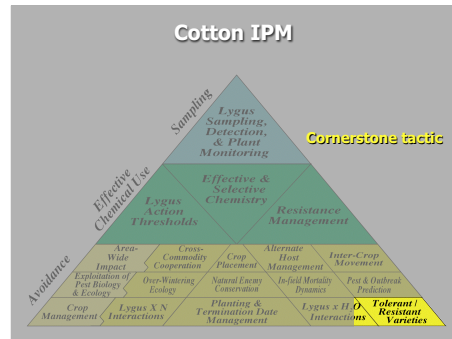


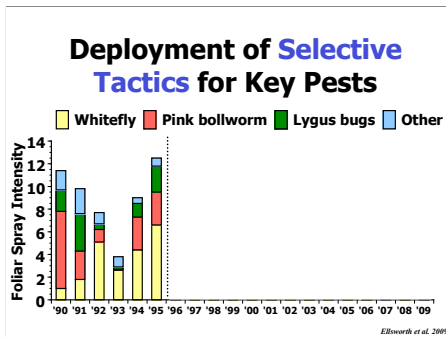
The following work presented represents a collaboration between the University of Arizona and Bayer CropScience.

15 minutes; 50+.

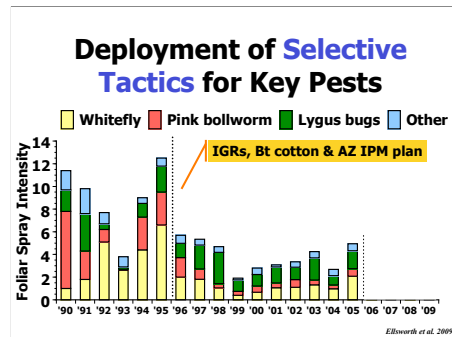
This annotated presentation is available at: <http://cals.arizona.edu/crops/presentations/11BeltwideTwinlinkvF3lo.pdf>



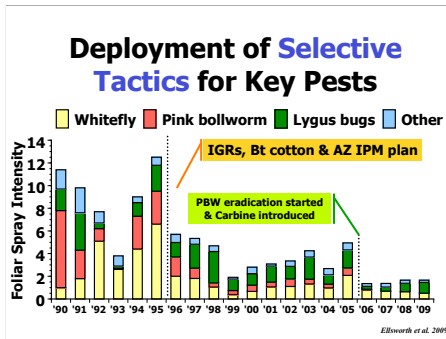
I like to begin any discussion by reviewing the overall structure of Cotton IPM as a means to understanding the potential role a new tactic may play in the system. As we all know, the cornerstone to IPM is resistant varieties. It shapes the foundation for all else that we do in the production of cotton. Bt cotton for us in Arizona has been an all-important selective control tactic for pink bollworm, our key lepidopteran pest.



I also like to start by reviewing the history of deployment of selective tactics against key pests in our Arizona system. It is a striking history, where we can see the no. of foliar insecticides used to control each of 3 key pests over time, whitefly, pink bollworm and Lygus bugs.

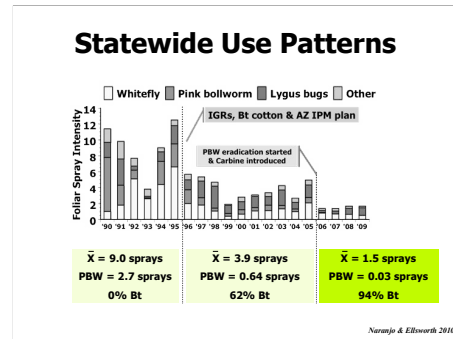


The results have been striking. A watershed of change occurred in 1996 with the introduction of very safe and selective Insect Growth Regulators (IGRs) for whitefly control, and transgenic Bt cotton, along with an IPM plan for whitefly management and comprehensive outreach campaign that consisted of extensive grower and pest manager education.

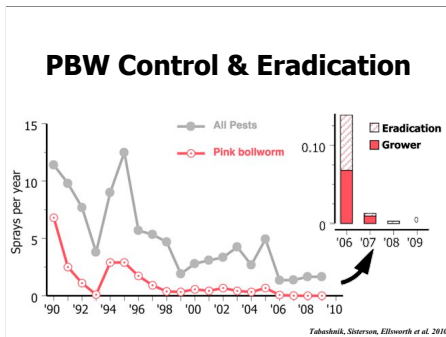


More recently, growers in collaboration with state agencies began PBW eradication in 2006. At the same time, we introduced flonicamid (Carbine) in 2006 as our first fully selective control agent, a feeding inhibitor, for Lygus.

