

# SUSTAINABILITY OF TRANSGENIC INSECTICIDAL CULTIVARS: Integrating Pest Genetics and Ecology

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## ABSTRACT

This review examines potential impacts of transgenic cultivars on insect population dynamics and evolution. Experience with classically bred, insecticidal cultivars has demonstrated that a solid understanding of both the target insect's ecology and the cultivar's performance under varied field conditions will be essential for predicting area-wide effects of transgenic cultivars on pest and natural enemy dynamics. This experience has also demonstrated the evolutionary capacity of pests for adaptive response to insecticidal traits in crops. Biochemical and genetic studies of insect adaptation to the *Bacillus thuringiensis* (Bt) toxins expressed by currently marketed transgenic cultivars indicate a high risk for rapid adaptation if these cultivars are misused. Theoretical and practical issues involved in implementing strategies to delay pest adaptation to insecticidal cultivars are reviewed. Emphasis is placed on examining the "high dose"/refuge strategy that has become the goal of industry and regulatory authorities.

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## INTRODUCTION

In the summer of 1996, over 1.8 million acres of farmland were planted to genetically engineered cotton that expressed a gene derived from the bacterium *Bacillus thuringiensis* (Bt). This gene codes for production of a protein that is toxic to some of the most important lepidopteran pests of cotton. Genetically

engineered corn and potato cultivars with insecticidal Bt transgenes were also planted in 1996 but on a smaller scale. Transgenic cultivars that produce other insecticidal proteins are under development (12).

The Environmental Protection Agency's (EPA) actions that permitted commercial use of transgenic insecticidal cultivars (TICs) in 1996 was viewed by some environmentalists and scientists as premature (87, 90, 130). Their main concern was that we lacked information necessary to use TICs in ways that would avoid rapid genetic adaptation by target pests. They argued that in addition to losing utility of the transgenic crops, we would risk loss of the most successful microbial insecticide, Bt (134), which is currently the mainstay of many organic farmers (36, 70, 90). Advocates for commercialization held that it was necessary to plant TICs on large tracts of land to gain the information on pest ecology and genetics needed to develop approaches for thwarting pest adaptation (87). Additionally, they pointed to environmental and economic benefits that would be lost by not using TICs (87).

In the next five years, acreage planted to TICs in the United States is likely to increase dramatically (12, 70), and use of TICs is expected to spread throughout the developed and developing world (59, 105). The purpose of this review is to assess what we currently know about the potential impact of TICs on pest population ecology and the potential for delaying pest adaptation to TICs. Gaps exist in our knowledge of the genetics of pest adaptation to TICs, but our tools for predicting the impact of TICs on pest population dynamics appear to suffer from even more limitations. In this review, I make some recommendations for remedying this situation. The critical challenge is to optimize the benefits of TICs to pest management without selecting for rapid pest adaptation.

Although TICs are a new innovation, they also represent an extension of one form of classical host-plant resistance (HPR) that is often called antibiosis (84, 100). Because pest managers have had long-term experience with integrating antibiosis into cropping systems, I draw on the historical record of insect- and pathogen-resistant cultivars as a means of anticipating some challenges that may be encountered during commercialization of TICs.

Because all commercialized TICs are based on expression of toxins derived from Bt, and because a growing number of theoretical and empirical studies are focused on specific genetic and ecological interactions between target pests, natural enemies, and Bt, I use Bt-based TICs as a model for discussion.

## THE CLASSICAL BACKGROUND

In 1969, Luginbill & Knipling (77) published results of a population dynamics model of the wheat stem sawfly, *Cephus cinctus*, that examined the potential impact of a new resistant wheat cultivar on the population density of this sawfly.

By using survival and reproduction parameters derived from field observations of sawflies on susceptible and resistant wheat cultivars, the model predicted that 6 years after continuous planting of resistant wheat, the sawfly population would decline from 100,000 per acre to 98 per acre. The resulting pest population would be expected to infest only 1 out of 1000 stems, and it would be almost undetectable, even on susceptible cultivars. Luginbill & Knipling suggested that sawflies could be kept in check by rotating resistant and susceptible wheat, with 2 years of resistant wheat followed by 3 years of susceptible wheat.

The introduction of sawfly-resistant wheat did not proceed as smoothly as the model predicted (138). First of all, yields of the resistant varieties were often lower than those of other cultivars, so farmer acceptance was a problem. In addition, seed had to be purchased before sawfly densities could be predicted, so farmers always faced an uncertain payoff. Beyond that, the solid stem trait that was responsible for sawfly resistance was not completely expressed under all environmental conditions (138). Although there were some reports of areas where sawfly populations decreased dramatically after release of the resistant cultivar (77), it is difficult to determine from published data if the population declines were due to the cultivar or other environmental factors.

One important variable that was not incorporated into the Luginbill-Knipling model was density dependence in sawfly mortality caused by natural enemies. It is very possible that as the populations decreased following deployment of resistant wheat, there was a concomitant decrease in the impact of the sawfly's specialized parasites (94). This decrease in parasites would result in a new population density that could be orders of magnitude higher than anticipated by the Luginbill-Knipling model. Some lessons for the future that can be gleaned from experience with wheat stem sawfly are the need to (a) understand details of natural population regulation in the target pest, (b) understand field performance of TICs under varied environmental conditions, and (c) institute long-term rigorous monitoring of pest population dynamics before and after introduction of TICs so that impacts of TICs can be objectively assessed.

Another case in which area-wide pest densities were predicted to decrease based on intensive planting of an antibiotic cultivar was Hessian fly (*Mayetiola destructor*) in wheat. The best set of published data on the relationship between percentage of wheat with resistance to Hessian fly and the fly's population densities comes from Foster et al (35). They report the percentage of Hessian fly infestation of nonresistant wheat from 1956–1988, a period that includes 7 years before resistant cultivars began to dominate the Indiana landscape. A graphical analysis of their data indicates that the relationship between the percentage of resistant wheat planted and Hessian fly density is not direct (see Figure 1 at <<http://www.AnnualReviews.org>> in the Supplemental Materials Section).

