Revised Guidelines for Research in Transgenic Plants & 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

Printed at :
Public Printing (Delhi) Service
C/80, Okhla Industrial Area Phase-I
New Delhi-110020

CONTENTS

FOREWORD

As we advance our research efforts in developing transgenics in plants, the scientists would require safety guidelines for understanding such endeavours. Accordingly a Committee of Experts under the Chairmanship of Prof. V.L. Chopra, National professor, ICAR, has revised the earlier (1994) guidelines. The revision is the consequence of enormous experience the country has gathered from the use of transgenic plants from early nineties. The first transgenic field experiment was started at the instance of the Department in early 1995. After watching the actual risks from the use of certain transgenic plants, a need was felt that the earlier guidelines could be simplified for the benefit of researchers in the country. In addition, the biosafety aspects of transgenic plants were required to be highlighted. The need for strengthening the monitoring and evaluation mechanism was felt and a Monitoring-cum-Evaluation Committee (MEC) comprising experts from user Ministries have been incorporated in the Revised Guidelines. A Green House design concept which is expected to be guiding the institutes in installing their own safe Green House/Net House for use in research utilizing transgenic plants has also been developed. The Review Committee on Genetic Manipulation (RCGM) of the DBT has approved the document. Detailed guidelines for toxicity and allergenicity evaluation of transgenic seeds, plants and plant parts as have been finalized by the present RCGM in consultation with Industrial Toxicology Research Institute (ITRC), Lucknow are annexed. The necessity of this arose from the food safety evaluation of transgenic seeds and plants, as such plants code for various proteins, which are not native in them, have been made to be produced through human intervention utilizing recombinant DNA technology. While the expression of many transgenes is considered to be a low risk, in many situations their presence in the food may trigger allergic reactions. Therefore, the safety evaluation of transgenic foods is very relevant. The RCGM has done a commendable job and I feel that this document will be extensively utilized by the researchers, the scientific community and the industry who are involved in the development of transgenic seeds and plants that have potential for entrance into the human food chain.

New Delhi

Manju Sharma

(Manju Sharma)
Secretary to the Government of India
Department of Biotechnology

REVISED GUIDELINES FOR RESEARCH IN TRANSGENIC PLANTS

1998

1. INTRODUCTION:

The revised present document is meant for the researchers in the country who are involved in recombinant DNA research on plants. Earlier the Department of Biotechnology in January 1990 issued a compendium of guidelines under the title "Recombinent DNA Safety Guidelines". A revision was made in 1994 under the title "Revised Guidelines for Safety in Biotechnology. The current guidelines have been developed in the light of enormous progress that has been made in recombinant DNA research and its widespread use in developing improved microbial strains, cell lines and transgenic plants for commercial exploitation.

2. COVERAGE OF THE REVISED GUIDELINES

The current guidelines cover areas of recombinant DNA research on plants including the development of transgenic plants and their growth in soil for molecular and field evaluation. The guidelines also deal with import and shipment of genetically modified plants for research use only.

3. STATUTORY BODIES DEALING WITH THE RECOMBINANT DNA WORK:

In accordance with the Notification No. GSR 1037 (E) dated 5th December, 1989 of the Ministry of Encironment & Forests which empowers the Review Committee on Genetic Manipulation (RCGM) to bring out manuals of guidelines specifying procedure for regulatory process with respect to activities involving genetically engineered organisms in research use and applications including industry with a view to ensuring environmental safety, the present changes in the procedures are being made. These changes are made reiterating the powers conferred on the RCGM to lay down procedures restricting or prohibiting production, sale, importation and use of genetically engineered organisms or cells as are mentioned in the attached schedule of the above mentioned notification.

A. ISBC (Institutional Biosafety Committee):

i. The IBSC is nodal point for interaction within an Institute/University/Commercial Organization involved in r-DNA research for the implementation of the recombinant DNA guidelines. As such, in the first instance, it is necessary that the organizations intending to carry out research activities involving genetic manipulation of microorganisms, plants or animals should constitute their IBSC in accordance with the procedures in vogue and as informed to the public through the above nofification. All recombinant research carried out by the organization should have a designated Principal Investigator (P.I). It would be the duty of the P.I. to apprise its IBSC about the nature of the experiments being carried

out. Depending upon the category of the experiments as narrated later on in the present guidelines the P.I. can inform the IBSC about the recombinant experiments, seek permission of IBSC before starting the experiments or seek the permission of the RCGM through its IBSC in cases where the risks involved in the experiments are considered to be of higher magnitude having the potential of polluting/endangering the environment, the biosphere, the eco system, the animals and the human beings.

The Department of Biotechnolgy in January 1990 has enumerated the duties of the SC in pages 15-16 of the original ``Recombinant DNA Safety Guidelines".

RCGM (Review Committee on Genetic Manipulation):

i. The RCGM is functioning in the Department of Biotechnology to monitor the safety-related aspects of ongoing research projects involving genetically engineered organisms.

ii. The RCGM shall include representatives of a) Department of Biotechnology; b) Indian Council of Medical Research; c) Indian Council of Agricultural Research; d) Council of Scientific and Industrial Research; and e) others experts in their individual capacity. RCGM may appoint subgroups to monitor specific projects.

iii. The RCGM would review all the reports of all approved on-going research projects involving high-risk category and controlled field experiments.

iv. The RCGM or its constituted subgroups shall visit the site of experimental facilities periodically, where projects with biohazard potential are being pursued and also at a time prior to the commencement of the activity to ensure that adequate safety measures have been taken as per the guidelines.

v. The RCGM would issue the clearance for import/export of etiologic agents and vectors, transgenic germplasms including transformed calli, seeds and plant parts for research use only.

vi. The RCGM shall meet at least twice in a year

vii. For research in recombinant DNA work-involving risks categorized as category-III and above in this revised document the permission of the RCGM through the Department of Biotechnology must be obtained by the P.I. before conducting the research work.

viii. RCGM can authorize applicants (P.I.s) to conduct limited field trails in multi locations in the country. The design of the trial experiments is either provided by the RCGM or it may approve the protocol designed by the P.I. The protocol will seek answers to relevant and necessary questions on environmental hazards including risks related to animal and human health. Data should also be generated on economic advantage of the transgenics over the existing varieties.

ix. RCGM can, if required, direct the applicants to generate toxicity, allergenicity and any other relevant data on transgenic materials in appropriate systems. RCGM may design or approve a protocol for conducting experiments to seek answers to the above.

x. The RCGM can put such conditions as would be required to generate long term environmental safety data from the applicants seeking release of trangenic plants into the open environment, and who have complied with initial safety evaluation.

4. CATEGORIES OF GENETIC ENGINERRING EXPERIMENTS ON PLANTS AND THEIR NOTIFICATIONS:

A. CATEGORY I, routine recombinant DNA experiments:

This category includes routine cloning of defined genes, defined non-coding stretches of DNA and open reading frames in defined genes in E. coli or other bacterial and fungal hosts which are GENERALLY CONSIDERED AS SAFE (GRAS) to human, animals and plants. A list of such microorganisms will be prepared by the RCGM and shall be made available to the P.I. on request.

This category inovlves experiments in the lab in contained environment and includes the following.

i. Routine cloning of defined DNA fragments of microbial, animal and plant origin in GRAS organisms.

ii. Transfer of defined cloned genes into Agrobacterium;

iii. Use of defined reporter genes to study transient expression in plant cells to study genetic transformation conditions;

iv. Molecular analysis of trangenic plants grown in-vitro.

Categories I experiment need only intimation to the IBSC in the prescribed proforma (available with the RCGM secretariate).

