

THE ROAD TOWARDS DISCOVERY OF A NEW PLANT EXTRACT (PLANT X) FOR MANAGING COTTON PESTS: Part 1: EFFICACY AGAINST *HELICOVERPA* SPP.

ROBERT K. MENSAH¹, NICK WATTS³, PETER GLENNIE⁴, ALISON YOUNG¹, ANGELA SINGLETON¹, LEAH AUSTIN¹, and HO DANG¹

¹ NSW Department of Primary Industries, Australian Cotton Research Institute, Locked Bag 1000, Narrabri, NSW 2390; * Corresponding author E-mail: robert.mensah@dpi.nsw.gov.au

² School of Biological Sciences, The University of Queensland, St Lucia Campus, Brisbane, Queensland 4072

³ Growth Agriculture Pty Ltd, Wee Waa, NSW

⁴ Peter Glennie & Sons, Norwood, Moree

SUMMARY

The study determined the chemical components of cotton and alternative host plants that influence acceptance or rejection of such plants by adults and larvae of *Helicoverpa* spp. Fractionalised extracts of Plant X were assessed for bioactivity towards both adult and larvae of *Helicoverpa* spp. in the laboratory. The results showed that Plant X have shown oviposition and feeding deterrence towards *Helicoverpa* spp. and high toxicity towards larvae. In addition, different modes of action of Plant X extracts have been identified. We conclude that natural chemical tools such as Plant X can be used as part of IPM to manage *Helicoverpa* spp. in cotton.

INTRODUCTION

Cotton crops in Australia like other parts of the world continue to face pest problems in both conventional and transgenic (genetically engineered) crops. Many insect species have been recorded in Australian cotton but only 6 are regarded as major pests, with another 17 considered minor pests (Hearn and Fitt, 1992; Fitt, 1994). The key pests include *Helicoverpa* spp. (*Helicoverpa armigera* and *H. punctigera*); two spotted mites (*Tetranychus urticae*); green mirid (*Creontiades dilutus*); thrips (*Thrips tabaci*); green vegetable bugs (*Nezara viridula*), silverleaf whiteflies (*Bemisia tabaci*) and aphids (*Aphis gossypii*).

Nonetheless the introduction of transgenic cotton crops and the transition from conventional to transgenic (Bt) cotton has given the cotton industry the platform to undertake a true integrated pest management (IPM) program to avoid over-reliance on synthetic insecticides on both conventional and Bt cotton crops.

One of the approaches with greater potential to revolutionise the way insect pests are managed in broadacre crops such as cotton, is the use of natural plant chemical compounds or plant extracts. The natural plant extracts or secondary plant compounds (SPCs) in general can influence the behaviour of insects by functioning as cues stimulating an insect's "interest" or deter insects from infesting a particular host plant (Rhoades and Coates 1976). Many SPCs have evolved in plants to actually protect the plants against pest infestation (Rhoades and Coates 1976). Some SPCs extracted from non-host plants and then sprayed on host plants can change the behaviour of a pest, particularly moths, which then avoid the host plant (Tingle and Mitchell 1984). Unfortunately, numerous studies into pest management has focussed on chemical compounds that kills the pest rather than behaviour modifying compounds (Tingle and Mitchell 1984, Mensah and Moore, 1999). Consequently, potentially useful compounds with more subtle modes of action that could lead to novel products have been overlooked (Mensah and Moore, 1999). Such compounds attract or repel pests over considerable distances; or stimulate or deter both feeding and egg-laying following contact. Deterrent compounds directly suppress oviposition and feeding by insects (Mensah, 1996, Mensah *et al.* 2000), they are considered more important than stimulants and in fact a deterrent effect is more commonly noted in SPCs (Bernays and Chapman 1994).

The aim of the study was to (1) identify plants that may contain chemical components that influence acceptance or rejection of such plants for oviposition and feeding by adults and larvae of *Helicoverpa* spp. and other pests, (2) determine possible insect-host interactions and efficacy of bio-active compounds or fractions of target plants, and (3) develop strategies for exploiting these bio-active compounds or fractionalized extracts of the target plant against *Helicoverpa* spp.

