ANNEXURE 6.3.3(a)

Assessment of the allergenicity of protein extracts from seeds of Bollgard II™ cotton relative to those of conventional cotton, as tested in Brown Norway rats

FINAL REPORT

(COPY No. 3/3)

ASSESSMENT OF THE ALLERGENICITY OF PROTEIN EXTRACTS FROM SEEDS OF BOLLGARD II® COTTON RELATIVE TO THOSE OF CONVENTIONAL COTTON, AS TESTED IN BROWN NORWAY RATS

STUDY No.: 3777/03

SPONSORED BY

MAHARASHTRA HYBRID SEEDS COMPANY LIMITED
RESHAM BHAVAN
4th FLOOR 78, VEER NARIMAN ROAD
MUMBAI 400 020
INDIA

TEST FACILITY

TOXICOLOGY DEPARTMENT
RALLIS RESEARCH CENTRE
RALLIS INDIA LIMITED
POST BOX No. 5813, PLOT Nos. 21 & 22
PEENYA II PHASE, BANGALORE - 560 058
INDIA

ADDITIONAL TEST SITE 1

MONSANTO COMPANY REGULATORY
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MAIL CODE U4A
ST. LOUIS, MISSOURI 63167
USA

DATE OF SUBMISSION: JULY 24, 2004

TABLE OF CONTENTS	PAGE

contd.

TABLE OF CONTENTS **PAGE**

TABLE OF CONTENTS contd.

LIST OF TABLES **TABLE** **PAGE**

LIST OF APPENDICES **APPENDIX** **PAGE**

LIST OF APPENDICES **APPENDIX PAGE**

LIST OF ANNEXURES **ANNEXURE PAGE**

QUALITY ASSURANCE STATEMENT

The Study No.: 3777/03 entitled "Assessment of the Allergenicity of Protein Extracts from Seeds of Bollgard II® Cotton Relative to those of Conventional Cotton, as tested in Brown Norway Rats " has been inspected in accordance with the OECD Principles of Good Laboratory Practice for the testing of chemicals [C(97)186/Final].

This study was inspected and findings reported to Management and to the Study Director on the dates shown below:

INSPECTIONS DATE	PHASE	REPORTING DATE
	INITIATION PHASE	
20.01.2004	Study plan review	20.01.2004
29.03.2004	Review of Amendment No.1 to study plan	29.03.2004
	IN LIFE PHASE	
27.01.2004	Acclimatization and feed mixing	03.02.2004
28.01.2004	Initial body weight, cage change, feed input and body marking	03.02.2004
30.03.2004	Authenticity of extracts, test item administration (intradermal injection) – challenge and necropsy	05.04.2004
	REPORTING PHASE	
15.06.2004	Review of statistical report of Active Cutaneous Anaphylaxis assay	15.06.2004
17.07.2004 to 21.07.2004	Draft report review	21.07.2004
24.07.2004	Final report review	24.07.2004

Inspections were performed according to the Standard Operating Procedures of the test facility's Quality Assurance Unit. The draft statistical report was reviewed only against the raw data values and the animal numbers. The report was inspected against the approved study plan and pertinent raw data and accurately reflects the raw data.

Date:

(Mr.SATISH MURTHY. V)
Head, Quality Assurance Unit
Rallis Research Centre, Bangalore

STATEMENT OF CONFIDENTIALITY

This report contains confidential and proprietary information of Maharashtra Hybrid Seeds Company Limited, Resham bhavan, 4th floor, 78, Veer Nariman Road, Mumbai 400 020, INDIA which will not be disclosed to anyone except the employees of this company or to persons authorised by law or judicial judgement without the expressed or written approval of Maharashtra Hybrid Seeds Company Limited, Resham bhavan, 4th floor, 78, Veer Nariman Road, Mumbai 400 020, INDIA.

STATEMENT OF GLP COMPLIANCE

This study was performed in compliance with the OECD Principles of Good Laboratory Practice [C(97) 186/Final] with two exceptions. Statistical analysis of the Active Cutaneous Anaphylaxis (ACA) challenge data was performed in compliance with Good Laboratory Practice Standards as specified in 40 CFR Part 160 (U.S. E.P.A.) and the production and characterization of the test article will be accomplished in a non-GLP facility, as per this mutually agreed to by the sponsor and the test facility. The Revised Guidelines for Research in Transgenic plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant Parts, Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998, envisages the conduct of a Passive Cutaneous Anaphylaxis (PCA) using Guinea pig to assess the allergenicity of genetically transformed products by dermal sensitization. The present experiment was conducted using oral sensitisation of Brown Norway Rats followed by a challenge by ACA, which is deemed comparable to the Guinea pig model.
This study was performed as per the mutually agreed study plan signed by the Study Director and Monitoring Scientist on 21.01.2004 and 27.01.2004, respectively, Amendment No.1 to study plan signed by the Study Director and Monitoring Scientist on 30.03.2004.

