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Research

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Field and Forage Crops

Lethal and sublethal effects of cotton expressing single and pyramided proteins of *Bacillus thuringiensis* (Bt) on *Helicoverpa armigera* (Lepidoptera: Noctuidae), *Spodoptera litura* (Lepidoptera: Noctuidae), and *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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The susceptibility to proteins from *Bacillus thuringiensis* (Bt) can vary among lepidopteran pest species. While Bollgard 3 cotton (BG3) effectively controls the primary pest *Helicoverpa armigera* (Hübner) in Australia, its effectiveness against other pests, such as *Spodoptera litura* (Fabricius) and *Spodoptera frugiperda* (J. E. Smith), is unknown. This laboratory study assessed the survival and development of *H. armigera*, *S. litura,* and *S. frugiperda* larvae when fed foliage from a non-transgenic cotton variety (CC) and 3 transgenic cotton varieties: Bollgard (BG1) expressing Cry1Ac, Bollgard II (BG2) expressing Cry1Ac and Cry2Ab, and Bollgard 3 (BG3) expressing Cry1Ac, Cry2Ab, and Vip3A. Pyramided Bt cotton had greater negative effects on survival and development of all species compared with CC or BG1. The proportion of *H. armigera* that eclosed as adults was very low when larvae fed on BG2 or BG3 compared with BG1. Eclosion rates of *S. litura* and *S. frugiperda* on BG3 were much lower compared with BG2 and BG1. This study demonstrates that BG3 has greater efficacy against a wider lepidopteran pest complex compared with previous Bt cotton products. Despite efficacy in the laboratory, *S. litura* larvae are reported to be surviving in BG3 fields, suggesting other factors are influencing field efficacy. As BG3 production expands across tropical northern Australia, preserving the susceptibility of *S. litura* and *S. frugiperda* to BG3 proteins is crucial. This study identifies the need for further research on field survival and resistance management strategies for secondary pest species.

Keywords: Old World bollworm, fall armyworm, Bollgard, tropical cotton production

Introduction

Transgenic crops which express insecticidal proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) are relied upon for pest management across the world (Fitt 2003a, Bravo et al. 2011, Naranjo 2011, Wilson et al. 2018, Horikoshi et al. 2021). However, the susceptibility of lepidopteran species to Bt proteins varies (Reisig et al. 2021, Tay et al. 2022), presenting challenges as Bt crops are introduced into new regions, where secondary lepidopteran pests are also exposed to Bt proteins (Reisig et al. 2021).

In Australia, *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) are major economic pests of cotton, with larvae damaging all plant parts, particularly fruiting structures (Downes and Mahon 2012). Bt cotton has provided the foundation for sustainable cotton production (Wilson et al. 2018), with 3 Bt cotton products expressing different Bt protein combinations consecutively released to specifically target *H. armigera* and *H. punctigera* (Knight et al. 2021). Bollgard, marketed as Ingard in Australia, and expressing only Cry1Ac, was released in 1996 (Fitt, 2003a); however, protein expression was variable

Table 1. Cotton types tested, their respective Bt traits, and supplier for experiments on *H. armigera*, *S. litura,* and *S. frugiperda*.

Cotton type	Variety	Bt proteins expressed and traits	Supplier
Non-transgenic cotton (CC)	Sicot 620[a]	No insecticidal or herbicide resistance traits	Cotton Seed Distributors, Wee Waa NSW
Bollgard (BG1)	Breeding line[b]	Cry1Ac + Roundup Ready (CP4 EPSPS)	Commonwealth Scientific and Industrial Research Organisation (CSIRO), Narrabri NSW
Bollgard II (BG2)	Breeding line[c]	Cry1Ac + Cry2Ab + Roundup Ready (CP4 EPSPS)	Bayer Crop Science, Toowoomba QLD
Bollgard 3 (BG3)	Sicot 748B3F[a]	Cry1Ac + Cry2Ab + Vip3A + Roundup Ready (CP4 EPSPS)	Cotton Seed Distributors, Wee Waa NSW

[a]Commercially available to Australian producers.
[b]Not commercially available to Australian producers; breeding line produced to express Cry1Ac protein and CP4 EPSPS gene.
[c]Not commercially available to Australian producers; breeding line produced to express Cry1Ac and Cry2Ab proteins, and CP4 EPSPS gene.

between different plant structures and temporally, with expression declining during the boll maturation period (Fitt et al. 1994, Constable et al. 1998). In particular, the decline in expression over time necessitated insecticide applications in Bollgard from mid-season onwards for control of *Helicoverpa* spp. larvae (Doyle et al. 2002, Pyke 2003). In 2003, Bollgard was replaced by Bollgard II, expressing Cry1Ac and Cry2Ab (Fitt 2003b, Knight et al. 2013). Bollgard II was highly efficacious, providing season long expression of Cry2Ab and reducing insecticide use for *Helicoverpa* spp. by more than 90% (Knight et al. 2021). However, due to concerns about the ability of *H. armigera* to evolve resistance, particularly late in the season when only Cry2Ab is expressed at efficacious levels, Bollgard II was superseded by Bollgard 3 in 2016 (Knight et al. 2021). Bollgard 3 expresses Cry1Ac, Cry2Ab, and Vip3A, and the expression of Vip3A and Cry2Ab is maintained throughout the crop cycle, offsetting the risk associated with the decline of Cry1Ac expression during boll filling (Knight et al. 2021).