Methodology

Experiment 1: Responses of *Helicoverpa* spp. to different alternative crops interplanted in cotton in commercial fields

The study was conducted on a 6 ha irrigated cotton field at Norwood near Moree from 22 September 2001 to 30 March 2002. The alternative crops evaluated were lucerne, pigeon pea, sorghum, sweet corn, cotton and Plant X. Most of these crops are grown in monoculture as refuge crops in the district. Each of the crops were planted in strips, 8 m (or rows) wide and 150 m long adjacent to and separated by cotton strips 20 m (or rows) wide and 150 m long. In total four strips of each crop were planted across the cotton field. The alternative crops were planted at the same date as the cotton crop. All crops were irrigated as the same time as cotton; irrigation depended on the soil moisture level.

Visual counts of *Helicoverpa* spp. eggs and larvae on cotton and the alternative crops were carried out every fortnight throughout the cotton season. Plants were assessed in randomly selected 1 – m lengths of row of each treatment replicate, i.e a total of 4 m per treatment. Counts were separated into *Helicoverpa* eggs, very small and small larvae and medium and large larvae. Data are expressed as numbers per metre and numbers per metre per sample date for each treatment.

Experiment 2. Oviposition responses of *Helicoverpa* spp. larvae to different alternative crops in the laboratory - Cage experiment with plants

Following the results of experiment 1, potted plants of different refuge crops and cotton genotypes were arranged in a circle in a 2m x 2m x 1.5m cage, replicated in three cages. Care was taken to ensure that the potted plants of different species in the cage had comparable biomass and height. Thirty pairs of three-days- old moths were released into each cage. Egg counts were made at four days after infestation. Screening for oviposition preference was carried out among the cotton genotypes first, then three chosen cotton genotypes were tested together with other refuge crops.

Experiment 3: Determination of toxicity of plant structures to *Helicoverpa* spp. 2nd instar larvae

Following experiment 1 and 2 field and laboratory results that revealed that *Helicoverpa* spp. infestation on Plant X was significantly lower than that on cotton and other alternative crops, we investigated the efficacy of various structures of Plant X on *Helicoverpa* spp. larval feeding.

Plant X structures or parts (viz; new, middle, old leaves; whole and cut pods; green (immature), mature seeds; crushed immature seeds and crushed mature seeds) were prepared and placed individually on a filter paper moistened with 100 µl of distilled water in a petridish 12 mm in diameter.

One second instar larva of *Helicoverpa* spp. was placed on each plant structure in each petri dish and sealed. Each treatment was replicated four times. The dishes were then placed into a Labec incubator running at 25°C (±2°C) with 14 hours light / 10 hours dark and checked daily for mortalities for up to 9 days.

Control treatment using newly opened cotton leaf, square, seed as compared to Plant X's leaf, fruit and seed was set up. Larval mortality on cotton (control mortality) was used for calculation of corrected mortality $[(C-T) / (C+T)] \times 100$ at days 1-2, 3-4 and 5-9 days.

Experiment 4: Efficacy of Plant X fractions on oviposition of *Helicoverpa* spp. on cotton plants in the mesh house (No-choice test), 2004-2005

Solid phase extraction (SPE) technique was used to fractionate extracts and to provide fractions for biological assays against insects. Six various fractions (1-6) from Plant X were sequentially eluted from an SPE cartridge as solvent polarity is increased. The experiment was conducted in the mesh house at the Australian Cotton Research Institute (ACRI) from 30 December 2004 until 7 January 2005 to determine oviposition deterrent activities of fractions isolated from Plant X. The Plant X fractions tested was (1) Fraction 1 (2) Fraction 2, (3) Fraction 3, (4) Fraction 4, (5) Fraction 5 and (6) Fraction 6, (7) Crude Plant X extract (control 1) and (8) Untreated (control 2). Each treatment was replicated 4 times in a complete randomized design. Plants used in the experiment were grown in 8 cm diameter pots in black soil (from the field) and watered three times a week. The plants were fertilized once. Potted plants were kept and maintained in the mesh house which allowed a greater degree of exposure to the natural environment but protected them from potential pest infestation. Once the plants had reached the 4-true-leaf stage, 0.25mL of extract of each treatment was placed on each leaf (1 mL in one pot) and spread evenly over the surface. The plants were then covered and three mated (5 day post emergence) female moths were released into the cages. Eggs were counted three days after treatment. The Oviposition Deterrent Index (ODI) was calculated as follows: $ODI = 100 \times (C-T)/(C+T)$ where C = total eggs laid in control; T = total eggs in treated filter paper.