DECLARATION

The Study Director hereby declares that the work was performed under his supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Date:

(Dr.S.M.SULAIMAN)
Study Director

STUDY DETAILS

Study Title	:	Assessment of the Allergenicity of Protein Extracts from Seeds of Bollgard II® Cotton Relative to those of Conventional Cotton, as tested in Brown Norway Rats
Study Number	:	3777/03
Sponsor	:	Maharashtra Hybrid Seeds Company Limited Resham Bhavan 4th Floor 78, Veer Nariman Road MUMBAI 400 020
Monitoring Scientist	:	Dr. Richard E. Goodman Manager, Allergy Program Product Characterization Center Monsanto Company Regulatory 800 North Lindbergh Blvd. Mail Code U4A St. Louis, Missouri 63167 e-mail: richard.e.goodman@monsanto.com
Sponsor's Nominee	:	Dr.M.K.Sharma General Manager Maharashtra Hybrid Seeds Company Limited Resham bhavan, 4th floor, 78, Veer Nariman Road, Mumbai 400 020 INDIA
Test Facility	:	Toxicology Department Rallis Research Centre Rallis India Limited Post Box No. 5813, Plot Nos. 21 & 22 Peenya II Phase, Bangalore - 560 058, INDIA

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Note : The Principal Investigator for the additional site-1 is Dr. Richard E. Goodman, who is also the Monitoring Scientist for this study. Hence, the terminology " Monitoring Scientist " was used in the study.

Additional Test Site 1	:	Monsanto Company Regulatory 800 North Lindbergh Blvd. Mail Code U4A ST.Louis, Missouri 63167 USA

Study Schedule

Acclimatization	:	Start : 21.01.2004 End : 27.01.2004
Sensitization	:	Start : 28.01.2004 End : 30.03.2004
Observations	:	Start : 28.01.2004 End : 31.03.2004
ACA challenge	:	30.03.2004 and 31.03.2004

STUDY PERSONNEL

The following personnel participated in the conduct of the study.

Name	Signature	Date
Dr.S.M.SULAIMAN M.V.Sc., Study Director Haematology, Clinical Biochemistry and Metabolism Section	_____	_____
Mr.P.J.PRAKASH M.Sc., Technical Co-ordinator Acute and Non-Rodent Section	_____	_____
Dr.K.VENUGOPALA RAO M.V.Sc., Study Veterinarian Acute and Non-Rodent Section	_____	_____
Mr. M.VENKATESULU B.Sc., Data entry, Documentation, Data Analysis and Report compilation EDP Section	_____	_____

ASSESSMENT OF THE ALLERGENICITY OF PROTEIN EXTRACTS FROM SEEDS OF BOLLGARD II® COTTON RELATIVE TO THOSE OF CONVENTIONAL COTTON, AS TESTED IN BROWN NORWAY RATS

SUMMARY

This study was conducted to assess the allergenicity of protein extracts of Bollgard II® cotton relative to conventional cotton seeds. Seven to eight week old Brown Norway rats were randomly selected and assigned to 3 groups: G1 (control group), G2 (5% cotton seed diet) and G3 (10% cotton seed diet) and fed with control (commercial) diet and diet fortified with 5 and 10 % cotton seed, respectively for 63 days. The animals were observed daily for signs of toxicity and preterminal deaths. Body weights were recorded weekly and feed consumption was recorded three times per week at every change. There were no clinical signs of toxicity or mortalities and all the animals gained body weight.

At the end of feeding (sensitisation) period, 8 animals from each group were selected and challenged by intradermal injections of protein extracts from transgenic hybrid -Bollgard II cotton seeds, 4 non-transgenic cotton seed hybrid – MECH 12, MECH 162, MRC 6014 , MRC 6018 and rice grain. Systemic injection of Evan's blue (1% w/v) by intracardiac route provided an area of extravessation at the dermal challenge sites as an indicator of immune or inflammatory response.

Based on the statistical analysis of size of the induced vascular leakage (blue spot) at the challenge sites, the allergenicity and inflammatory characteristics of transgenic cotton seed Bollgard II were similar to non-transgenic cotton seed varieties. Based on these data, it is concluded that there is no biological difference between the allergenicity of the cotton seed meal from Bollgard II cotton seeds and the 4 non-transgenic cotton seed hybrids.

Date:

(Dr.S.M.SULAIMAN)
Study Director

ASSESSMENT OF THE ALLERGENICITY OF PROTEIN EXTRACTS FROM SEEDS OF BOLLGARD II® COTTON RELATIVE TO THOSE OF CONVENTIONAL COTTON, AS TESTED IN BROWN NORWAY RATS

INTRODUCTION

The Objective of this study was to assess the relative allergenicity of cotton seed proteins in Bollgard II cotton compared to the allergenicity of cotton seed proteins in conventional cotton hybrids, as measured by active cutaneous anaphylaxis in cotton seed-fed Brown Norway Rats.

MATERIAL AND METHOD

1. ANIMALS	:	Brown Norway rats (BN / SsNo1aHsd)
Source	:	Toxicology Department Rallis Research Centre Bangalore – 560 058, INDIA
No. of groups	:	Three groups: Control diet group, 5% cotton seed diet group and 10% cotton seed diet group.
No. of rats/group	:	10 females. (nulliparous and non-pregnant).
Date of birth	:	04.12.2003 to 07.12.2003
Age at start of treatment:		7-8 weeks
Identification	:	Rat accession number, cage card, crystal violet (temporary marking, before acclimatization) and Turmeric solution (body marking ,after grouping) was used for colour body markings.
Acclimatization	:	After veterinary examination the animals were acclimatized for 7 days before treatment.
Body weight range at start of treatment (g)	:	G1: 130 ± 6.25 G2: 127 ± 6.51 G3: 125 ± 6.92

2. GROUPING

Ten female rats/group were selected by an in-house method of body weight stratification and distribution. At the end of the acclimatization period, the animals procured for the study were weighed and grouped into body weight ranges: example: 121-130, 131-140, 141-150 g etc., and distributed to all the groups in equal numbers. Animals with extreme body weights were discarded.

