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CANEGRUB RESISTANT PLANTS
CONTAINING ANTIMETABOLIC COMPOUNDS

by

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SUMMARY

Transgenic sugarcane plants engineered to express either the potato proteinase inhibitor II or the snowdrop lectin gene show increased antibiosis to larvae of *Antitrogus consanguineus* in pot-based glasshouse trials.

Canegrubs feeding on the transgenic line UP87, transformed with the potato gene, gained as little as 4.2% of the weight of canegrubs fed on untransformed control plants. Similarly, larvae feeding on the roots of transgenic line G87, transformed with the snowdrop gene, gained only 20.6% of the weight of grubs feeding on the non-transgenic control plants. Overall, 22% of the tested transgenic plant lines engineered with either the potato or the snowdrop constructs resulted in a statistically significant reduction in gain of weight by canegrubs feeding on roots. Weight gains of insects were compared to those of larvae feeding on the roots of either non-transgenic control plants, or non-transgenic plants regenerated after passage through the tissue culture system.

Plants transformed with a proteinase inhibitor from an ornamental tobacco showed no statistical effect on the weight gain of the grubs. This result was unexpected, as the proteinase inhibitor from tobacco was predicted to be as effective as the PI from potato. Further analysis of the gene construct by sequencing established that the gene construct was faulty in contrast to the original restriction analysis that had indicated that the plasmid was correctly constructed. Protein could not be produced by the faulty construct, and this is reflected in the negligible effect on weight gain of the grubs. The plasmid was re-constructed and transformed into sugarcane. Plants containing this construct are being grown for testing.

Having now established that canegrub resistance can be engineered into sugarcane, the next phase is to test the efficacy of these transgenes against other species of canegrub larvae and to further develop a commercial product.

1.0 BACKGROUND

Canegrubs, the larvae of endemic melolonthine beetles, are the most important insect pests affecting the sugar industry in Australia. The 19 species of canegrub are placed in four genera, *Dermolepida, Lepidiota, Antitrogus and Rhopaea*. Collectively, canegrubs cost the industry about $12 million a year in damage and control costs, although uncontrolled populations could cause such severe damage as to leave parts of the industry unviable (Robertson *et al.,* 1995). Management of canegrubs is highly dependent on a controlled-release formulation of chlorpyrifos, suSCon® Blue, as plant resistance to canegrubs has not been a breeding or selection criterion. However, the failure of suSCon® Blue in some highly alkaline soils (Robertson *et al.,* 1998), combined with the difficulty of reapplying insecticide into ratoon crops to provide control over high insect populations, has focused research into investigating options to increase plant resistance. Research to investigate the level of natural resistance in sugarcane and some related germplasm is being pursued for potential application in conventional breeding programs (Allsopp *et al.,* 1995, 1996b, 1997; SRDC project BSS132).

Another option is to engineer insect-resistance genes from other plant species into sugarcane. The first phase of this approach is to use biochemical or feeding assays to identify candidate compounds which increase the level of resistance to canegrubs, and then to transfer the genes encoding these compounds into sugarcane. Potential compounds, such as proteinase inhibitors, toxins from *Bacillus thuringiensis*, lectins, avidin and α-amylase inhibitor, were tested and suitable candidates identified (McGhie *et al.,* 1995; Allsopp *et al.,* 1996a, 1997, Allsopp and McGhie, 1996; SRDC project BSS95) during these screens. Genes for the potato proteinase inhibitor II (Murray and Christeller, 1994), the *Nicotiana alata* proteinase inhibitor (Atkinson *et al.,* 1993) and the lectin gene of the snowdrop plant, *Galanthus nivalis* (van Damme *et al.,* 1987), were obtained, constructed into expression plasmids and transformed into sugarcane (Allsopp *et al.,* 1995).

This project builds on project BSS95, which successfully identified antimetabolic compounds that are toxic to or inhibit development of canegrubs, demonstrated that genes for these compounds could be transferred into sugarcane plants and developed experimental procedures for testing and gene transfer that will be used in the new project. It seeks to develop canegrub-resistant sugarcane plants and commence a process for delivery of these plants to canegrowers. These plants should provide alternatives to synthetic insecticides and improve options for canegrub control through the use of novel host-plant resistance.

2.0 OBJECTIVES

The project aimed to provide an alternative to synthetic insecticides for controlling canegrubs. This would be achieved by transgenically incorporating compounds that are detrimental to the development and survival of canegrubs into sugarcane plants, and releasing these plants to canegrowers.

