July 93 - June, 1995

93/145

Cotton *Research and Development Corporation*

Plain English Summary of:

IDENTIFICATION OF PROTEINS ORALLY TOXIC TO THE GREEN MIRID

Project Number: CSE38C

Commonwealth Scientific and Industrial Research Organization
Division of Entomology

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A FINAL REPORT PREPARED FOR THE COTTON RESEARCH AND DEVELOPMENT CORPORATION

Plain English Summary of "Identification of proteins orally toxic to the green mirid"

Aim

The aim of this proposal was to identify insecticidal proteins for use in transgenic control strategies targeted at the green mirid, *Creontiades dilutus*.

Benefits to the Cotton Industry

The identification of proteins orally toxic to the green mirid would provide a foundation for the development of transgenic cotton with increased resistance to mirid attack. The major benefit of this is reduced dependence of chemical insecticides for cotton production. By reducing the use of chemical insecticides, the cotton industry receives the following benefits:

(1) reduced insecticide costs
(2) reduced environmental contamination
(3) decreased incidence of resistance to chemical insecticides in pest insects
(4) improved sustainability of the cotton industry

Background Justification

One of the main challenges in cotton production is to be able to reduce the use of chemical insecticides while still achieving necessary control levels of insect pests. One strategy to achieve this is the development of transgenic cotton that will have an increased resistance to insect attack. The recent development of transgenic cotton plants expressing *Bacillus thuringiensis* (B.t.) toxin means that less chemical insecticides will be needed for the control of *H. amigera*. Although this promises to be a tremendous breakthrough in the control of lepidopteran pests, it is not a panacea because there is no known B.t. toxin that is active against insect pests such as mirids. As such, insect protection of transgenic B.t. cotton grown in areas with serious mirid infestation will still require substantial amounts of chemical insecticides. Clearly, there is a great need for research aimed at developing transgenic cotton with increased resistance to mirid attack. The first objective that must be met to achieve this goal is the identification of insecticidal proteins which are orally active against the mirid.

The objective of this proposal was to identify insecticidal proteins that are orally active against mirids. To date, most investigations aimed at finding orally-active insecticidal proteins have used chewing insects such as Lepidoptera and Coleoptera to look for toxic activity. These insects generally have high levels of proteolytic activity in their gut and a relatively impermeable stomach wall. As a result of this, only a handful of the many protein toxins that have been screened against these insects have oral insecticidal activity. The mirid gut, on the other hand, has relatively gentle proteolytic activity and a more "porous" structure. Although possibly a disadvantage with some gut-adhering toxins such as B.t., these characteristics may carry an advantage in that insecticidal proteins could pass through the gut and act upon internal targets in the mirid body. Studies on mirids carried out in other laboratories has shown that viruses and large dye particles can pass through the gut and enter the haemolymph. In light of this, we implemented a mirid feeding bioassay to screen for insecticidal proteins. In one

study mirids were fed a solution of a scorpion insect-selective toxin that has not been found to be orally active against lepidopterans. After ingesting the toxin, test mirids showed inhibited feeding and had a higher mortality rate than control mirids. This demonstrated that not only can the toxin pass through the gut and interact with the nervous system, but that it can arrive there intact and still possessing biological activity. This finding strongly supports the need for testing other toxins against the mirid.

Research Summary:

The aim of this study was to complete the development of a mirid feeding bioassay to facilitate screening and characterization of proteinaceous toxins. The types of protein toxins that are available for screening influenced the assays that were developed. Acute toxins, such as the scorpion neurotoxin, can be assayed using adults and scoring for mortality or paralysis. However, there is a very limited number of these acute toxins available. Much more numerous and available are inhibitors of digestive enzymes, lectins and similar proteins that are likely to have a more subtle effect on mirid feeding, growth and development. Assessment of the deleterious effect of these proteins necessitated the development of a feeding bioassay using nymphs.

Objective 1. Develop a laboratory rearing method to supply mirids for biological assays.

Availability of a sufficient number of healthy insects is essential for any screening project. We tested numerous procedures to try to maintain a laboratory colony of mirids. Although we were not successful in rearing mirids in a continuous culture in the laboratory, we have developed and characterised a protocol for obtaining adults and nymphs in the lab from field-collected insects.

Objective 2. Characterize adult feeding bioassay.