B. CATEGORY II

This category includes lab and green house/net house experiments in contained environment where defined DNA fragments non-pathogenic to human and animals are used for genetic transformation of plants, both model species and crop species and the plants are grown in green hous/net house for molecular and phenotypic evaluation.

This category includes the experiments described below

i. Transgenics with constitutive, tissue specific and chimeric promoters used for experimenting expression of defined DNA fragments

ii. Marker genes extensively used in genetic transformation of plants in lab and green house/net house experiments.

iii. Lab and green house/net house experiments with plants with herbicide resistance conferring genes;

iv. Lab and green house/net house experiments with plants using heterologous genes which confer resistance to biotic and abiotic stresses (i.e. genes like chalcone synthase, heat shock proteins, chitinase, protease inhibitors etc.);

v. Lab and green house/net house experiments with genes from plants, animals and microbial sources that would confer resistance to plant pathogens.

vi. Lab and green house/net house experiments on transgenics with genes for the production of antibodies.

vii. Green house/net house experiments with transgenics with transposable elements for gene tagging in crop species or model species.

Permission for performing Category II experiments will be provided by IBSC. The decision of the IBSC would be intimated to the RCGM before execution of the experiments and RCGM would put this information on record.

C. CATEGORY III & ABOVE

This category pertains to high risk experiments where the escape of transgenic traits into the open environment could cause significant alterations in the biosphere, the ecosystem, the plants and animals by dispersing new genetic traits, the effects of which can not be judged precisely. All experiments conducted in green house and open field conditions not belonging to the above Category I and Category II types, would fall under Category III risks. Such experiments could be conducted only after clearance from RCGM and notified by the Department of Biotechnology.

5. CONTAINMENTS:

Different levels of containment are prescribed for the three different categories of rDNA experiments.

i. Category I experiment should be performed using routine good laboratory practices (See Appendix I for details).

ii. For Category II experiments dealing with evaluation of transgenics in green house/net house, the designs for the contained facility shall be as described in Appendix II. The transgenic experiments of Category II risks will have to be carried out in green house/net house, the specification of which is significantly stringent to ensure arrest of transgenes within the contained facility.

iii. For Category III experiment rin green house / net house, the later needs to be designed as indicated broadly in Appendix II. The specifications of the green house / net house have been designed to ensure near complete isolation of the facilities from the open environment; care has also been taken to prevent the entry of insects into the green house / net house facility.

For limited field experiments in the open environment, the RCGM would provide for and / or would approve the design of the experimental field plots.

6. MONITORING AND EVALUATION MECHANISMS FOR GREEN HOUSE / NET HOUSE EXPERIMENS AND LIMITED FIELD TRIALS IN THE OPEN ENVIRONMENT :

The RCGM can bring out manuals of Guidelines specifying procedures for regulatory process with respect to activities involving genetically engineered organisms in research

and applications to ensure enironmental safety. To monitor, over a period of time, the impact of transgenic plants on the environment, a special Monitoring cum Evaluation Committee of the following constitution will be set up by the RCGM. The Committee shall have the following constitution.

a)	Chairman of the Committee	: Secretary, DBT & Secretary, DARE shall jointly discuss and elect a leader of the committee.
b)	Eminent Plant Biotechnologists	: To be nominated by RCGM, 3-4 Nos.
c)	Seed Technologists	: To be nominated by ICAR, 2-3 Nos.
d)	Plant Breeders	: To be nominated by ICAR, upto 2 Nos.
e)	Plant Ecologist/Environmentalist	: To be nominated by RCGM, up to 2 Nos.
f)	Nominee of NBPGR	: To be nominated by ICAR.
g)	Nominee of MoE&F	: To be nominated by the Chairman, GEAC
h)	Member-Secretary	: Member-Secretary, RCGM.

This committee will undertake field visits at the experimental site/s. The committee shall be guided by the RCGM on the design of field experiments and on the preparation of formats for collecting scientific information on plants in green house / net house conditions as well as in limited field trials. Based on the on-the-spot situation the committee can suggest remedial measures to adjust the original trial design and assist the RCGM in collecting, consolidating and analyzing the field data for evaluating the environmental risks emanating from the transgenic plants. This committee shall also collect or cause to collect the information on the comparative agronomic advantages of the transgenic plants. From time to time, the committee shall advise the RCGM on the risks and benefits from the use of the transgenic plants put into evaluation. Trials will be done for at least one year with minimum four replications and ten locations in the agroecological zone for which the material is intended. The biological advantage of transgenics will have to be clearly enumerated by the applicant, the Institution, the University or the Industry. The latter would recommend those transgenics, which would be found to be environmentally safe and economically viable by the RCGM, to the Genetic Engineering Approval Committee for consideration for release into the environment

7. BIOSAFETY ASPECTS OF THE TRANGENIC PLANTS

Experiments are designed to systematically identify the hazards, to access to risks and to take step to manage the risks by applying logically valid strategies, to systematically identify the hazards and to assess the risks; the information on the following aspects would be required to be generated.

I. Characteristics of the donor organisms providing the target nucleic acids. These may include the following :

   a. Name of the donor organisms with its identification characteristics with relevant reference to published information if any.

   b. Pathogenically and toxicity characteristics to plants and animals.

c. Allergenicity characteristics to human alongwith of the allergenic substances, wherever possible.

d. The geographical origin of the organism, its distribution pattern and survival mechanism.

e. The method of transfer of its genetic materials to other organisms.

II. Characteristics of the vectors used: These may include the following :

a. The origin, identity and habitat of the vectors used.

b. The sequence, frequency of mobilization, specificity and marker genes if any, present in the vectors.

c. The abilities of the vectors to get established in other hosts; the hosts are also to be specified.

III. Characteristics of the transgenic inserts : These may include the following :

a. the specific functions coded by the inserted nucleic stretches including the marker gene inserts.

b. The expression of the nucleic acid products and their activities/properties.

c. The toxicity of the expression products on the host plant, if any.

d. The toxicity and allergenicity of the nucleic acid products to human and animals.

IV. Characteristics of the transgenic plants : These may include the following :

a. Methods of detection of the transgenic plant in the environment.

b. Methods of detection and characterization of the escaped transgenic traits in the environment.

c. Toxicity and pathogenicity of the transgenic plants and their fruits to other plants in the ecosystem and the environment.

d. Possibility of and the extent of transgenic pollen escape and pollen transfer to wild near relatives, and the consequences to the environment.

e. Pathogenicity, toxicity and allergenicity of the transgenic plants and their fruits to human and animals.

Information on many of the above questions may already be available. Many questions may however be required to be investigated and answers found out, for which appropriate new experiments would have to be designed to gather data. For generating toxicity and allergenicity data, standard protocols devised by international agencies could be used. The Indian national toxicological laboratory like the Industrial Toxicology Research Centre, Lucknow could be consulted to generate appropriate protocol for these purposes.

For minimizing the risk arising from the limited release of transgenic plants, the following may be taken into consideration :

a. Special separation for isolation, for preventing reproduction/fertilization and seed setting.

b. Biological prevention of flowing by making use of sterility properties etc.

c. Human intervention for the removal of reproductive structures of flowers.

d. Controlling the reproductive structures of transgenic plants like the seeds and the plant propagules from unaccounted spread.

e. Controlling and destroying volunteer plants from the experimental field.

f. To take into account the proximity to human activity in case the transgenic plants have allergenic properties to human and animals.

g. Appropriate training of field personnel responsible for handling the transgenic plants.

h. Plans for handling unexpected events.

i. Documentation of previous published information, if any, including any documented evidence of effects of release to ecosystem.

Thorough comparison with national checks for productivity and susceptibility/resistance to biotic and abiotic stresses will have to be made.