GROUP ALLOCATION, NUMBER OF RATS AND DOSE

Group	Dose	No. of rats	Sex	Rat numbers	
				From	To
G1	Control (normal diet)	10	F	Rc1031	Rc1040
G2	5% cotton seed diet Sensitizing diet	10	F	Rc1041	Rc1050
G3	10% cotton seed diet Sensitizing diet	10	F	Rc1051	Rc1060

F : Female

3. HUSBANDRY

Room Number: Laboratory Room No. SC-18

Conditions:

Animals were housed under standard laboratory conditions, air conditioned with 12-15 filtered fresh air changes/hour. Environment: temperature 21-23°C, relative humidity 30-70%, with 12 hours fluorescent light and 12 hours dark cycle.

Housing:

The rats were individually housed in standard suspended polypropylene cages (size: L 290 x B 220 x H 140 mm) with stainless steel top grill having facilities for powdered feed and drinking water in glass bottles. Bedding: steam sterilized paddy husks were used and changed three times per week during the sensitization period.

Diet *ad libitum*:

Control feed: Ssniff Rats/Mice powdered food (maintenance meal) - low in germs manufactured by Ssniff Spezialdiäten GmbH., Ferdinand-Gabriel-Weg 16, D-59494 Söest, Germany. This feed was used as the base diet for the preparation of the sensitising diets containing 5 or 10% finely ground cotton seed powder. During the acclimatization period, all the rats were fed with control feed (powder).

Water:

Protected water: Deep bore-well water passed through an activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter cum-purifier manufactured by Eureka Forbes Ltd., Mumbai - 400 001, INDIA was provided to animals in glass bottles with stainless steel sipper tubes. Water analysis report is given as Annexure 4.

DIETARY SENSITIZATION MATERIAL, TEST AND CONTROL ITEM INFORMATION:

Sensitization material (powdered cotton seed meal) information (as furnished by the Sponsor):

Sensitizer	:	Powdered cotton seeds
Name to be used in the report	:	Powdered cotton seeds
Code by test facility	:	088/1-BGC
Batch No.	:	25-10-03
Prepared and supplied by	:	Monsanto Research Centre Bangalore

Date of preparation	:	25.10.2003
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	05.11.2003
Purity to be stated in the report	:	Not applicable
Physical appearance	:	Grey colour, fine powder
Storage conditions	:	deep frozen (Below - 20°C)

Extract No .1 Information (as furnished by the Sponsor):

Name	:	Cotton seed hybrid MECH 12 (Control)
Name to be used in the report	:	Cotton seed hybrid MECH 12 (Control)
Lot No.	:	2004/01
Prepared and supplied by	:	Monsanto Research Centre Bangalore
Date of preparation	:	12.03.2004
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Light colour liquid
Protein content	:	4 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Extract No .2 Information (as furnished by the Sponsor):

Name	:	Cotton seed hybrid MECH 162 (Control)
Name to be used in the report	:	Cotton seed hybrid MECH 162 (Control)
Lot No.	:	2004/02
Prepared and supplied by	:	Monsanto Research Centre Bangalore
Date of preparation	:	12.03.2004
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Light colour liquid
Protein content	:	4 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Extract No .3 Information (as furnished by the Sponsor):

Test item	:	Cotton seed hybrid Bollgard II in a MECH 162 genetic background
Name to be used in the report	:	Protein extracts from Bollgard II cotton seeds
Code by test facility	:	088/2-BGC.E
Lot No.	:	2004/03
Prepared and supplied by	:	Monsanto Research Centre Bangalore

Date of preparation	:	12.03.2004
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Light colour liquid
Protein content	:	4 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Extract No .4 Information (as furnished by the Sponsor) :

Name	:	Cotton seed hybrid MRC 6014 (Control)
Name to be used in the report	:	Cotton seed hybrid MRC 6014 (Control)
Lot No.	:	2004/04
Prepared and supplied by	:	Monsanto Research Centre Bangalore
Date of preparation	:	12.03.2004
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Light colour liquid
Protein content	:	4 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Extract No .5 Information (as furnished by the Sponsor) :

Name	:	Cotton seed hybrid MRC 6018 (Control)
Name to be used in the report	:	Cotton seed hybrid MRC 6018 (Control)
Lot No.	:	2004/05
Prepared and supplied by	:	Monsanto Research Centre Bangalore
Date of preparation	:	12.03.2004
Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Light colour liquid
Protein content	:	4 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Extract No .6 Information (as furnished by the Sponsor):

Name	:	Rice grain extract (Control)
Name to be used in the report	:	Rice extract (Control)
Lot No.	:	2004/06
Prepared and supplied by	:	Monsanto Research Centre Bangalore
Date of preparation	:	12.03.2004

Date of expiry	:	1 year from the date of preparation
Date of receipt at test facility	:	25.03.2004
Physical appearance	:	Colourless liquid
Protein content	:	1 mg/ml
Storage conditions	:	Deep frozen (Below –20°C)

Sensitization material (powdered cotton seed meal) composition:

Ground cotton seed meal was incorporated into the diets of the Brown Norway Rats as a sensitizing dose to stimulate cotton seed specific IgE antibodies. The sponsor arranged to have Monsanto India Limited provide the cotton seed meal as a single bulk sample for inclusion in the diets, as described in the next section.