To challenge adults, the solution to be tested is prepared in a 7.5% sucrose solution and delivered to the mirids using a membrane-based feeding assay. Adult mirids survive well on sucrose and can be assayed for up to 2 weeks. Sucrose stabilizes most proteins as well as acting as a phagostimulant. By adding a known amount of radioactivity to the solution, we found that adults ingest approximately 4 ul of sucrose per day compared to only 1 ul of water.

Objective 3. Develop and characterize a feeding bioassay for nymphs.

Since nymphs do not survive on sucrose, we developed an assay using an artificial diet. When reared on this diet, weight gain of nymphs from the first to fourth instar is fairly linear and shows little variation between insects. As such, this assay should permit identification of proteins that have an anti-feedant or otherwise deleterious action that affects weight gain.

Objective 4. Use the above assays to screen for proteins that were orally toxic to the green mirid.

Compounds with a range of biological activities have been tested. To date, the scorpion toxins and proteinase inhibitors show the most promising and have supported the feasibility of using these assays to identify insecticidal proteins. However, the availability of insects for bioassays severely limited the number of assays that could be done.

Summary:

The green mirid is a major pest of cotton during the seedling and pre-squaring stages of growth. Current control procedures require intensive spray regimes of chemical insecticides which are both costly and a major source of environmental contamination. Biotechnology approaches to insect control constitute a rapidly growing commercial interest. The identification and isolation of insecticidal proteins and their corresponding genes is a critical and integral part of this approach. Insecticidal gene products have already been implemented against lepidopteran pests of cotton. Isolation of proteins toxic to the green mirid will widen this approach.

The green mirid is a particularly suitable insect with which to look for orally active gene products. As it is a large sucking insect with a fluid diet, it has less vigorous digestive processes and a more 'porous' gut than those of chewing insects such as *H. armigera.* Viruses and dyes have been shown to pass through the gut. In this study we have characterized a mirid rearing procedure and feeding bioassay which will allow us to screen for orally toxic proteins.

Introduction:

The green mirid, *Creontiades dilutus* (Stal), is becoming an increasingly important factor in the cost of growing cotton in Australia. This hemipteran feeds on fluids present in the phloem of young cotton plants at the seedling and pre-squaring stages. Infestation results in an extended maturation period for the plant, which, in turn, leads to reduced yield and increased damage by other pests and weather. Because the damage is indirect and largely due to vagaries such as weather, the economic impact of the green mirid is difficult to calculate but is regarded as substantial. In the northern growing areas it is considered the second most important pest of cotton after *H. armigera* (B. Pyke, G. Walter and I. Titmarsh, personal communications). Its control requires intensive spray regimes of chemical insecticides, with spray cycles of four to five days being required in the Dawson-Callide Valley. In addition to the expense, such control measures are causing concern for two reasons. Firstly, the chemical control of the mirid may interfere with the insecticide resistance management program for *H. armigera.* Secondly, the environmental damage caused by chemical insecticides is becoming more evident. An alternative to chemical control of the green mirid is therefore highly desirable.

A promising alternative is provided by recent advances that allow cotton plants to express the protein products of exogenous single genes. The presence of gene products in the phloem fluid of cotton plants that would disrupt the feeding of mirids in some manner would provide an ideal means of control. Already it has been demonstrated that control of *H. armigera* is possible with plants genetically engineered to express the proteinaceous toxins of *Bacillus thuringiensis* (B.t.).

The major requisite for the control of the green mirid by such means is the identification of single gene products that are able to act orally against the feeding mirid in a manner similar to the action of B.t. toxins against *H. armigera.* However, it is unlikely that a B.t. strain will act against mirids as these toxins are specific for certain members of the dipteran, lepidopteran and coleopteran orders. No B.t. strains have been identified that act against hemipterans.

In certain respects, the mirid is a promising insect to control using genetically engineered plant strategies. Lacking a peritrophic membrane, it has a 'porous' gut and a variety of plant viruses and large molecular weight dyes have been shown to cross

into the haemocoel after ingestion (Gibb and Randles, 1989). The mirid also has very low protease activity in both its salivary gland and gut (Colebatch, unpublished data). Both of these factors increase the likelihood that ingested protein toxins could reach an internal target site functionally intact

This assumption was tested using *Androctonus australis* insect toxin (AaIT), an unmodified protein of 8000 Da that is specifically toxic to insects (Zlotkin et al., 1972). Ingestion of this neurotoxin had a significant effect upon mirid feeding and mortality. The effects upon mirid feeding depended on the concentration of the toxin. At a concentration of 0.5 mg/ml, an inhibitory effect on feeding occurred with an increased incidence of death. At an order of magnitude lower concentration (and below), the toxin markedly stimulated mirid feeding. Both effects were destroyed after exposing the toxin to heat indicating that they are not due to nonproteinaceous factors.

