuges because pigeon pea can produce twice as many moths as cotton. We tested this assumption on commercial farms using eggs and pupae of both *Helicoverpa* species as measures of attractiveness and productivity respectively.

We found that pigeon pea attractiveness and productivity is inconsistent across the season and that, compared to cotton, higher egg densities in pigeon pea were concentrated at the end of the season. We discuss the implications of these data in terms of the pros and cons of following the Genetic Dilution strategy and maintaining pigeon pea refuges for as long as possible, versus destroying pigeon pea refuges at the end of the season as part of a Season Quarantining strategy.

1. Introduction

In Australia, cotton (*Gossypium hirsutum* L.) is grown commercially from the tropics (15°S) to the temperate zones (35°S). Throughout this range, *Helicoverpa* spp. are the most destructive pests in Australian cotton (Whitehouse et al., 2009b; Fitt and Wilson, 2012). There are two main species of *Helicoverpa* in Australian cotton: *H. armigera* (Hübner) which is cosmopolitan and *H. punctigera* (Wallengren) which is endemic. *H. armigera* in particular is difficult to manage because of its ability to develop insecticide resistance rapidly. For example, since the 1960s, *H. armigera* has developed resistance to nearly every insecticide used for its control within 5–8 years (Whitehouse et al., 2009b; Zalucki et al., 2009; Wilson et al., 2013).

To control *Helicoverpa* spp damage, *Bt* cotton was introduced to Australia in 1996. *Bt* cotton contains insecticidal genes that target Lepidoptera. The first generation, Ingard, contained one insecticidal gene (Cry1Ac); the second generation, Bollgard II (introduced in 2003) contained Cry1Ac and Cry2Ab. The third generation, Bollgard 3 was introduced in 2016 and includes Cry1Ac, Cry2Ab and Vip3A. To slow the development of resistance by *H. armigera* and *H. punctigera* to *Bt* cotton, a Resistance Management Plan (RMP) was put in place when *Bt* cotton was first introduced in 1996 (Wilson et al., 2013).

The RMP has been very successful. It was established 20 years ago and there has been no statistically significant increase in resistance levels to *Bt* toxins (Downes et al., 2016). This is despite Bollgard II forming over 90% of the cotton crop (Tabashnik et al., 2013).

However, despite the introduction Bollgard 3, the pressure on *H. armigera* and *H. punctigera* to develop *Bt* resistance remains high. Firstly, there is a relatively high level of existing resistance in *H. armigera* and *H. punctigera* to Cry2Ab and a high underlying

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baseline resistance for Vip3A (Downes et al., 2016). Secondly, the expression of Cry1Ac and Vip3A toxins in Bollgard 3 is not optimal as levels appear to drop towards the end of the season (reviewed in Downes et al., 2016). Thirdly, although resistance to Cry1Ac remains low in Australian pest populations, in other parts of the world resistance to Cry1Ac toxin by *H. armigera* is so prevalent that crops have succumbed to pest pressure (Tay et al., 2013). Finally, *Helicoverpa* spp can complete six generations in the tropics and four generations in temperate regions during the warmer months (Baker et al., 2016a) so multiple generations are exposed to *Bt* cotton each season. Because of the high threat of resistance, it is important to keep testing the efficacy of strategies used in the RMP.

The RMP strategies adopted in Australia for *Bt* cotton can be categorized into two groups: "Season Quarantining" and "Genetic Dilution". Season Quarantining is where the genetic contribution of moths emerging from *Bt* cotton is restricted between seasons. A Season Quarantining technique is "pupae busting" where cultivation destroys diapausing *H. armigera* and *H. punctigera* (hereafter referred to as *Helicoverpa*) pupae in the soil during winter (Fitt and Forrester, 1987; Wilson, 1987; Daly and Fitt, 1990; Murray and Zalucki, 1994). It is used only in temperate regions where the climate is cool enough for *Helicoverpa* to enter diapause.

Pupae busting was first used effectively in the late 1980s to limit the transfer between seasons of genes conveying resistance to chemical insecticides (Fitt and Daly, 1990). In the late 1980s, resistance by *H. armigera* to chemical insecticides increased during the season, so that at the end of the season, the diapausing *Helicoverpa* pupae were highly resistant. These pupae were then killed by cultivation, so that at the beginning of the following season, the level of resistant *H. armigera* in cotton was lower (Fitt and Daly, 1990). Where there was no pupae busting, the level of resistance was as high at the beginning of the following season as it had been at the end of the preceding season (Fitt and Forrester, 1987; Forrester et al., 1993; Murray and Zalucki, 1994).

Trap crops can be a Seasonal Quarantining technique. Trap crops are designed to lure pests away from the target crop by presenting them with a more attractive host that can then be destroyed, killing the pests (Hokkanen, 1991; Tillman and Mullinix, 2004; Ratnadass et al., 2014). As such, they are usually used in pest management to reduce the number of pests in the target crop. In warmer regions of Australia where *Helicoverpa* do not reliably diapause, a trap crop of pigeon pea (*Cajanus cajan* (L.)) is planted at the end of the cotton season to attract the last generation of moths produced in cotton (Maas, 2014). This lineage of *Helicoverpa* could have been exposed to *Bt* cotton over six consecutive generations. The aim is to destroy as many offspring of the 6th generation as possible to reduce the risk of this exposed lineage (that could be carrying resistance genes) contributing to the following season's *Helicoverpa* population.