The specific objectives were:

- Introduce proteinase inhibitor and lectin genes into selected new cultivars and commence registration for field release to canegrowers.

- Determine the production, storage and fate of foreign antimetabolic compounds in sugarcane cells.

- Screen further candidate antimetabolites for activity against canegrubs.

The rationale behind this approach is that a wide range of strategies to control canegrubs need to be developed and available for integration into pest management systems. This is particularly important when the only current feasible control method fails to work or is no longer acceptable to use because of economic or environmental factors. Developing canegrub-resistant transgenic sugarcane is a very feasible approach. Compounds that are detrimental to canegrubs have already been identified and the technology is available to successfully transfer genes that code for these compounds into sugarcane plants. Such plants should be accepted readily by canegrowers.

3.0 OUTCOMES/OUTPUTS

- Production of transgenic plants that produce novel lectins and proteinase inhibitors.

- Established that resistance to canegrubs in sugarcane can be significantly enhanced by the use of transgenes encoding antimetabolites such as lectins and proteinase inhibitors.

4.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

4.1 Introduction of proteinase inhibitor and lectin genes into selected new cultivars

Embryogenic callus of cultivar Q117 was produced essentially as described by Franks and Birch (1991) on MS medium (Murashige and Skoog, 1962) supplemented with 3 mg/L of the synthetic plant-growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D). Callus was maintained in the dark at 27°C. Three plasmids containing canegrub-resistance genes were constructed. All constructs contained the *Emu* (Last *et al.,* 1990) or the maize ubiquitin promoter, *Ubi*, (Christensen and Quail, 1996), and the *Nos* termination sequence. The plasmid p*UbiPinII* contained the proteinase inhibitor II gene from potato (*Solanum tuberosum*) (Murray and Christeller, 1994), p*UbiNaPI* contained the proteinase inhibitor from the ornamental tobacco *Nicotiana alata* (Atkinson *et al.,* 1993), and p*UbiGNA* contained the lectin gene from *Galanthus nivalis,* the snowdrop plant (van Damme *et al.,* 1987). The plasmids were co-transformed into callus by microprojectile bombardment with pUKN, an antibiotic selectable marker gene construct containing the *Ubi* promoter, the neomycin phosphotransferase gene and a *Nos* termination sequence for selection of transformed callus on geneticin-supplemented media. Following bombardment, the callus recovered for two weeks on MS-2,4-D medium, and then selection occurred over nine weeks on a geneticin supplemented MS-2,4-D medium.

Antibiotic-resistant callus pieces were then transferred to a 2,4-D-free MS-geneticin medium and shoot regeneration initiated by placing the transgenic callus in the light. When shoots were about 10 cm tall and the plantlets had a well-established root system, they were transferred to 15 cm pots in a PC2 containment glasshouse. They were then grown to plants about 1-2 m tall and single-eye bud cuttings were taken for testing of activity in pot-based bioassays.

Table 1. Production of transgenic plants containing various genes for canegrub resistance

Construct introduced	Cultivar	Number of plants
EmuPinII	Q117	13
	Q153	3
	Q155	4
UbiPinII	Q117	223
UbiNaPI (damaged construct)	Q117	58
UbiNaPI (correct construct)	Q117	90
UbiGNA	Q117	106
UbiPinII+*UbiNaPI*(dc)+*UbiGNA*	Q117	1
UbiPinII+*UbiGNA*	Q124	104
UbiNaPI(cc)+*UbiGNA*	Q117	108
Total		**710**

The *UbiPinII* and *UbiGNA* constructs were sequenced to determine whether the gene was in frame and that no deletions/mutations from the original sequence had occurred during construction of the plasmid. A gene that was not in frame, or had deletions/mutation would not be expressed correctly. Both genes were found to be in frame and completely intact.

The *UbiNaPI* construct was not sequenced, as the gene consists of multiple same-sequence domains, which makes sequencing difficult without examining each individual domain. Attempts were made to express the construct in sugarcane protoplasts, but the results were inconclusive. The construct was checked by restriction mapping and appeared to be correct and, due to the technical problem of sequencing through the domains, it was decided that sequencing was not necessary. When results from the pot trials indicated that plants containing this gene construct were not affecting the weight gain of the test grubs, the construct was rechecked. Even in the relatively short time frame of this project, significant advances in sequencing technology had been made including the introduction of commercial automated sequencing facilities. The *UbiNaPI* construct was partially sequenced at the ends of the gene only and found to be incorrectly assembled. The construct was remade and the ends of the new construct were sequenced. This construct (*UbiNaPI*(cc)) was found to be correct and in frame and was used to produce more plantlets.