All the information as above are to be documented in the form of a document which would be called the registration document. An outline of the ingredients of a registration document is enclosed at Appendix III The guidelines for the evaluation of toxicity and allergenicity of transgenic seeds, vegetable and leaves have been placed at Appendix IV.

8. IMPORT AND SHIPMENT OF TRANSGENIC GERMPLASM FOR RESEARCH PURPOSES :

Clearance for import of trangenic material, for research purposes would be provided by the RCGM. The RCGM will issue an import certificate after looking into the documents related to the safety of the material and the national need. The RCGM will take into consideration the facilities available with the importer for in soil tests on the transgenic material. The importer of a transgenic material may import the material accompanied by an appropriate phyto-sanitary certificate issued by the authority of the country of export, and such import may be routed through the Director, NBPGR on the basis of the import permit issued by the DBT, based on the recommendations of the RCGM. The import certificate would be cancelled if NBPGR would not provide the phyto-sanitary certificate. NBPGR will provide information on the time that is required for phyto-sanitary evaluation. These evaluations will be done in a time-bound manner in presence of the agents of the institutes or the commercial organizations that are importing the material, if they so desire. Parts of the seed material will be kept at NBPGR in double lock system in the presence of the importer. This lot of seed will act as a source material in case of any legal dispute.

APPENDIX I

GOOD LABORATORY PRACTICES

i. Use a pipettor for all the solution transfers. No mouth pipetting.

ii. Plug pipettes with cotton.

iii. Do not blow infectious material out of pipettes.

iv. Do not prepare mixtures of infectious material by bubbling expiratory air through the liquid with a pipette.

v. Before and after infecting an animal, swab the site of injection with a disinfectant.

vi. Sterilize discarded pipettes and syringes in pan where they were first placed after use.

vii. Before centrifuging, inpect tubes for cracks. Inspect the inside of the Turin cup for rough walls caused by erosion or adhering matter., Carefully remove all bits of glass from the rubber cushion.

viii. Use centrifuge tunnion cups with screw caps or equivalent.

ix. Avoid decanting centrifuge tubes; if you must do so, wipe off the outer rim with a disinfectant. Avoid filling the tube to the point that the rim ever becomes wet with culture.

x. Sterilize all contaminated material before discarding.

xi Periodically, clean out deep-freeze and dry-ice chests in which cultures are stored to remove broken ampules or tubes. Use rubber gloves and respiratory protection during the cleaning.

xii. Avoid smoking, eating and drinking in the laboratory.

xiii. Do not reuse plasticware that has been used for PCR, recombinant DNA work and plant transformation work.

xiv. Sterilize all the plasticware before discarding it.

xv. Burn all the transgenic material in an incinerator after observations have been taken.

APPENDIX II

MODEL PLAN FOR THE CONSTRUCTION OF A GREEN HOUSE/NET HOUSE FOR EXPERIMENTS USING TRANSGENIC PLANTS

Frame Structure : The structure should be made from galvanized mild steel designed to with stand wind loading of not less than 100 km/hour. The method of affixing the polythene film cover to the frame should be strong enough to with stand similar wind velocities. The base may be constructed with bricks and cement or with any durable structure up to a height of 1½ to 2 feet from the ground level so as to isolate the land inside the framed structure from the out side land.

Optimum size of unit : The recommended minimum size of the unit would be 1000 to 1500 cubic meters. In dimensions each such unit may be 30 meters long 13 meters wide and having an under the gutter height of about 3 to 4.5 meters from the base. The plan view as well as the side view of a multi span unit with double door entry recommended for an optimum size unit is enclosed along with this appendix-II. It is recommended that all the green house structures should have double door entry as indicated in the enclosed drawings, and the span of the area for the double door entry can be kept as 5 to 6 meters in length and about 3 meters in width along with height maintained commensurate with the main structure of the unit. The main entrance may be optionally be provided with an air curtain. The outer door shall be only one panel of flush door opening inside the buffer area and the inside doors may be more than one (two sliding doors have been shown in the drawing). In case sliding doors are not installed, the inside doors should be of one panel each, opening inside the buffer area only. The entry wall can be utilized for housing the suction fans as shown in the drawing while the opposite wall can be mounted with evaporation pads (shown in the drawing). The optimum sized unit recommended above would provide a growing area of about 350 sq. meters, allowing 10% for path ways. This unit would have a volume of about 1100 – 1200 cubic meters. Such a unit would be able to maintain a stable temperature, the desired humidity with adequate and ample air circulation.

Plastic film covering : It is recommended that the area covering the frame should be of 200 micron (800 guage) thickness, UV stabilized polymer film. Such materials are expected to have a life span of 4 to 5 years. All coverings should be double film covering on all surfaces to give double UV filtration and a more stable temperature control. The roof covers are likely to be inflated by the action of blower fans, thus maintaining a cavity throughout the unit. In addition to it is suggested that an internal separation wall can be constructed to bifurcate the spans if there are more than one, which can be done by fixing the plastic films to the securing rails. With in the whole unit facilities can thus be provided for separate crop studies.

Fan, Pad system and Filter screens : An evaporative cooling system will be required to enable the maintenance of stable temperature gradient from the site of evaporating pad to the suction end. The surface are of the cooling unit will depend upon

the over all size of the structure. If the unit exceeds 30 meters in length then the temperature variation through out the length of unit may be such that an even temperature may not be maintainable even with the introduction of turbo circulation fans. The dimensions of the evaporation pad required to obtain a temperature 15 degree centigrade below ambient for a give volume of green house can be calculated from the following approximate equation.

Pad area (P) = Length X Width X Height, the whole divided by 94.85
Where P is in sq. Meter area.

As an example it is stated that a unit having the dimensions of 30 meters X 13 meters X 3 meters requires a pad area of not less than 12.35 Sq. meters. As most pad units are constructed to order, it is expected that it would not be difficult to have the pad areas of correct size.

All external surfaces of the pad should have filter screens of at least a 40 X 40 mesh net covering made from durable plastic material.

The fans required for a unit of above dimensions, to be housed at the other end of the unit should be about 61 centimeters (24 inch) in diameter with low noise and high C.u. ft. /min (CFM) air circulation capacity, with four numbers to be installed per unit. It is recommended that motors with 1.5 H.P. with three phase may be installed which is slightly over designed but which is expected to have more life span and therefore substantial saving on replacements. Compromises can be made by installing 1 H.P. three phase motors, but this may need more maintenance. The fan units should have 40 X 40 mesh durable plastic screen fitted to the out side of the external surface. Each motor unit can be connected to one semi automatic temperature controlled which should shut down the fan as and when the temperature drops below the required levels. Such designs are available in the market.

Blower fans are required to be fitted on to each roof section which will inflate the top roof sheet. These fans must also to be fitted with 40 X 40 mesh durable plastic screen on the induction side to prevent any pollen evacuation. As these fans are expected to be constantly in operation it is recommended that these should be fitted with bearings and not with bush type.

It is essential to have circulation fans within the green house to ensure that a uniform temperature is maintained through out the growing area. The number and the positioning will however depend upon the external conditions and therefore may vary from place to place. The manufacturer may be consulted for selecting the correct number.

Irrigation : Full over head irrigation systems are available and can be installed. In smaller houses it would be advisable to carryout the watering manually as regulation of humidity is difficult to maintain through over head irrigation system because any extra watering will increase the humidity level. In line feeding units can be installed to take care of the nutrient requirements of the plants. A water tank needed to supply water to the pads and irrigation may be installed slightly below the ground level to avoid direct influence by sun or solar heat. The water will therefore remain cool.