Another result from the use of resistant wheat cultivars was the evolution of Hessian fly populations unaffected by one or more antibiotic traits. Hessian flies adapted to antibiosis genes H3, H5, and H6 within 15, 9, and 22 years, respectively, of commercialization in Indiana (35). However, examination of data on percentage of wheat planted with a specific antibiosis gene gives a different picture. If one counts only years when a specific antibiosis gene was in more than 50% of the wheat acreage, a conservative estimate of “time until fly adaptation” to the H3, H5, and H6 genes is 8, 7, and 3 years, respectively. (The relationship between intensity of planting cultivars with a Hessian fly antibiosis gene and the rate of pest adaptation is illustrated by Figure 1 at <<http://www.annurev.org>> in the Supplemental Materials Section.)

The history of Hessian fly adaptation to one antibiotic factor after another is certainly not unique. Breeding programs for crop resistance to greenbug (20, 140), alfalfa aphid (96, 120), and others (39, 101) constantly struggle to keep one step ahead of pest evolution. Plant pathologists, who have relied more extensively on crop cultivar resistance than entomologists, have also faced this problem. Many pathogen-resistant cultivars have been adapted to by the target fungus, virus, or bacterium in less than 5 years, but in some cases, intensively planted pathogen-resistant cultivars have maintained their resistance for over 50 years (66, 72, 131, 132).

Some plant pathologists feel that the durability of a resistant cultivar can be predicted based on factors such as the number of genes that control the resistance trait, the way the trait impacts the pathogen, and the ecological context in which the resistance factor originally evolved (112, 131, 132; also see 26, 110). Although these factors have some predictive power, notable exceptions exist (66).

Before we increase the use of TICs that produce Bt toxins, it is worth asking whether Bt toxins have any characteristics that might predispose them to unusually long or short durability. When Bt was introduced, some researchers felt that resistance would not be an issue (see 5, 122). Insects and Bt had coexisted for millions of years, and Bt had been used as an insecticide for many years. If insects could have adapted to Bt, they would have done so long ago. This assessment overlooks the fact that Bt is a soil-inhabiting microbe that is poorly understood from an ecological perspective (89). We do know that naturally infected insects are rarely found in the field and that many insects targeted for control with Bt toxins are unlikely to encounter Bt naturally during their active feeding stages (89). Furthermore, Bt sprays break down quickly, so large segments of treated pest populations are never exposed to the toxins. Perhaps the historical lack of Bt resistance is simply the result of limited exposure. When Bt toxins are expressed constitutively in commercialized TICs, pest exposure often

becomes much more common. The next section, therefore, addresses what we know about the genetic and physiological capacity of insects for adapting to Bt in the face of more intensive exposures.

## INSECT RESISTANCE TO Bt TOXINS

Most Bt strains produce a number of related toxins, each coded for by a single gene (75). Each toxin has a very specific target site within the insect (38). Both the classical host-plant resistance and pesticide resistance literature indicate that toxic factors that impinge only on a single, specific target site may offer less of an evolutionary barrier than toxins with multiple effects (39, 53, 97). Furthermore, since 1981, considerable research has demonstrated the potential of insects to adapt to Bt toxins. This research has been comprehensively reviewed (5, 38, 122). I present a brief overview of this research because an understanding of the physiology of insect adaptation to Bt toxins is of fundamental importance in designing sustainable approaches for deployment of Bt-based TICs.

There are at least five points in the Bt toxicity pathway where genetic change in an insect could decrease effects of Bt formulations: decreased solubilization of the Bt crystal, decreased cleavage of the full-length Bt protein into an active fragment, increased proteolytic digestion of the active fragment, decreased binding of the active fragment to the midgut epithelium, and decreased functional pore formation (5, 38). Each of these changes could be accomplished in more than one way.

Fewer targets exist for insect adaptation to TIC-produced Bt because within a TIC, the toxin is not expected to be in crystal form [P Lavrick (Monsanto, St. Louis), personal communication], and many of the Bt gene constructs in transformed plants directly produce the active toxin moiety (102). From an evolutionary perspective, understanding of these potential physiological changes allows us to judge the following: 1. whether adaptation is likely to occur based on a change in a single gene; 2. whether the adaptive trait in the insect is likely to have dominant, additive, or recessive inheritance; 3. whether adaptation that is caused by selection with one toxin is likely to result in cross-adaptation to other Bt toxins; and 4. whether the adaptive trait is likely to have an associated fitness cost.

Heckel (56) pointed out that because proteolysis of the toxic fragment involves a gain of function, it is less likely to be inherited recessively than a decrease in toxin binding to the midgut epithelium, which is likely to result from a loss of function. Heckel concluded that proteolytic detoxification is likely to result in cross-resistance to many Bt toxins if the protease cleaves

within a conserved region of the Bt-toxin molecule, whereas alteration in the midgut binding site would lead only to limited cross-resistance because many Bt toxins have different binding sites (135).

It is useful to combine our physiological understanding of potential adaptive mechanisms with empirical findings. The first documentation of resistance to Bt in a crop pest was reported by McGaughey in 1985 (86). The *Plodia interpunctella* strain that he examined had developed approximately 100-fold resistance to Bt strains that produced a number of toxins. This resistance was inherited in an almost completely recessive manner. Follow-up biochemical studies indicated that the resistant strain had decreased toxin binding (136). A recent study of resistant *P. interpunctella* larvae (99) produced evidence that, in addition to changes in binding, there may be a decrease in cleavage of the full-length Bt protein to its toxic form (92). This decrease could be due to a loss of protease function, so recessive inheritance is consistent with the potential physiological change.

Biochemical studies of a *Plutella xylostella* strain with >200-fold resistance to the Bt-toxin CryIAb demonstrated that there was decreased toxin binding to the midgut epithelial cells (27) and that lack of binding was a recessive trait (82). [Studies with other strains of *P. xylostella* produced conflicting results regarding changes in toxin binding (25, 83).] Biochemical analysis of a *Heliothis virescens* strain with over 1000-fold resistance to CryIAC and cross-resistance to some other CryI toxins revealed a decrease in toxin binding to one of the three membrane-bound proteins to which CryIAC binds (73). Resistance in this strain is partially recessive and is controlled mostly by a single locus [or a set of tightly linked loci (50, 57)].