In Australia's tropical regions, cotton production is expanding due to the high control efficacy of Bollgard 3 under a more diverse and abundant lepidopteran pest complex (Strickland et al. 2003) than in temperate and sub-tropical areas where cotton is typically grown. In addition to *H. armigera*, the tropical pest complex of cotton includes *H. punctigera*, *Spodoptera litura* (Fabricius), and *Pectinophora gossypiella* (Saunders) (Strickland et al. 2003). *Spodoptera frugiperda* (J.E. Smith) established in Australia in 2020 (Piggott et al. 2021) and is now well-established in the tropical and sub-tropical production areas of northern Australia. This species is considered a pest of transgenic cotton in Brazil and the United States, and foliar-applied insecticides are sometimes required to control larvae (Barros et al. 2010, Hardke et al. 2015, Yang et al. 2022).

The availability of Bollgard 3 has renewed grower interest in tropical cotton production in Australia, and it is viewed as a potential solution to previous difficulties with lepidopteran pest control (Phillip 2019). Among the species complex in northern Australia, *S. litura* and *S. frugiperda* potentially pose the greatest risk for tropical cotton production due to high populations during the summer wet season (Strickland et al. 1998, 2003) when the crop is grown (Grundy et al. 2012). Since Bollgard 3 cotton production commenced, *S. litura* larvae of all instars have been observed intermittently surviving in fields across northern Australia. *Spodoptera frugiperda* egg masses have also been found on cotton plants in Kununurra in northern Western Australia (Spafford, personal observation). Additionally, evidence from overseas suggests that *S. frugiperda* has the propensity for field-evolved resistance to Bt proteins (Storer et al. 2010, Huang et al. 2014, Yang et al. 2017), while *S. litura* has demonstrated laboratory-evolved resistance to Bt proteins (Barkhade and Thakare 2010).

The control efficacy of Bollgard and Bollgard II against *H. armigera* has been well documented in Australia (Daly and Fitt 1998, Doyle et al. 2002, Fitt 2003b, Lu et al. 2011), but no such information is available for the efficacy of Bollgard 3 against Australian populations of *S. litura* and *S. frugiperda* and how this compares to efficacy against *H. armigera*. Such data would be valuable for informing pest management strategies as Bt cotton production expands into new geographic regions in Australia.

The objective of this study was to determine the survival, growth, and development of *S. litura*, *S. frugiperda*, and *H. armigera* feeding on non-Bt cotton and Bt cotton that either expressed Cry1Ac alone or expressed pyramided Bt proteins. Our study aimed to provide a better understanding of the relative susceptibility of a laboratory population for each pest species to Bt cotton and how pyramided proteins contribute to pest mortality. The pest management risks for Bt cotton production in northern Australia and elsewhere in the world, where similar pest complexes exist, can then be better defined.

Materials and Methods

Insects

Neonate larvae (< 24-h old) from laboratory cultures of *S. litura*, *S. frugiperda,* and *H. armigera* were used in all experiments. Laboratory cultures were established from 2020 to 2021 field collections of *S. litura* larvae from crops in Kununurra, Western Australia (–15.65°, 128.70°), *S. frugiperda* larvae from crops in Walkamin, northern Queensland (–17.13°, 145.42°), and *H. armigera* from crops in Lockyer Valley, southeastern Queensland (–27.55°, 152.27°). All cultures were supplemented with field-collected specimens annually to minimize inbreeding and maintained at the Queensland Department of Primary Industries in Toowoomba in southeastern Queensland. Similar rearing methods were used for all 3 species, based on the methods of Volp et al. (2022) (Supplementary Table S1). The cultures were maintained in an environmentally controlled room (25 ± 2 °C, 12:12-h light:dark cycle, 60% relative humidity).

Cotton Plants

Cotton types expressing no Bt protein (control; CC), a single Bt protein (Cry1Ac; BG1), 2 Bt proteins (Cry1Ac and Cry2Ab; BG2), or 3 pyramided Bt proteins (Cry1Ac, Cry2Ab, and Vip3A; BG3) were used in the experiment (Table 1). The term "cotton types" referred to all 4 cotton types tested, while "Bt cotton" referred to plants expressing Bt proteins. All cotton types share a common genetic background and have a similar growth habit (Stiller, personal communication). While the Bt cotton also expressed a glyphosate-resistant

trait, the control cotton did not. Previous research demonstrated that glyphosate-tolerant crops had no detrimental effects on non-target organisms, including insects (Carpenter 2001, Talyn et al. 2019).