Proposed positioning : The location and the orientation of the unit is of significant importance. The fans should not be positioned in a manner that they blow directly towards

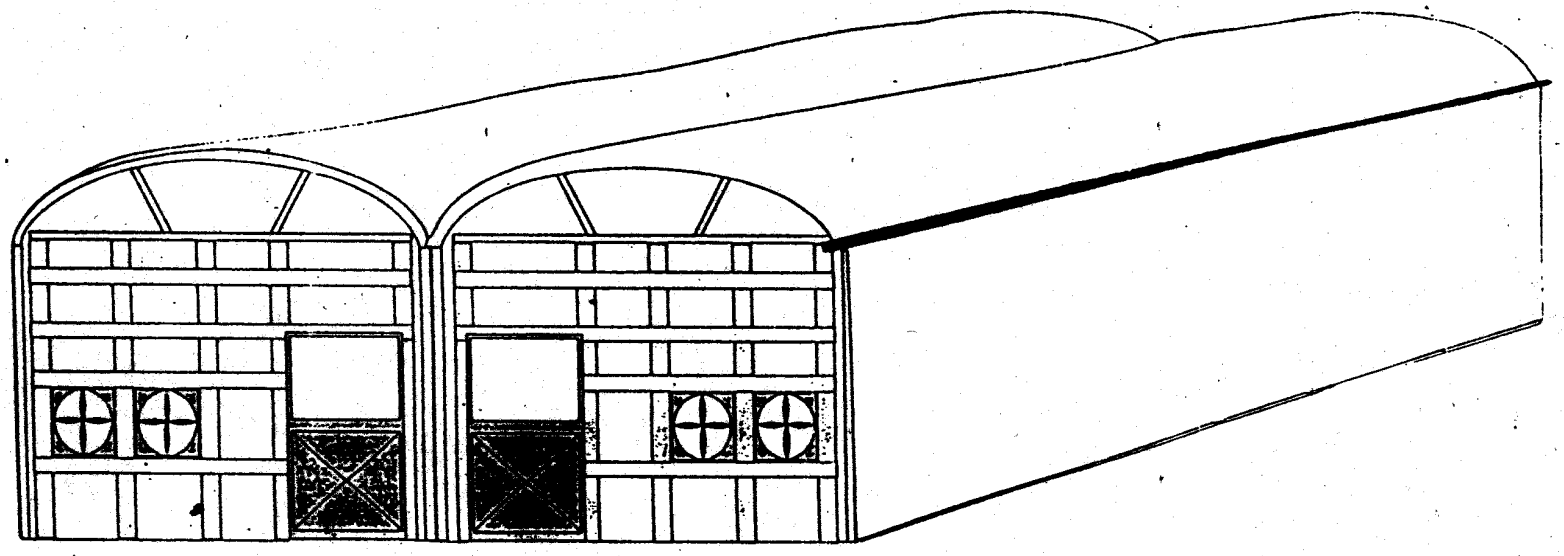
the plants. Electricity and water are continuously required. Therefore these must be positioned within a reasonable reach of the unit to keep costs down. The area selected for the unit must be flat, and as far as possible leveled to accommodate the unit plus approxmately two meters off around the out side. It would be useful to provide a drainage system around the unit at suitable lower levels to enable the drainage of extra water. A suitable drain off area is also recommended to enable the extra water running off from the gutters; the drain off area may be more than 10 meters away from the unit.

Views showing the different aspects of Polyhouse/Greenhouse : Five diagrams showing schematically one recommended unit of the dimension 30 meters X 13 meters X 3 meters (Length X Breadth X Gutter height, excludng the dome height) are appended at enclosures I to V. The installers can install units bigger than the one suggested above. However, they have to ensure that all the safety precautions namely, installation of double doors, use of durable structures for the framework, use of at least 200 micron (800 gauge) plastic films in double coverings are used in the construction. Further, all the outlets would have to be secured by applying 40 X 40-mesh durable plastic coverings as indicated above.

Enclosure-I

DOUBLE SPAN ENCLOSED UNIT FOR TRANSGENIC PLANT PRODUCTION

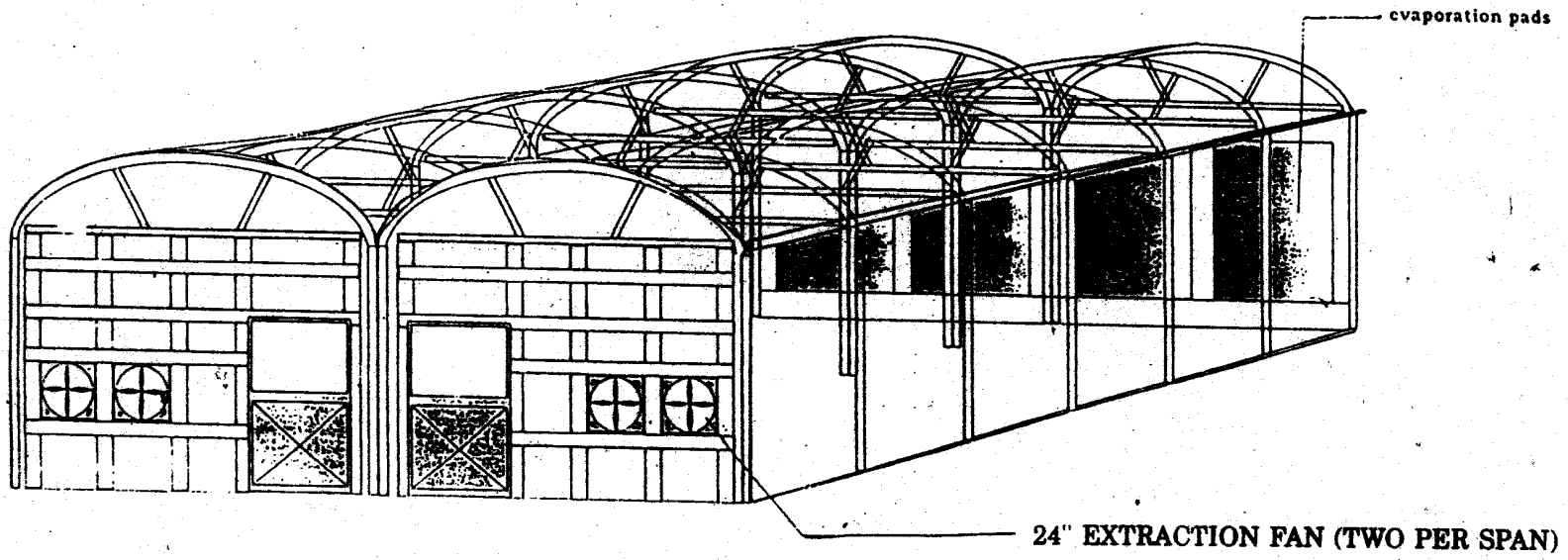
With Fan & Pad Cooling System



DOUBLE POLY SKIN ON ROOF AND ALL WALLS.
FANS AND BLOWERS FITTED WITH 40/40 MONO FOIL MESH NETTING
DOORS FITTED WITH AIR RETENTION BRUSHES
ENTRANCE THROUGH DOUBLE DOOR (AIRLOCK TYPE) SYSTEM