4.2 Determination of the production, storage and fate of foreign antimetabolite compounds in sugarcane cells

Production of antimetabolites

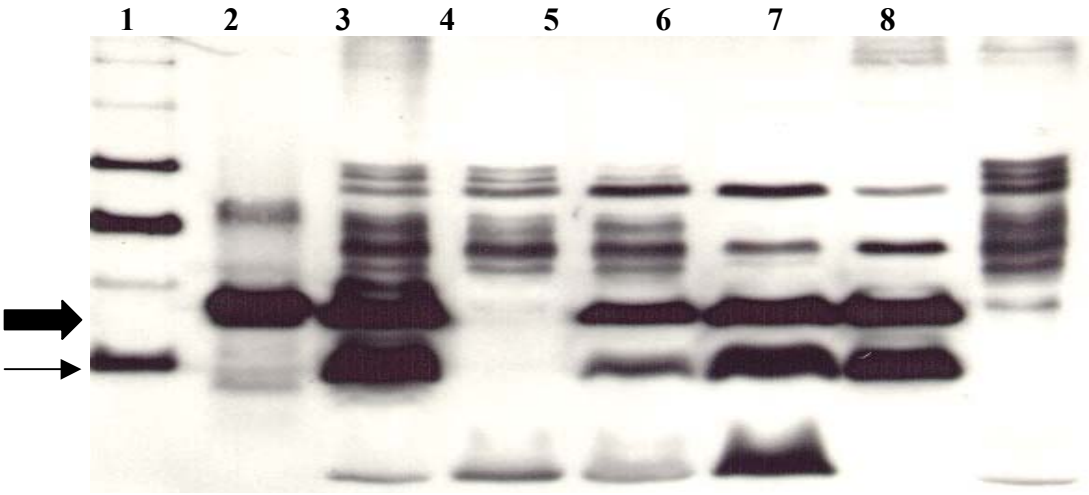
Plants were analysed for the presence of the various transgenes by the polymerase chain reaction (PCR) using a modification of the system described by Smith and Gambley (1993). Southern and western blots were performed essentially as described by Sambrook *et al.* (1989). Results of the Southern hybridisations were used to identify possible transformation clones so that plants originating from different transformation events were selected for propagation and testing. Protein concentrations were measured by the Bradford colourimetric assay (Bio-Rad) as described in the manufacturer's instructions.

Gene integration was confirmed using Southern hybridisation analysis for 20 *EmuPinII* and 26 *UbiNaPI* plants. This has revealed that transformants containing *EmuPinII* belong to six individual lines (three Q117, one Q153, two Q155) with low (<6) copy numbers of the gene integrated into the genome. The *UbiNaPI* transformants appear to belong to six individual lines with gene integration at quite high (>6) copy numbers. Fourteen *UbiGNA* plants from a single shooting event were examined. These plants were found to belong to 10 individual lines, with varying copy numbers (low-high). There is some preliminary evidence that copy number can be correlated with transgene expression. Of these 14 plants, the lower copy number plants produced more GNA protein than those with high copy numbers. Plants with the highest copy number (ie 10+) produced very little GNA protein, or none at all.

Measurement of the PinII protein in the transgenic lines has been difficult, although ELISA data indicate that the PinII protein is being expressed in plants at approximately 0.001-0.003% of total extractable soluble protein.

A number of the *UbiGNA* transgenic lines express the snowdrop lectin to approximately 0.05% of total extractable soluble protein in both leaves and roots (Figure 1). However, extractable soluble protein is only one constituent of roots and leaves, and comprises approximately 0.5% of the mass of sugarcane roots and 1.0% of leaf mass as measured by the Bradford assay. The range of expression of GNA lectin in the different transgenic lines was 0 to 0.05% of total extractable soluble protein. However, there was a poor statistical correlation between the weight gain of canegrubs feeding on the roots, and the estimated level of expression of the lectin in either roots (R^2=0.19) or leaves (R^2=0.23). The reason for this poor correlation is also unknown. The western blot (Figure 1) suggests that the 13 kD lectin may be undergoing processing to a smaller 6.5 kD fraction in sugarcane, and this may be affecting the concentration of biologically active lectin in the roots. The lectin molecule also may not be folding correctly to the active tertiary structure, but why this occurs in some transgenic lines and not others is uncertain. At present, this poor correlation between the *in vitro* lectin assays and the *in planta* results is limiting the efficient screening of regenerated lines for selection for the pot-based assay. This is one area of research that requires further investigation as part of the effort to efficiently produce canegrub-resistant transgenic lines of commercial value. Initial studies to determine the functionality of the expressed lectin by an agglutination assay suggest this approach may be feasible to rapidly screen transgenic plants, but further research is necessary.