Adapted from Naranjo & Ellsworth 2009.

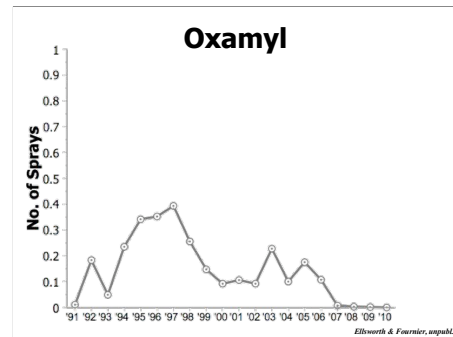


If we draw out information from these critical periods, we can see rather dramatic declines in overall insecticide use, as well as huge declines in PBW sprays made by growers. Bt cotton adoption rose to 94% over this last period, and even higher over the last 2 years shown, ca. 98.25%.

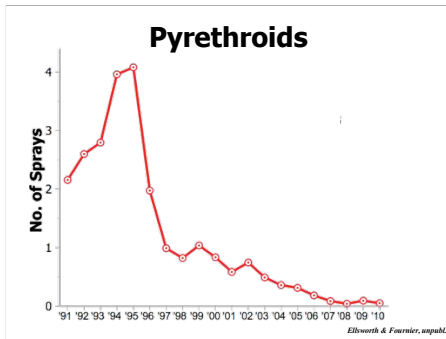


Re-organizing the PBW data, we can see just how low our spray numbers have gone since eradication. For the first time in over 40 years, Arizona cotton growers did not make a single spray against PBW in 2008–2010. Even when considering the programs over-sprays, we can see that no sprays of any kind have been made since 2009!

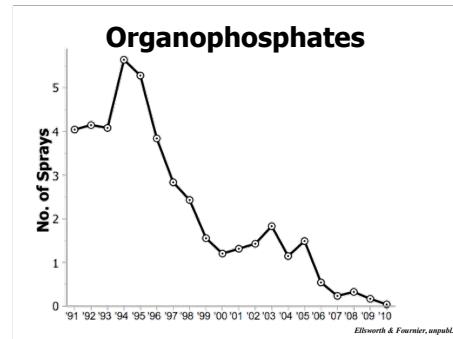
The credit we take for any part of this is shared with many, many others, but the result has been over \$220M saved cumulatively since 1996.



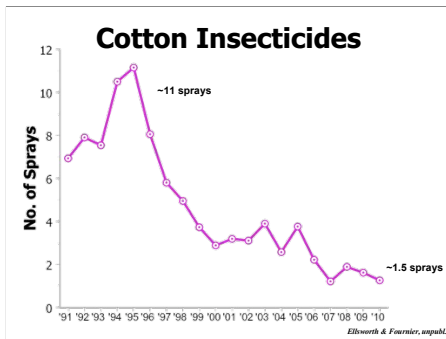
We can also examine patterns of use for specific groups of chemistry that were very important in PBW control at one time. The carbamate, oxamyl or Vydate C-LV, was at one time a popular early season control measure for PBW moths. It should be noted here that all the modern lepidopteran chemistry developed over the last 2 decades is essentially ineffective against PBW, because control programs must target the moth on the wing rather than the cryptic and protected larvae or eggs.



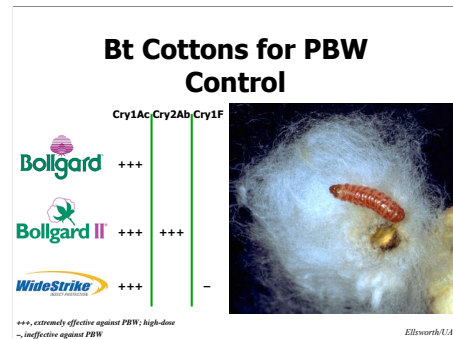
Pyrethroids, too, were used and became very important in whitefly control; however, their usage has declined almost to zero in cotton here.



Organophosphates are another important group of insecticides used to control PBW, especially chlorpyrifos and methyl-parathion. This group has also declined to almost nothing. Carbine introduction has been very important to this continued trend in recent years as a selective Lygus feeding inhibitor (since 2006).



From a 30-yr high in 1995 of nearly 11 sprays used on average statewide for arthropod control to just 1.5 sprays in recent years. And virtually all pyrethroids, most organophosphates, all carbamates, and nearly all endosulfan uses have been eliminated in cotton in favor of reduced risk chemistries, mainly neonicotinoids, flonicamid (feeding inhibitor), ketoenols (lipid inhibitors, i.e., spiromesifen or Oberon), and IGRs, all of course, used over the top of Bt cottons.