A number of other resistant strains display no significant decrease in toxin binding. For example, two other strains of *H. virescens* that were 16- and 50-fold resistant to the Bt toxin CryIAC had no biologically meaningful changes in specific binding (52, 78). One of these strains was broadly cross-resistant to other Bt toxins, and resistance was inherited as a nearly additive trait (52).

## INITIAL FREQUENCY OF TOXIN-RESISTANCE GENES

Simple population genetics theory predicts a close relationship between the initial frequency of toxin-resistance alleles and the time it takes for those alleles to predominate in a pest population. Any empirical estimate of this initial frequency would therefore help in resistance-risk assessment. Many attempts to select insects for resistance to single Bt toxins have resulted in strains with >100-fold resistance (5, 122), but there have been cases of limited or no response to selection (5, 122). Given the small number of insects that are used to start a selection experiment (<100–1000), the initial frequency of resistance (R)

alleles must be at least 0.005–0.0005 in order to be found in these small samples (50, 122). In some cases, the insects used in selection have already developed low levels of resistance in the field (86, 126) or have at least been exposed to effective Bt applications (139), so the allele frequencies for resistance may have been higher than in a completely unexposed population. Nevertheless, these experiments indicate that the initial frequency is likely to be higher than the conventional expectation of  $10^{-6}$ .

Recent work by Tabashnik et al (127) determined that one of their control laboratory colonies of *P. xylostella* had a resistance frequency of 0.12. This is a startling finding with dire implications for resistance management, so it is tempting to find ways to dismiss these data. However, the 0.12 frequency may not be unrealistic if the resistance alleles are held in a polymorphic state based on a primary physiological function unrelated to resistance. In support of this possibility is an earlier study of a *P. xylostella* strain from the Philippines, which was resistant to CryIAb but not resistant to the Bt formulation, Dipel<sup>®</sup>, used to control it in the field (27). This strain may have been derived from a biotype or subspecies of *P. xylostella* that had a different common allele for the midgut protein to which CryIAb binds.

Another recent study directly estimated the frequency of a major Bt-resistance allele in field populations of *H. virescens* (48). Field-collected *H. virescens* males were mated to females of a Bt-resistant laboratory colony, and F<sub>1</sub> and F<sub>2</sub> offspring were then examined for Mendelian segregation of resistance alleles from the field. The initial frequency of resistance alleles in the field-collected males was estimated to be  $1.5 \times 10^{-3}$ , much lower than for the *P. xylostella* lab colony but much higher than the conventional  $10^{-6}$ . Alstad & Andow (2) recently suggested an efficient sib-mating method for estimating initial allelic frequencies in field populations that does not require use of a resistant laboratory strain.

## RESISTANCE MANAGEMENT FOR VULNERABLE BT GENES

Based on assessment of insect traits that could result in resistance and on empirical findings, Bt toxins appear vulnerable to pest adaptation. In the following section, I briefly review a number of approaches that have been proposed for husbanding Bt genes in TICs, and then I focus in more depth on the “high-dose”/refuge strategy that is the current goal of industry (30, 31) and regulatory authorities (24). (More detailed reviews of other strategies can be found in References 1, 42, 43, 46, 49, 88, 109, 115–117, 122.)

In 1988, four basic strategies were outlined that could be used to delay insect adaptation to TICs (42).

1. Mixtures of toxic and non-toxic cultivars (refuge approach);
2. Stacking of two or more toxins in each TIC plant within a mixture;
3. Low doses of toxins that act in concert with natural enemies to decrease pest populations;
4. Tissue-, time-, or signal-dependent expression of toxins.

Absent from this list was the high-dose approach, which was not even considered until 1991 when Monsanto (St. Louis, MO) scientists demonstrated that they could produce plants with toxin titers that were much higher than that needed to kill 100% of susceptible genotypes of target insects (103). Subsequently, attention of industry, universities, and regulatory authorities has focused primarily on the use of high-dose TICs grown in proximity to non-toxic cultivars. This narrowing of focus to the high-dose approach was in part due to (a) potential technological feasibility, (b) practicality from a marketing and farming perspective, and (c) the promise of greatly prolonging the useful life of Bt toxins. As discussed below, the technological feasibility of the high-dose approach remains uncertain.

### *Mixtures of Toxic and Non-Toxic Cultivars*

The perspective that spatial mixing of TICs with non-toxic cultivars could increase sustainability of the TICs came from a number of disciplines. Scientists working with conventional insecticides advocated this approach in terms of a “refuge” population of insects in non-sprayed areas (14, 15, 19, 114, 124, 128). Plant pathologists and entomologists working with conventional host-plant resistance had also investigated this approach (42, 43, 72, 74). The basic goals of the mixture strategy are twofold: 1. reduce the difference in fitness between susceptible and resistant insects, and 2. reduce the degree to which a resistant insect can pass on its phenotypic trait to its offspring (46).

Some simple mathematical calculations are useful in illustrating how mixtures reduce the fitness differential between insect genotypes. For simplicity, and based on empirical findings discussed above, I assume that resistance can be a single gene trait. The allele R codes for resistance, and allele S codes for susceptibility. On a toxic plant, individuals that are homozygous for the resistance trait (RR) are assumed to be unaffected by the toxin and have a fitness of 1.0. The initial R allelic frequency,  $q$ , is assumed to be 0.001. Assuming random mating, the expected frequency of the RR genotype is  $q^2$  or  $10^{-6}$ . (Because of their low frequency, no matter what their fitness, RR individuals generally do not have a significant impact in the crucial early stages of resistance development.) The homozygous-susceptible individuals (SS) have a fitness of  $1.0-t$ ,

where  $t$  is related to the toxicity of a specified TIC and could vary from 0 to 1. Heterozygotes have a fitness of  $1 - [(1-h)t]$ , where  $h$  reflects the dominance of the resistance trait. If the resistance trait is completely dominant,  $h = 1.0$  and RS heterozygotes have a fitness of 1.0. If the resistance trait is completely recessive,  $h = 0.0$  and heterozygotes have a fitness of  $1 - t$ .