Objectives:

The aim of this study was to complete the development of a mirid feeding bioassay to facilitate screening and characterization of proteinaceous toxins. The types of protein toxins that are available for screening influenced the assays that were developed. Acute toxins, such as the scorpion neurotoxin, can be assayed using adults and scoring for mortality or paralysis. However, there is a very limited number of these acute toxins available. Much more numerous and available are inhibitors of digestive enzymes, lectins and similar proteins that are likely to have a more subtle effect on mirid feeding, growth and development. Assessment of the deleterious effect of these proteins necessitated the development of a feeding bioassay using nymphs.

The original objectives for this project were:

Year 1: Preliminary screening for candidate insecticidal proteins that will be orally active against mirids.

Year 2: Dose response and mode-of-action studies on the most promising toxins.

During the course of the study, these objectives were modified to the following:

Objective 1. Develop a laboratory rearing method to supply mirids for biological assays.

Objective 2. Characterize adult feeding bioassay.

Objective 3. Develop and characterize a feeding bioassay for nymphs.

Objective 4. Use the above assays to screen for proteins that were orally toxic to the green mirid.

Materials and Methods:

Insects

Mirids were collected from flowering lucerne in Cowra, NSW using an insect net. Adults and nymphs were separated from other insects and flora with an aspirator. Rearing of adults was conducted in a glasshouse at 25 $\pm$ 6°C . Relative humidity was uncontrolled. Natural light was supplemented with overhead fluorescent lights to give 14 hours of light each day. Two populations of approximately 160 adults were placed

in separate rectangular metal and mesh cages (approximately 500cm x 500cm x 300cm). Folded paper towel was placed in the cage to provide shelter. Green beans (1 per 20 adults) were placed on top of the cage and served as both food source and oviposition site. Beans were changed daily.

For rearing nymphs, the number of eggs on each bean was counted and the beans placed in 2 litre plastic freezer boxes with mesh lids. Nymphs were reared in a constant temperature room at 25°C with 60% humidity and 14 hours of light. Green were added to the box to feed emerging nymphs. Food was changed every other day or earlier if fungus was observed.

Feeding Assays
The feeding assay is based on one described by Gibb and Randles (1989). Briefly, a substance to be tested is brought up in a sucrose solution and sandwiched between two pieces of parafilm that is then used to cover a glass vial containing the mirid. Feeding was occasionally monitored by adding food dye to the test sample and quantified by adding radioactive label.

Results:

Objective 1. Develop a laboratory rearing method to supply mirids for biological assays.

1a. Characterization of mirid rearing on green beans.

Laboratory-reared insects minimize the variability found in field collected insects by providing specimens of uniform age and nutritional background. To determine if the number of mirids required for biological assays could be produced in a laboratory culture, rearing procedures using green beans, whole bean plants and lucerne plants were tested.

Growth and development of the mirid on green beans at 25°C has been characterized (Foley and Pyke, 1985). The egg hatches, on average, 8 days after oviposition. The nymph then progresses through 5 instar stages, requiring approximately 15 days, before it reaches adulthood. The aim of this study was to determine the reproductive capacity of mirids grown under these conditions and to determine if sufficient numbers of adults could be produced to permit both maintenance of the colony and biological assays.

Two cages of 160 field-collected adults were established. Previously, it was found that field collected adults routinely produce eggs for approximately 4 to 5 weeks. Therefore, the number of eggs produced in four 1-week intervals and the subsequent number of adults reared from these eggs was determined. The results are shown in Table 1.

The total number of adults produced in this 4 week study was 413, a 1.29-fold increase over the number of original adults. Theoretically, using the same number of adults to keep the colony at it's current level, approximately 120 adults (0.29X total adult output) would be available for biological assays during the 4 week interval. Although this number may be sufficient for low-level screening, it works on the assumption that egg numbers produced by the lab reared adults will be similar to those of field-collected adults.