As per the Certificate of Analysis provided by the sponsor (Annexure I), the cotton seed meal represents an equal mixture of ground, full-fat cotton seed from the following non-transgenic cotton seed hybrids:

1. Mech-12
2. Mech-162
3. MRC-6014
4. MRC-6018

The Certificate of Analysis (Annexure I) includes summaries of the composition data (gossypol and aflatoxin content and pesticide residues) from samples of the individual seed collections that were used to prepare the ground cotton seed meal. The same ground cotton seed materials were used to produce the control extracts and the sensitizing diets.

PREPARATION OF THE CONTROL AND COTTON SEED MEAL-FORTIFIED (SENSTIZING) DIETS

The Powdered (ground) cotton seed meal provided to Rallis by Monsanto was divided into sub-lots, which were stored at less than –20°C. The sub lots were taken from the freezer and used for the preparation of cotton seed meal fortified diets as described below.

Diets were prepared weekly; by mixing sub lots of ground cotton seed meal with the same powdered Ssniff diet that was used to feed the control group. Each prepared diet was distributed as 3 aliquots and a small retention sample. Aliquots from each of the dose groups were immediately distributed into the individual rat feed dispensers and the remaining aliquots were stored below –10°C until being distributed into the individual rat feed dispensers. Uneaten diet in the dispenser was discarded prior to the addition of fresh diet.

G1 group (Control diet): 500 grams of the powdered control feed was mixed manually in a stainless steel container for 2 minutes. This premix was added in portions to the remaining 1500 grams of the feed and mixed manually in a stainless steel container for 4 minutes.

G2 group (Sensitizing diet, 5% cotton seed): 100 grams of the ground cotton seed meal was mixed with 500 grams of the feed by manual mixing in a stainless steel container for 2 minutes. This premix was added in portions to the remaining 1400 grams of the feed and mixed manually in a stainless steel container for 4 minutes. The ground cotton seed meal in the final diet was equal to 5% of the total weight of the diet.

G3 group (Sensitizing diet, 10% cotton seed): 200 grams of the ground cotton seed meal was mixed with 500 grams of the feed by manual mixing in a stainless steel container for 2 minutes. This premix was added in portions to the remaining 1300 grams of the feed and mixed manually in a stainless steel container for 4 minutes. The ground cotton seed meal in the final diet was equal to 10% of the total weight of the diet.

The proximate analysis of the prepared diets (Control [normal] diet, 5% cotton seed diet and 10% ground cotton seed diet) was performed at Rallis Research Centre on 2 occasions: on the day of the first diet preparation and on the day of the last diet preparation (Table 1, Page 37).

Treatment (Sensitization)

The prepared diets were provided to animals *ad libitum* for 2 or 3 days before changing to new aliquot (three feed inputs/week). Each time, the left over feed in the hopper was weighed, then discarded and replenished with fresh feed. The animals were fed the prepared diets for 63 days (until the ACA challenges).
The first day the sensitising diets were fed to the rats was defined as " Day 1".

Note: The proximate analysis of the powdered cotton seed and the analysis of gossypol, aflatoxins, and pesticide contaminants in the cotton seed powder was performed in an external laboratory (Non-GLP) and the results are presented in the report as Annexure 1.

ACTIVE CUTANEOUS ANAPHYLAXIS (ACA) CHALLENGE

Animals were challenged by intradermal injection of soluble extracts of test and control materials to determine if proteins are other soluble molecules from the test material (Bollgard II cotton seed) would induce greater mast cell degranulation than soluble molecules from the control materials (non-transgenic cotton seed or rice grain), as measured by the diameter of extravasations of Evans Blue dye.

Animals were selected for the ACA during the last week of the feeding period (day 62 of the experiment). There were no clinical signs of toxicity or mortalities. There were 10 animals available for selection in each group (control, 5% and 10% cotton seed dietary groups) at the end of sensitisation period. The lowest and the highest body weight animals in each group were not challenged.

The remaining eight animals from each group were challenged by ACA within a 48-hour period, between experimental days 63 and 64. Each animal was challenged with four concentrations of four different challenge materials (test and control extracts), as well as a positive histamine control. Animals within dietary Group 1 were randomly assigned a number, 1-8. Animals within dietary Group 2 (5% cotton seed diet) were randomly assigned a number, 9-16. Animals within dietary Group 3 (10% cotton seed diet) were randomly assigned a number, 17-24. Challenges were sequentially administered to an animal from each dietary group (e.g., the first animal of Group 1, the first animal of Group 2, the first animal of Group 3, then the second animal of Group 1 and proceeded similarly until all animals are challenged). Challenge dose material identity numbers had been assigned to each animal number using the FREQ Procedure of SAS (SAS Institute, Inc., Cary, NC, USA) as presented in Appendix 4. Additionally, the location of the test and control injection sites were randomly assigned to one of four rows from head to tail.

PREPARATION OF EXTRACTS FOR INTRADERMAL INJECTION

Extracts of ground cotton seed and rice were thawed and diluted freshly for intradermal injection on each challenge day. The extracts were diluted based on the protein concentration in the extracts as analysed and provided by the sponsor (Annexure 2).

Cotton seed hybrid MECH 12 (Control) : Extract No. 1

Protein Concentration: 4 mg/ml

- Stock Solution: 3 ml of the extract was diluted to 30 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].
- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].
- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

Cotton seed hybrid MECH 162 (Control) : Extract No. 2

Protein Concentration: 4 mg/ml

- Stock Solution: 3 ml of the extract was diluted to 30 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].

- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].
- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

Cotton seed hybrid Bollgard II in MECH 162 (Test item) : Extract No. 3

Protein Concentration: 4 mg/ml

- Stock Solution: 3 ml of the extract was diluted to 30 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].
- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].
- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

Cotton seed hybrid MRC 6014 (Control) : Extract No. 4

Protein Concentration: 4 mg/ml

- Stock Solution: 3 ml of the extract was diluted to 30 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].
- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].
- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

Cotton seed hybrid MRC 6018 (Control) : Extract No. 5

Protein Concentration: 4 mg/ml

- Stock Solution: 3 ml of the extract was diluted to 30 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].
- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].

- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

Rice grain extract (Control) : Extract No. 6

Protein Concentration: 1 mg/ml

- Stock Solution: 8 ml of the extract was diluted to 20 ml with sterile normal saline to a final protein concentration of 400ng/µl (20µg/50µl) [Concentration : A].
- 5 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 100ng/µl (5µg/50µl) [Concentration : B].
- 1.25 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 25ng/µl (1.25µg/50µl) [Concentration : C].
- 0.31 ml of the stock solution was diluted to 20 ml with sterile normal saline to a final protein concentration of 6.2ng/µl (0.31µg/50µl) [Concentration : D].

The specific test and control challenge dose administration order was pre-selected to randomize the order of extracts and the order of challenging animals within each feeding group (Appendix 4 - as provided by the sponsor). Challenges were performed alternately on one animal from each feeding group, with one-half of the animals from each group challenged on day 63 and one-half on day 64. Intradermal injections (50 microliters) were administered and recorded in the specified order. Histamine (positive control) was also injected intradermally. The intradermal injections were performed as per the schematic diagram for each animal (Appendix 5).

Evan's Blue dye: Sigma Chemicals, USA: For Intracardiac injection

Evan's blue dye crystals – 1% (w/v) of Evan's blue solution.

Histamine free base: Sigma Chemicals, USA: For Intradermal injection

A concentration of 0.5 µg /µl histamine was injected with 20 µl/site.

Pentabarbital sodium anaesthetic: Sigma Chemicals, USA

A concentration of 10 mg/ml of pentobarbital was administered by intraperitoneal route at the dose of 32.5 mg (3.25 ml) / kg body weight.

METHOD OF INTRADERMAL INJECTION:

The predetermined sites for intradermal injections were marked with ink and the injections were performed using 26 gauge needles (½ inch) fitted to 1 ml syringes (1/100 graduations).

After the animals were anaesthetized with pentabarbital by the intraperitoneal route, they were injected intradermally with diluted extracts. Fifteen to twenty seven minutes later they were injected with Evan's blue by the intracardiac route. Ten to thirteen minutes later they were injected intradermally with histamine. Ten to eighteen minutes later the animals were euthanized, the skin excised, inverted and the diameter of blue spots were measured, recorded and photographed.

AUTHENTICITY OF EXTRACTS:

The protein concentration of the extracts were provided to Rallis in a Certificate of Analysis prior to initiation of the ACA Challenge (Annexure 2).

The identity of the test and control extracts supplied by the sponsor were authenticated by the Monitoring Scientist at Rallis Research Centre. This was accomplished using commercially available lateral flow immunoassay sticks (Strategic Diagnostics, Inc. USA) to detect the presence or absence of the Cry 1Ac and Cry 2Ab proteins that are expressed due to the insertion of the Bacillus thuringiensis *cry 1Ac* and *cry 2Ab* genes in Bollgard II cotton.

Principle of the method using Lateral Flow Strips(LFS):

Lateral flow strips have a lower cotton pad between plastic layers containing unattached anti-Cry1 or anti-Cry2 IgG antibodies that are labelled with immunogold. As the strip is dipped in the extract solution, any Cry1 or Cry2 proteins present in the solution will be bound by the specific IgG. All soluble proteins will be wicked up the strip. About half way up, there is a capture antibody (anti-Cry1 or anti-Cry2) so that the Cry protein will attached at that line. If it is also bound by the Immunogold labelled anti-Cry1 (anti-Cry2), a red line will appear. Near the top of the strip, below the large cotton pad, is another antibody strip of anti-IgG. This is a control capture antibody that will capture any IgG that has reached this level, and any immunogold labelled IgG will make a red mark at that level, whether the test solution was water, or extract of non-transgenic cotton or Bollgard II cotton.

Performance of the test:

The extracts were diluted into 1 ml microcentrifuge tubes, i.e. 150 µl of extract diluted with 300µl of distilled water and mixed. For each sample, an aliquot of 200 µl of diluted extract was transferred to another microcentrifuge tube and the LFS was inserted in the test solution up to the line below the arrowheads on the end, which does not have a cotton pad. The LFS was allowed to remain in the fluid for 10 minutes. After developing for 10 minutes, the strips were removed, interpreted, and labelled with the name of the extract and photographed. Each of the cotton seed and the rice extracts were freshly prepared and tested on both challenge days using Cry1 and Cry2 LFS.

Interpretation:

1. The control line, should always develop approximately 1 cm below the reservoir pad. A red line in this position indicates that the device is functioning properly.
2. A red line appearing below the control line indicates a positive result. If the test strip displays 2 red lines (control line and test line), the test is complete and the sample is positive.

OBSERVATIONS

1. Daily:

Rats were observed once daily for signs of toxicity and pre-terminal deaths.

2. Body weights:

Individual body weights were recorded weekly.

3. Feed consumption:

The feed input was done three times a week day 1, 3 and 5 of each week. At each input, residual feed was weighed, recorded and discarded so that only fresh feed was returned to the cage. The weekly feed consumption, expressed as "g/rat/day" was calculated by adding all feed inputs and subtracting residual feed and wastage for each week of the study.