Experiment 5. Efficacy of Plant X fractions on the feeding of *Helicoverpa* spp. 2nd instar larvae on leaf discs of cotton plants in the laboratory (No-choice test)

The experiment was conducted in the laboratory from the 3 January 2005 until 6 January 2005 using cotton leaf in a no-choice test. *H. armigera* 2nd instar larvae were used for the feeding bioassays. The Plant X fractions tested was (1) Fraction 1, (2) Fraction 2, (3) Fraction 3, (4) Fraction 4, (5) Fraction 5, (6) Fraction 6, (7) Crude Plant X extract (control 1) and (8) Water-treated (control 2). Each treatment was replicated 4 times in a complete randomized design. A 3 day-old cotton leaves taken from plants grown in the glasshouse were used for the study. The leaves were treated with 1 mL of extract of each treatment. To prevent preference for or avoidance of the extract, 0.5mL was applied to each side of the leaf surface. The leaves were allowed to air dry in the fume hood for 1 hour.

Once air dried, a 25mm disc of each treated leaf was cut out, weighed and placed in a 55mm petri dish. The filter paper was moistened with 100 μ L of distilled water to prevent the disc from drying out. One larva of the desired size was weighed and placed into each dish. The dishes were then placed into a Labec incubator running at 25°C (\pm 2°C) with 14 hours light / 10 hours dark for 48 hours.

Forty-eight hours after treatment, both the leaf discs and the larvae were then weighed. The differences in weights of both the leaf discs and larvae before and after the experiments were calculated for each treatment and control to determine any antifeedant effect of the treatments.

Analysis of data

All experimental data were analysed using the ANOVA procedures of Instat, version 2.03 (Graphpad Instat Software Inc., San Diego, California, USA). Tukey-Kramer multiple comparison tests were used to separate the means

Results:

Experiment 1: Responses of *Helicoverpa* spp. to different alternative crops interplanted in cotton in commercial fields

Egg- and larval-densities on cotton and refuge crops

Figure 1 presents the number of *Helicoverpa* spp. eggs and larvae recorded on the refuge crops and cotton under field conditions. Among the six crops, cotton and pigeon pea were highly preferred for egg-lay and Plant X was the least preferred.

Throughout the study period (from December 2001 until March 2002) only a small number of very small and small larvae were recorded on Plant X. No medium and large larvae were recorded on this plant compared with other refuge crops tested, indicating that it caused high mortality rate to the larvae (Figure 1).