Plants were grown in a 2:1:1 mix of Searles Premium potting mix, sand, and perlite, with slow-release fertilizer (NPK 15.3: 2: 12.6). Plants were germinated and grown for 5 wk in 1.6-L pots in environmentally controlled rooms (29 ± 2 °C, 12:12-h light:dark cycle, 55% relative humidity) before being transferred to a controlled temperature glasshouse (27 ± 4 °C) and natural photoperiod for the remainder of their growth and development. Plants were watered regularly and no additional fertilizer was applied. The experiment required flowering cotton plants (approximately 65 to 75 d after sowing). Weekly plantings were undertaken to ensure continuity of plant material for experimentation.

Neonate Survival on Different Cotton Types

The survival of *H. armigera*, *S. litura*, and *S. frugiperda* larvae was assessed when fed on leaf material collected from flowering CC, BG1, BG2, and BG3 cotton plants. Bioassays were conducted as part of a large experiment designed to compare the 12 factorial combinations of cotton type × species. Leaf discs (10-mm diameter) were cut from fully expanded mid-canopy leaves and placed in 22-ml food-grade plastic containers with a 2% water-agar base to maintain leaf moisture. Newly hatched neonate larvae were selected without bias for the experiments. Using a fine paint brush, a neonate larva was placed onto a leaf disc in each container and secured with a lid. One hundred neonates were prepared this way for each cotton type × species treatment during a bioassay, with each larva considered a replicate. The experiment was conducted across 4 bioassays on 28 October and 11 November 2022, and 20 and 23 January 2023. *Helicoverpa armigera* and *S. litura* larvae were evaluated in all 4 bioassays while *S. frugiperda* larvae were only evaluated in the final 2 bioassays. Insects were maintained in the laboratory under environmentally controlled conditions (28 ± 2 °C, 12:12-h light:dark cycle, 60% relative humidity). Insect survival was assessed daily from day 3 until day 10. Larvae were considered dead if they failed to respond to prodding with a fine paint brush, based on the mortality criteria outlined by Bird and Drynan (2023). Leaf material was replaced with fresh leaf discs every 3 d or sooner, if needed, ensuring continual availability of edible leaf material.

Larval Growth and Development on Different Cotton Types

On day 10 of each bioassay, approximately 20 surviving larvae of the initial 100 larvae from each cotton type × species treatment were randomly selected for continued assessment of growth and development through to adult eclosion, with each larva considered a replicate. Eighty replicates were prepared per cotton type × species treatment across the 4 bioassays, with the exceptions of *H. armigera* on BG2, where 77 larvae were tested, *S. frugiperda* on CC, BG1, and BG2, where 40 larvae were tested, and *S. frugiperda* on BG3, where 12 larvae were tested. Reduced numbers of larvae were due to low larval survival to day 10 or *S. frugiperda* being evaluated in 2 bioassays. Each larva was held in a 70-ml food-grade plastic container with a 2% water-agar base and leaf material (3 × 3 cm) of the respective cotton type. Larvae were monitored daily, and larval and pupal development periods and pupal mass (measured 24 h after pupation) were recorded.

Statistical Analysis

All analyses were conducted in Genstat Version 24 (VSN International 2024). The survival of neonates of *H. armigera*, *S. litura,* and *S. frugiperda* on the 4 cotton types were compared using Kaplan Meier tests (Kaplan and Meier 1958). Survival data were right censored at day 10. Survival curves for the 12 cotton type × species treatment combinations were analyzed using a log-rank test and were reported per species.

Larval development period, the period from neonate hatching to adult eclosion, and pupal mass were analyzed using a linear mixed model framework. Cotton type, species, and their interactions were fitted as fixed terms in the models, while bioassay was included as a random term. Heterogeneous residual variance was fit for each species, using a diagonal variance structure, based on residual diagnostic plots and a significant likelihood ratio test. Predictions of the fixed effects were obtained from the model as empirical best linear unbiased estimates (eBLUEs). All linear mixed models were fitted using the linear mixed model function, whereby variance components were estimated via residual maximum likelihood (REML) (Patterson and Thompson 1971). Bonferroni tests ($P < 0.05$) were then used for multiple mean comparisons.

The proportion of individuals reaching pupation and eclosing as adults was analyzed using a generalized linear model with a binomial distribution and logit link function. The dispersion parameter was also estimated, as opposed to set at a fixed value of 1. Cotton type, species, and their interactions were fitted as factors in the model. Significance testing of the main effects and their interaction was completed using an analysis of deviance, with the deviance ratio used as the *F*-statistic. This test is analogous to an analysis of variance (Welham et al. 2014). Predictions were obtained from the regression model, which are estimated mean proportions; these data are presented as percentages for reporting. For each cotton type × species treatment, the estimated percentage of neonates that could eclose as adults was calculated by multiplying the probability of neonate survival on day 10 with the percentage of surviving larvae from day 10 that eclosed as adults.

