Preliminary Evaluation of the "Next Generation" of *Bt* Cotton

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Abstract

The next generation of Bollgard® cotton was evaluated for agronomic and insecticidal efficacy under central Arizona growing conditions. Two novel lines were compared with their recurrent parents, DP50 and DP50B. There were no season-long differences observed among the varieties in most plant development and insect parameters. However, DP50 had significantly lower emergence than the other lines tested (possibly related to seed quality). The lower plant population may have been responsible for greater whitefly abundance observed on two dates mid-season. During early-season ratings of secondary "pests" (15 DAP) (scaled on damage and/or presence), the two test lines received lower ratings for thrips and flea beetle when compared with DP50, DP50B and DP50Bu (untreated for Lepidoptera). However, these difference are likely as a result of the difference in seed treatments that the two test lines received (Gaucho®) and the others did not. This seed treatment does have known activity against thrips and beetle pests. In mid-season, the two test lines received lower ratings for beet armyworm when compared to DP50, DP50B and DP50Bu (although, not significantly different from DP50B or DP50Bu). Efficacy against pink bollworm (PBW) was assessed one time at the end of the season (we were limited to this time, so as to not affect yield), and DP50 was the only variety in which PBW exit holes were observed and PBW larvae collected. However, the low Lepidoptera pressure experienced during the season limited assessments of the two novel lines' efficacy toward PBW. There was no significant difference in yield (bale/A) among the varieties. Although, one of the test lines had a lower lint turnout than each other variety. The two novel Bollgard lines performed well under our growing conditions, but continued evaluations will be necessary under more conditions and more insect pressures before "varietal" performance and gene efficacy can be assessed adequately.

Introduction

Transgenic plants expressing insecticidal proteins, such as the endotoxin derived from the crystalline bacterium, *Bacillus thuringiensis* var. *kurstaki* HD-1, have made positive impacts on current agricultural production such as: (1) worker and environmental safety through lower conventional insecticidal use, (2) selectivity toward caterpillar pests (e.g., *Pectinophora gossypiella*, PBW) while minimizing the potential disruption of other pests, (3) reduced conventional insecticide use leading to enhanced conservation of natural enemies, and (4) in Arizona, removal of concern for PBW control transferring management focus on other pest species (e.g., *Bemisia* and *Lygus*) (Ellsworth et al. 1995a). This technology is widely used in Arizona cotton production. Transgenic cotton expressing the *Bt* Cry1Ac toxin (Bollgard®) constituted approximately 65% of the acreage grown in Arizona in 1998 (Williams 1999). With so much cotton acreage dependent on the expression of a single toxin, the potential for resistance development cannot be

underestimated (Tabashnik et al. 1990, Bartlett 1995, Bartlett et al. 1997, Simmons et al. 1998, Patin et al. 1999). Moreover, efforts to maximally suppress PBW regionally with even greater adoption of *Bt* cottons (in areas with high levels of PBW) could increase the selection pressure on PBW populations, possibly resulting in rapid development of resistance.

The rotation and/or mixture of different classes of conventional insecticides are common methods of forestalling or overcoming resistance to their chemistries (e.g., Prabhaker et al. 1996). Availability of multiple transgenic toxins may help maintain the current efficacy of this valuable technology (McGaughey & Whalon 1992, Gould 1998). There have been numerous Lepidopteran active crystal protein genes identified (Gill et al. 1992), and such genes could be used to produce different transgenic toxins for a rotation or multiple toxin management approach.

The "next" generation of Bollgard cotton lines active against Lepidopteran pests are currently under development. Monsanto has modified transgenic cotton lines to express two proteins from *B. thuringiensis* var. *kurstaki*. These lines were generated from the commercial Bollgard variety, DP50B. These lines are "pyramided." That is, they contain both the Bollgard gene (Cry1AC) and a new gene ("CryX"). It is imperative to evaluate these new lines under low desert conditions to ensure their performance in the Arizona production system. A comparative evaluation of transgenic lines against their recurrent parents is a good approach to verify maintenance of varietal integrity (Ellsworth et al. 1995b, 1996; Silvertooth & Norton 1998). Furthermore, the insecticidal capacity of the new lines needs to be compared with the Bollgard gene now in use. The objective of the current study was to determine the agronomic and insecticidal efficacy of next generation *Bt* transgenic lines. These trials mark the first ever evaluations of these experimental lines in the field. Seed availability was extremely limited, and the test was constrained by this and other regulatory requirements.

Materials and Methods

The test plots were planted in 2 rows x 15 ft, separated by same size internal buffer (i.e., DP50) plots, in a RCBD design with four replicates. The two test lines expressing the novel *Bt* transgenic toxin, MONS1 and MONS2, were compared against their recurrent parental lines, DP50B and DP50. The comparisons were equivalent with the following exceptions. The two test lines came from greenhouse-produced 'winter' production, while the recurrent parents were from commercial channels. Also, the two test lines were seed-treated with an insecticide, Gaucho®, which has some short-residual activity on thrips and beetle pests. All lines were to be protected against pest insects including Lepidopterans with insecticides. Thus, an additional treatment of DP50B was incorporated into the experimental design to observe the effects of Lepidopteran insecticides (designated, DP50Bu). Replicates were separated by 4-row buffers, and were surrounded by two, 12-row lateral and 55 ft "capping" buffers. All buffers and post-harvest residues were destroyed according to regulatory requirements.

The field was planted with a two-row cone planter on 2 June with a 40-inch width in dry soil and watered up. Percent emergence was observed 7, 14 and 30 days after planting (DAP). Crop growth and development parameters were collected on approximately 14-day intervals beginning 7 August (66 DAP). Plant height, number of mainstem nodes, node of first fruiting branch, aborted sites at positions one and two, and the number of nodes above top white flower (NAWF) were recorded (Silvertooth & Norton 1998). From these measurements we calculated height to node ratios (HNR) and percent fruit retention (%FR). Additional plant growth parameters were also recorded: number of blooms every 3 days for a one month period (once blooming was first observed; 31 July, 59 DAP), and the number of cracked bolls every 7 days for a one month period (once cracked bolls were first observed; 8 September, 98 DAP).