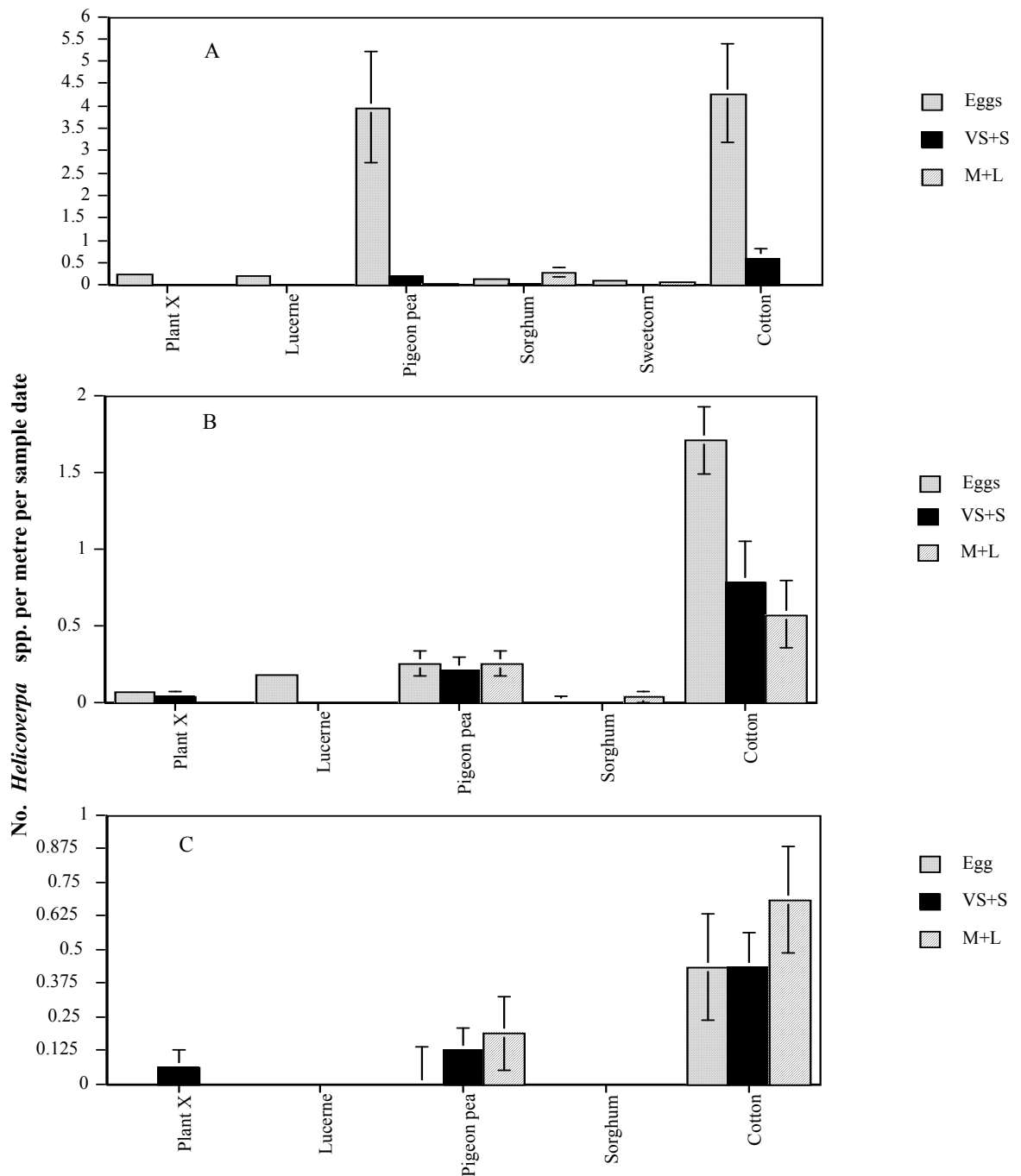


Figure 1. Number of *Helicoverpa* spp. recorded on refuge crops interplanted in cotton during early (A), middle (B) and late (C) seasons in commercial irrigated cotton farm at Norwood near Moree, 2001-2002.

Experiment 2. Oviposition responses of *Helicoverpa* spp. larvae to different alternative crops in the laboratory - Cage experiment with plants
Oviposition tests

Results of oviposition preference tests in cage recorded at 48 hours after infestation (HAI) on different cotton genotypes and refuge crops is presented in Table 1. MHR11 was the most attractive host for egg lay among the tested cotton genotypes (Table 1). Plant X was the least preferred crop (Table 1). Maize, sorghum and chick pea were more preferred than lucerne and cotton. It is noteworthy (Table 1) that Plant X proved to be significantly deterrent under

very high egg pressure (number of eggs on maize and sorghum were 1680 and 1885 per plant respectively, compared with only 18 on Plant X).

Table 1: Oviposition preference of *H. armigera* on cotton and refuge crops in cage experiment.