Results

Neonate Survival on Different Cotton Types

Cotton types affected the survival of *H. armigera*, *S. litura,* and *S. frugiperda* neonates to day 10 (log-rank = 1573.4; df = 11; $P < 0.001$; Fig. 1). For all 3 species, survival was high on both CC and BG1 but low on BG3 (Fig. 1). There were no differences in larval survival among species on CC and BG1, with survival ranging from 88% to 95% on day 10. Larval survival was lower on BG2 for all species compared with CC or BG1 from day 3. There was no difference in the survival curves or survival on day 10 of *H. armigera* neonate larvae fed BG2 or BG3, with survival rates of 38% and 47%, respectively (Fig. 1A). For *S. litura* and *S. frugiperda,* survival on day 10 was lower when neonates were fed BG3 (33% and 6%, respectively) compared to when they were fed BG2 (72% and 40%, respectively). *Spodoptera frugiperda* neonate survival on BG3 showed a greater immediate decline compared to the other species, with a survival of 31% on day 3, compared with 62% and 57% for *H. armigera* and *S. litura*, respectively (Fig. 1).

Larval Growth and Development on Different Cotton Types

Larval development period was affected by species ($F = 76.2$; df = 2, 324; $P < 0.001$) and cotton type ($F = 260.1$; df = 3, 438.4; $P < 0.001$). There was also an interaction between species and cotton type ($F = 49.3$; df = 5, 355; $P < 0.001$). Larvae of all 3 species that fed on BG2 and BG3 took longer to develop than those fed on CC