Bt cottons have been pivotal to our ability to stabilize the control system, starting with Bollgard, which is no longer marketed and based in the highly effective Cry1Ac protein, followed by the 2-gene Bollgard II where Cry2Ab is also highly effective against PBW, and finally Widestrike which produces once again the highly effective Cry1Ac protein.



This brings us to TwinLink cottons, which produce Cry1Ab and Cry2Ae proteins. Developed by Bayer CropScience, we hope to see registration for 2013. Starting in 2007, we initiated field tests to examine PBW control dynamics.

One important QC/QA procedure we followed was labeling every plant used in our bioassay tests uniquely with weather-resistant Tyvek labels. This permitted us to re-locate plants for trait integrity testing and exclusion of off-type plants from our results.

High Dose / Refuge Strategy

- 25x toxin concentration to kill susceptible larvae (US-EPA, SAP, 2008)
- Enough to kill nearly all heterozygous larvae (LC95 ?)
- Designed for 1-gene systems
- Guidelines have not been revised for 2-gene systems

Ellsworth/UA

Insect Resistance Management, thus far, for Bt transgenic cottons has been based in "High Dose" and structured or unstructured refuges. While we respect the need for refuges in certain systems, the goal of this research was to examine the utility of TwinLink cottons for high dose expression of proteins toxic to PBW.

EPA suggested that high dose is equivalent to the 25 times the toxin concentration needed to kill susceptible larvae. This in essence was a proxy or estimate based on the need for high-dose control of nearly all larvae heterozygous for Bt resistance. This might be equivalent to an LC95. However, these suggestions were designed around 1-gene control systems, not 2.

Efficacy & High Dose Dynamics

- Field grown plants
 - 2007–2009 (& 2009–2010, AZP-R)
- 4 lines
 - Coker check line
 - Cry1Ab
 - Cry2Ae
 - TwinLink (TL)
- 11 artificial field infestations
 - 3900 bolls
 - 61067 larvae assayed

Dr. Shujuan 'Lucy' Li

We grew plants in field trials conducted in 2007–2009. At the end of this talk, I will conclude with information from field & lab trials conducted based on field plantings of TwinLink cottons in 2009 and 2010.

In each field trial, 4 lines were examined. A Coker check or non-Bt line, the TL comparative, and then near isogenic lines containing 1 of the 2 genes encoding for Bt proteins, Cry1Ab or Cry2Ae.

We conducted 11 artificial infestations in the field, examined nearly 4000 bolls and assayed over 60,000 larvae. Dr. Lucy Li referred to these larvae as "her babies"; however, she is within days of giving birth to her own and first human baby this week! Lucy conducted the bulk of these assays, which are laborious to do.

Field Procedures

- US-EPA Method 4
- Flowers tagged (incl. plant/plot designations)
- Ca. 100 susceptible PBW eggs, stapled to bracts of bolls (15–19 d old)
- Bolls harvested ca. 7 d later
- Held under lab conditions (up to 21 d)

Ellsworth/UA

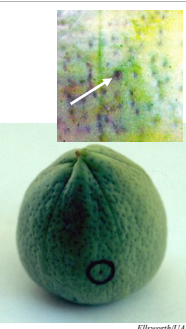
Our overall approach is derived from US-EPA Method 4, which involves the testing of susceptible PBW larvae. This is a surrogate for understanding how candidate lines control target pests that are heterozygous for a resistance gene.

We tagged flowers in order to select bolls of uniform age for the assays. Every plant was marked with a Tyvek label impervious to weather and water, which allowed us to track back plant material for QA/QC purposes, if needed.

PBW eggs from a USDA-ARS susceptible PBW colony were used throughout. Bolls were harvested ca. 7 d after infestation with pharate 1st instar larvae (i.e., eggs about to hatch). They were then held under laboratory conditions for up to 21 d.

Lab Procedures

- Estimate attack rates (= larvae assayed)
 - Count no. entry holes under microscope
- Dissections 10–28 d > infestation
 - Sufficient time to permit development to 'large' larvae (\geq 3rd instar = "survivors")
 - All living & dead PBW extracted & instars determined




Ellsworth/UA

To estimate attack rates or the no. of larvae being assayed, we counted the no. of entry holes under the microscope. Some may not be familiar with the biology of PBW. Once 1st instar larvae hatch, they immediately crawl onto the boll exterior and bore directly into the boll within a matter of hours. This is one of the cryptic behaviors that makes them insensitive to most foliar insecticides.