Crop type	Number of eggs / plant
MHR 11 (cotton)	812 bc ¹
OGF (cotton)	119 cdc
Lumein (cotton)	168 c
Plant X	18 f
Maize	1680 a
Sorghum	1886 ab
Chick pea	1388 ab
Lucerne	214 c

¹/ Means in a column followed by common letter are not significantly different

Experiment 3: Determination of toxicity of plant structures to *Helicoverpa* spp. 2nd instar larvae

Tables 2 present the mortality of *Helicoverpa* spp on different parts of Plant X. The results show that toxin(s) in Plant X are located primarily in the leaves and *H. punctigera* was found to be more susceptible to the toxin(s) in Plant X than *H. armigera* larvae and mortality caused to second stage larvae was higher than to first stage larvae. This may be due to the ability of the second stage larvae to feed more than those of the first stage. Thus the second stage ingests a more toxic dose than first larvae in the course of their feeding.

Table 2. Mortality of second instar larvae of *Helicoverpa* spp. on different structures of Plant X in the laboratory at ACRI in Narrabri, 2003

Species	Plant Structure	Part	Percent Mortality ¹			
			Day1-2	Day3-4	Day5-9	Total
<i>H. armigera</i>	Leaf	New	20.0	10.0	20.0	50.0
		Middle	10.0	30.0	0	40.0
		Old	20.0	30.0	31.1	81.1
	Whole seed	Green	31.1	0	20.0	51.1
		Mature	63.3	-	-	63.3
	Crushed seed	Green	20.0	11.1	40.0	71.1
		Mature	0	41.1	20.0	61.1

<i>H. punctigera</i>	Leaf	New	88.9	-	-	88.9
		Middle	77.8	-	-	77.8
		Old	88.9	-	-	88.9
	Whole seed	Green	12.5	0	10.0	22.5
		Mature	50.0	-	-	50.0
	Crushed seed	Green	44.4	0	10.0	54.4
Mature		11.1	62.5	-	73.6	

^{1/} Control treatment: Newly opened cotton leaf as compared to Plant X's leaf; Cotton square as compared to Plant X's pod and cotton seed as compared to plant X's seed. Larval mortality on cotton (control mortality) was used for calculation of corrected

Experiment 4: Efficacy of Plant X fractions on oviposition of *Helicoverpa* spp. on cotton plants in the mesh house (No-choice test), 2004-2005

Free choice tests conducted under laboratory conditions using *H. armigera* adult females confirm that Plant X fraction 3 and fraction 4 (Table 3) had significantly lower ($P < 0.001$) number of eggs per plant laid on them than all the other fractions tested (Table 3). Thus the two fractions may contain oviposition deterring compounds.

Table 3. No-choice oviposition test of *Helicoverpa armigera* females on filter papers treated with Plant X fractions 1-3 at ACRI in Narrabri, 2004-05

Treatments	No. eggs/ plant \pm SE	¹ Oviposition Deterrent Index (ODI)
Fraction 1	32.75 \pm 25.39 a	20.1 a
Fraction 2	41.00 \pm 14.41 a	9.1 a
Fraction 3	17.50 \pm 7.84 b	47.6 b
Fraction 4	6.50 \pm 1.19 a	42.2 a
Fraction 5	58.25 \pm 22.96 b	-56.9 b
Fraction 6	35.00 \pm 10.40 b	-37.3 b
Control (water)	49.25 \pm 17.21 a	0.0 a

Means within columns followed by the same letter are not significantly different ($P > 0.05$) (Tukey-Kramer Multiple comparison test).

¹Oviposition Deterrent Index (ODI) was calculated as follows:

$ODI = 100 \times (C - T) / (C + T)$; where C = Total eggs laid in control; T = total eggs in treated filter paper.

Experiment 5. Efficacy of Plant X fractions on the feeding of *Helicoverpa* spp. 2nd instar larvae on leaf discs of cotton plants in the laboratory (No-choice test)

In no-choice bioassays conducted using the fractions of Plant X and *H. armigera* 2nd instar larvae, leaves treated with fractions 2, 4 and 6 were consumed at lower levels and resulted in lower weight gains by the larvae compared to the other fractions and the control tested (Figure 2). Fraction 2 appears to have a stronger deterrent effect than fraction 4 and 6 so much so that the 2nd instar resulted in a weight loss (Figure 1).