If  $t = 0.90$  and  $h = 0.5$  (additive inheritance), then fitness of RR, RS, and SS genotypes feeding on the TIC is 1.0, 0.55, and 0.10, respectively. On non-toxic plants, all genotypes are assumed, for simplicity, to have a fitness of 1.0. In a 1:1 mixture of toxic and non-toxic plants, fitness of the RR genotype remains 1.0. The RS genotype fitness becomes  $(0.5)(0.55) + (0.5)(1.0) = 0.775$ , and fitness of the SS genotype becomes  $(0.5)(0.10) + (0.5)(1.0) = 0.55$ . In a pure TIC stand, the RS genotype is  $0.55/0.10 = 5.5$  times more fit than the SS genotype, whereas in mixed plantings it is only  $0.775/0.55 = 1.4$  times more fit. This decrease in the fitness differential between RS and SS due to the mixture has a major impact on evolution. In this specific case, evolution of resistance is about four times slower in the mixture until the frequency of R reaches 0.10. As the frequency of R becomes higher than 0.10, a significant portion of homozygous RR individuals are produced. Because these individuals are 10- and 1.8-fold more fit than SS individuals in the pure stand and mixture, respectively, they increase the rate of evolution as they become common.

If the resistance trait is partially recessive as in many of the empirical studies above, the refuge has more of an effect. If  $h$  is 0.1, then fitness of the RS genotype is  $1 - [(1-0.1)(0.9)] = 0.19$ . The RS/SS fitness ratio in pure toxic stands would be  $0.19/0.10 = 1.9$ , and in the mixture it would be  $0.595/0.55 = 1.08$ . Starting with an initial R frequency of 0.001, a frequency of 0.1 is reached after 8 generations in the pure stand and after 54 generations in the mixture. Again, the decrease in fitness differential between RS and SS individuals explains the impact of mixtures on the rate of evolution. (Note that for multivoltine insects there may be 4 or more generations per year.)

If resistance is completely recessive ( $h = 0$ ), there is never any difference between RS and SS genotypes, so resistance is driven only by the initially rare RR genotypes. In this case, it takes 116 generations for R frequency to increase from 0.001 to 0.10 in a pure stand and over 1000 generations in the mixture.

A 50% refuge is higher than considered practical by most farmers. If a 20% refuge is used and the R trait is partially recessive ( $h = 0.10$ ), the RS/SS fitness ratio in the mixture increases to  $0.352/0.28 = 1.26$  and the R frequency reaches 0.1 in about 20 generations instead of 54. If the 4% refuge imposed by EPA for cotton is used, the 0.10 frequency is reached in only 10 generations.

For an insect with a sessile feeding stage, a 1:1 seed mixture of a TIC and a non-insecticidal nearly isogenic cultivar is expected to translate to a 50% refuge. However, in many cases it is not simple to translate from the ratio of

TICs versus non-insecticidal cultivars (on a seed, plant, or acreage basis) to an effective refuge percentage. If we assume recessive inheritance, randomized mating, and tightly linked population dynamics of insects that develop on toxic and non-toxic plants, the effective size of a refuge can be computed as follows:

$$\text{Refuge \%} = \frac{A}{(A + B/q^2)} \times 100\%,$$

where  $A$  is the number of susceptible adult insects produced in the refuge in a specified generation,  $B$  is the number of RR-resistant insects produced in the TIC, and  $q$  is the frequency of resistance alleles. ( $B/q^2$  is comparable to the number of insects that would be produced in the TIC if all the insects were unaffected by the toxin, and there was no density-dependent survival.)

For a simple case, assume that seed of a cultivar identical to the TIC, but lacking the toxin, is mixed with TIC seed in a 1:4 ratio; the feeding stage is sessile; there is no density-dependent mortality;  $q = 0.001$ ; and  $A$  insects are produced on non-toxic plants. There are four times as many TIC plants as non-toxic plants, but only  $q^2$  of the insects are entirely resistant, so  $B/q^2 = (4A) \times (0.001^2)$ . The equation reduces to  $(A/5A) \times 100 = 20\%$ , which directly reflects the percentage of non-toxic seed.

A somewhat more complex situation occurs if the 20% non-toxic seed is planted in a separate field that is subsequently sprayed with an insecticide that kills 90% of the pests. In this case, we would multiply the original  $A$  by 0.10, but  $B$  would remain unchanged, so the refuge would decrease from 20 to  $\sim 2.4\%$ . If cultivars or wild plants that constitute the refuge are 50% as suitable for SS insects as the TIC was for RR insects, the effective refuge size would decrease from 20 to 11.1%.

While  $q$  is small, pest density in the refuge is expected to be much higher than in the TICs. If density-dependent population dynamics are present, 50% higher mortality of larvae could occur on refuge plants and effective refuge size would again become 11.1%. Alstad & Andow (1) and Ives (61) pointed out that if the refuge fields were planted in a manner that made them less attractive to ovipositing females, the effective refuge size could decrease significantly.

Assessment of refuge size described above assumes enough movement between the refuge and TIC fields to couple the population dynamics in both areas. If movement is only 1–10% per generation, the refuge may develop independent population dynamics, which makes assessment of refuge size much more complex (11, 41, 101a). In some cases, for example, refuges may be much more effective if the same fields are planted with the susceptible cultivar every year than when farmers rotate fields used as refuges (41).