Fig. 1. Expression of snowdrop lectin in transgenic plants transformed with the p*UbiGNA* construct. Western blot of protein extracts. Lane 1: Molecular weight markers, 2: lectin standard 25 ng, 3-7 extracts from transgenic plants 8: extract from untransformed Q117. 100 ng of total protein loaded in lanes 3-8. Samples in lanes 3, 5, 6 and 7 are positive, sample in lane 4 is negative for expression of the snowdrop lectin. Large arrow indicates position of 13 kD lectin positive control in lane 2. Small arrow indicates position of 6.5 kD lectin fragment in transgenic sugarcane extracts.



Effect of transgenic plants on canegrubs

The effects of transgenic lines on canegrub growth and survival were tested in five pot trials:

- the first (ES97-11a) compared
    - 2 *EmuPinII* lines based on Q155,
    - 7 *EmuPinII* lines based on Q117,
    - field-grown Q155 plants,
    - and field-grown Q117 plants;

- the second (ES97-11b) compared
    - 10 *UbiNaPI* lines based on Q117,
    - and field-grown Q117 plants;

- the third (ES98-1) compared
    - 15 *UbiPinII* lines based on Q117,
    - 8 *UbiGNA* lines based on Q117,
    - field-grown Q117 plants,
    - and Q117 tissue-cultured plants (regenerated from untransformed embryonic callus);

- the fourth (ES98-12) compared
    - 5 *UbiPinII* lines based on Q117,
    - 13 *UbiGNA* lines based on Q117,
    - 3 *UbiNaPI* lines based on Q117,
    - 1 line of NPT (kanamycin resistance, NPTII) based on Q117,
    - 1 line of PNG (*UbiPinII*+*UbiNaPI*(dc)+*UbiGNA*) based on Q117,
    - field-grown Q117 plants,
    - and Q117 tissue-cultured plants;

- the fifth (ES98-13) compared
    - 20 *UbiPinII* lines based on Q117,
    - 4 *UbiGNA* lines based on Q117,
    - 2 *UbiNaPI* lines based on Q117,
    - 1 line of NPT (kanamycin resistance, NPTII) based on Q117,
    - and field-grown Q117 plants.

Single-eye setts from each line were established in 20-L pots in a PC2 glasshouse at the Southern Sugar Experiment Station, Bundaberg. When the plants were about 80-90 cm high, each pot was infested with three (trials 1 and 2) or four (trials 3-5) third-instar larvae of *Antitrogus consanguineus,* the southern one-year canegrub. All grubs were weighed prior to infestation of plants; all pots contained grubs of similar weights (± 10 mg). All trials originally consisted of four replicates of each line in randomised complete-block designs, although the poor growth of some plants meant that these pots were excluded from the analyses. About six weeks after infestation the larvae were recovered and the weight gain and number of surviving grubs determined.

In trial 1, there were no significant differences in grub weight gains among *PinII* lines derived from Q155 ($F=2.38$, df=2,6, $P=0.17$) (Table 2) or among *Pin II* lines derived from Q117 ($F=0.94$, df=7,20, $P=0.50$) (Table 3). At the time these plants were produced *Emu* was one of the first promoters to be developed for transgene expression in monocots, and there was little information available on its performance in sugarcane. The *Emu* promoter is now generally regarded as a poor promoter for sugarcane genetic engineering, and these results are not in conflict with this consensus.

Table 2. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q155 and engineered with a gene to express the potato proteinase inhibitor II using the *Emu* promoter

Line	Weight gain (mg)
Emu194	1370.5
Emu197	1690.0
Q155	1433.7

Table 3. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the potato proteinase inhibitor II using the *Emu* promoter

Line	Weight gain (mg)
Emu161	1157.4
Emu176	1084.0
Emu177	1087.2
Emu178	1228.2
Emu183	1207.1
Emu186	1041.0
Emu191	1197.0
Q117	945.2

In trial 2, there were no significant differences in grub weight gains among *UbiNaPI* lines derived from Q117 (F=1.54, df=10,30, P=0.17) (Table 4). In reflection, this result is consistent with expectations of gene expression from a damaged construct.