Incubation time was more than sufficient to permit development to 3rd instar or larger in check and Bt lines. Comprehensive dissections of each boll involved locating all living PBW as well as PBW cadavers. Given that some die in the first days of the assay, cadavers may have had up to 3–4 weeks to decompose while still in a growing boll. This is very difficult work, but work that Lucy and our other works are very proficient at.

Survival / Mortality Criteria

- \geq 3rd instar ('large') = "survivors"



- Excluded bolls
 - Moldy
 - Bolls without larvae
 - Bolls without entry holes
 - Putative transgenic bolls with "survivors" that failed QA/QC tests (ELISA, PCR; nulls & hemizygous plants)
 - Check bolls with \geq 20 entry holes (2008–2009)

Ellsworth/UA


Live larvae reaching 3rd instar or larger were considered "survivors" for this bioassay.

Several classes of bolls were excluded from the mortality calculations, including those that failed QA/QC testing.

In 2008 & 2009, we changed the protocol to exclude check bolls containing more than 20 entry holes, for the following reason.

Carrying Capacity of Boll

- Max. surviving PBW from 1 boll = 16 (previous studies)
- Seed feeder & cannibalistic
- Max. seed / boll, 20–30
- Max. attack rate in check line = 73 (!!!)
- 2008–2009 assays limit check assays to \leq 20 entry holes.



Ellsworth/UA

The PBW carrying capacity of a cotton boll is finite. It is bounded by the resource available. In 15 years of bioassay testing, the largest number of \geq 3rd instar larvae surviving in non-Bt bolls was 16. [That number was bested in this study and reached 18]. PBW are essentially seed feeders, with only 1 larvae developing per seed; they also have the capacity for cannibalism. While the number is variable, a boll might produce maximally about 20–30 seed. Yet the maximum number of larvae attacking check bolls in 2007 was 73! Since this number cannot survive to large larval stage, check mortality becomes inflated in a density-dependent manner.

So in 2008 and 2009, we continued assays in bolls of the check line when there were \leq 20 entry holes.

Mortality Assessments (%)

- Apparent mortality (ave. per boll)
- For each boll population,
 - $1 - (\text{large larvae} / \text{entry holes}) \times 100$
 - Mean \pm 95% Confidence Interval
- Corrected mortality
 - Performed on aggregate data

Ellsworth/UA

Mortality assessments included calculation of apparent mortalities on a per boll basis. Means \pm 95% CI were calculated for each boll population.


In addition, we calculated check-corrected mortality from aggregate boll data as might be expected in traditional bioassay procedures.

However, it would seem counterintuitive to make these assay corrections, since mortality associated with candidate lines is observed directly and this parameter is used directly in modeling resistance. In addition, check line mortality is biased higher than would normally be considered acceptable in lab bioassays.

Off-Types

- 2007: 4 plants
 - 3 with 12 bolls, Null
 - 1 with 4 bolls, Hemizygous
- 2008: 10 plants
 - 5 with 11 bolls, Null
 - 5 with 7 bolls, Hemizygous
- 2009: 3 plants
 - 1 with 3 bolls, Null
 - 2 with 9 bolls, Null

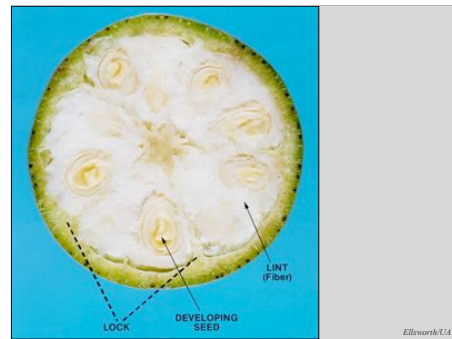
When considering measurements of rare events, even low rates of undetected contaminants can skew results



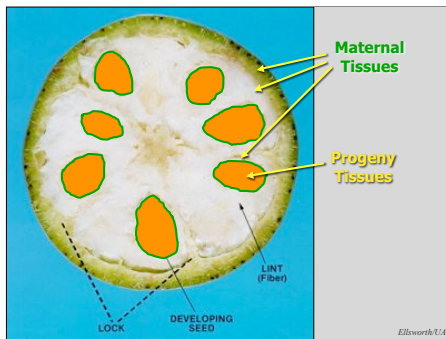
Ellsworth/UA

Over the course of 3 years and thousands of bolls from hundreds of plants examined, we retrieved "survivors" from putative Bt lines (plants from within Bt plots). However in 17 cases, the plants turned out to be off-type, and either null or hemizygous for the target trait(s). Because of our ability to back-track to specific plants in our design enabled by our in-field labeling of each plant, we were able to exclude these boll assays from our findings.