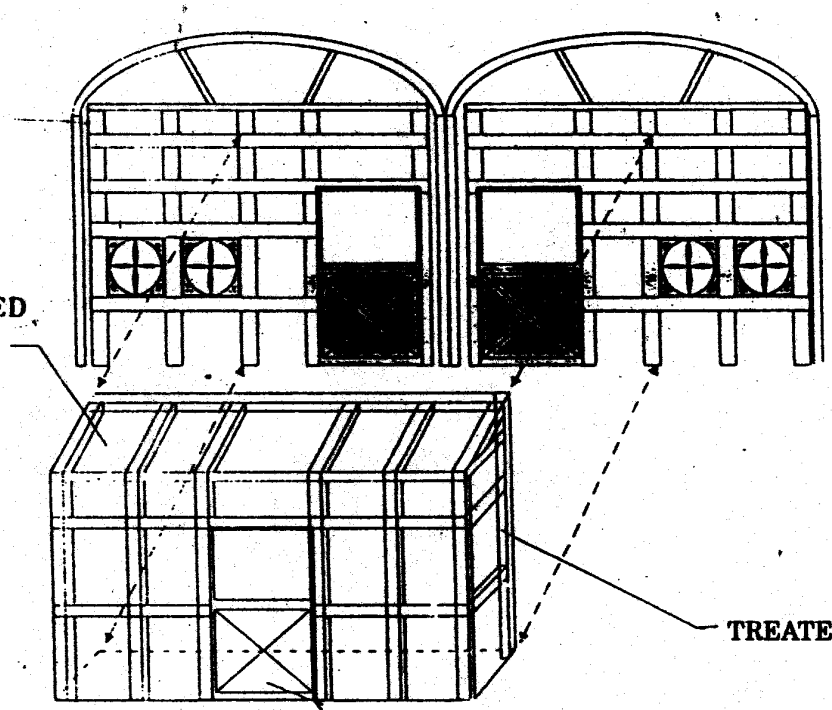
Enclosure-II

VIEW SHOWING MULTI-SPAN UNIT WITH COOLING PADS & FANS FITTED



Enclosure-III

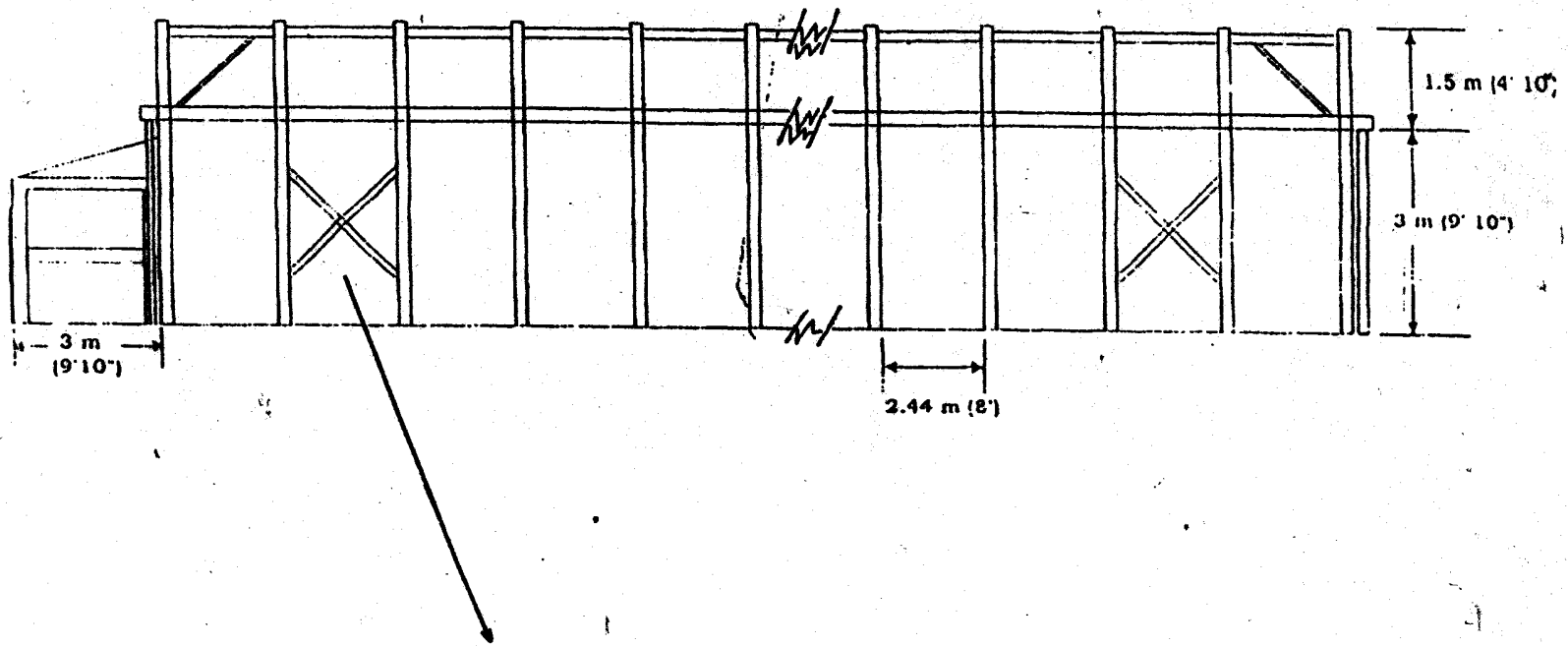
VIEW SHOWING DOUBLE DOOR ENTRY



SINGLE DOOR EXTENSION UNIT

Enclosure-IV

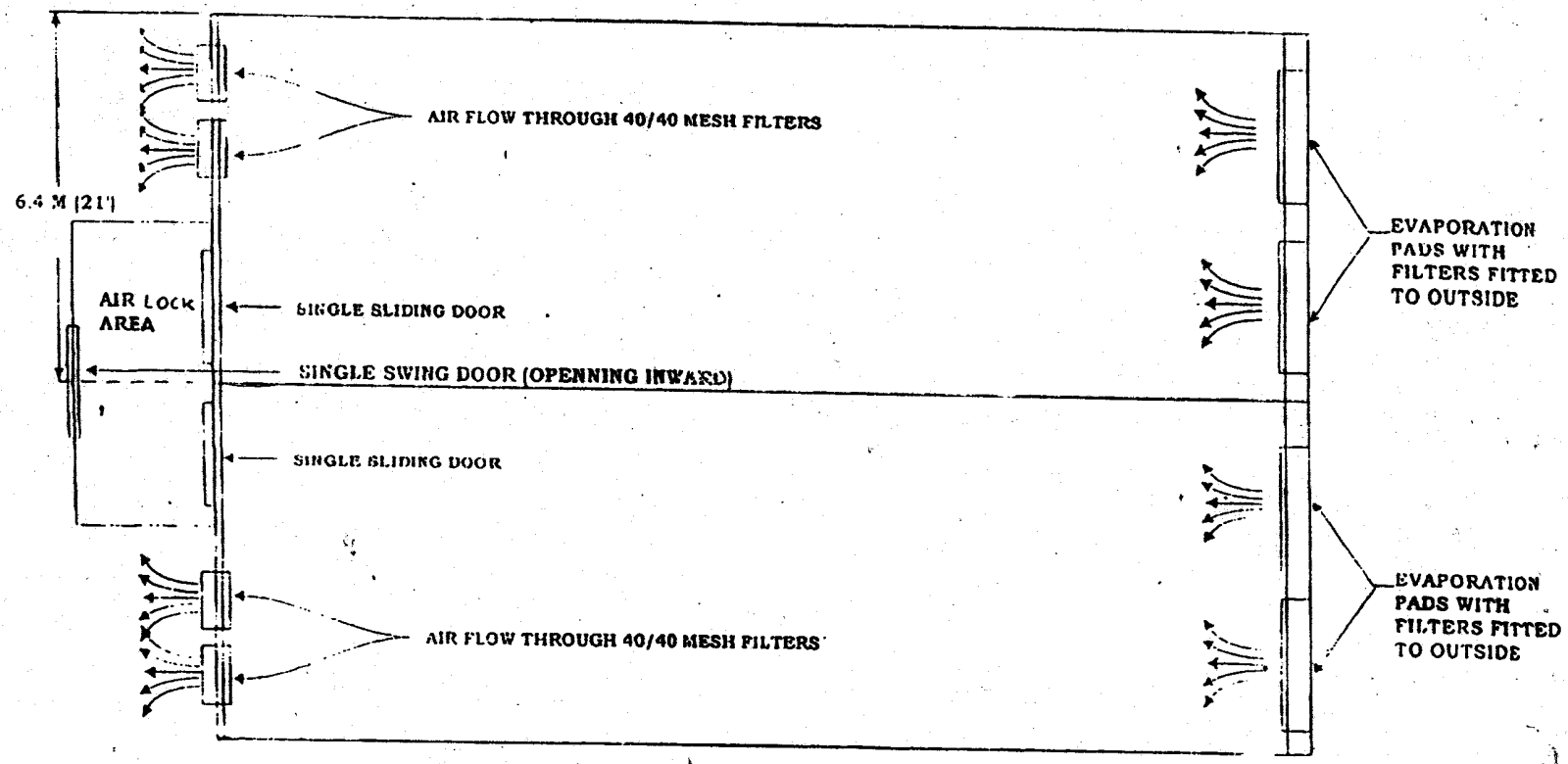
SIDE VIEW OF MULTI SPAN UNIT WITH DOUBLE DOOR ENTRY



CROSS BRACES FITTED TO ALL SPANS BOTH ENDSS AS SHOWN

Enclosure-V

PLAN VIEW OF GREEN HOUSE STRUCTURE



RECOMMENDED MINIMUM GROWING AREA 13 M X 30 M (2 X 6.4 M SPANY 12 X 2.44 M BAYS)

POTENTIAL PLANT CAPACITY - APPROX. 1,000 14" CLAY POTS, OR 2,000 LITRE POTS

APPENDIX III

APPLICATION SEEKING NON-REGULATED STATUS IN INDIA UNDER THE ENVIRONMENT (PROTECTION) ACT-1986 FOR TRANSGENIC PLANTS

CERTIFICATION

(A certificate to the following effect be submitted by the applicant)

I / we..... Son(s) / Daughter(s) of..... certify that to the best of my knowledge and belief the information provided in the application includes all information and views on which a judgement can be made to decide about the status of the product under the Indian Environment (Protection) Act, 1986 and that it includes all relevant information and data known to me / us.

Signature of the Applicant(s)
Name of the Applicant(s) _____
Representing _____
Full Address of the Applicant & the representative
Organization _____
_____

Place :

Date :

EXPLANATORY NOTE APPENDED FOR THE REVIEW

(This should justify the design of the work, the citation of the past literature if any on the subject with proper referencing, the authentication of the gene/s and the gene product/s by method/s to be stated if unpublished, and any other relevant information published in the literature with proper citation. The idea is to enable the reviewers to appreciate the special features of the product/s being reviewed).

PARTICULARS OF THE APPLICATON SEEKING NON-REGULATED STATUS UNDER ENVIRONMENT (PROTECTION) ACT, 1986

Name of the Project :

Submitted by under Section/s of the EPA-1986	:	Name & Address of the Applicant indicating telephone No., Fax No., E-mail No. etc., and the Section/s under which application is submitted.
Document Prepared by, and responsible for further responses	:	Name & Address of the person who is responsible for furnishing replies to subsequent queries.
Names & Addresses of the Contributor to the substantive parts of the document	:	1. _____ 2. _____ 3. _____ etc.

ABBREVIATIONS USED IN THE APPLICATION

Abbreviations/symbols used	Expansion of the Abbreviations/symbols with explanations if required

CONTENT OF THE CHAPTERS

(Data required for the appraisal of Environmental Risks and Safety from the use of Transgenic plants)

Part : A

Chapter I : **Introduction**

1. Rationale for the development
2. Benefits - Economic Benefits, Agronomic benefits etc.
3. Regulatory approvals required for, and earlier approvals obtained if any for specific purposes in India with approval No. etc.
4. Other relevant information including status of regulation in the country of origin with documentation, and status of regulation in other countries.
5. Discussion and conclusions.
6. Referencing.

Chapter II : **Biology of the Plant System**

1. Relevance of the plant in India.
2. Taxonomy, genetics, pollination pattern etc.
3. Description of the near relatives of the plant in the ecosystem.
4. Methods of pollen dispersal in target plants & in near relatives.
5. Others including documented references.
6. Conclusions.
7. Referencing.

Chapter III : **Molecular Biology of the Plant and Transformation Methods**