Insect management was conservatively protective so as to eliminate any stress from insects. Knack® (pyriproxyfen), Monitor 4E® (methamidophos) and Lannate LV® (methomyl) were sprayed on 31 July (59 DAP) for whiteflies, *Lygus*, beet armyworm, cabbage looper, and cotton bollworm; Vydate C-LV® (oxamyl) and Steward 1.25SC® (indoxacarb) on 13 August (72 DAP) for *Lygus* and beet armyworm; Orthene® 90S (acephate) and Zephyr 0.15EC® (abamectin) on 26 August (85 DAP) for *Lygus* and mites; and, Vydate and Applaud® (buprofezin) on 18 September (108 DAP) for *Lygus* and whiteflies. The DP50Bu plots did not receive Lannate or Steward.

Trial plots were rated periodically for susceptibility to secondary pests beginning 17 June (15 DAP). Only non-

destructive methods of sampling could be employed in the small trial plots. Insect caused or induced damage and/or their presence were the rating criteria, using the scale of 0 to 3. Abundance of whitefly adults, nymphs and eggs were monitored on approximately 14-day intervals beginning 16 July (44 DAP). PBW impact was determined 2 days prior to harvest, choosing appropriately-aged bolls (i.e., young enough to harbor PBW larvae and without interfering with yield estimates). PBW and *Lygus* pressure was determined for the entire field by sampling external buffer regions only at approximately 7-day intervals for *Lygus* beginning 15 July (43 DAP) and 21-day intervals for PBW beginning 8 August (98 DAP).

Harvest was conducted 18 November (169 DAP) using a Hydrostatic International, model 622 CP, two-row picker. The spindle wrap, heads and cotton chute were manually cleaned-out between plots to ensure integrity of samples. Each run consisted of harvesting "external" and "internal" buffer plots as well as the trial plots. Additional two-row "plots" were harvested from external buffer locations lateral to test line plots (i.e., "lateral check"). All additional buffer plots were measured to equal the length of their reference test line plot, some having received the Lepidoptera-specific sprays and others not. The seed cotton was ginned at the Maricopa Agricultural Center's GLP-compliant Short Staple Cotton Gin facility. Percent turnout, bales (480 lb/ A), and seedcotton (lb / A) were determined for each variety.

All data were analyzed by ANOVA, and means separated by orthogonal contrasts ($P<0.05$). T-tests were conducted for additional comparisons of yield data ($P<0.05$). All data were normalized, where appropriate, and raw means presented for interpretation.

Results

Prebloom, Early Season

DP50 had lower seedling emergence (59.0 ± 4%) ($P<0.06$, df = 1,12) at final count (2 July; 30 DAP) than all other lines (72.6 ± 1.5%). During this early period, thrips, banded-winged whiteflies, pale-striped flea beetles, white-marked fleahoppers, grasshoppers and leaf miners were observed in the plots and rated accordingly. On 17 June (15 DAP), the two test lines had significantly lower thrips ratings (0.12 ± 0.02) ($P<0.05$, df= 1,12) and flea beetle (mainly pale-striped flea beetle) ratings (0.06 ± 0.02) ($P<0.04$, df = 1,12) when compared with each variety (thrips: 0.37 ± 0.05; flea beetle: 0.29 ± 0.04). On 2 July (30 DAP) white-marked fleahopper ratings were higher for MONS1 (0.12 ± 0.04) ($P<0.008$, df = 1,12) when compared with each variety (0.02 ± 0.01).

Mid-season

By 7 August (66 DAP), MONS1 was significantly taller (77.5 ± 0.8 cm) ($P = 0.002$, df = 1,12) than DP50 (73.4 ± 0.9 cm), resulting in a significantly larger HNR (1.49 ± 0.03) ($P = 0.006$, df = 1,12) than DP50 (1.38 ± 0.04) (fig. 1). There were no further significant differences in height or HNRs (fig. 1) (16 July, 11 & 25 August), nor differences in %FR (fig. 2) or NAWF (fig. 3) among varieties in all mid-season assessments (7, 11, & 25 August). There were no significant differences in white bloom production for the first month of observation (fig. 4). However, on the final date of observation (i.e., ca. one month since first bloom, 31 August) MONS2 had significantly more white blooms (18.4 ± 2.0 / plot) ($P = 0.006$, df = 1,12) when compared against DP50B (12.5 ± 2.6 / plot). There were no significant differences observed among varieties in node of first fruiting branch during the mid-season.

For adult whitefly abundance on the first date, 16 July, there were no significant differences among varieties (fig. 8). By the next date, 31 July, DP50 had more WF eggs (38.7 ± 5.0 / disk) ($P<0.02$, df = 1,12) than each other line (14.6± 1.7 / disk) (fig. 5), and higher small nymph numbers (7.9 ± 1.0 / disk) ($P<0.03$, df = 1,12) when compared to MONS1 (2.9 ± 0.4 / disk), MONS2 (3.6 ± 0.6 / disk) and DP50B (4.7 ± 0.7 / disk) (fig. 6). There were no significant differences among varieties for WF adults or large nymphs on this date or for all WF stages on 7 August. By 11 August, DP50Bu had higher WF egg numbers (14.8 ± 1.7 / disk) ($P<0.01$, df = 1,12) when compared with MONS1 (7.4 ± 1.4 / disk) and MONS2 (6.5 ± 1.0 / disk) (fig. 5). Furthermore, DP50Bu had 10-times more small nymphs (0.7 ± 0.4 / disk) ($P<0.03$, df = 1,12) than each other trial line (0.07 ± 0.03 / disk), and higher large nymph numbers (1.7 ± 0.8 / disk) ($P<0.03$, df

= 1,12) than each other trial line (0.14 ± 0.05 / disk) (figs. 6–7). On 17 August, we found higher WF adult numbers for DP50 (1.8 ± 0.3 / leaf) (P = 0.007, df = 1,12) when compared with MONS1 (0.6 ± 0.2 / leaf) (fig. 8).