Without this ability, these few bolls bearing large larvae would have extremely skewed our mortality calculations and assessments of high-dose.



When excluding hemizygous plants, it was necessary to review my basic botany.



A boll is made up of progeny tissue, the seed endocarp, and maternal tissues, the boll exo- and endo-carp as well as the seed coat. Recall that PBW are ultimately seed-feeders and are seeking out what is progeny tissue of the boll.

Hemizygous for Trait

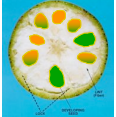
(selfed: 1:2:1 ratio trait expression in seed)

Maternal
Hemizygous

x

Paternal
Hemizygous

=



Hemizygous for 2 traits, selfed = 1:2:1:2:4:2:1:2:1

Ellsworth/UA

Thus, a plant that is hemizygous for a given trait, when selfed, produces bolls with a specific ratio of 1:2:1 in trait expression. So in a 1-gene system, there would be a 25% chance that a PBW would encounter a null seed in what would otherwise be a Bt-expressing plant.

The system is even more complex for a 2-gene system where 1/16th of the seed population is null for both traits.

Incidentally, this system of selfed hemizygous plants is one of the current standards for breeding and production in the Indian cotton system. This would seem to be a risky practice with respect to control and resistance dynamics for PBW there.


Ellsworth, Li, Tabashnik, Holloway & Humphries

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Summary Results

- **Attack rates (entry holes / boll)**
 - Mean: 7–27
 - Max: 73, Check line; 95–97, Bt lines
- **Assay efficiency (recovery rates, PBW / boll)**
 - Mean: 6.1 – 22.3
 - Max: 61%, Check; 73–92, Bt's

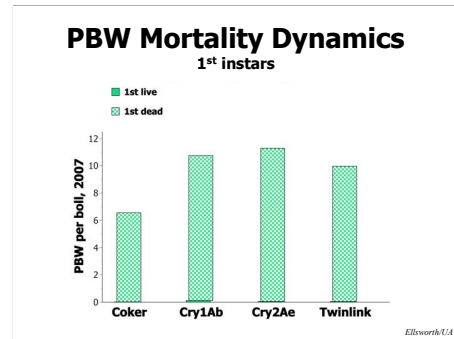
*density-dependent mortality



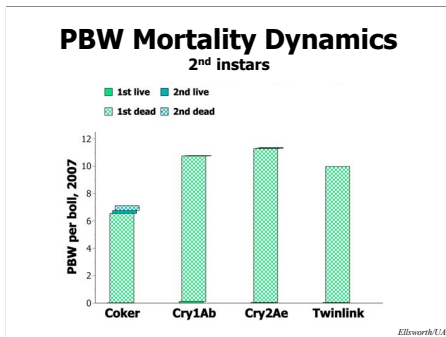
Just a few summary results:

The mean no. of entry holes ranged from 7–27 per boll. The maximum observed was 73 (from 2007 for the check line before we constrained this to ≤20 per boll) and 95–97 for the Bt lines, which represents tremendous larval pressure on a single boll.

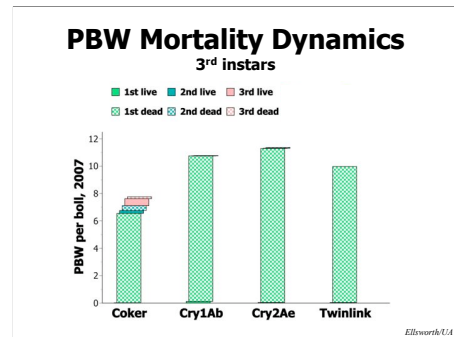
Our assay efficiency really depends on how well Lucy and other workers were able to extract cadavers from bolls. In general recovery rates reached 80–90% in 2008–2009, and is extraordinarily high given the difficulty in detecting tiny 1st instar cadavers that had been dead for up to 10–28 d.



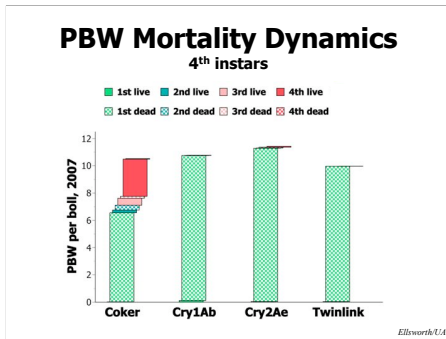
Now let's examine a series of charts that depict PBW mortality dynamics measured from the bioassays for each plant line. Each chart builds upon the last. This is a chart of live and dead 1st instars that were present by the end of the assay period. There are two important insights here. First, PBW die as 1st instars even in the Coker check line. However, live 1st instars are still present (and very much stunted) in the Bt lines.