1. Description of the plant materials to be transformed.
2. Source of the gene and the cloning strategy followed.
3. Characteristics of the plant expression vector.
4. Characteristics of the inserted genes with sequence details.
5. Characteristics of the vectors and the transformation system employed with description of sequences used.
6. Genetic analysis including insert No, Copy No., Insert Integrity, Segregaton, Stability of the gene transfer, Description of the expressed gene, Biochemistry of the expressed gene products, authentication of the gene products by physical, chemical, immunological and biological methods etc.

7. Discussion and conclusions.
8. Referencing.

Part : B

Chapter IV : **Field Trial Plans :**

1. Field test permit, locations and design of trial
2. Plant growth and specific observations required to be made including the extent of gene escape, persistence of escape etc.,
3. Strategy adopted for determining efficacy of the transgenics in the field trials and plan for presentation of data.
4. Sumary and conclusions highlighting expectations from the trail.
5. References.

Chapter V : **Result of Phenotype of the Transformed Plant & Fruits / Seeds**

1. Germination and vigour results of the transgenic line in field & in the lab.
2. Description of the Phenotype of the transformed plant.
3. Composition and quality of the transformed plant and the seeds / fruits of the plants and comparison with non-transgenic phenotypes.
4. Competitive Toxicant analysis of the transformed plant and potential for weedinss in cases of uncontrolled release of transgenic plants.
5. Risks during the processing / handling of the transformed plant / fruits.
6. Susceptibility of the plant products / fruits to diseases and pests
7. Long term influence of the plant pests to the transformed plants, fruits and seeds.
8. Gene transfer to non-transgenic lines including near relatives and percentage of transfer under specific field conditions.
9. Out-crossing potential including pollen transfer to cultivated genotypes, and wild species and its implications.
10. Implication of transfer of genetic information to species to which it can inter breed.
11. Possible impact on environment on over all assessment.
12. Summary and conclusions.
13. Referencing.

Part : C

Chapter VI : **Consequences to the Environment**

1. Environmental consequence of introduction of transformed cultivars

2. Statement of unfavourable grounds
3. Effect on non-target organism including non-target insects, non-target birds and fish and non-target animals including mammals and wild life on extensive exposure of transgenics.
4. Impact on endangered species
5. Response plans for controlling unfavourable grounds in the environment.
6. Plans for protecting human and animal health from undesirable effects.
7. Summary and conclusions.
8. Referencing.

Part : D

Chapter VII : **Food Safety Evaluation :**

1. Evaluation of food safety assessment in approved protocol including nutritional studies (anti-nutritional factors if any and substantial equivalance studies etc.), sub acute and chronic toxicity studies & allergenicity status if any etc.
2. Classical animal feeding trials
3. Immunotoxicological studies
4. Gut toxicological studies.
5. Fundamental molecular biological studies including gene integration, gene regulation, gene expression and effects of transgenic proteins.
6. In vitro hemolytic behaviour of the transgenic proteins if any and its relevance to in-vivo studies in target animals.
7. Summary and conclusions.
8. Referencing.