The most prevalent secondary lepidopteran pest recorded was beet armyworm (BAW). Some plants exhibited severe BAW damage (e.g., drilling of terminal), and some plants showed only minor to no defoliation dependent on the variety. On 12 August, DP50 received a significantly higher BAW ranking (0.6 ± 0.1) (P = 0.005, df = 1,12) when compared with each test line (0.07 ± 0.04). Although, the two test lines did not receive a significantly lower BAW ranking than DP50B (0.25 ± 0.9) or DP50Bu (0.25 ± 0.06) (P>0.27, df = 1,12). Cabbage loopers were only observed on DP50.

Late-season

On the last two plant evaluation dates (9 & 29 September), there were no significant differences observed among varieties in plant height, HNR, node of first fruiting branch, and NAWF (fig. 1, 3). On 9 September, MONS2 had a slightly lower %FR (26.9 ± 1.1%) (P<0.09, df = 1,12) than both DP50B (29.5 ± 1.5%) and MONS1 (31.1 ± 3.3%) (fig. 2). Compared to baseline growth estimates for Arizona cotton, %FR (fig. 2) was quite low for all varieties (which could be attributed to the intense *Lygus* pressure experienced during the season, see fig. 9), cutout occurred quicker than the baseline estimates (fig. 3), and HNRs followed a normal pattern (fig. 1). There was never a significant difference in number of cracked bolls among the varieties (fig. 4).

There were no significant differences in WF abundance among varieties in the late-season (figs. 5–8).

During the later portion of the season (i.e., 8 September – 4 November), 10–70% of bolls sampled from external buffer regions displayed PBW damage (e.g., mining, warting, etc.), but no PBW larvae were ever found. Thus, PBW populations never reached levels to warrant an application for this pest specifically. In the final PBW assessment (16 November, 167 DAP), we observed PBW exit holes and collected large larvae (3rd–4th instar) only from DP50 (exit holes: 5.0 ± 2.6%; large larvae: 22 ± 6%). However, there was no significant difference among varieties in number of PBW hits (i.e., carpal wall warting, mining, and/or seed staining) (P = 0.97, df = 4,12).

Yield

The yields were not significantly different among the varieties (1.58 ± 0.05 bales/A; 1659 ± 48 lbs seed / A) (fig. 10). However, MONS1 had a significantly lower lint turnout (29.7 ± 0.4%) (P<0.003, df = 1,12) than each other variety (31.8 ± 0.2%) (fig. 11). MONS2 had slightly higher yields (1.58 ± 0.08 bale/A) when compared with its lateral check (1.39 ± 0.06 bale/A) (P = 0.08, df = 9) and external buffer plots (1.35 ± 0.06 bale/A) (P = 0.1, df = 20). MONS1 had significantly lower lint turnout (29.7 ± 0.4%) when compared with either the internal (32 ± 0.2%) (P = 0.0001, df = 18) or external buffer plots (31.3 ± 0.3%) (P = 0.06, df = 20), regardless of treatment with Lepidopteran-active insecticides. In all comparisons, application of Lepidopteran-active insecticides did not enhance yield.

Discussion

Through the majority of the evaluations, the two test lines developed normally when compared with their recurrent parent lines, DP50B and DP50. However, the DP50 plots appeared less uniform and vigorous by general observation when compared to the other trial lines. We do not know whether the seed received was of lower quality (e.g., green-house grown), but such a possibility cannot be dismissed. Also, seed germination under the extreme soil temperatures present in June may not be reflective of typical agronomic conditions. Compared against the Arizona cotton baselines (Silvertooth & Norton 1998), however, we can draw the conclusion that all the varieties behaved approximately the same in HNR, NAWF and %FR. Fruit retention was lower than normal overall and likely as result of the intense *Lygus* pressure experienced throughout the season, in spite of the four *Lygus* sprays made.

The insect ratings allowed us to observe and estimate impacts of various secondary insects on the trial lines. The test lines had lower thrips and flea beetle ratings than the other lines. However, this was a very early season difference (15

DAP) that was likely due to the use of an insecticidal seed-treatment for the test lines but not for the comparative, recurrent lines. On one date, MONS1 received a higher rating for white-marked fleahopper, when compared against each other trial line. The small plot size, the small magnitude of the differences, and the subjectivity of rating must be considered in any interpretation made about the efficacy of the test lines against secondary insects. A new protein could potentially have some direct toxicity to these insects or some other indirect effect on arthropod diversity. In this case, however, the CryX gene has no known activity outside of Lepidoptera. Further quantitative studies are necessary to validate the sources and determine the precise mechanism(s) of these changes in arthropod diversity.

The seasonal abundance of whiteflies was lower than that of years past (Ellsworth 1999, Ellsworth & Naranjo 1999). Regardless, there were a few dates that produced significant differences among the varieties. On two dates, DP50 had a greater abundance of WFs than all or some of the trial lines. The poorer stand establishment of DP50 lead to lower and less uniform plant populations and less canopy closure, resulting in greater potential for heat stress on the plants. Plant stress and plant density effects of increasing WF abundance are widely accepted. On another date, the DP50Bu line had greater WF abundance than all or some of the varieties. At this time, the DP50Bu had not received the Lannate spray contrary to the remaining varieties which received Knack + Monitor + Lannate 11 days earlier. Knack is known to have no direct lethal action against adult whiteflies, but non-pyrethroid combinations, like Monitor + Lannate can have significant adult knockdown activity. Either non-pyrethroid alone, like Monitor used alone on the DP50Bu plots, has relatively few, if any effects, on whitefly dynamics. Thus, the additional tank-mix of Lannate sprayed on the other varieties is likely to be the source of differences observed in WF abundance here.