### *Stacking of Two or More Toxins in Each TIC*

As with the mixture strategy, the idea of inserting genes for production of two or more biochemically distinct toxins in each TIC has its origin both in classical plant breeding literature (17, 37, 40, 41, 95) and in insecticide resistance management literature (10, 16, 18, 81, 114, 121). The basis for this strategy is sometimes referred to as “redundant killing” because insects adapted to one toxin still die because of the second toxin, and a totally susceptible insect “dies twice.” If two toxins in a TIC are so distinct that there is little potential for cross-resistance, then it would at least require resistance alleles in the insect at two independent gene loci ( $R$  and  $R'$ ) to permit high survival on the TIC. In a diploid insect, this results in nine potential genotypes, and only one of these,  $RR R'R'$ , is highly fit on a two-toxin plant unless the inheritance of each trait is additive to dominant. These double homozygotes are expected to be very rare when the TIC is initially commercialized. If the  $R$  and the  $R'$  alleles have an initial frequency of 0.001, then the frequency of double homozygotes is  $0.001^4 = 10^{-12}$ . No matter what the fitness of these  $RR R'R'$  genotypes, they will contribute little to the evolution of adaptation in early phases of selection because they are so rare, if they exist at all. Therefore, as with the single toxin case above, any type of planting that minimizes the differential in fitness between the more common genotypes will slow resistance.

A number of models have examined this two-toxin-per-plant strategy (17, 40, 41, 44, 116, 117). Gould (44) examined a case where  $t$  for each toxin was 0.9 and initial  $R$  and  $R'$  frequencies were both 0.01. Only 7 generations were necessary for resistance to evolve in pure stands if  $h$  was approximately 0.01 and the interaction between toxicity of the two toxins was multiplicative. If a 30% refuge was added to this system, resistance evolved in 123 generations. When inheritance of resistance to each toxin was additive ( $h = 0.5$ ) and the interaction between toxins was also additive, the result changed dramatically. Even with a 30% refuge, resistance could evolve in 14 generations.

Roush (117) modeled a two-toxin system where  $t = 1.0$ ,  $h = 0.30$ , and initial  $R$  and  $R'$  frequency was 0.001. With a 10% refuge, more than 50 generations were necessary for adaptation to a TIC with two toxins, but only about 5 generations were necessary for adaptation to a TIC with one toxin.

### *Low Doses of Toxins that Act in Concert with Natural Enemies to Decrease Pest Population Density*

A well-developed literature in entomology demonstrates that “partial resistance” in crops that causes a low level of pest mortality but also alters behavior and slows growth of immatures can enhance the effectiveness of natural enemies (6, 8). van Emden (133) was the first to develop a theoretical framework

demonstrating that in cases where neither biocontrol nor partial host-plant resistance alone would control a pest, their combined action could offer success. The potential for this general result has been demonstrated in greenhouse and field plot tests for both classically bred cultivars (6, 8, 55) and for TICs with low levels of toxin expression (63).

A genetic model examining the low-dose approach found that the interaction between the pest and its natural enemies could either decrease, increase, or have no effect on the rate of pest adaptation (51). The outcome depended on details of the ecological interaction between the pest and its natural enemies. In some cases, the interaction was expected to increase the selective differential between RS and SS genotypes and in other cases to decrease the difference. Recent experimental work in field plots (62) and in the laboratory (64, 76) found both results, and in one selection experiment where the selective differential was increased by a natural enemy, more rapid adaptation to a TIC occurred in three of four experimental trials (65). Clearly, effects of this strategy on the rate of pest adaptation must be more closely examined.

### *Tissue-, Time-, or Signal-Dependent Expression of Toxins*

The degree of yield reduction caused by a pest population is dependent on its density, as well as on when and where on the plant insects feed. Expression of toxin-coding genes could be limited to vulnerable plant parts, and times when toxicity is most needed (43, 109, 116, 117). If a pest causes no damage when it feeds on mature leaves but causes severe stunting when it feeds on buds and developing leaves, then toxin production only in buds would be useful. As the leaves expand, toxin titer could rapidly decrease below levels that affect the pest. Such a strategy could be problematic if the toxin level in bud tissue started out well above the 99% lethal dose ( $LD_{99}$ ) of the pest and decreased slowly as leaves expanded because periods would occur when leaves had a moderate dose (98, 116). Even with the problem of titer decline, this strategy can have an advantage, especially if vulnerable tissues are present in only one of the seasonal generations of a multivoltine insect (e.g. corn silks or cotton bolls).

For multivoltine insects, TICs that decrease reproductive output of an early season generation of a pest may face lower densities of later pest generations, even if plants are not toxic later in the season (67).

## THE HIGH-DOSE APPROACH

The literature on insecticide resistance management has often discussed the theoretical potential for spraying crops with extremely high doses of one or more insecticides (15, 19, 114, 124, 128). As shown in the section on mixtures,

the smaller the selective differential between SS and RS individuals, the slower the rate of evolution. When an insecticide spray kills 95% of SS individuals, the survival of RS individuals is likely to be significantly higher unless the alleles governing resistance happen to be phenotypically recessive (i.e. the RS and SS insects are physiologically identical). Instead of hoping that resistance is phenotypically recessive, the high-dose approach attempts to make resistance alleles “effectively recessive” even if they are not phenotypically recessive (see Figure 2 at <<http://www.AnnualReviews.org>> in the Supplemental Materials Section).

As the dose of insecticide increases above the LD<sub>99</sub> of susceptible insects, it can greatly reduce the fitness of RS individuals even if resistance is inherited as a phenotypically additive trait (e.g. 107, 128). Taylor & Georghiou’s (128) results show that if the LD<sub>95</sub> for the SS individuals is 0.001 ppm of toxin and the LD<sub>95</sub> of the RS individuals is 0.09 ppm, no toxin-induced mortality of RS individuals is likely to occur when there is 99% mortality of SS individuals (see Figure 2 at <<http://www.AnnualReviews.org>> in the Supplemental Materials Section). If the dose of insecticide is increased to 0.09 ppm, 95% mortality of RS individuals occurs and mortality of SS should approach 100%. If the dose is 0.09 ppm and there is a 10% refuge, the ratio of overall survival of the two genotypes becomes  $0.1045/0.100 = 1.045$ , so the initial rate of adaptation is very slow. If an extremely high dose causes nearly 100% mortality of both SS and RS genotypes, then the only driving evolutionary force is in the rare RR individuals (as described above for phenotypically recessive traits). As long as the R allelic frequency is low, most individuals are SS, so rare RR individuals and RS individuals are expected to mate mostly with SS individuals and produce effectively susceptible RS individuals.