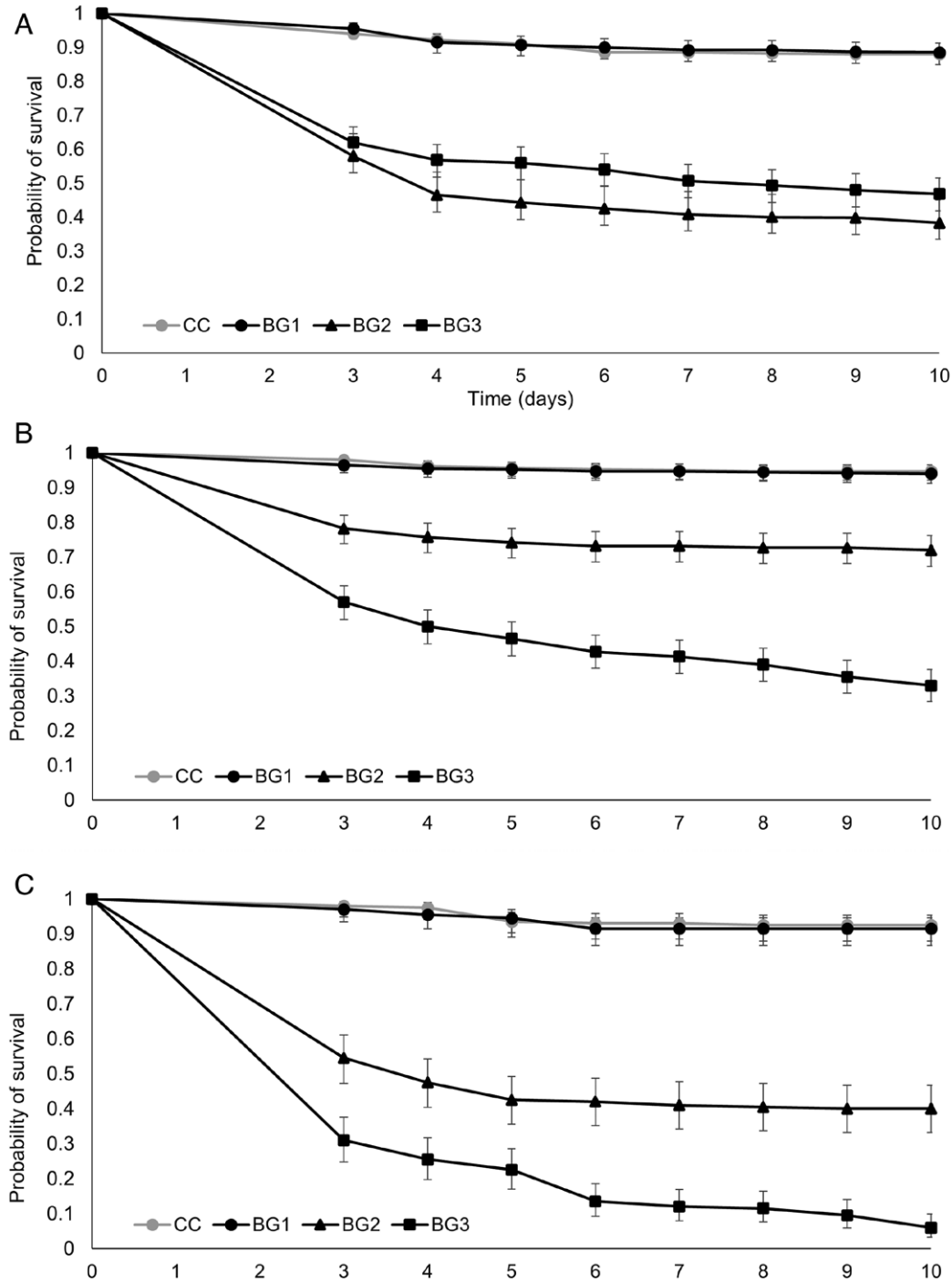

Fig. 1. Survival probability for *H. armigera* (A), *S. litura* (B), and *S. frugiperda* (C) larvae as neonates placed on CC (no Bt protein, gray circle), BG1 (Cry1Ac, black circle), BG2 (Cry1Ac and Cry2Ab, black triangle), or BG3 (Cry1Ac, Cry2Ab and Vip3A, black square) leaf discs. Error bars represent the upper and lower boundaries of the 95% confidence interval. **A) *Helicoverpa armigera* B) *Spodoptera litura* C) *Spodoptera frugiperda***

or BG1 although the duration varied (Table 2). In the case of *S. frugiperda*, larvae were unable to complete development on BG3. *Helicoverpa armigera* took 3.9 d longer to reach pupation on BG1 compared with CC. There was no difference in larval development time between *S. litura* larvae that fed on CC and BG1, whereas *S. frugiperda* larvae that fed on BG1 had a shorter larval development period by 2.6 d compared with larvae that fed on CC (Table 2).

Pupal mass was affected by species ($F = 818.6$; df = 2, 214.1; $P < 0.001$) and cotton type ($F = 56.4$; df = 3, 371.8; $P < 0.001$). There was also an interaction between species and cotton type ($F = 34.7$; df = 5, 389.8; $P < 0.001$). Pupae of *H. armigera* that developed from larvae fed on Bt cotton were 14% to 45% lighter than those that developed on CC (Table 2). The pupal masses of *H. armigera* reared on BG2 and BG3 were approximately 35% less than those that developed on BG1. There was no difference in pupal mass when *S. litura* larvae fed on CC and BG1, but when larvae fed on BG2 or BG3, pupal masses were 19 or 49% less, respectively, than pupae developing from larvae fed on CC (Table 2). *Spodoptera frugiperda*

Table 2. Mean (± SE) of larval development period, pupal mass and period between neonate hatching and adult eclosion for *H. armigera*, *S. litura*, and *S. frugiperda* fed CC (no Bt protein), BG1 (Cry1Ac), BG2 (Cry1Ac and Cry2Ab), and BG3 (Cry1Ac, Cry2Ab, and Vip3A). Means in a column followed by the same letter are not significantly different (Bonferroni tests, $P > 0.05$). *Spodoptera frugiperda* larvae did not complete development on BG3.

Pest species	Cotton type	Larval development period (days)	Pupal mass (mg)	Period from neonate hatching to adult eclosion (days)
H. armigera	CC	18.7 ± 1.1 d	383 ± 7 b	30.3 ± 1.3 de
	BG1	22.6 ± 1.1 c	328 ± 7 c	33.7 ± 1.3 c
	BG2	34.6 ± 1.2 a	212 ± 15 de	45.5 ± 1.5 a
	BG3	34.5 ± 1.5 a	216 ± 25 de	46.5 ± 1.7 a
S. litura	CC	18.8 ± 1.1 d	425 ± 7 a	27.9 ± 1.3 f
	BG1	18.2 ± 1.1 d	420 ± 7 a	27.2 ± 1.3 f
	BG2	24.4 ± 1.1 b	345 ± 8 c	33.9 ± 1.4 c
	BG3	32 ± 1.7 a	217 ± 29 de	42.6 ± 2.5 ab
S. frugiperda	CC	22.3 ± 1.1 c	193 ± 6 e	31.2 ± 1.4 d
	BG1	19.7 ± 1.1 d	218 ± 6 d	28.8 ± 1.4 ef
	BG2	25.7 ± 1.2 b	198 ± 6 de	35.2 ± 1.4 bc
	BG3	-	-	-

larvae fed on BG1 developed into pupae that were 13% heavier than those that developed on CC (Table 2). Larvae of *S. frugiperda* fed on BG2 had similar mass to those that developed on CC, and those that fed on BG3 were unable to successfully pupate.

The period from neonate hatching to adult eclosion was affected by species ($F = 163.8$; df = 2, 311.6; $P < 0.001$) and cotton type ($F = 184.1$; df = 3, 381; $P < 0.001$). The interaction between species and cotton type also affected the period from neonate hatching to adult eclosion ($F = 32.8$; df = 5, 337.6; $P < 0.001$). *Helicoverpa armigera* larvae that fed on Bt cotton took longer, 3.4 (BG1) to 16.2 d (BG3), to eclose compared with larvae fed on CC (30.3 d). There were no differences in developmental duration between *H. armigera* larvae that fed on BG2 or BG3 (Table 2). For *S. litura*, there was no difference in the duration from neonate hatching to adult eclosion when fed on CC or BG1, whereas BG2 and BG3 increased this duration by 6 and 14.7 d, respectively, compared with larvae developing on CC (Table 2). *Spodoptera frugiperda* larvae that fed on BG1 had a shorter developmental duration, by 2.4 d, than larvae that fed on CC. Larvae of *S. frugiperda* that fed on BG2 took 4 d longer to reach adult eclosion than larvae feeding on CC.