ACTIVE CUTANEOUS ANAPHYLAXIS RESPONSE

The diameters of the extravasated blue dye spots in the excised and inverted skin were observed, measured and photographed. Photography was performed using a Digital camera and a SLR camera using photographic film. Each photograph has the Group number, Animal number, date and time of challenge.

STATISTICAL ANALYSIS

Analysis of body weight, net body weight gain and feed consumption data was performed by Rallis. The data were compared by Bartlett's test for homogeneity of intra-group variances prior to evaluation by one-way Analysis of Variance. Since the intra-group variances were not heterogeneous, the ANOVA was performed using the raw data. A Dunnett's "t" test was used to evaluate differences in body weight, net body weight gain and feed consumption between dietary groups using a significance level of $P \leq 0.05$.

Rallis provided Monsanto Company (St. Louis) with exact copies of the raw data from the ACA challenges of all animals. Statistical analysis of the ACA assay results was performed by Monsanto Company. Monsanto Company prepared a draft statistical report of the ACA analysis, which was reviewed by the Rallis QAU only for raw data and animal numbers prior to finalization (signature by the Monsanto statistician). The ACA statistical report (Annexure 3) and statistical data will be archived at Rallis.

The ACA assay data was analyzed using repeated measures of variance on the diameters of the ACA spots using SAS (SAS Institute, Inc., Cary, NC). This analysis was used to evaluate the significance of each of the individual components of variability (diet, extract dose, and cottonseed protein extract source).

The repeated measures model used was of the following form:

$D_{capd} = M_{cpd} + A_{ca} + E_{capd}$

Where:

D_{capd} = diameter obtained for protein extract p, dose d, animal a, and cottonseed diet c.

M_{cpd} = true mean diameter for dose d of protein extract p for animals fed diet c.

A_{ca} = random error for animal a fed cotton seed diet c.

E_{capd} = random within-animal error for each observed diameter.

The Mixed procedure in SAS was used to fit this mixed effect linear model to the data. The variability among the M_{cpd} were broken down into separate components representing main effects and interactions.

RESULTS AND DISCUSSION

a. **TOXIC SIGNS AND PRE-TERMINAL DEATHS:**

There were no clinical signs of toxicity and pre-terminal deaths (mortalities).

b. **BODY WEIGHT:** Table 2 App. 1

The weekly mean body weights increased in all the groups during the observation period. There was no statistically significant intergroup difference between control and treatment groups except for statistically significant lower body weights of the G3 group during weeks 3 to 9 ($p \leq 0.05$), as analyzed by one-way-ANOVA.

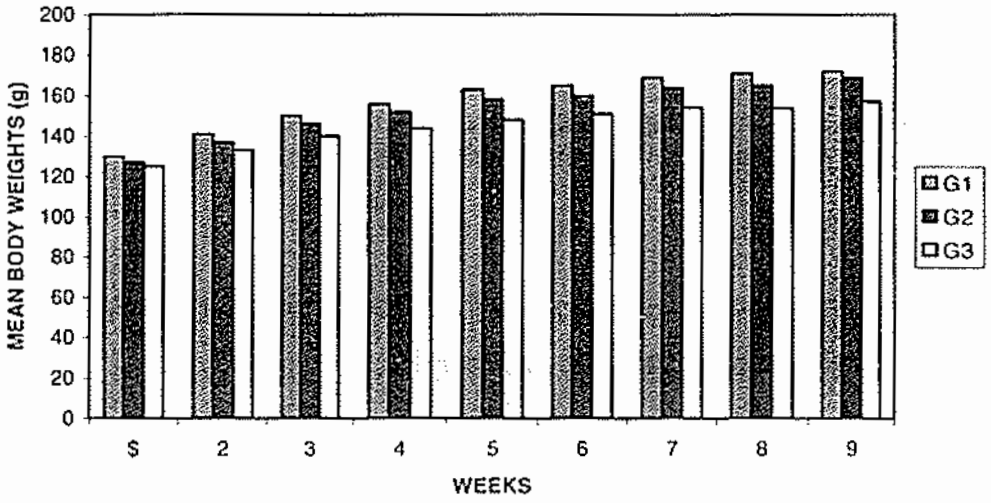


Figure 1: Mean body weights for each of the treatment groups of Brown Norway rats, at weekly intervals.

Net body weight gain: Tables 3, App. 2

The mean cumulative net body weight gain in control, 5% cotton seed diet and 10% cotton seed diet were 42, 42 and 32 grams respectively. The cumulative net body weight gain was significantly lower in the G3 group.

Percentage Cumulative Net body weight gain: Tables 3, App. 2

The percentage cumulative net body weight gain was 32, 33 and 26 % in comparison with the initial body weight. The percentage cumulative net body weight gain was significantly lower in the G3 group.