Part : E

Chapter VIII : **Supportive Evidences for All The Chapters**

1. Supporting evidences in statements of annexures cataloguing Chapter No. and Annexure No.
2. Supportive evidence providing lists of figures cataloguing Chapter No. and Figure No.
3. Supportive evidence providing lists of tables cataloguing Chapter No. and Table No.

Part : F

Chapter IX : **Summary and Conclusions**

1. Executive summary and overall conclusions.

ACUTE ORAL TOXICITY TESTS OF TRANSGENIC SEED IN RAT

ADOPTION : OECD 401

Application and limitation of tests:

Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a test chemical or multiple doses given within 24 hrs. It is the initial step to find out median lethal dose (LD50) value which serve as basis for classification and labelling of the compound. It also forms a basis for selection of dose for subchronic studies. It will provide information on target organs toxicity after single exposure.

Principle:

The test compound is administered orally by gavage in numerical doses to groups of animals, one dose per group. Signs of toxicity and death of animals are observed during 14 days observation period. The dead animals are necropsied during and the surviving animals are sacrificed and necropsied after the 14 days observation period for gross pathology. Vital tissues of moribund and sacrificed animals are put for histopathological studies, clinical biochemistry and haematological examination.

DESCRIPTION OF THE TEST PROCEDURE:

Animals :

Healthy animals kept under standard animal husbandry conditions are used. At least 10 animals (male/female) are dosed. The weight variation of animals does not exceed 5-10g.

Animal Maintenance

Animals are acclimatized to the experimental animal room having temperature 75 ±2°F, humidity 30-70% and 12 : 12 hrs. light dark condition. Animals are caged with maximum of 2 animals in each polypropylene cages. Standard animal diet and water *ad libitum* is given to animals.

Preparation of dose:

Test sample i.e. fine powder of transgenic seed dissolved/suspended in groundnut oil is administered to rats fasted overnight. The volume does not exceed 1 ml/100 g body weight. At least four doses of the test sample spaced in geometrical factor are selected. The treatment schedule is as given below.

Group 1 - Control (normal diet)
Group 2 - Non transgenic seed
Group 3 - Transgenic seed

Limit test dose

If a test sample at 5000 mg/kg body weight produces no mortality, then other doses are not essential.

Observations:

The dosed animals are observed twice daily for 14 days to record the signs of poisoning and death of animals. The signs of poisoning include tremor, convulsion, salivation, diarrhoea, lethargy, sleep, coma, dyspnea, nasal bleeding etc. The time of death of animals is recorded. The body weight, food and water intake is recorded daily and monitored weekly. All the animals (moribund/live) are sacrified after 14 days and examined for gross and histopathological changes, clinical biochemistry and haematological examination.

Pathology

The liver, kidney, gonads, adrenals, spleen and brain are weighed immediately after autopsy. All animals are subjected for gross pathological changes. The vital organs like liver, kidney, brain, gonads, spleen, adrenal, thyroid, lungs, heart, stomach, duodenum, jejunum, colon, uterus, prostate are fixed in formal saline solution and tissues embeded in parafin wax and section cut at 6 um on rotary microtome. The prepared slides are then stained in haematoxylin eosin for microscopic examinations.

Haematology

Haematology is carried out in oxalated blood using standard methods of Wintrobe and Landsberg 1935 and Kolmer et. al. 1951. Blood is analysed for WBC, RBC, Hb differential leucocytes, clotting and prothrombin time and ESR.

Clinical Enzymes

Serum and blood are analysed for:

(i) Glutamic oxaloacetic transaminase (GOT), (ii) Glutamic pyruvic transaminase (GPT), (iii) Alakline phosphatase (Orthophosphoric monoester hydroxylase ALP), (iv) Bilirubin (v) Blood glucose (vi) Blood urea nitrogen, (BUN) (vii) Non protein nitrogen, (NPN) by the method of Wootton (1982), (viii) Acetylcholinesterase (AchE) by the method of Hestrin 1949 and (ix) Protein by the method of Lowry et.al. 1951; (x) Serum histamine level.

Calculations

LD50 values and its range are calculated by the procedure of Weil 1952 and toxicity rating is done by Gleasons et.al. 1969. All observed are recorded and calculated by appropriate methods. The statistical evaluation is done by Fisher's student `t' test. The results are summerised in tabular form.

References

Weil, C.S., tables for convenient calculation of median effective dose (LD or ED) and instruction in their use. Biometrics, 8, 249, 1952.

Gleason, M.N., Gosselin, R.E., Hodge, H.C. and Smith, R.P. Clinical toxicology of commercial products. Acute poisoning 3rd ed. Williams and Williams, Baltimore, Maryland.

Report on Acute oral toxicity

Test Animal.....

Rats..... Sex : Male/Female.....

Test Sample
Solid, Liquid, any other

Nature of vehicle
dist. water, peanut oil, corn oil, any other

Date of experiment started .....

Date of experiment terminated .....

LD50.....mg/kg; Range..... to .....mg/kg

Dosage (mg/kg)	Animals Died/Dosed	Death	Symptoms of toxicity
1. Control			
2.			
3.			
4.			

Statistical Method

Gross Pathology

Observations

Conclusions

Toxicity Rating

Report on Acute Oral Toxicity

Test animal : Rat.

Test Chemical : Solid, Liquid, any other

Nature of vehicle: dist. water, peanut oil, corn oil, any other

Date of expt. started....., date of expt. terminated.....

FOOD (G) WATER (ML) INTAKE OF MALE OR FEMALE ANIMALS EXPOSED

TO.....FOR 14 DAYS

Dosage (mg/kg/day)	**Days**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MALE														
1. Control														
2.														
3.														
4.														
FEMALE														
1.														
2.														
3.														
4.														

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated : :.....

RELATIVE ORGAN WEIGHT OF MALE OR FEMALE ANIMALS EXOSED TO ..... FOR 14 DAYS

Dosage (mg/kg/day)	Liver	Kidney	Adrenalm	Heart	Spleen	Brain	Pituitary	Test is Ovary	Epididymis Uterus	Cervix	Vagina
MALE											
1. Control											
2.											
3.											
4.											
FEMALE											
1.											
2.											
3.											
4.											

*Organ weight / Body weight x 100

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated :.....

BLOOD PICTURE OF MALE OR FEMALE ANIMALS EXPOSED TO:..... FOR 14 DAYS

Dosage (mg/kg/day)	RBC (x 10 mm)	WBC (x 10 mm)	Hb	PVC	platelet	Differential Leucocyte count (%)			
						Neutrophils	Lymphocytes	Monocytes	Eosinophils
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1.									
2.									
3.									
4.									

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated:.....

BIOCHEMICAL CHANGES IN MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 14 DAYS

Dosage (mg/kg/day)	Blood Sugar	Alk. Phos.		Protein		GOT		GPT	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1. Control									
2.									
3.									
4									

Title : SUBCHRONIC (90 DAYS) ORAL TOXICITY TEST OF TRANSGENIC SEED IN RAT

Adoption OECD 408

Application and Limitation of Test

Subchronic oral toxicity is the adverse effect occuring as a result of repeated daily oral dosing of a chemical to the animals. In the evaluation of toxic characteristics of a chemical the subchronic oral toxicity provides information on possible health hazards due to repeated exposure over a limited period of time. It will provide the information on target organ and the possibility of cumulation and for the selection of dose level for chronic studies.

Principle

The test sample is orally administered in three doses to animals for a period of 90 days. The animals are observed for any signs of toxicity and death during the period of exposure. Vital tissues of moribund and sacrificed animals are put for histopathological studies. Clinical biochemistry and haematological examinations are also made.

Description of the test Procedure

Rat is the preferred rodent model for subchronic oral toxicity studies. Healthy animals kept under standard animal husbandry conditions are used. At least 20 animals of 6-8 weeks old are used per group for three dose levels. The weight of the animals does not vary + 20 g.