For surviving larvae (ie those that reached day 10), the interaction between species and cotton type affected the percentage of larvae that could successfully pupate ($F = 12.8$; df = 6, 756; $P < 0.001$) and eclose as adults ($F = 13.6$; df = 6, 757; $P < 0.001$) (Fig. 2). The dispersion parameter estimated for larvae that could successfully pupate and eclose as adults was 0.37 and 0.51, respectively. The analysis of deviance tables are provided in Supplementary Table 2 and 3. All species had a higher percentage of larvae successfully pupating and eclosing as adults on CC and BG1 cotton, ranging from 89% to 99% (Fig. 2). For *H. armigera*, the percentage pupating on BG2 and BG3 decreased to 16% (12 out of 77 individuals) and 5% (4 out of 80 individuals), respectively (Fig. 2A). The percentage of *H. armigera* that eclosed as adults on BG2 was 13% (10 out of 77 individuals), while all larvae that successfully pupated on BG3 (5%) eclosed as adults (Fig. 2B). For *S. litura* and *S. frugiperda*, 90% of surviving larvae could successfully pupate on BG2 (72 out of 80 individuals and 36 out of 40 individuals, respectively). However, only 78% and 81% of individuals eclosed as adults, for *S. frugiperda* and *S. litura*, respectively (31 out of 40 individual and 65 out of 80 individuals) (Fig. 2B). Only 5% of *S. litura* larvae (4 out of 80 individuals) were able to successfully pupate on BG3, with 2.5% eclosing as adults (2 out of 80 individuals) (Fig. 2). No *S. frugiperda* larvae were able to successfully pupate on BG3.

The estimated percentage of neonates that could eclose as adults on cotton expressing no or different combinations of Bt proteins was highly variable (Table 3). All species experienced low survival on BG3, with 2.3% and 0.8% of *H. armigera* and *S. litura* neonates, respectively, successfully eclosing as adults, while no *S. frugiperda* neonates eclosed as adults (Table 3). *Helicoverpa armigera* experienced low survival on BG2, with 5% of neonates eclosing as adults. However, for *S. litura* and *S. frugiperda*, neonate survival to adult eclosion increased on BG2 to 58.5% and 31.0%, respectively (Table 3). All species were predicted to have higher neonate to adult eclosion survival on CC and BG1, ranging from 78.6% to 91.4% (Table 3).

Discussion

Our study demonstrated that BG3 cotton caused the highest mortality in all 3 species, with very few to no individuals successfully eclosing as adults (Fig. 1, Table 3). For *H. armigera*, BG3 did not significantly increase the already high mortality provided by BG2 (Fig. 1, Table 3). In contrast, the survival of *S. litura* and *S. frugiperda* was significantly lower on BG3 compared with BG1 and BG2 (Fig. 1, Table 3). Although this study did not assess individual proteins in isolation, the increased mortality recorded on BG3 for *S. litura* and *S. frugiperda* is likely due to the inclusion of Vip3A in the Bt trait package. This concurs with surface overlay toxin bioassays conducted by Tay et al. (2022) that demonstrated *S. litura* and *S. frugiperda* were more susceptible to Vip3A than Cry1Ac and Cry2Ab. The addition of Vip3A to the Bt trait package in BG3 was anticipated to provide greater protection against a range of lepidopteran pests including *Spodoptera* species (Knight et al. 2021). Consequently, BG3 was expected to be better-suited for production in northern Australia (Knight et al. 2021), with its more diverse and abundant lepidopteran pest complex (Strickland et al. 2003), a conclusion supported by our study.

Larval survival across the 3 species was similar on both CC and BG1 cotton (Table 3). Despite Australian populations of *H. armigera* remaining susceptible to Cry1Ac (CottonInfo 2024) and the high efficacy of BG1 until boll set and senescence (Fitt et al. 1994, 1998), our study showed no differences in *H. armigera* survival on CC and BG1. This result mirrors the inconsistent and often variable efficacy of cotton varieties expressing Cry1Ac in Australia, where larval survival has often required the use of foliar-applied synthetic insecticides from mid-season onwards (Doyle et al. 2002, Pyke 2003). Variable expression of Cry1Ac has also been reported in soybeans (Yu et al.