With the limited Lepidopteran pressure experienced this season and the requirement for protective oversprays, the test lines could not be fully evaluated for their potential efficacy against PBW. The test lines did not, however, permit PBW development, same as the Bollgard recurrent parent, DP50B. Given that the number of "hits" were not significantly different among the trial varieties, we can also conclude that there was approximately equal PBW "pressure" across the lines, with DP50 being the only line permitting PBW development. Even for DP50, however, rates of large larvae were low (< 30%), even in November. This was most likely because of the very late planting date which escaped early season buildup of PBW. We were not able to assess PBW damage in the trial lines during the season, because this would have required destructive sampling (thus, affecting yields). DP50B did receive a higher BAW rating than both test lines, although not statistically significant. Such a difference in efficacy against BAW should be examined more closely with further quantitative studies.

The yield potential of the entire field was influenced by the late planting date and extensive *Lygus* pressure experienced throughout the season. The test lines may have been further limited by higher rates of seed dormancy due to their greenhouse origin. The small plot size and alleys in the test contributed to slower canopy closure and potentially higher heat stress. Regardless of these effects, the two test lines yielded as much as their recurrent parent reference lines (i.e., DP50 and DP50B).

Monsanto has produced at least two cotton lines with novel *Bt* toxin(s) that performed well under our Arizona growing conditions. Cotton lines expressing *Bt* toxins divergent from those currently in wide use will be a fundamental component of a resistance management program for transgenic cotton. Though the potential for cross-resistance cannot be ruled out (McGaughey & Whalon 1992), the need for novel *Bt* toxins will likely intensify, if *Bt*-cottons continue to be used over such large acreages. Continued evaluations, such as those reported here, will be necessary under more conditions and more insect pressure before "varietal" performance and gene efficacy can be assessed adequately.

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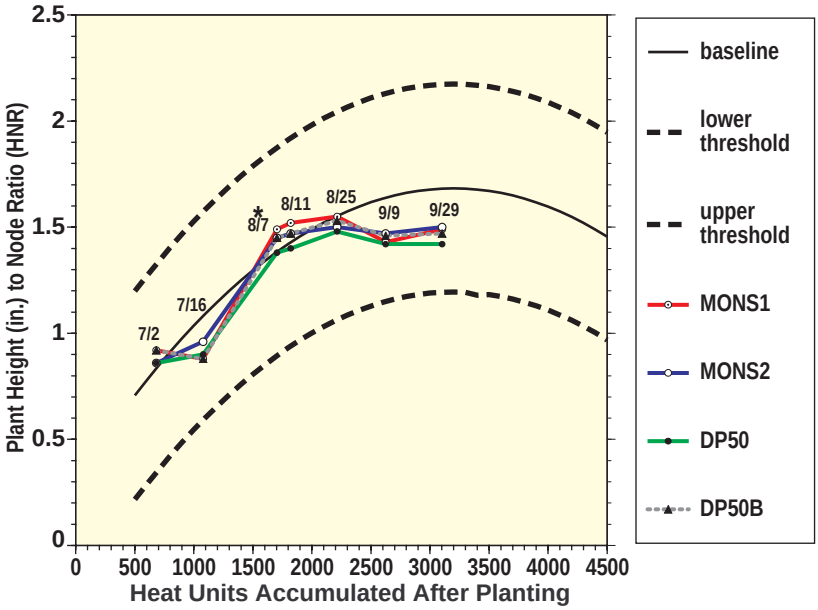

Figure 1. Plant height (in) to node ratios (HNR) in relation to heat units after planting. Average responses (baseline) with threshold levels for upland cotton in Arizona are established by Silvertooth & Norton (1998). Dates marked with an '*' have a significant variety effect ($P < 0.10$). The two test lines (MONS1, MONS2) developed similarly to their recurrent parent varieties (DP50, DP50B) and well within the thresholds for development of Arizona cotton varieties.

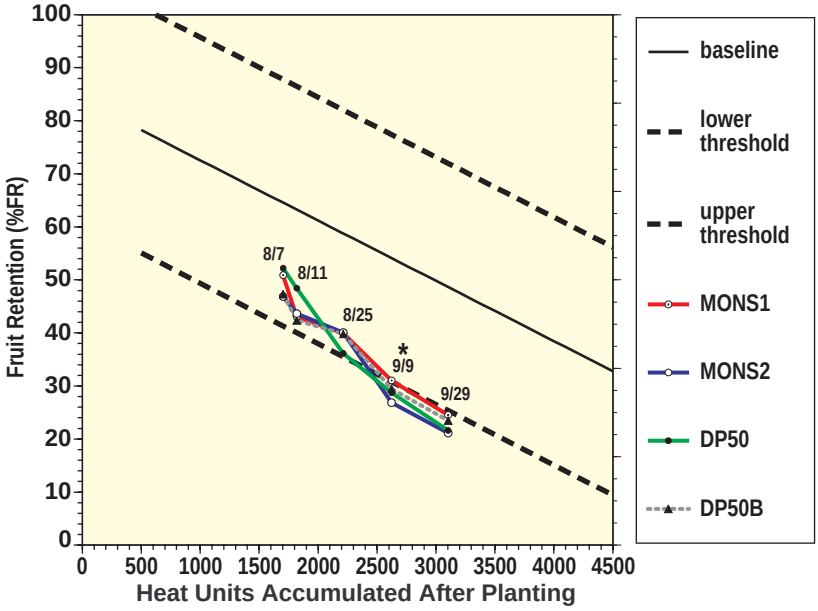

Figure 2. Fruit retention (% of first two fruiting sites retained on all branches; %FR) in relation to heat units after planting. Average responses (baseline) with threshold levels for upland cotton in Arizona are established by Silvertooth & Norton (1998). Dates marked with an '*' have a significant variety effect ($P < 0.10$). The two test lines (MONS1, MONS2) had levels of %FR similar to their recurrent parent varieties (DP50, DP50B), though all varieties were somewhat lower than normal thresholds of %FR for Arizona cotton varieties. The low %FR was due to heavy and persistent *Lygus* infestations.