Three important practical problems specific to insecticides have made commercial use of this idea unrealistic: 1. Increasing the dose of insecticide is expensive; 2. use of high doses could have negative effects on natural enemies and other non-target organisms; and 3. as the insecticide residue decays, part of the insect population is likely to be exposed to a dose that has more of an effect on SS than on RS individuals for phenotypically non-recessive traits (114, 125, 128).

If a toxin could be consistently produced by a plant at a highly toxic concentration without having a negative effect on yield, and the toxin did not affect non-target organisms, these constraints on the high-dose strategy would be lifted. Until 1991, this condition did not seem feasible because molecular biologists were unable to express Bt toxins at high concentrations. In 1991, Perlak et al (103) demonstrated that specific alterations could be made in the DNA sequence of Bt toxin genes, which resulted in dramatic increases in toxin production. This discovery opened the exciting possibility of generating TICs

with toxin titers more than 20 times higher than those needed for killing 99% of SS individuals. Such concentrations of toxin would be expected to kill almost all RS individuals even if resistance was not phenotypically recessive. As an example, Metz et al (91) found that Bt-expressing broccoli plants that caused about 10% mortality of *P. xylostella* neonates from a resistant strain caused 100% mortality of a susceptible strain and an F<sub>1</sub> hybrid.

If a very high dose is used in combination with a refuge, resistance could evolve rapidly only if it was governed by a single dominant allele. With Bt, only one published case exists of completely dominant resistance [25-fold resistance to CryIIA (50), so there would still be significant mortality on high-dose plants].

If two unrelated toxins are produced at very high concentrations, resistance is expected to develop slowly even if refuges are small and initial allelic frequencies are high. Assuming that partially recessive alleles code for resistance to toxins A and B at an initial frequency of 0.05, a deterministic model predicted that adaptation to double toxin plants would take 221 generations if there was a 10% refuge (47). If the fitness cost to resistance alleles is 5% when the pest is feeding in the refuge, this model predicted that resistance might never evolve (47). A stochastic model of the same situation is currently being developed (S Peck, F Gould, S Ellner, unpublished data).

## MAKING THE HIGH-DOSE/REFUGE STRATEGY WORK

The high-dose strategy with one or two toxins is extremely attractive from a simple theoretical perspective, but in the real world a number of factors could interfere with its success. Mallet & Porter (80) pointed out that if the spatial scale at which TIC plants and normal plants were mixed was so small that the pest's feeding stages could move between plants (e.g. seed mixtures), these feeding stages would consume varying proportions of toxic and non-toxic food. Instead of ingesting a high dose of toxin or no toxin at all, which is the basis of the high-dose strategy, these insects would often consume intermediate doses. Effective recessiveness of the resistance trait that is crucial to success of the high-dose approach is expected to decrease in this situation unless the resistance trait is phenotypically recessive. Even in "pure" stands of TICs, polyphagous pests may move between TICs and weedy hosts within the field, which could interfere with the high-dose strategy (RL Hellmich, personal communication). Seed producers generally expect to have up to 3% off-types in their commercially sold seed. Because these off-types do not produce toxin and larvae move between plants, the high-dose strategy could be compromised.

In contrast, if the spatial scale of mixing is very large relative to adult movement of a target pest species (e.g. farm to farm), most RR insects from toxic areas might not move far enough to find SS individuals with which to mate (and

vice versa). Because a key to success of the high-dose strategy is having RR individuals that mate almost exclusively with common SS individuals, such non-random mating would compromise the utility of this strategy (11, 41, 45, 116).

Another factor that could interfere with random mating is temporal asynchrony between RR and SS individuals. The RR individuals that feed on high-dose TICs may survive, but they may develop more slowly than SS individuals that were feeding on totally non-toxic cultivars. This differential in development time could result in most SS individuals completing mating and oviposition before RR adults emerged (45, 68, 123). Even if RR individuals were receptive for mating when SS individuals were present, they might be smaller or in other ways less attractive to SS individuals (54). It has been shown that nutritionally stressed larvae of *H. virescens* become small adults, but these adults appear to be successful at mating with larger adults (68).

All of the above factors must be considered in developing a workable high-dose approach. These concerns are leading to new research initiatives aimed at understanding more about movement and mating behavior of a few major target pests, but to date our understanding is inadequate. *H. virescens* and *Helicoverpa zea* larvae are known to move between plants, so seed mixtures might not work. However, in choice situations, *H. virescens* feeds mostly on diets that lack Bt toxins (49), so this problem could be moderated.

First-instar European corn borers (*Ostrinia nubilalis*) normally disperse among plants (113) and spin down and away from TICs after “tasting” plant material (F Gould et al, unpublished data; P Davis, RT Roush, unpublished data). However, this initial contact causes a decrease in larval fitness and, therefore, argues against seed mixtures of high-dose TICs (45, 117). The Colorado potato beetle, *Leptinotarsa decemlineata*, feeds on foliage as a larva and as an adult. Work with seed piece mixtures suggests that beetles move between plants and receive an intermediate dose of toxin (116). The situation may be different for another target of TICs, *Pectinophora gossypiella*, whose larvae generally remain on fruiting plants where they hatch (9).

In terms of adult movement, *H. virescens* males and females can move >7.5 km in the spring (118). Of males that moved away from a release area, about half as many moved ~5 km as the number that moved ~2 km (118). Recent work indicates that these moths may move less in summer generations (M Caprio, unpublished data). *O. nubilalis* has been observed moving to the periphery of corn fields before mating (119) and can move between large farms in a single generation (13), but this may not always be the case (21). *P. gossypiella* and *L. decemlineata* can fly long distances (22, 129) but are not expected to do so when suitable crops are available (33). When farmers rotate potato fields, *L. decemlineata* adults must move between fields in the spring. Unfortunately, *L. decemlineata* adults mate before they overwinter, so even though females may move to new fields and can mate before laying eggs in the spring, some of

those eggs will have been fertilized by males from the old field (7, 29). Even if 10% of the eggs are fertilized by prewinter matings, the high-dose strategy could be significantly compromised. In most cases, data on insect movement give distances moved over the entire adult life span or until egg-laying begins (108). For determining optimal spatial scales of high-dose mixtures, we need data on how far adults move before they mate. As indicated by the work on *L. decemlineata*, these distances could be quite small.