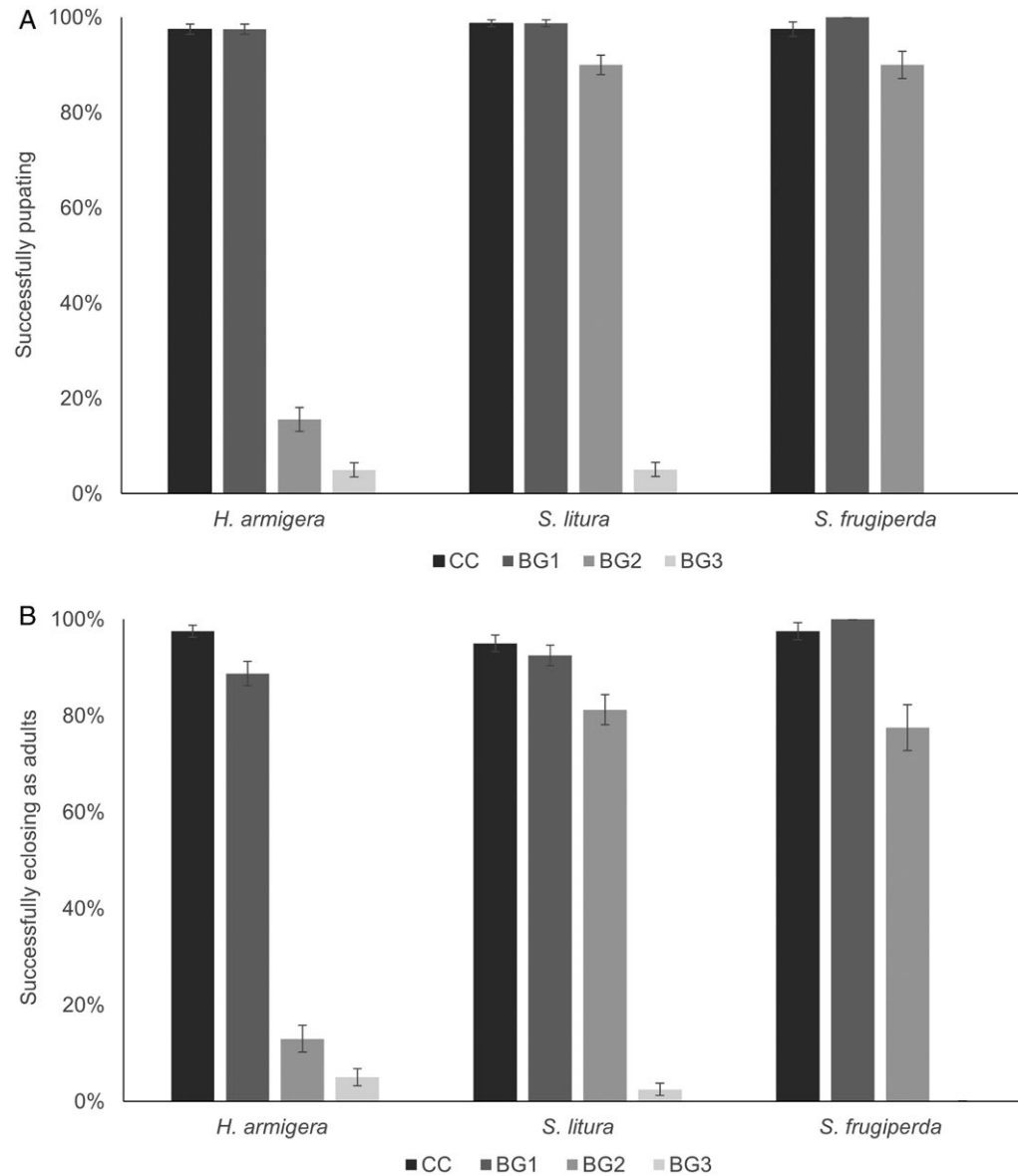

Fig. 2. Mean percentages (+ SE) of surviving *H. armigera*, *S. litura,* or *S. frugiperda* that pupated (A) and reached adult eclosion (B) after 10 days of feeding exposure to leaf discs of CC (no Bt protein), BG1 (Cry1Ac), BG2 (Cry1Ac and Cry2Ab), or BG3 (Cry1Ac, Cry2Ab and Vip3A). No *S. frugiperda* successfully pupated on BG3. **A) Pupated B) Eclosing as adults**

Table 3. Estimated proportion of neonates that would successfully eclose as adults for *H. armigera*, *S. litura,* and *S. frugiperda* when feeding on 4 cotton types: CC (no Bt protein), BG1 (Cry1Ac), BG2 (Cry1Ac and Cry2Ab), or BG3 (Cry1Ac, Cry2Ab and Vip3A). This was calculated using probability of larval survival on day 10 (Fig. 1) multiplied by the percentage of larvae survivors (from day 10) that successfully eclosed as adults (Fig. 2).

	Proportion of neonates eclosing as adults (%)			
Species	CC	BG1	BG2	BG3
H. armigera	85.8	78.6	5.0	2.3
S. litura	90.0	87.0	58.5	0.8
S. frugiperda	90.2	91.4	31.0	0.0

2013, 2014) and rice (Zhang et al. 2011) at different phenological stages. Concerns over the inconsistent efficacy of Cry1Ac in cotton and the risk of resistance development led to the rapid introduction of BG2 varieties in Australia, which expressed dual proteins to better manage resistance (Roush 1998) and offer greater crop protection (Knight et al. 2013, 2021).