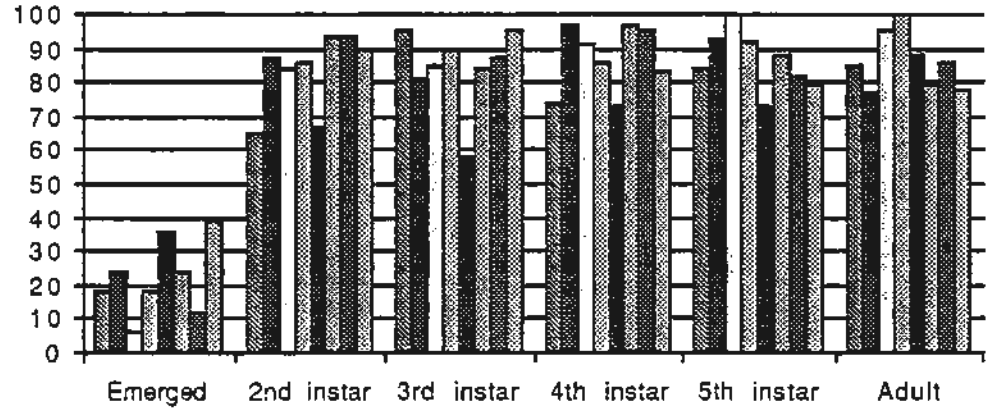
Table 1. Reproductive capacity of field collected mirids under laboratory conditions.

Cage 1 Week	#eggs	1st instar	2nd instar	3rd instar	4th instar	5th instar	Adult
1	781	139	86	82	61	51	45
2	546	129	112	91	88	82	63
3	540	31	26	22	20	20	19
4	199	36	31	28	24	22	22
Total-1	2066	335	255	223	193	175	149
Cage 2 Week							
1	108	39	26	15	11	8	7
2	500	120	113	95	92	81	64
3	823	101	95	83	79	65	56
4	806	314	283	271	224	176	137
Total-2	2237	574	517	464	406	330	264

To determine if adults reared in culture produced as many eggs as field-collected mirids, the reproductive capacity of 211 lab-reared mirids was determined over a four week period. Over this time 549 eggs were produced, 201 successfully hatched and 103 survived to adulthood. The number of eggs produced was only about one-quarter of the number produced by field-collected adults and the total number of adults produced was 0.5x that of the original population.

One way to overcome reduced oviposition is to increase the output from the eggs that are produced. To identify were improvements would be most beneficial, the survival of each developmental stage from the previous stage was calculated from the data in Table 1. The percentage of eggs that successfully hatched to first instars and the percent survival of nymphs between subsequent instars is shown graphically in Fig. 1.

Figure 1. Percent survival of each developmental stage.



The survival of nymphs through each instar was relatively high and, overall, 48± 15% of first instar nymphs reached adulthood. The number of eggs that successfully hatched to form first instars was much lower and variable with emergence at 25 ± 15%.

The major deterrent to high levels of emergence is the conflict between the humid conditions required for hatching and the problems of fungal growth under these conditions. We tried treating the beans containing eggs with 0.03% and 0.06% bleach solution. Although this reduced the appearance of fungus, emergence rates were highly variable and ranged from 0% to 29%. The best results were obtained by wrapping the bean loosely in paper towel. Emergence was still variable but was generally around 50% and, on occasion, went as high as 80%. The toweling probably increased local humidity around the bean as well as minimizing its exposure to airborne spores. Since mirids are cannibalistic, the paper towel may also have protected the emerging nymph from older nymphs.

1b. Rearing on different food sources.

Problems associated with rearing mirids on green beans included fungal growth and pesticide residues. To circumvent these, we examined the utility of a rearing procedure involving artificial diets. These were tested using a parafilm membrane feeding assay. A chemically defined diet used successfully with aphids was tested but was not attractive to mirids. We then tested a diet that had previously been used successfully for *Lygus* bugs (Debolt, 1982). The liquid diet was presented to the mirids through parafilm and changed every 3 days. Green beans were still required as oviposition sites because, although mirids preferred to feed on the diet, we could not encourage egg-laying on the diet packages. This procedure eliminated much of the insect handling, and gave high rates of nymph survival (75% and greater). However, after reaching adulthood these nymphs did not lay many eggs, suggesting that the diet was lacking in some essential nutrient(s).

Attempts to culture mirids on alfalfa plants and green bean plants was unsatisfactory. Caged plants presented handling difficulties, provided low yields of adult insects and were prone to fungal growth.