Table 4. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the *Nicotiana alata* proteinase inhibitor using the *Ubi* promoter

Line	Weight gain (mg)
UN3	956.1
UN7	812.0
UN8	956.3
UN10	923.9
UN12	934.3
UN17	858.6
UN20	1181.8
UN23	1048.2
UN24	801.5
UN25	1042.5
Q117	945.2

In trial 3, there were significant differences in grub weight gain among the *UbiPinII-Q117* plants (F=3.53, df=16,31, P=0.0013) (Table 5) and among the *UbiGNA-Q117* plants (F=3.84, df=9,18, P=0.0073) (Table 6).

Grubs feeding on two of the eight *UbiGNA* lines (G1, G87) and three of the fifteen *UbiPinII* lines (UP87, UP260, UP302) had statistically lower weight gains than those feeding on either type of Q117. Feeding on the most resistant line, UP87, transformed with the p*UbiPinII* construct, resulted in grubs gaining only 4.2% of the weight gain of grubs fed on Q117. Feeding on the most resistant *UbiGNA* line, G87, resulted in grubs gaining 20.6% of the weight gain of grubs on Q117.

Table 5. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the potato proteinase inhibitor II using the *Ubi* promoter

Line	Weight gain (mg)*
UP45	409.2 abc
UP73	472.6 ab
UP87	19.8 e
UP88	503.8 ab
UP92	373.6 abcd
UP100	476.8 ab
UP104	237.8b cde
UP120	611.1 a
UP140	460.0 abc
UP153	197.0 cde
UP158	416.8 abc
UP260	113.8 de
UP263	478.3 ab
UP276	345.5 abcd
UP302	20.7 e
Q117	467.7 ab
Q117 – tissue cultured	460.5 ab

* Means followed by the same letter are not significant difference at the 5% level

Table 6. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the snowdrop lectin using the *Ubi* promoter

Line	Weight gain (mg)*
G1	122.8 c
G17	301.9 bc
G18	817.3 a
G51	244.0 bc
G87	96.5 c
G103	539.9 ab
G128	338.0 bc
G151	259.6 bc
Q117	467.7 b
Q117 – tissue cultured	460.5 b

*Means followed by the same letter are not significant difference at the 5% level

In trial 4, there were significant differences in grub weight gain among the lines tested (F=1.72, df=24, 72, P=0.041 – analysis on ln(weight gain)), with significantly lower weight gain on UN29 (*UbiNaPI*), G42 (*UbiGNA*) and NPT3 (kanamycin resistance, NPTII) than on Q117 (Table 7).

Table 7. Mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the snowdrop lectin (G plants), the potato proteinase inhibitor II (UP plants), kanamycin resistant control, NPTII (NPT plants) or potato proteinase inhibitor + *Nicotiana alata* proteinase inhibitor + Snowdrop lectin (PNG plants) using the *Ubi* promoter

Line	Weight gain (mg)*
G3	251.6 abc
G4	271.8 ab
G9	291.3 abc
G11	160.7 abc
G37	190.5 abc
G42	44.1 cd
G44	272.2 ab
G52	127.3 abc
G55	281.0 a
G58	103.4 abc
G60	195.1 abc
G62	225.3 abc
G68	240.2 abc
UP40	285.4 ab
UP153	226.2 abc
UP245	122.8 abc
UP246	118.3 abc
UP258	231.5 abc
UN13	124.3 abc
UN15	61.1 abc
UN29	25.4 bc
PNG3	90.4 abc
NPT3	-35.4 d
Q117	343.3 a
Q117 – tissue culture	214.8 abc

*Means followed by the same letter are not significant difference at the 5% level following ln-transformation

In trial 5, there were no significant differences in grub weight gain among the lines tested (F=0.80, df=27, 81, P=0.74 – analysis on ln(weight gain)). However, we consider that this test is unreliable as when the grubs were introduced the plants were much smaller than in previous trials and many plants died subsequent to infestation.

4.3 Screen of further candidate antimetabolites for activity against canegrubs

One test of a range of new potential antimetabolites was carried out during the project.