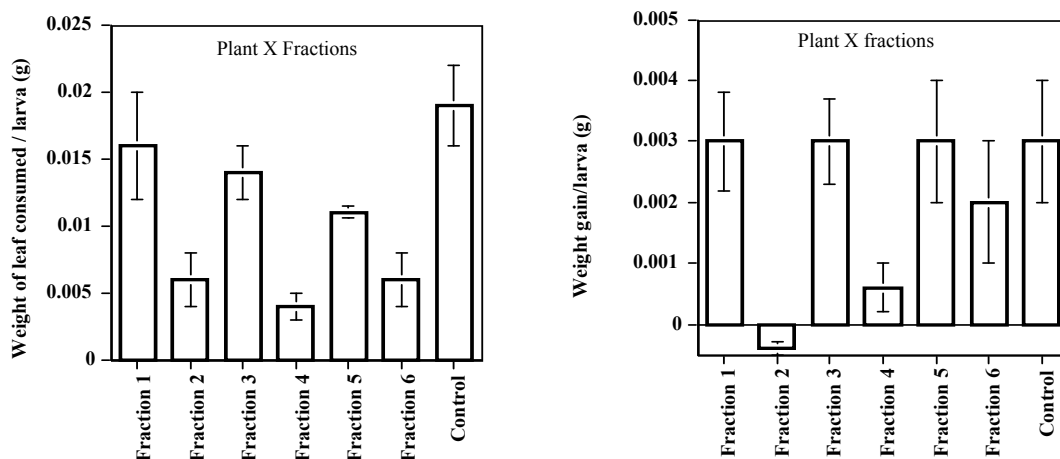


Figure 2. Feeding response of *Helicoverpa armigera* 3rd instar larvae on cotton leaves treated with Plant X fractions (no-choice tests) at ACRI in Narrabri, 2004-05

DISCUSSION:

In these studies Plant X fractions and several compounds have been found to influence not only the oviposition behaviour of pest species (adults) but also compounds that modify the feeding behaviour of larvae. The reduced numbers of eggs laid on Plant X treated plants is a consequence of host selection (pre-alighting behaviour) and host acceptance (post-alighting behaviour).

In cage experiments where the sources of attractants/ repellents for oviposition of various host species are in a limited space, semiochemicals that affect host selection become less prevalent to oviposition than those affecting host acceptance. The significantly lower number of eggs on Plant X (105 fold less than that of sorghum) indicates that host rejection factors in Plant X can be detected by moths and other pests. A similar observation on *Heliothis virescens* was reported by Ramaswamy *et al.* (1987). These compounds have the potential to be developed as commercially viable “tools” in IPM programs, utilizing the push-pull (Pyke *et al.* 1987) or attract-repel techniques.

Plant X fraction 3 or 4 were found to have oviposition repellent properties and may be applied to an economic crop (in this case cotton) to drive out or repel the adult pest, thus reducing or eliminating oviposition. Plant X fraction 2 was found to be quite toxic to the very small larvae of *Helicoverpa armigera*. The potential development and application of feeding deterrents offers a level of protection to economic crops, that if oviposition does occur, the level of damage sustained from larval feeding is reduced. By applying oviposition stimulants/attractants to crops that contain feeding deterrents and / or toxins it may be possible to manipulate pest behaviour to oviposit on the “masked” unsuitable crops. Thus consequent hatchlings face reduced development or mortality.

These studies were part of a larger study that has exploited the biological activities of Plant X to develop a new environmentally sound semiochemical product to be commercialised by Growth Agriculture Pty Ltd in the Australia Cotton Industry. These studies were particularly interested in secondary plant compounds (SPCs) and fractionalised extracts from Plant X effective against *Helicoverpa* spp. and other pests. These SPCs and Plant X fractions have been tested to determine the effect they have on direct mortality and modifying the behaviour of pests specifically *Helicoverpa* spp. and sucking pests in cotton. Such compounds and fractions have been developed and in the process of being commercialised as semiochemical product (code-named “Plant X now Sero-X) by Growth Agriculture to be used as part of an Integrated Pest Management (IPM) program in the Australian cotton industry. This will ensure the sustainability and economic viability of the Australian cotton industry and also minimize the negative impacts of pesticides on the environment.

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