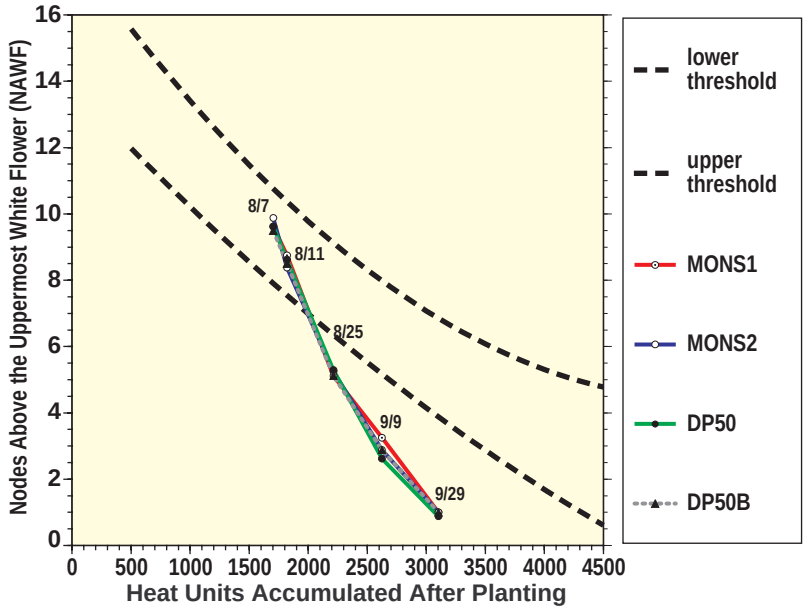

Figure 3. Number of nodes above the uppermost white flower (NAWB) in relation to heat units after planting. Upper and lower threshold levels for upland cotton in Arizona are established by Silvertooth & Norton (1998). The two test lines (MONS1, MONS2) moved towards cutout at the same rate as their recurrent parent varieties (DP50, DP50B).

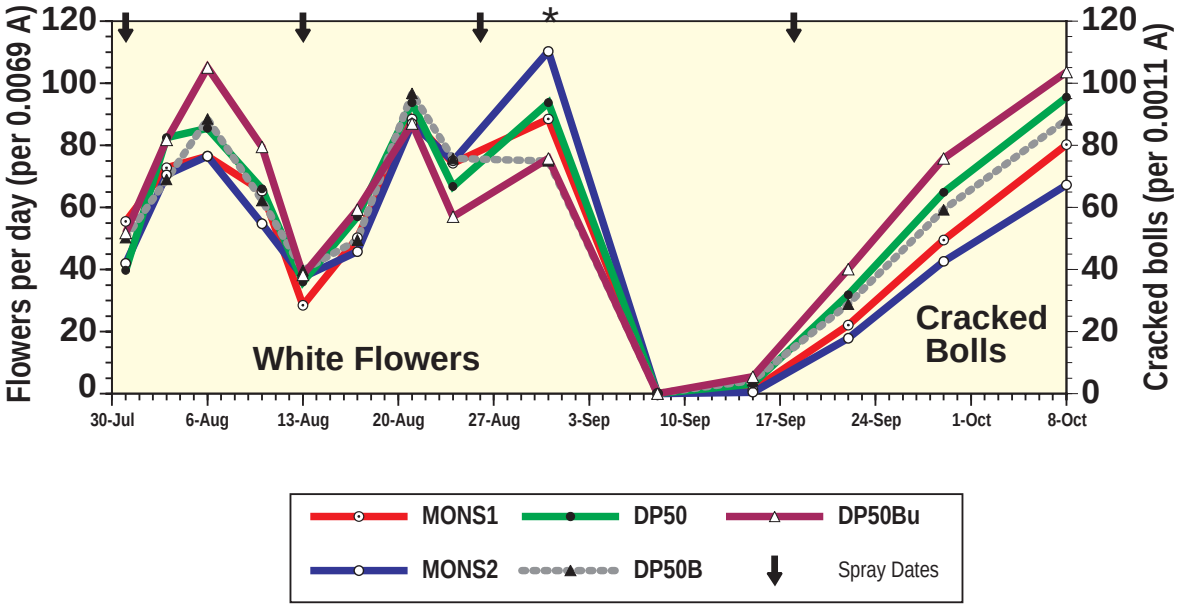

Figure 4. Number of new flowers per day and, later, of cracked bolls per date for the season. Dates marked with an '*' have a significant variety effect ($P < 0.10$). The two test lines (MONS1, MONS2) developed similarly to their recurrent parent varieties (DP50, DP50B). MONS2 had significantly more flowers on one date.