The limited data we have for *H. virescens*, *H. zea*, and *O. nubilalis* on plant-to-plant movement by larvae, and field-to-field movement by adults, suggest that field-to-field mixtures of high-dose TICs and normal cultivars might be a reasonable spatial scale. This scale should generally result in larvae either getting a very high dose of toxin or no toxin. It should also permit mating between SS individuals from non-toxic fields and rare RR individuals emerging in high-dose TIC fields, although we cannot assume that there would be completely random mating. For *P. gossypiella*, a within-field refuge (e.g. seed mixture or row by row mixing) would be best because of limited larval and adult movement. For *L. decemlineata*, neither a random seed piece mixture nor a field-to-field mixture can reconcile the limited among-field premating adult movement and the within-field movement of feeding stages. Within-field refuges of small patches may be the only potential solution. More detailed field studies on each of these target pests are needed before the optimal spatial scale for a mixture can be determined. If two target insects such as *H. virescens* and *P. gossypiella* infest the same crop, the optimal refuge spatial scale must consider the biology of both species.

Another major problem for the high-dose approach arises when crops have multiple pests and/or when pests are polyphagous. For example, *H. zea* may be a target pest on crop #1, so that crop is engineered to produce a high dose of a toxin that is very effective against *H. zea*. If *H. zea* feeds on crop #2 but is not a major pest, crop #2 may be engineered to express a high dose of a related toxin that is appropriate for a more important pest of crop #2 but is not very toxic to *H. zea*. This would expose *H. zea* to an intermediate dose of toxin and would interfere with the utility of the high dose in crop #1 if there was cross-resistance to the related toxins (45, 117).

## THE IMPORTANCE OF POPULATION DYNAMICS

As indicated in the section on classical plant breeding, one result of area-wide planting of cultivars with antibiotic traits could be a decrease in target pest densities, but our ability to orchestrate such an outcome has been limited. Most recent research related to commercialization of TICs is concerned with resistance

management. Less attention has been focused on population dynamics of target pests (but see 1, 28, 41, 58, 61, 116, 117). For example, recent resistance management models typically measure the time it takes for resistance to develop in terms of when the population reaches a specific frequency of resistance alleles (e.g. 41, 117). This measurement tells us when resistance is becoming common within a pest population, but it does not necessarily tell us when the resistant population begins to cause damage. By the time a population adapts to a high-dose approach without a refuge, that population may have suffered 99.9% toxin-related mortality for 10 generations. Even if the resistance allele frequency was 1.0 after these 10 generations, the density of resistant individuals might be only one per acre.

How long would it take for this population of resistant individuals to build up to damaging densities? We know that when overuse of broad-spectrum insecticides leads to resistance, the resistant individuals often have high replacement rates because natural enemy populations are generally decimated by the same insecticides (85, 125). Because Bt-based TICs do not directly affect most natural enemies, we do not know what kind of replacement rates to expect for resistant pest genotypes. A drastic drop in the pest's density due to TICs could cause local extinction of species-specific parasitoids and pathogens, so any resistant pests could be free of their suppressive effect. However, if a pest population is mostly held in check by generalist natural enemies or other factors that are weakly density dependent or entirely density independent, recovery from the heavy toll taken by high-dose TICs may be slow.

In addition to altering the rate of return of a resistant pest population to damaging densities, natural enemies can affect the effective size of the refuge population and thus impact the rate at which resistance develops (45). Arpaia et al (3) conducted laboratory and field experiments to test this possibility with *L. decemlineata* and a major egg predator, *Coleomegilla maculata*. In this case, the percentage of eggs killed was inversely density dependent, and the predator had the impact of multiplying A in the equation for computing refuge size by 2.5 (see "Mixtures of Toxic and Non-Toxic Cultivars" above). They predicted that in a 1:1 Bt:non-Bt plot-by-plot mixture, adding this natural enemy could decrease the rate of resistance evolution 2.27-fold.

## THE STATUS OF CURRENTLY COMMERCIALIZED TICs

The EPA and some large private companies have committed themselves to sustainable use of Bt-based TICs (24). They have stated clearly that the high-dose/refuge approach is their goal (30, 31). It is, therefore, useful to examine current

TIC resistance management practices and performance of the few currently commercialized TICs.

Tests of cotton TICs that produce CryIAC indicate that it induces 100% mortality of *H. virescens*, and laboratory tests with heterozygous resistant larvae indicate that 90% die within 8 days (F Gould, L Carter, L Stelman, unpublished data). A fair conclusion is that a high dose has been achieved. These same cotton TICs only cause 75–90% mortality of susceptible *H. zea* larvae, relative to cotton cultivars without the toxin (71, 79). A high dose has not been achieved in this case. Data on *P. gossypiella* (23, 32) indicate that the Bt-toxin level in commercial cotton cultivars acts as a high dose for this insect. Information from the 1997 season in Australia indicates that Bt-expressing cotton planted there causes far less than 90% mortality of *Helicoverpa armigera* and *Helicoverpa punctigera* in some fields (34).

Two genetically distinct corn TICs produce CryIAB. One type has the CryIAB gene linked to a constitutive promoter (99a). TICs derived from this type of engineered gene appear to produce toxin in all plant parts all season long and always seem to cause more than 99% mortality of *O. nubilalis* larvae (99a). In contrast, *H. zea* larvae feeding on ears of these corn TICs typically experience less than 90% mortality, but development of the survivors is slower than on non-toxic corn (F Gould, unpublished data; G Dively, personal communication). The second type of engineered corn (69) produces high levels of toxin in green leaf tissue, but toxin is produced at low levels in some plant parts such as silks, kernels, and stalks. These plants seem to offer a high dose early in the season, but later in the season mortality is ~75% (99a). Because levels of toxin in silks or kernels are so low, *H. zea* is not significantly affected by this corn after silking begins (F Gould, unpublished data). Later stages of neither corn TIC provide a high dose for the southwestern corn borer (99a).