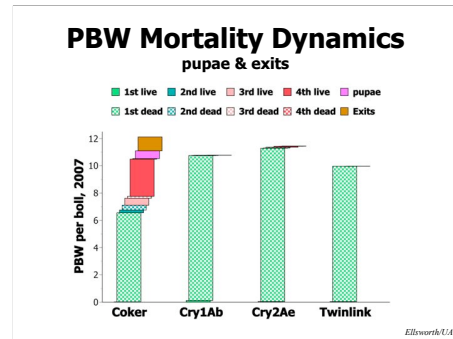
There are not many 2nd instars, dead or live, by the end of the assay.



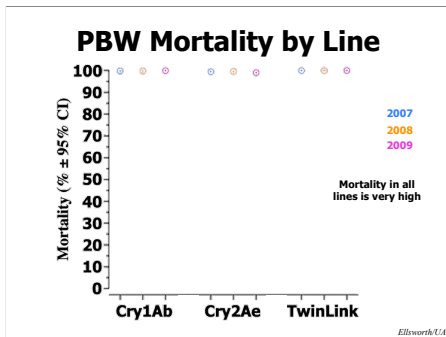
Recall that the assay is designed to permit enough time for larvae to develop to the 3rd instar or larger. These live individuals are scored as "survivors". Generally, we do not see a great deal of mortality from this point onward.



There are more than 2 live 4th instars per boll in the non-Bt check line.

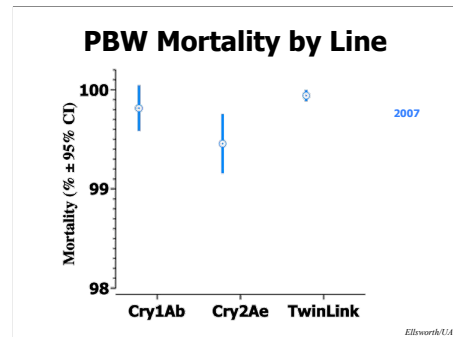


Pupae and exited larvae are all live individuals and found almost exclusively in the non-Bt check line, though a small sliver of survival is seen especially in the Cry2Ae line.



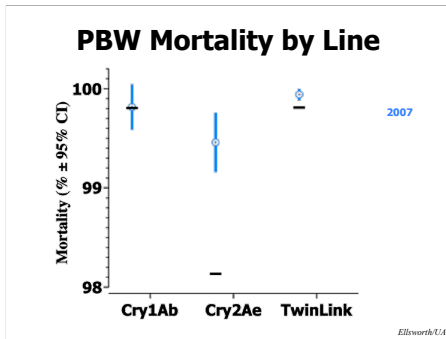
The following charts depict mortality as a % ± 95% CI, by plant line and year.

This chart shows all the data from this 3-yr study. Mortality is exceptionally high for all 3 lines in all 3 years. It is so high that it is difficult to discern differences among lines. So in order to better depict these differences, I will use some visual trickery to explode the scale shown, isolating the y-axis on the 98–100% interval. Bear in mind that this perceptual shift is a distortion of the data, which ultimately and more properly looks as shown here. I.e., the differences among lines are so slight as to be nearly imperceptible.

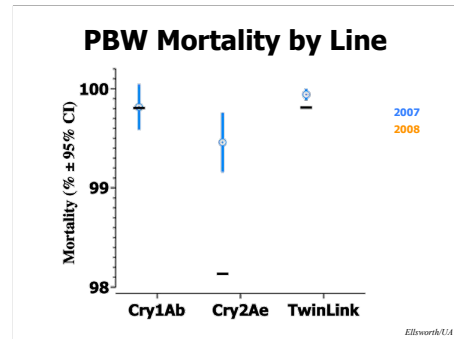


The following charts depict mortality as a % ± 95% CI. Note the scale is enlarged to show just 98 to 100% mortality for each plant line.

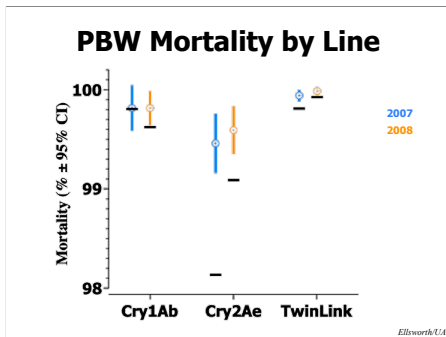
These apparent mortalities are very high, and highest with the least variation in the TwinLink line.