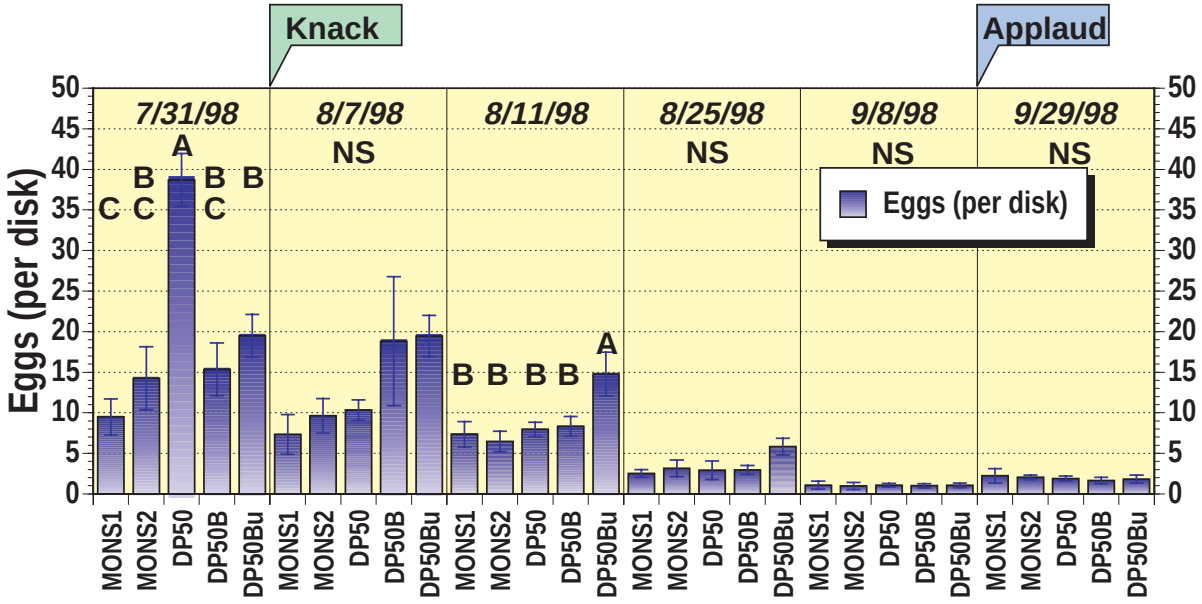

Figure 5. SWF eggs (per 3.88 cm^2 disk) by variety. Two SWF-specific sprays (Knack & Applaud) were timed as shown above. Bars not sharing a letter are significantly different by orthogonal contrasts (P≤0.05). Initially (7/31), DP50 harbored more eggs than the other lines most likely as a result of a thinner plant population. Later (8/11), DP50Bu had higher egg numbers than the other lines. DP50Bu did not receive both parts of a non-pyrethroid combination that was made on the other varieties.

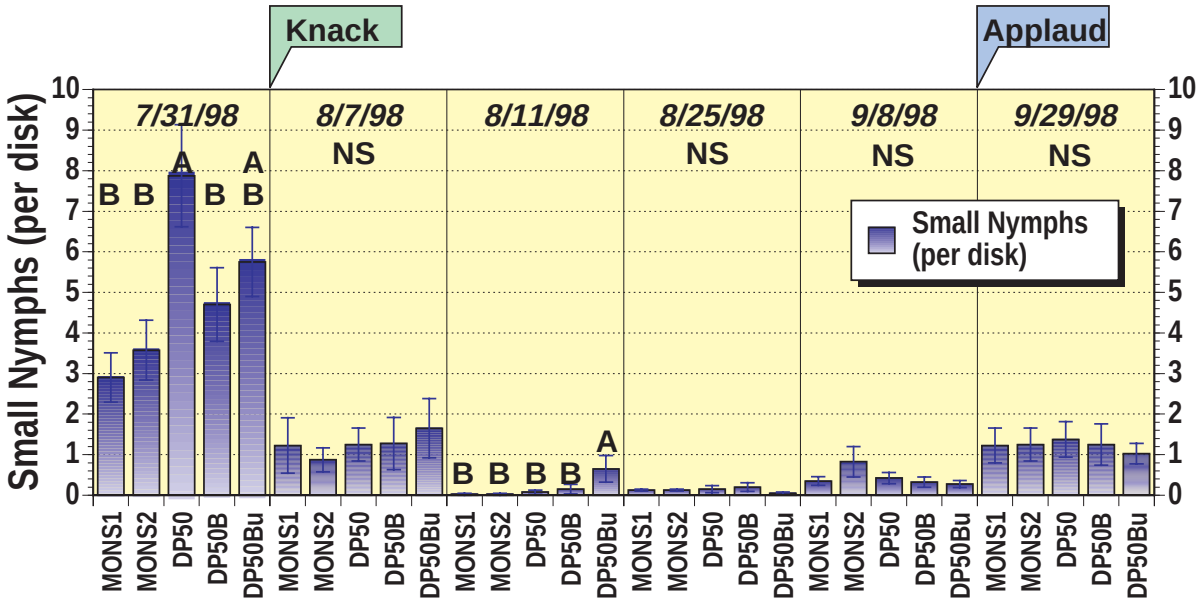

Figure 6. SWF small nymphs (instars 1 & 2 per 3.88 cm^2 disk) by variety. Two SWF-specific sprays (Knack & Applaud) were timed as shown above. Bars not sharing a letter are significantly different by orthogonal contrasts (P≤0.05). Small nymph numbers followed the same trends in differences on two dates (7/31 & 8/11) as the egg numbers. Season-long, the two test lines (MONS1 & MONS2) did not differ significantly from their recurrent parent, DP50B.