Potato TICs express CryIIIA toxin at levels that are at least 50-fold higher than the LC<sub>99</sub> of susceptible *L. decemlineata* neonates (104). These plants cause adults to cease feeding and reproduction. Second instars of a *L. decemlineata* strain with over 1000-fold resistance to CryIIIA cannot grow on leaf material from these plants (137).

Although the goal of the private sector was to develop TICs with high-dose expression (30, 31), this goal has been achieved for only some pest/TIC interfaces. The EPA's original requirement for a 4% effective refuge was based on results of models that predicted that a 4% refuge could significantly hamper evolution of resistance when there was a high dose and there was random mating. These same basic models indicate that when toxin titers only cause moderate mortality of susceptible insects (80–95%), refuges as small as 4% will have little impact on resistance (117; see "Mixture of Toxic and Non-Toxic Cultivars"

above). If we are to use TICs with moderate expression in a sustainable manner, refuge size must be significantly increased. With some generalists such as *H. zea*, wild hosts and other crops could serve as part of a larger refuge, but we lack data on the contribution of these hosts to overall pest population size in different geographic areas.

Although refuge size with moderate dose TICs must be larger, some of the spatial requirements for these refuges are not as strict as they are for high-dose TICs. Because advantages of high-dose TICs are so tightly tied to maintaining effective recessiveness, almost all RR individuals must mate with SS individuals and very limited larval dispersal can occur between TIC plants and non-toxic plants. With moderate dose TICs, larval movement is not expected to reduce recessiveness significantly, so seed mixtures are not problematic. Unless resistance is phenotypically recessive, the need for mating of RR and SS individuals is also relaxed.

In 1994, Roush (115) argued that using Bt in plants could hamper resistance more than intensive use of Bt sprays. Roush's argument was based on the presumption that plants have high-dose expression. Moderate-dose plants will certainly need to be deployed in a very judicious manner to outlast the alternative of Bt sprays.

## CAN EFFECTIVE REFUGE SIZE BE INCREASED?

In public discussions of refuges, it is often pointed out that farmers will not accept more than 4–10% non-toxic seed because yield reduction will be too great. Although this may be true in the first year or two after wide-scale planting of TICs, it is not clear that a refuge will incur high pest damage in subsequent years. If the predictions of Painter (100) and more recent authors (1, 45, 77, 116, 117) are correct, wide-area deployment of TICs could depress overall populations of target pests to the point that refuges incur little damage.

Luginbill & Knipling (77) and Roush (116) found that without density-dependent mortality, some TIC deployment strategies could cause local pest extinctions. Extinction would be much less likely in systems that include specialized natural enemies that act in a density-dependent fashion, but pest densities might still be suppressed to below economic thresholds (2, 51). In systems where use of broad-spectrum insecticides has suppressed natural enemy populations, decreased use of insecticides expected to accompany the introduction of TICs could increase the effectiveness of biocontrol and result in lower pest densities (58). If appropriate use of TICs does decrease the damage potential of target pests, it will be economically reasonable for farmers to plant substantial portions of their land to conventional cultivars, unless the price of TICs

[or the price of individual traits within transgenic cultivars with multiple traits (93, 106)] decreases dramatically.

The best way to predict the impact of TICs on target pest population dynamics involves field experiments that monitor pest densities over time in response to various refuge sizes and natural enemy complexes. One such experiment (111) examined how a 16.7 and 50% refuge affected population dynamics of *P. xylostella*. Replicated 0.09-hectare field plots were set up in which 0, 50, or 83.3% of the collard hosts were toxic to *P. xylostella*. Population dynamics of *P. xylostella* and its natural enemies were monitored for over two generations. In completely non-toxic plots, *P. xylostella* densities continued to rise throughout the 1994 season. In plots with 50% toxic plants, *P. xylostella* densities generally leveled off after about 8 weeks. When 83.3% of the plants were toxic, *P. xylostella* densities increased initially, but populations crashed by the end of the season. Interestingly, parasitism rates and spider densities did not appear to be affected by the treatments.

This experiment followed arthropod dynamics for only one season, but if the impact of the 16.7% refuge continued in the next season, *P. xylostella* would not have caused significant damage to non-toxic plants. If results in this system can be extrapolated to other pests, the economically feasible refuge size may be larger than currently envisioned. Longer term experiments of this type with target pests of TICs could help to determine the optimal percentage and the spatial scale of refuges that would maximize impacts of natural enemies in decreasing pest population density.

Long-term experiments of this kind may not be possible with most target pests, but if we maintain good records of pest and natural enemy population densities in a number of regions before and after introduction of TICs, we may be able to gather similar information on impacts of varied percentages and spatial scales of refuges. This information will be important because much of what we learn about the impact of Bt-based TICs on pest populations will be applicable to future TICs that are based on other toxins.

## GENERAL CONCLUSIONS

Population genetic models indicate that unless TICs produce very high doses of toxin(s) relative to the target pest's  $LD_{99}$ , and are planted in a manner that allows high levels of mating among pest genotypes, while not permitting movement of feeding stages between toxic and non-toxic plants, effective refuge size needs to be much larger than the current standard of 4%.

Population dynamics theory and experience with classically bred insecticidal cultivars suggest that widespread planting of TICs could result in the decrease in damage status of target pest populations over a 1- to 4-year period. If this

occurs, it may be economically feasible for farmers to maintain effective refuge percentages in excess of 20%. We currently lack data needed to predict how TICs will alter regional population dynamics of target pests. Therefore, small-scale experiments aimed at learning more about pest population dynamics, as well as careful monitoring of pest and natural enemy population dynamics, are essential to programs aimed at limiting pest exposure to TICs while decreasing pest densities.

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