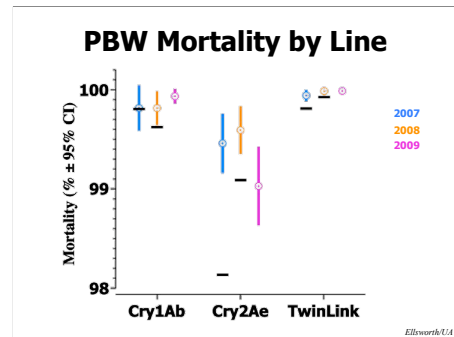
The black line represents the check-corrected mortalities for each line. The impact is significant, especially when considering the Cry2Ae results. Nevertheless, mortality is still very high.



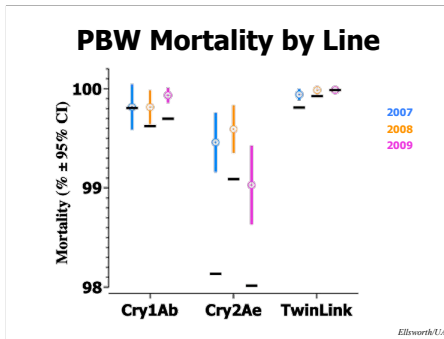
The orange lines represent the 2008 results. There are very similar results throughout. TwinLink mortality levels are exceptionally high.



Again, the check line mortalities impacted the results, though recall that we better controlled for density-dependent mortality in the check line starting in 2008. This reduced check mortality by about 10%, though still resulting in check mortalities around 50%.



The 2009 data (pink) show no survival in the TwinLink line.



The important point is that these are very high levels of mortality in PBW, regardless of plant line. However, it appears that the Cry1Ac line is somewhat higher and more consistent in PBW mortalities.

Criteria for High-Dose?

- “The SAP discussed... high-dose definition. In general, the Panel indicated that *no single number could be rigorously defended as the cut off for a high dose for any key pest species.... Any definition of high dose must therefore be imprecise.*”

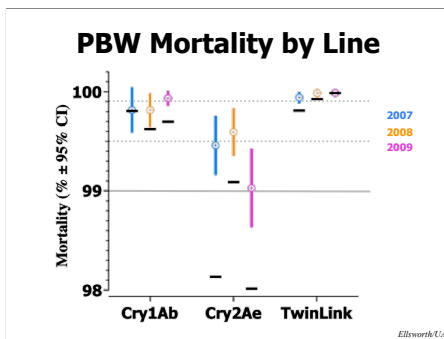
– SAP Report No. 2000-07, 3/2001

Ellsworth/UA

So what should be the criteria for assigning “high-dose” to a new technology?

The EPA Scientific Advisory Panel (SAP) very adroitly pointed out that no single number was appropriate and that any definition would have to be imprecise.

Given the huge differences among systems, budworm in Southern cotton, bollworm in cotton or corn, vs. pink bollworm in Western cotton, it is such that we should assess these things on a case by case basis.



By most standards, an LC99 should be sufficiently high to be considered high-dose, and all three lines satisfy this metric. Others might be interested in the 99.5% or 99.9% level. Regardless, the commercial product, TwinLink cotton, controls PBW well above this highest level.

New High-Dose Control Option for PBW

- ‘Apparent’ mortality in an ecological context is best
 - TL & its constituent genetic parts control most PBW as 1st instars
 - TL & its constituents produce toxins, sufficient to be considered “high-dose” for resistance management
- | | |
|--|---|
| | TwinLink, 99.98%
or ca. 1 in 5000 |
| | Cry1Ab, 99.87%
or ca. 1 in 900 |
| | Cry2Ae, 99.37%
or ca. 1 in 200 |

Ellsworth/UA

The actual (‘apparent’) observed mortality (uncorrected) would seem to be the most appropriate predictor of the ecological context simulated in most models. If PBW cannot survive in a candidate line or only survive at exceptionally low levels, it is immaterial what the control mortality is. No or just a few PBW can survive and create no or very little opportunity for resistance evolution and development. Most of the Bt mortality occurs in the 1st instar.

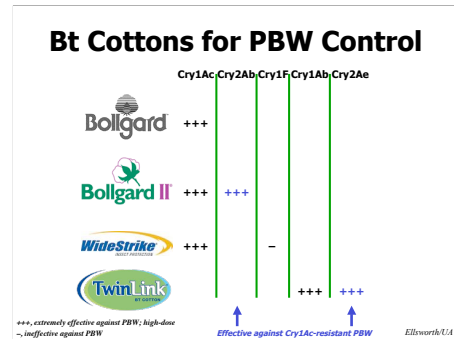
TL & its constituent genetic parts produce toxin concentrations consistent with a high-dose designation for PBW. Furthermore, while each line delivers very high levels of control, there is a clear benefit of TL over each constituent line, going from 1 in 200 survival in the Cry2Ae line to 1 in 5000 survival in the TL line.