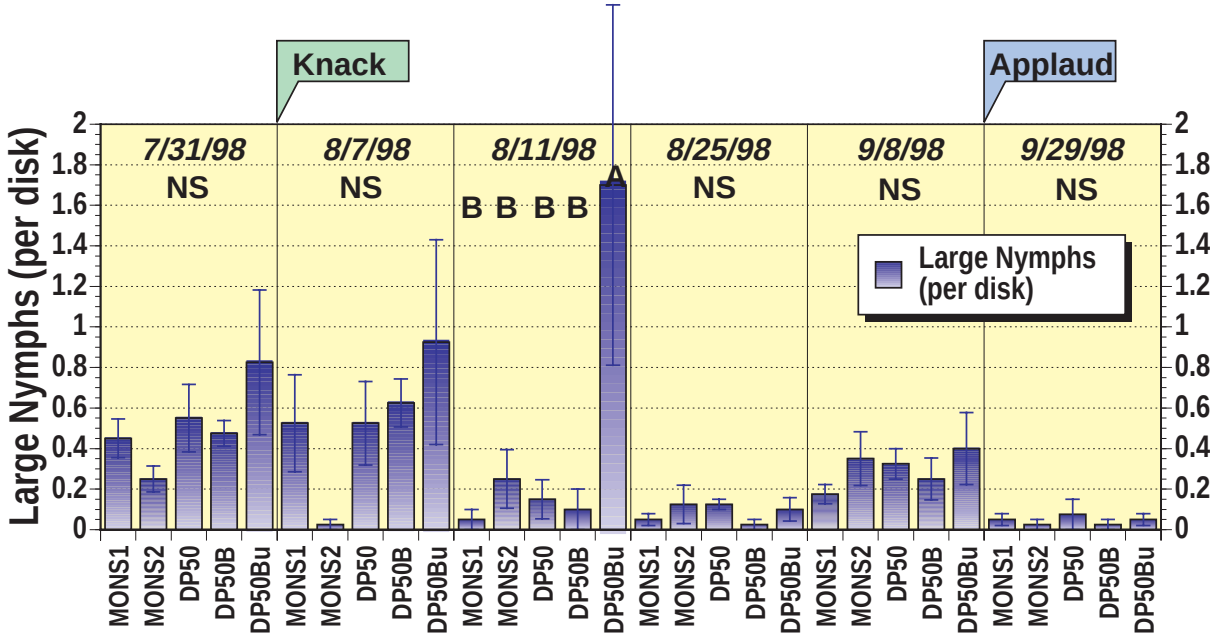

Figure 7. SWF large nymphs (instars 3 & 4 per 3.88 cm^2 disk) by variety. Two SWF-specific sprays (Knack & Applaud) were timed as shown above. Bars not sharing a letter are significantly different by orthogonal contrasts (P≤0.05). Large nymph numbers followed the similar trends in differences on one date (8/11) as the egg and small nymph numbers. Season-long, the two test lines (MONS1 & MONS2) did not differ significantly from their recurrent parent, DP50B.

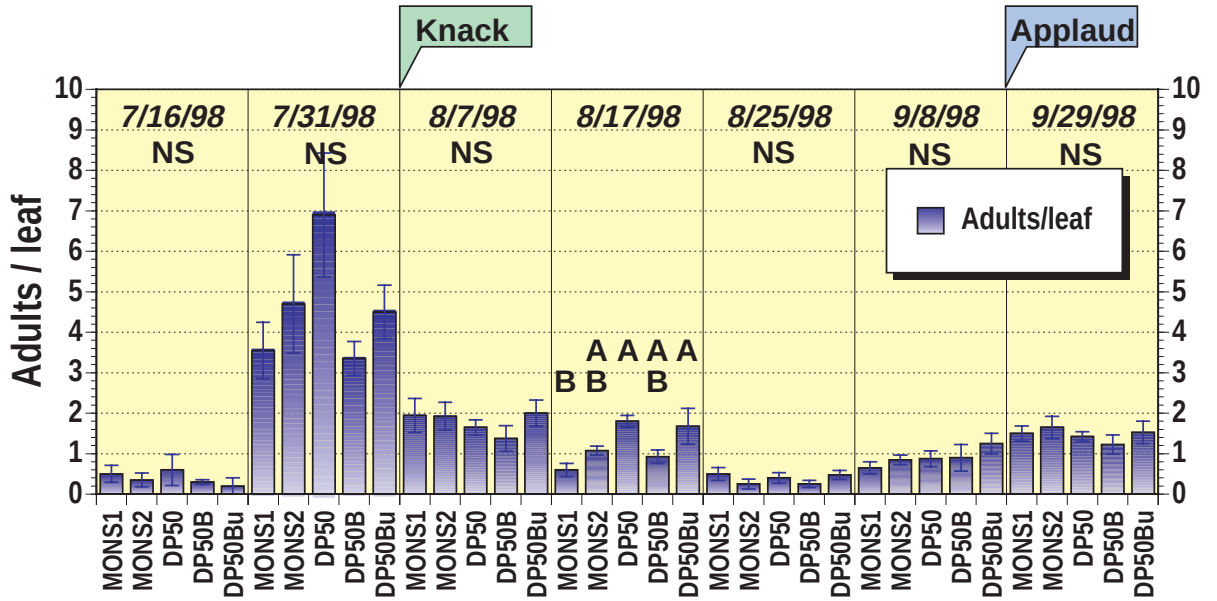

Figure 8. SWF adults (per leaf) by variety. Two SWF-specific sprays (Knack & Applaud) were timed as shown above. Bars not sharing a letter are significantly different by orthogonal contrasts (P≤0.05). Season-long, the two test lines (MONS1 & MONS2) did not differ significantly from their recurrent parent, DP50B.