4.3.1 Materials and methods

Proteins from 19 sources (the name of the supplier and the nature of the materials can not be divulged owing to confidentiality agreements) were tested under quarantine conditions. The samples were frozen, airfreighted in dry ice to Brisbane and then taken by road to Bundaberg, again in dry ice. They were stored at Bundaberg in a freezer until needed.

Proteins were tested in a diet developed by Allsopp (1994) and used to test a range of other antimetabolites (Allsopp, 1994; Allsopp *et al.,* 1996a, 1997; Allsopp and McGhie 1996). The diet was prepared by adding 2 g of methyl-4-hydroxybenzoate and 25 g of agar to 325 mL of boiling water and heating and stirring until the agar dissolved. The following were added to 400 mL of water and blended: 2.5 g linseed oil; 30 g wheat germ; 35 g vitamin-free casein; 35 g sucrose; 10 g Wesson salts; 0.5 g cholesterol; 1 g choline chloride; 0.4 g inositol; 1 g sorbic acid; 0.1 g α-tocopherol acetate. This was mixed together and allowed to cool to 45°C. Then 4 g of ascorbic acid, 2 mL of 40% formaldehyde and 0.033 g of a B-vitamin mixture were stirred in. Ingredients for the B-vitamin mixture were: 10 mg niacinamide; 2.5 mg folic acid; 0.2 mg biotin; 5 mg riboflavin; 2.5 mg thiamine hydrochloride; 30 mg calcium pantothenate; 20 μg vitamin B_{12}; 2.5 mg pyridoxine hydrochloride. The test protein solutions (or the buffer solutions) were added at 250 mg/kg while the diet was at 45°C, and the mixture was cooled rapidly. Diet was made up on the day of use.

Young third-instar larvae of greyback canegrub (*Dermolepida albohirtum*) were collected from sugarcane fields near Ayr, northern Queensland and airfreighted to Bundaberg. They were held before use for 7 d at 25°C without food to ensure that only healthy larvae were used. Each healthy larva was weighed (mean 1.48 g) and placed in a 100 mL cup half-filled with moist sand. About 8 g of diet was added and the cup filled with moist sand, capped and held at 25°C. There were 24 replicates of each diet. Weights of grubs and deaths were recorded at 7, 14 and 28 days after establishment. New diet was added at day seven and diet was replaced by grass-seedling sprouts at day 14. No test material was left at day 14.

As well as the 19 test proteins there were three 'control' groups; two consisted of the artificial diet made to 250 mg/kg with one of two buffer solutions that were used to prepare the proteins; one contained the artificial diet made to 250 mg/kg with water. A further treatment consisted of larvae given no food.

Differences in weight gains between treatments were tested with the Kruskal-Wallis one-way analysis of variance, and means were separated by the procedure outlined in Conover (1980). The χ^2 statistic (with Yates' correction) in 2x2 tables was used to test for differences in larval mortality.

4.3.2 Results and discussion

Larvae fed on all of the diet mixtures gained weight rapidly to day 14 and then gained little further weight (Table 8); this is similar to results in other feeding assays (eg Allsopp, 1994). As expected, larvae in the no-food treatment gained very little weight (Table 8) and were excluded from further analyses of weight gain. There were no significant differences ($P< 0.05$) in weight gains at day 7, 14 or 28 of larvae on the 'blank' diet, the

added-protein diets or the added-buffer diets (day 7 T=11.49, n=501, P=0.95; day 14 T=17.78, n=491, P=0.66; day 28 T=26.72, n=485, P=0.18).

There was no significant mortality (Table 8) during the course of the experiment in any of the groups fed on diet, and, what mortality there was, did not differ significantly between diets (P>0.11).

The test procedure has been successful in past experiments in demonstrating antimetabolic effects (reduced growth rates and reduced survival) on canegrubs (Allsopp, 1994; Allsopp and McGhie, 1996). The absence of any significant antimetabolic effects in this experiment indicates that none of the proteins are useful against Australian canegrubs.