Objective 2. Characterize adult feeding bioassay.

To challenge mirids with proteins, we adopted a feeding assay based on delivering a fluid diet through a parafilm membrane (Gibb and Randles, 1989). In this assay, the protein to be tested for toxic activity is suspended in solution and presented to the mirid through the membrane. In order to optimize our assay we have tested a number of solutions. We have found that a 7.5% sucrose solution is the most suitable solvent to suspend proteins because first, proteins are relatively stable in sucrose and do not lose biological activity; second, adult mirids survive on 7.5% sucrose for over 2 weeks; and third, sucrose acts as a phagostimulant. Using radioactive tracer technique we determined that adult mirids ingest, on average, 4 ul of sucrose solution per day. Mirids fed on water only ingest approximately 1 ul per day.

Objective 3. Develop and characterize a feeding bioassay for nymphs.

Nymphs did not survive well on sucrose alone and generally died before the third instar. Therefore, to assay for proteins effecting nymph growth and development we used the *Lygus* artificial diet as the solution to deliver the proteins. Nymph development on the

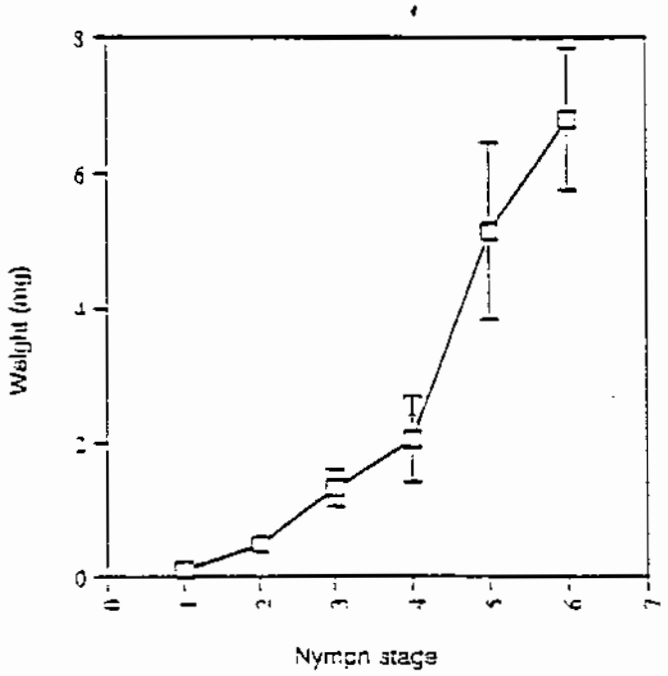
diet and compared to the rate of development on green beans (Foley and Pyke, 1985). The results are shown in Table 2. The developmental rate of nymphs grown on artificial diet is similar to that of nymphs reared on green beans but shows greater variation.

Table 2. Nymph development rate on artificial diet. **A**: nymphs grown on artificial diet, **B**: nymphs grown on green beans (Foley and Pyke, 1985).

	Length (in days) of nymphal stage			
First instar	**Second instar**	**Third instar**	**Fourth instar**	**Fifth instar**
A: 3.2 ± 1.2	3.2 ± 1.0	1.6 ± 0.6	3.6 ± 1.8	3.1 ± 1.8
B: 2.97 ± 0.13	2.2 ± 0.11	2.63 ± 0.12	2.48 ± 0.18	4.33 ± 0.19

To characterize nymph growth and weight gain on artificial diet, one day old first instar nymphs were weighed and placed on diet. Nymphs were then weighed one day after each molt. The results are shown in Figure 2. From the first to fourth instar stage, weight gain shows little variation between insects and is roughly linear. Moreover, weight increased approximately 20-fold during this period. As such, this assay should permit the identification of compounds that have an anti-feedant or otherwise deleterious action that affects weight gain.

Figure 2. Growth of mirid nymphs on artificial diet.



Lastly, to ensure against degradation of proteinaceous toxins while in the diet, diet was examined for endogenous protease activity. Quantitative protease assays using the general substrate azocasein were carried out at pH 8.0 (the pH of the diet). No observable protease activity was detected even after extended assay times of 36 hours.

Objective 4. Use the above assays to screen for proteins that were orally toxic to the green mirid.

Mirids were challenged compounds in 4 categories of biological activity:

Toxins: melittin, a neurotoxin purified from a parasitic wasp, toxin from an entomophagous bacteria, scorpion toxin and venom.