TL vs. Cry1Ac-Resistant PBW

- PBW Cry1Ac-resistance, 0.16 (Tabashnik et al. 2000a) **TwinLink, 100%**
- Cross-resistance to Cry1Aa, 1Ab (Tabashnik et al. 2009) **Cry1Ab, 80.28%**
- No cross-resistance to Cry1Ca, 1Da, 2Aa, 2Ab (Tabashnik et al. 2000b, 2009) **Cry2Ae, 99.95%**
- No losses of susceptibility to Cry1Ac in AZ PBW (Tabashnik et al. 2005, 2010)
- TL & Cry2Ae cottons control Cry1Ac-resistant PBW (AZP-R)

Ellsworth/UA

In studies conducted in field grown bolls in 2009–10, we challenged TL with Cry1Ac-resistant PBW (AZP-R). This culture was derived from field collected individuals by Tim Dennehy and colleagues in 1997. Tabashnik et al. (2000a) reported an R-allele frequency of 0.16 from that initial wild population. This finding challenged conventional thinking about the initial rarity of R alleles. Tabashnik et al. (2000b, 2009) went on to measure cross-resistance in this strain for Cry1A proteins, and lack of cross resistance among Cry1C-D, and Cry2 proteins. However, there have been no losses of susceptibility since in feral AZ PBW to Cry1Ac. Our tests show that TL & Cry2Ae lines control Cry1Ac-resistant PBW, whereas these same PBW can survive at higher rates (ca. 20%) on the Cry1Ab line, confirming Tabashnik’s earlier cross-resistance finding.

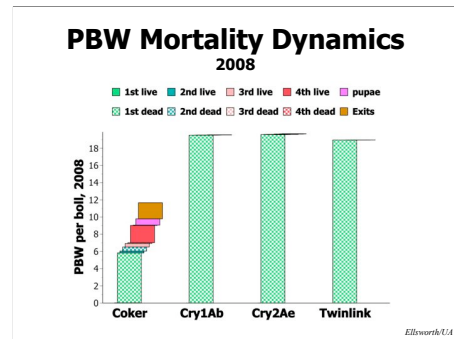


Mortalities measured in this study are very similar to studies conducted in our lab with Cry1Ac in Bollgard, BollgardII or Widestrike plant lines and to Cry2ab2 in BollgardII lines. Now Cry1Ab and Cry2Ae in TL cotton can also be considered highly efficacious, enough for the high-dose designation. Furthermore, the Cry2Ae protein developed for TL is 1 of only 2 candidates for control of Cry1Ac-resistant PBW. Given recent events in Indian cotton in Gujarat — Cry1Ac-resistant PBW in this western state of India have been found surviving in Bollgard cottons there (Dennehy et al. this volume) — we are fortunate to have such effective, high-dose alternatives to Cry1A proteins. As we wind up PBW eradication in the U.S., we hope to have avoided any Cry1A resistance and will benefit from having another 2-gene (both high dose) product to help prevent re-invasion and re-infestation in AZ cotton.



We wish to thank the Western IPM Center for grant support and the ACRPC for logistical support for measurement of pest management tactics use; USDA-ARS, ALARC, and TJ Dennehy / EARML for access to susceptible and Cry1Ac-resistant PBW, respectively. We thank BCS for early access to the TwinLink technology and acknowledge their funding support. The Arizona Pest Management Center (APMC) as part of its function maintains a website, the Arizona Crop Information Site (ACIS), which houses all crop production and protection information for our low desert crops, (<http://cals.arizona.edu/crops>), including a copy of this presentation.

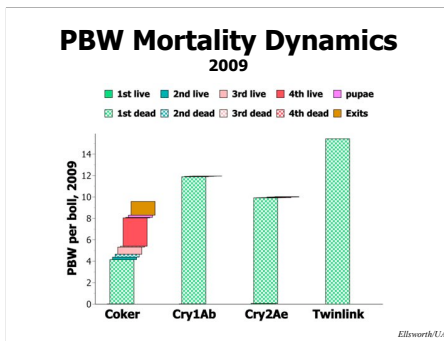
Photo credit: J. Silvertooth



Supplementary Data (not shown in the original presentation)
These results for the 2008 bioassays are very similar to the 2007 & 2009 (see next page) data.

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