Dual protein BG2 significantly reduced survival across all species, particularly for *H. armigera*, compared with survival on CC and BG1 (Fig. 1, Table 3). This is consistent with other studies that demonstrated BG2 provided improved control efficacy against a range of lepidopteran pests, including *H. armigera*, *S. frugiperda*, *S. litura*, *Spodoptera exigua* (Hübner), and *Helicoverpa zea* (Boddie), compared with BG1 (Adamczyk et al. 2001, 2008, Gore et al. 2001, Spafford et al. 2007, Downes and Mahon 2012, Ramanjali 2014).

When larvae survived on Bt cotton, the effects on their growth and development varied depending on species and the combination of Bt proteins (Table 2). Overall, Bt cotton negatively affected key developmental attributes of larval development period, pupal mass, and period from neonate hatching to adult eclosion compared with larvae reared on CC (Table 2). These findings are consistent with other studies assessing the effect of Bt crops on various lepidopteran pest

species, including *H. armigera*, *S. litura*, and *S. frugiperda* (Gould et al. 1991, Adamczyk et al. 2001, Fitt 2003b, Bilbo et al. 2018, Barcellos et al. 2023). However, there were some exceptions. For example, while larval survival across all species was generally high on BG1, *H. armigera* had increased development times and were smaller pupae on BG1 than on CC (Table 2). For *S. litura* larvae fed BG1, development times and pupal mass were similar to those on CC (Table 2), aligning with previous studies on *Spodoptera cosmioides* (Walker) showing no significant differences between larvae fed non-Bt and Bt cotton or soybean cultivars expressing Cry1Ac (Bernardi et al. 2014, Rabelo et al. 2020, Lutz et al. 2023). Larvae of *S. frugiperda* feeding on BG1 developed faster and into heavier pupae compared with those fed CC (Table 2), contrary to previous findings where *S. frugiperda* feeding on Bt soybean expressing Cry1Ac took longer to develop into pupae compared with those fed non-Bt cultivars (Bernardi et al. 2014). These differences may be attributed to factors such as host plant (Volp et al. 2022), Bt expression within the plant (Adamczyk and Sumerford 2001, Adamczyk et al. 2001), or variations in population susceptibility to different Bt proteins (Tay et al. 2022).

Our study shows, that while mortality of *S. litura* and *S. frugiperda* larvae was higher on BG2 compared to CC or BG1, mortality on BG3 was higher than on BG2 (Table 3). Despite the demonstrated high efficacy of BG3 against *S. litura* using glasshouse grown plants, larvae of all instars are frequently observed surviving in BG3 cotton fields in northern Australia (Grundy, personal observation). This suggests that, while BG3 causes greater mortality in *S. litura* compared with BG1 and BG2, other factors, such as larval behavior in the presence of Bt proteins (Luong et al. 2018, Visser et al. 2019, 2020) and variable Bt expression at different phenological stages or in different plant structures, may influence the field efficacy of BG3 (Gore et al. 2001, Dong and Li 2007, Bommireddy and Leonard 2008). This study was limited to a single population for each species, sourced from agricultural regions. Expanding future research to include field populations from across northern Australia could provide a better understanding of the widespread performance of BG3 cotton and any variation in regional population susceptibility that may also influence *S. litura* field survival. In addition, the adoption of Bt crops that express 2 or more proteins, along with refuge crops, is recommended as an effective strategy to delay the development of Bt resistance (Head and Greenplate 2012, Tabashnik et al. 2013). Thus, further research is required for *S. litura* on the genetics of resistance and insect behavior to inform potential resistance management strategies for this species.

The findings of this study highlight the importance of evaluating the effect of Bt crops on a range of lepidopteran pests, not just primary target species. Evaluating the effect of Bt crops expressing different protein combinations is essential for determining whether secondary pests could become more prominent in farming systems due to low susceptibility, as observed with *Spodoptera* species on BG1 (Bernardi et al. 2014, Rabelo et al. 2020, Lutz et al. 2023). Conversely, it is worth considering whether a Bt crop may provide high control efficacy and be better suited as a pest management tool in regions with certain lepidopteran pest complexes.

In conclusion, cotton expressing 3 Bt proteins (BG3) caused higher mortality in all 3 lepidopteran pest species tested in our study, offering a more effective Bt trait package for pest management as cotton production expands into tropical northern Australia. BG3 significantly reduces larval survival, growth, and development of *S. litura* and *S. frugiperda* larvae compared with BG1 and BG2, suggesting that Vip3A is making a considerable contribution to larval mortality. In contrast, there was no difference in the mortality of *H. armigera* larvae fed BG2 or BG3. However, despite the high efficacy of BG3 against *S. litura* demonstrated in our study, larvae are still frequently observed in fields, suggesting other factors may be influencing their survival which warrant further investigation.

Supplementary material

Supplementary material is available at *Journal of Economic Entomology* online.

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