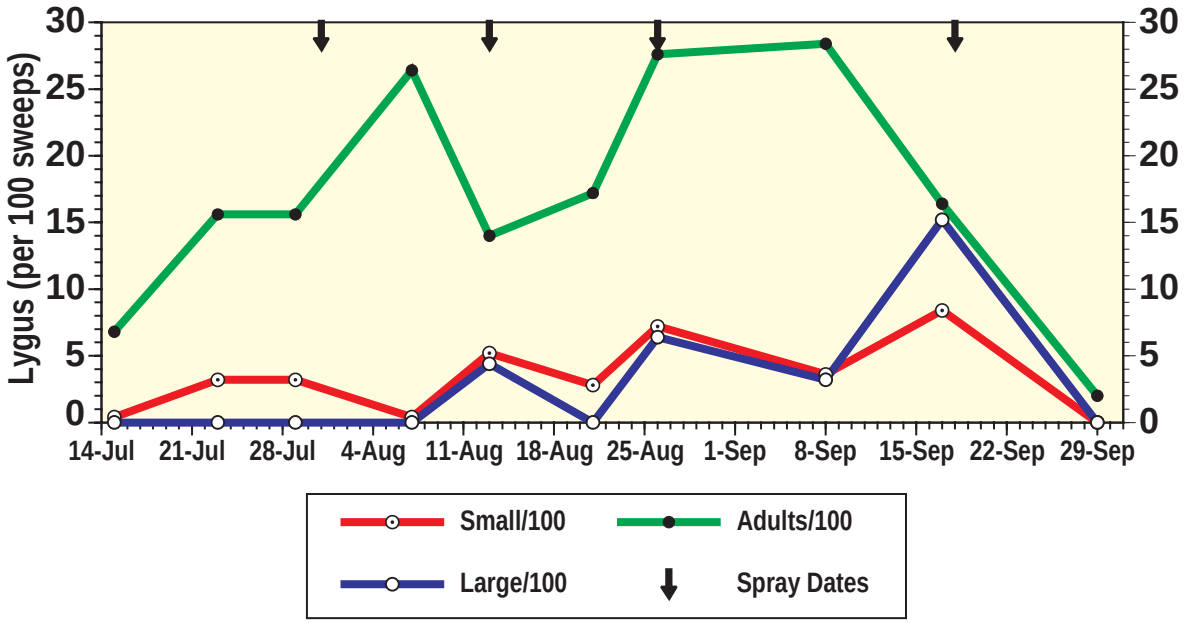

Figure 9. *Lygus* bugs (per 100 sweeps) including small (instars 1–3) and large (instars 4 & 5) nymphs, and adults monitored in buffer areas surrounding the experiment. A total of 4 *Lygus*-specific sprays were made through the course of the season as indicated by arrows. Adult numbers were refractory to the sprays, while nymph numbers consistently declined after spraying. A threshold of 15–20 total *Lygus* per 100 sweeps (with nymphs present) was reached on 23 July. Threshold levels were exceeded through most of the season until 29 September.

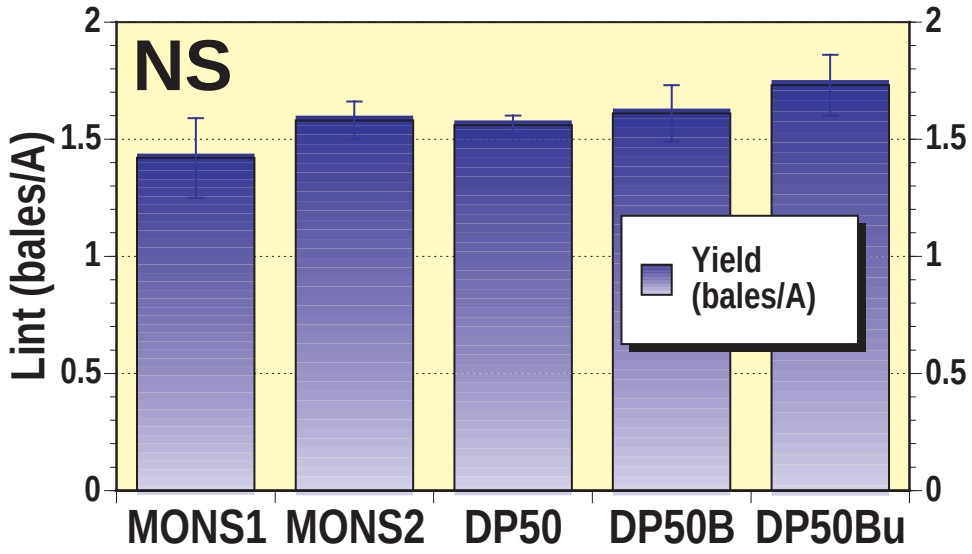

Figure 10. Lint yield (480 lb bales/A) by variety. There were no significant differences among varieties in yield ($P > 0.10$).

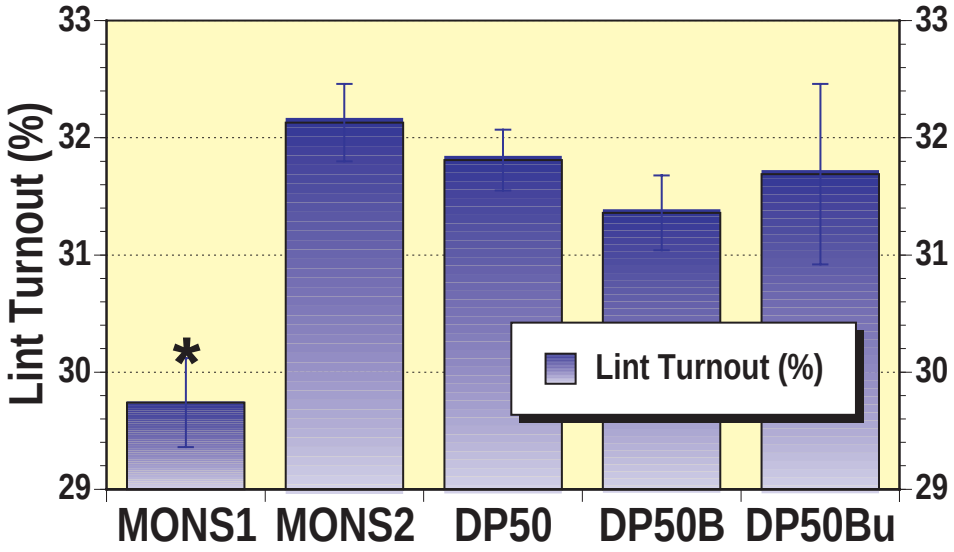

Figure 11. Lint turnout (%) by variety. Each plot was machine-picked and ginned in MAC's scaled version of a commercial gin. Turnouts were similar among varieties, except for MONS1 which had a significantly lower lint turnout ($P < 0.05$).