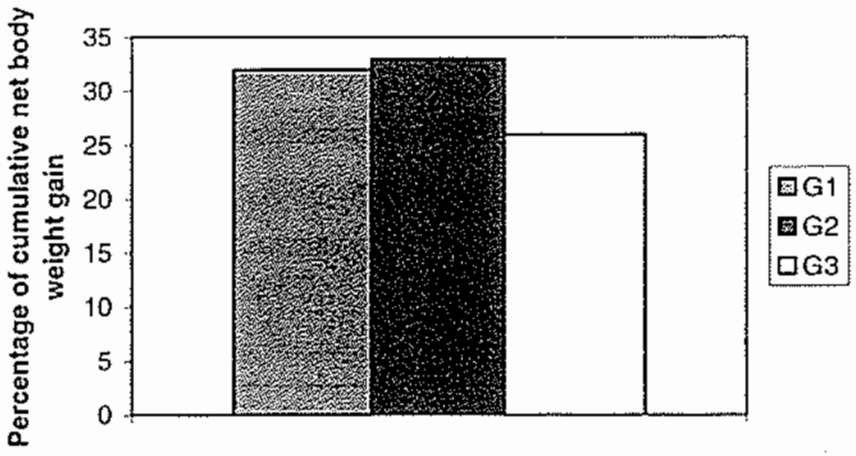


Figure 2: Percentage of cummulative net body weight gains in the three treatment groups of Brown Norway rats, over the complete 62 days feeding period.

c. FEED INTAKE: Tables 3, App 3

There were no significant differences in feed consumption between control and cotton seed diet treated groups.

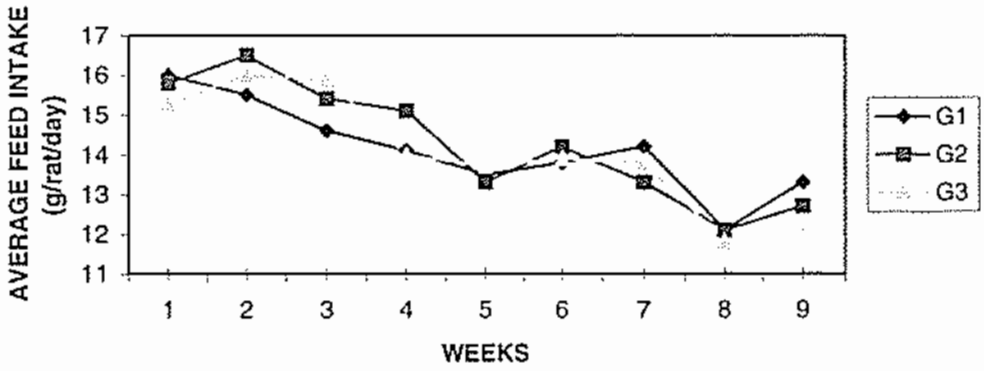
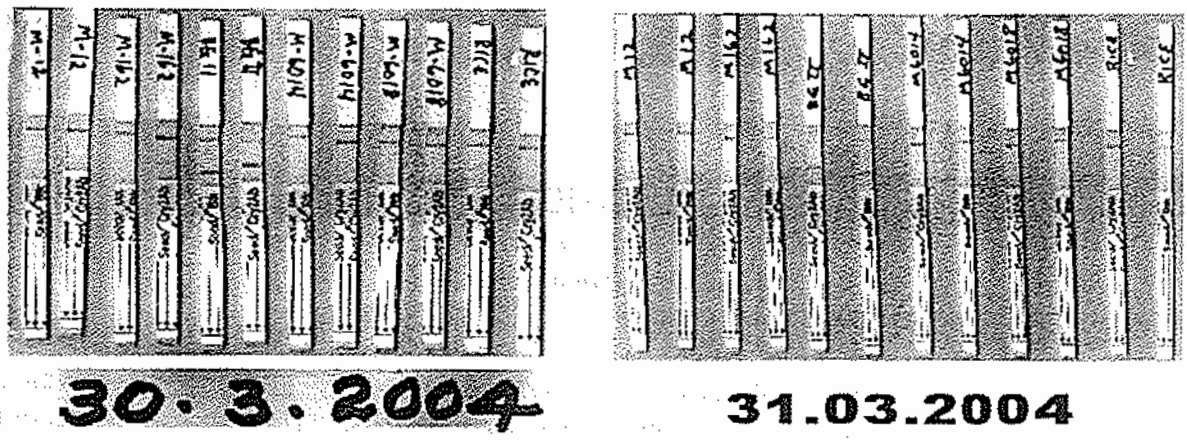


Figure 3: Average weekly feed intake in the three treatment groups of Brown Norway rats. The animals were provided with control diet (G1), 5% cotton seed diet (G2) and 10% cotton seed diet (G3) for the 62 days feeding period

Although the average body weight and weight gain of the 10% cotton seed diet fed group was significantly lower than the control and 5% cotton seed fed group, the animals continued to gain weight throughout the study and appeared to be in good health. Since the feed consumption was similar throughout, it appears that there was an anti-nutrient effect when 10% cottonseed is used in the diet. However, since the animals were in good health and the immune/inflammatory responses were similar between groups, it appears that there was no significant biological affect that would be assumed to alter the results of this study.

AUTHENTICITY OF EXTRACTS:

The identity of the test and control extracts supplied by the sponsor were authenticated by the Monitoring Scientist at Rallis Research Centre using commercially available lateral flow immunoassay sticks (Strategic Diagnostics, Inc. USA) to detect the presence or absence of the Cry 1Ac and Cry 2Ab proteins. The test item (Extract No. 3 : Bollgard II cotton in MECH 162) was positive for the presence of Cry 1Ac and Cry 2Ab proteins and all the other five extracts were negative.



Photograph 1: A positive result for the presence of Cry 1Ac or Cry 2Ab proteins in BG II cotton (Extract No.3 : Bollgard II cotton in MECH 162) is indicated by two red lines. A negative (single band) is indicated by one red line.