The aim of Genetic Dilution, as defined here, is to reduce the build-up of resistance within a season. Genetic Dilution is likely to be most effective when resistance genes are recessive (as is the case in Australia for *Bt* toxin resistance (Tay et al., 2015). It occurs when there are enough homozygous susceptible moths to dilute the genetic contribution to the next generation of homozygous resistant moths (Roush et al., 1998). For example, if a homozygous resistant moth (rr) mates with a homozygous susceptible moth (SS), their heterozygous offspring (rS) will be phenotypically susceptible and killed by *Bt* cotton, thereby removing the resistant gene from the gene pool (if they oviposit in Bt cotton).

Sources of Genetic Dilution are moths from outside the *Bt* cotton region, moths from other crops or unmanaged vegetation grown within the region, and moths from refuges grown as part of the RMP. Refuges are designed to produce large numbers of *Helicoverpa* moths throughout the season that have not been exposed to *Bt* cotton. Because they have not been exposed to *Bt* cotton, their larvae should be susceptible to *Bt* toxins and carry fewer resistance genes than those emerging from *Bt* cotton, where ideally any susceptible larvae have been killed. The refuge moths then mate with any resistant moths surviving on *Bt* cotton, thereby diluting any resistance genes in the next generation (Roush et al., 1998). The aim of refuges is to mitigate the advantage of resistance genes by diluting them with recessive genes within the season; whereas the aim of pupae busting and trap crops is to reduce the proportion of resistance genes transferring between seasons.

The rationale behind the use of refuges is based on models which indicated that if 10% of the *Helicoverpa* population was not exposed to *Bt* toxins, then sufficient numbers of unselected *Helicoverpa* moths would be produced to delay the development of resistance by 20 generations (Roush et al., 1998). The models are based on a number of assumptions. The first is that both *H. armigera* and *H. punctigera* developing in refuges and *Bt* cotton suffer the same level of non-*Bt* mortality. Obviously it is impossible to measure non-*Bt* mortality directly because *Bt* toxins kill insects. Consequently, the second assumption is that crop attractiveness (as measured by the number of eggs laid in the crop) is proportional to productivity (moths), if the larvae were not killed by *Bt* toxins. Based on these assumptions, the key to a refuge's effectiveness is its attractiveness to ovipositing moths relative to that of the neighbouring *Bt* cotton.

When *Bt* cotton was initially introduced, it contained only one gene (Cry1Ac) and only 30% of the crop could be planted in *Bt* cotton. That made the remaining 70% of the crop non-*Bt* cotton (although non-*Bt* cotton may contain genes to make it resistant to the herbicide glyphosate, we will be referring to non-*Bt* cotton as "conventional cotton" to distinguish it clearly from *Bt* cotton). When Bollgard II was introduced, cotton refuges were mandated at 10% of the *Bt* cotton area. When pigeon pea was adopted as a refuge, the aim was to maintain this same level of exposure. Because pigeon pea was twice as attractive as cotton to ovipositing moths (as measured by egg counts; Jayaraj, 1982) and on average throughout a season produced twice as many moths (as measured using pupae as surrogates; Baker et al., 2008), pigeon pea refuges only needed to be half the size of cotton refuges (5% of the *Bt* cotton area for Bollgard II).

However, there is some evidence that during the past decade pigeon pea refuges may not have performed as well as expected (Wilson et al., 2013). This could be in part caused by the quality of pigeon pea seed used in Australian refuges, which has not been maintained because it has not been a commercial crop in Australia since the mid-1990s. Pigeon pea management may be also less than ideal because these refuges are grown in systems set up to optimise cotton production, and there is no direct economic return from growing pigeon pea. While pigeon pea refuges have recently been monitored for moth productivity of both species (e.g., Baker et al., 2008, 2016b; Baker and Tann, 2014; Baker et al., 2016b), there are no recent comparisons of the attractiveness of commercial pigeon pea refuges versus their associated *Bt* crops.

A major challenge for pigeon pea attractiveness is the timing of flowering. It is known that *Helicoverpa* moths prefer flowering plants for oviposition (Cunningham and Zalucki, 2014). Although cotton begins flowering after 800° days (Constable and Shaw, 1988), which in the Namoi is late December, pigeon pea flowering is triggered by shortening daylengths after the summer equinox (R. Rachaputi, pers com.) and tends to start flowering in January. This delay could affect its relative attractiveness to *Helicoverpa*.

In northern New South Wales, Australia, we test the assumption that *Bt* cotton is as attractive as conventional cotton, and that cotton is half as attractive as pigeon pea to *Helicoverpa*. In particular, our aim was to establish: 1) if pigeon pea refuges on commercial farms