Enzymes: phosphodiesterase from snake venom, trypsin.

Enzyme inhibitors: proteinase inhibitors, amylase inhibitors.

Plant secondary compounds: lectins

Of these, scorpion toxins and proteinase inhibitors have shown insecticidal or growth-inhibiting properties.

Venom of the black scorpion, *Buthotus judaicus*, is known to contain at least two polypeptide neurotoxins that are selective towards insects. After ingestion of a solution of 1ug/ul venom, mirids became increasingly agitated and uncoordinated and did not survive as long as control insects. Using our estimate of feeding intake, we calculated that an ingested dose of 12 ug of whole venom resulted in death in 3 days. As the insect neurotoxins comprise only 2-3% of whole venom the effective dose may be much less. Incubation of venom with trypsin results in loss of activity indicating that this effect is due to a proteinaceous toxin.

Mirids challenged with artificial diet containing proteinase inhibitors active against serine and cysteine proteases have shown inhibited growth compared to control mirids. Although promising, these results are limited as nymphs were not available for more extensive testing.

Amylase inhibitors (types I, II and III) from wheat where also tested against a small number of nymphs. Growth of mirids challenged with these proteins was not significantly different from that of control nymphs. However, using a quantitative amylase assay we have determined that high levels of this enzyme are present in mirid salivary glands. As part of her post-graduate research, Ms Gill Colebatch will be able to use this *in vitro* assay to try to identify an inhibitor of mirid amylase and determine effective concentrations to be used in bioassays.

Discussion:

This study was founded on the premise that proteins could be orally toxic to the green mirid. Studies on the digestive physiology and biochemistry of the mirid have been carried out concurrently with this project by Ms. Gillian Colebatch, as part of the post-graduate research. Using the enzyme horse-radish peroxidase, she demonstrated that proteins can pass through the mirid gut and maintain activity. In addition, her characterization of the amylases and proteases present in the mirid salivary gland and gut have provided a foundation for the rational selection of compounds to test in bioassays. These results, along with the findings that scorpion toxins and proteinase inhibitors effect the mirid upon ingestion, continue to support the original premise and the feasibility of using transgenic plants to deliver insecticidal proteins to the mirid.

The major obstacle to developing this physiological phenomenon into a control strategy is the supply of mirids for biological assay and toxin characterization. The green mirid is a difficult insect to rear in continuous laboratory culture and, although colonies can be maintained, production of a continuous supply of healthy insects for biological assay is problematic. One way to overcome this problem is to continually replenish the laboratory colony with field-collected insects. However, in the ACT and nearby regions of NSW, mirids are found only during the months of February to April and do not appear in large numbers. Further north, at Cowra, mirids are found in increasing numbers from January to May. The unavailablity of field insects from June to December created a shortfall of insects for bioassays.

The utility of the adult biological assay has been demonstrated using a purified protein neurotoxin from scorpion venom as well as whole venom. Ingestion of small doses of this toxin had a insecticidal effect within 3 days. However, there is a severe shortage of fast-acting proteinaceous toxins suitable for the genetic engineering of plants. Our development of a biological assay to facilitate the identification of proteins that may have a more subtle effect, such as by retarding nymph growth and/or development, has increased the potential number of proteins that can be tested.

Conclusions and Recommendations:

To conclude, the biological assays developed in this study and the preliminary screening results obtained demonstrate the potential of finding a proteinaceous toxin suitable for the genetic engineering of cotton resistant to mirid attack.

The major obstacle faced by this project was the supply of insects. As mentioned earlier, the presence of mirids in the region is limited. As we have not been able to develop a suitable rearing procedure, this study would have benefited by being carried out in an area where mirids are more abundant.

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Communication of Resuits:

World Cotton Conference-1. (1994) Orally acting neurotoxins for controlling the green mirid, *Creontiades dilutus* (Stal) (poster).

Pest Management Research Workshop. Australian Cotton Research Institute, November 15-16, 1994.

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Appendix:

Budget:

Source	1994	1995	Total
CRDC (1)	37,692	39,113	76,805
CSIRO	118,960	118,960	237,920

(1) The CSIRO contribution is based on a multiplier of salary. The multiplier includes salary related on-costs and the costs of laboratory and all other facilities associated with this budget. For requested salaries, the CSIRO contribution is based on a multipler of salary that includes the costs of laboratory and all other facilities associated with this budget.