Table 8. Mean weight gain and survival of larvae fed different diets containing potential antimetabolites

Sample number	Mean weight gain (mg, SE)			Number surviving at day 28 (out of 24)
	Day 7	Day 14	Day 28	
1	610.1 (92.5)	769.1 (72.8)	963.7 (55.5)	21
2	553.1 (85.9)	734.9 (68.9)	914.9 (84.7)	21
3	387.0 (67.2)	578.2 (67.1)	771.7 (60.5)	22
4	586.6 (77.8)	747.5 (63.3)	922.3 (49.3)	23
5	480.5 (75.9)	669.3 (65.8)	861.9 (68.7)	21
6	418.3 (72.1)	612.1 (66.3)	813.4 (61.2)	22
7	515.9 (80.3)	672.0 (78.7)	864.8 (64.9)	22
8	474.2 (63.0)	601.3 (64.0)	816.7 (72.2)	21
9	466.5 (67.2)	608.2 (68.6)	775.2 (56.3)	23
10	501.1 (87.4)	627.2 (85.6)	784.4 (79.3)	21
11	471.8 (76.4)	598.1 (74.7)	747.2 (64.1)	22
12	555.2 (72.6)	669.9 (63.1)	793.4 (59.2)	24
13	533.0 (95.6)	733.2 (92.5)	915.1 (78.0)	23
14	505.4 (69.3)	693.1 (69.0)	892.8 (60.2)	22
15	389.5 (46.9)	526.6 (37.6)	728.7 (45.4)	21
16	500.2 (73.7)	677.3 (71.2)	925.0 (63.2)	23
17	459.0 (76.0)	646.1 (67.9)	847.0 (61.7)	22
18	443.3 (118.8)	644.4 (115.7)	821.7 (112.2)	23
19	491.7 (65.0)	650.4 (66.8)	836.9 (69.5)	22
Buffer 1	525.9 (84.4)	730.8 (73.0)	933.4 (67.2)	23
Buffer 2	461.7 (67.0)	676.9 (63.7)	910.6 (61.6)	20
Base diet	506.7 (82.7)	710.8 (62.8)	853.8 (56.0)	23
No food	51.4 (17.4)	21.6 (26.2)	7.5 (30.3)	17

4.4 General discussion

We have established that resistance to canegrubs in sugarcane can be significantly enhanced by the use of transgenes encoding antimetabolites such as lectins and proteinase inhibitors. The results from the pot assays indicate larvae feeding on the best transgenic lines gain almost no weight after six weeks. Whilst no significant mortality was seen in pot trials, the snowdrop lectin does kill *A. parvulus* larvae after four weeks when incorporated into an artificial diet (Allsopp and McGhie, 1996). If the trials had continued longer, we expect that significant mortality of the *A. consanguineus* larvae would have occurred. In the field, these underweight larvae may be more susceptible to natural enemies or pathogens such as *Adelina* spp. or *Metarhizium anisopliae* (Dall *et al.,* 1995), more susceptible to death at moults, or produce small adults with reduced egg-laying potential. Hence, we currently envisage transgenics as part of the pest management program for control of canegrubs and not the single solution to the problem. Combining various strategies, such as transgenics and natural pathogens, for canegrub control should limit the development of resistance in the canegrub populations and lead to long-term durable management options. The lectin transgenic plants may also show enhanced levels of resistance to sap-sucking insects such as aphids and planthoppers as well as nematodes based on reports from other groups (Gatehouse *et al.,* 1995). This lectin has low mammalian toxicity (Pusztai *et al.,* 1990), so these plants will probably have little effect on pests such as rats or wallabies, or indeed humans!

The next major challenge in this research is to test the performance of the transgenes against other species of canegrub such as greyback, *Dermolepida albohirtum.* The biological diversity of the 19 canegrub species makes selection and testing of transgenic lines potentially very difficult. Biochemical data indicate that the species selected for biochemical and feeding assays as being representative of this biological diversity *Antitrogus consanguineus, Lepidiota noxia* and *L. negatoria*, have very similar digestive enzymes (McGhie *et al.,* 1995), so these transgenes should afford control across all canegrub species. The in-field performance of these transgenes should be determined as part of the next phase of this program. Field trials of genetically-modified crops in Australia requires approval from the Genetic Manipulation Advisory Committee (GMAC), and an application to test the performance of genetically engineered canegrub-resistant sugarcane lines should be prepared under any new project.

5.0 RECOMMENDATIONS

We recommend that SRDC fund a new project to further develop this control strategy. The new project should:

- Negotiate intellectual property and research agreements to allow commercial release of transgenic plants.
- Further determine insecticidal performance of current transgenics expressing snowdrop lectin and protease inhibitors.
- Test additional products for antimetabolic effects on canegrubs and incorporate active ones into the program.
- Produce additional transgenic lines using snowdrop lectin and protease inhibitors and based on other cultivars.

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