ACTIVE CUTANEOUS ANAPHYLAXIS (ACA) RESULTS:

a. Photographs of the inner side of the animal skins showing the dermal blue dye staining at the ACA challenge sites on each animal are presented in Appendix 5. The responses to challenges with all the cotton seed extracts were dose dependent, with the exception of the rice extract. There were no apparent differences between the skin reactions of the same dose of each of the 4 extracts on the same animals. This observation suggests that there are no differences between the allergenic or inflammatory characteristics of the 5 cotton seed extracts tested in this study.

b. ACA spot diameters were measured immediately after euthanasia (Appendix 6). Comparison of the mean values obtained at each dose concentration indicates that the treatment means follow a dose-dependent response and there are no marked differences between the different cotton seed extracts used in the challenges.

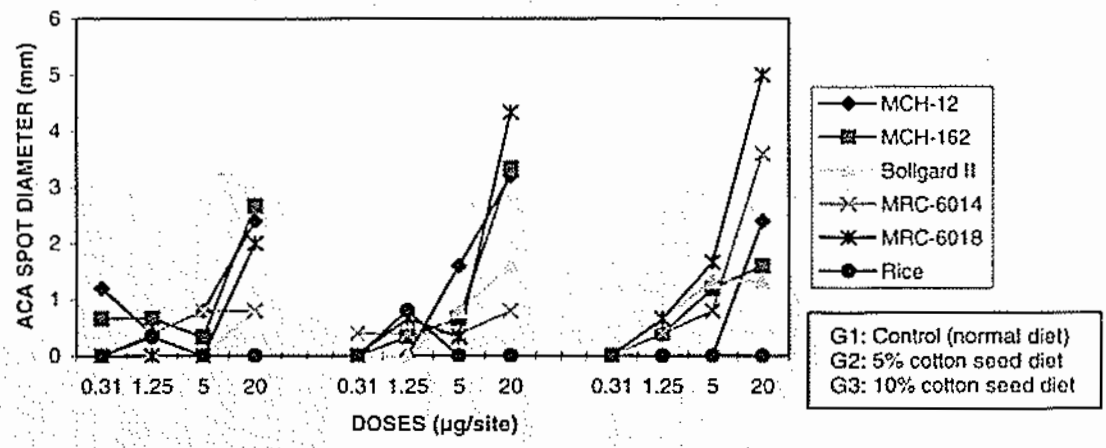


Figure 4: Mean ACA spot diameters (mm) after intradermal challenge with extracts at various doses to Brown Norway rats fed with control diet (G1), 5% cotton seed diet (G2), and 10% cotton seed diet (G3) for 63 days.

c. Statistical Analysis of ACA data (Annexure 3):

Table 1 of statistical analysis report shows that the two main effects, Extract and Dose, along with the two-factor interaction, Extract*Dose, are statistically significant, $p < 0.05$. There are no statistically significant differences among diets ($p = 0.62$), suggesting that much of the ACA response is an inflammatory response to mediators in the extracts rather than a memory immune response.

The pairwise comparisons in Table 3 of the statistical analysis report shows that MECH-12, MECH-162, MRC-6014, and MRC-6018 cotton extracts have similar mean ACA diameters; Bollgard II, MECH-162, and MRC-6014 have similar means; and Bollgard II and Rice are not statistically different.

The pairwise comparisons in Table 4 of the statistical analysis report show that a dose of 20µg is significantly higher than the other three doses; doses 1.25µg and 5µg have means which are not statistically different and doses of 0.31µg and 1.25µg have means which are not statistically different. . This dose effect demonstrates that components in the extracts were responsible for the ACA affect, rather than some mechanical injury or positional affect.

The pairwise comparison results in Tables 3 and 4 of the statistical analysis report are summarized by inclusion of the letters a, b, c, next to the means in Table 2. Means with the same letter are not statistically different at the 0.05 level of significance. Notice that, since the p-value for diets was greater than 0.05 ($p = 0.62$ in Table 1), means are not significant and the three diet means have the same letter "a" designation.

Figure 1 of the statistical analysis report shows that all the extracts except Rice have similar responses across doses, demonstrating the similarity of responses to the extracts of the five cotton hybrids.

This result of the statistical analysis leads to the conclusion that allergenic and inflammatory responses are similar for all hybrids of cotton, including Bollgard II cotton.

CONCLUSION

The study was conducted to assess the relative allergenicity of cotton seed proteins in Bollgard II cotton compared to the allergenicity of cotton seed proteins in conventional cotton hybrids, as measured by active cutaneous anaphylaxis (ACA) in cotton seed-fed Brown Norway Rats. The results from this study indicated that no biological differences were detected in comparing the allergenicity of the extracts of cotton seed meal from Bollgard II cotton and that of 4 non-transgenic cotton seed hybrids.

ARCHIVING

Rallis will archive data, reports and materials at the test facility for 15 years after completion of the study: study plan, raw data, ACA statistical report and the draft and final study reports. Photographs (including negatives) of the inverted skins of test animals will be archived. The specimens (inverted skins) will not be archived as the Evans Blue dye spots cannot be preserved in a way that would afford further evaluation. A sample of the test and control extracts was sent from the test item stores to the archives at the time of receipt of test item. This sample shall be stored for a period of 2 years from the date of this final report or till the next GLP inspection, whichever is later, however not beyond 30 years.

REPORT DISTRIBUTION

Archives: One signed final report in original, with high quality photographic renditions of the inverted skins (Copy No. 1/3).

Sponsor: Two exact copies of the final reports (Copy No. 2/3 and 3/3) with high quality photographic renditions of the inverted skins (not photocopies)