Animal Maintenance

Animals are acclimatized to the experimental animal room having temperature (75 + 2 F), humidity (30-70%) and 12 : 12 hr light : dark conditions. Animals are given commercial feed and water *ad libitum*.

Preparation of dose

Test sample i.e. fine powder of transgenic seed dissolved/suspended in peanut oil is orally administered by gavage to animals consequently (5 days/week) for 90 days. The selection of the dose is made on the basis of acute toxicity studies of the test sample. At least three dose level, one maximum, one minimum and one intermediate are used. Consideration is given that the highest dose may result toxic effects without causing excessive lethality and lowest dose may not produce any toxic effects. A group of vehicle control is also used.

Limit test dose

If a test at one dose level of at least 1000 mg/kg body weight (but expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects, then a full study using three dose levels may not be considered necessary. The treatment schedule is given below:

Group 1 - Control
Group 2 - Non transgenic seed
Group 3 - Transgenic seed

Observations

Animals are observed once daily to record the signs of poisoning, like tremor, convulsion, diarrhoea, lethargy, dyspnea and nasal bleeding etc. The time of death is also recorded. The body weight, food and water intake is recorded daily and monitored weekly. At the end of 90 days animals are weighed and sacrificed.

Pathology

The liver, kidney, gonad, adrenals, spleen and brain are weighed immediately after autopsy. All animals are subjected for gross pathological changes. The vital organs like liver, kidney, brain, gonads, spleen, adrenal, thyroid, lungs, heart, stomach, duodenum, jejunum, colon, uterus, prostate are fixed in formal saline solution and tissues embeded in parafin wax and section cut at 6 um on rotary microtome. The prepared slides are then stained in haematoxylin eosin for microscopic examinations.

Haematology

Haematology is carried out in oxalated blood using standard methods of Wintrobe and Landsberg 1935 and Kolmer et.al. 1951. Blood is analysed for WBC, RBC, Hb differential leucocytes, clotting and prothrombin time and ESR. Immunoglobulin profile (IGM, IGA, IGE).

Clinical Enzymes

Serum and blood are analysed for

(i) Glutamic oxaloacetic transaminase (GOT), (ii) Glutamic pyruvic transaminase (GPT), (iii) Alakline phosphatase (Orthophosphoric monoester hydroxylase ALP), (iv) Bilirubin (v) Blood glucose (vi) Blood urea nitrogen (BUN), (vii) Non protein nitrogen (NPN), by the method of Wootton (1982), (viii) Acetylcholinesterase (AchE) by the method of Hestrin 1949 and (ix) Protein by the method of Lowry et.al. 1951. (x) Serum histamine level.

Calculation and Evaluation of Data

All observed data are recorded and calculated by appropriate methods. The statistical evaluation in done by Fisher's student `t' test 1950. The results are summerised in tabular form.

References

Wintrobe, M. and Landsberg, J.W. A standard technique for blood sedimentation test. American J. Med. Sci. 189, 102, 1935.

Kolmer, K.A. Spaulding, E.H. and Robinson, H.W. Approved laboratory techniques Ves Scientific Book Agency Calcutta, India, 1951.

Wootton, I.D.P. microanalysis in Medical Biochemistry Sixth Edition, Churchill Ltd. London, 1982.

Hestrin, S.H. The reaction of Acetylcholine and other carboxylic acid derivatives with hydroxyl amine and its analytical applications J. Biol. Chem. 180, 249, 1949.

Lowry, O.H. Rosenburgh, N.J. Farr, A.L. and Randall, R.J. Protein measurement with the Folin Phenol reagent J. Biol. Chem. 193, 265, 1951.

Fisher, R.A. Statistical methods for research workers 11th edition Edinburgh Oliver and Boyd 1950.

Report on Subchronic Oral Toxicity

Test Animal : Rat.

Test Chemical : Solid, Liquid, any other

Nature of vehicle : dist. water, peanut oil, corn oil, any other

Date of expt. started :..... , Date of expt. terminated :.....

FOOD (G) WATER (ML) INTAKE OF MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	**Weeks**												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MALE													
1. Control													
2.													
3.													
4.													
FEMALE													
1.													
2.													
3.													
4.													

Report on Subchronic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

RELATIVE ORGAN WEIGHT OF MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Liver	Kidney	Adrenalm	Heart	Spleen	Brain	Pituitary	Testes	Epididymis	Cervix	Vagina	Ovary	Uterus
MALE													
1. Control													
2.													
3.													
4.													
FEMALE													
1.													
2.													
3.													
4.													

*$\frac{\text{Organ weight}}{\text{Body weight}} \times 100$

Report on Subchronic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

Blood Picture of Male or Female Animals Exposed to..... for 13 Weeks

Dosage (mg/kg/day)	RBC (x 10 mm)	WBC (x 10 mm)	Hb	PVC	platelet	Differential Leucocyte count (%)			
						Neutrophils	Lymphocytes	Monocytes	Eosinophils
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1.									
2.									
3.									
4.									

Report on Subchronic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

BIOCHEMICAL CHANGES IN MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Blood Sugar	Alk. Phos.		Protein		GOT		GPT	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1. Control									
2.									
3.									
4									

Title : PRIMARY SKIN IRRITATION TEST OF TRANSGENIC SEED IN RABBIT

Adoption : OECD 404

Application and Limitation of Test

The assessment and evaluation of the toxic characterestics of a substances', determination of the irritant effects on the skin of mammals is an important initial step. Information derived from the test serves to indicate the existance of possible hazard likly to arise from exposure of the skin to the test substance.

Principle

The substances to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed.

Description of the Test Procedure

Animals

At least three adult rabbit should be used. Additional animals may be required to clarify equivocal responses.

Animal Maintenance

Animals are acclimatized to the experimental animal room having temperature 75 + 2°F, humidity 30-70% and 12 : 12 hrs light dark cycle. Animals are caged with maximum of two animals in each cage. Standard animal diet and water *at libitum*.

Preperation of Dose and Limit Test Dose

Test sample i.e. transgenic seed at a dose of 0.5 ml. of liquid or 0.5 g. of solid is applied to the test side. The treatment schedule is given below:

Group 1 - Control
Group 2 - Non transgenic seed
Group 3 - Transgenic seed

Observations

Animals are ovserved for signs of erythema and oedema and the responses scored at 30-60 minutes, and then at 24, 48, 72 hours and 7 and 14 days after patch removal. Dermal irritation is scored and recorded as per the grades given in the table below.

References

Draise, J.H. The Appraisal of Chemicals in Foods, Drugs, and cosmetics pp. 46-48. Association of Food and Drug Officials of United States, Austin, Texas 1959.

Draise, J.H. Appraisal of the Safety of chemicals in Foods, Drugs and Cosmetics; pp. 46-59. Association of Food and Drugs official of the United States, Topeka, Kanasas 1965.

Evaluation of Skin Reaction

Erythema and Eschar Formation	***Value***
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Maximum possible - 4

Oedema Formation	
No Oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre) and extending beyond area of exposure)	4

Maximum Possible - 4

Title : IRRITATION TO MUCOUS MEMBRANE TEST OF TRANSGENIC SEED IN FEMALE RABBIT

Adoption : OECD 405

Application and Limitation of Test

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant effects on the mucous membrane of the rabbit is an important step. Information derived from this study serves to indicate the existence of possible hazards likely to arise from exposure on the mucous membrane to the test substance.

Principle

The substance is tested is applied in a single dose to the mucous membrane of the experimental animals. Simultaneous animals in the control group are also taken. The degree of irritation is read and scored at specific intervals. The complte evaluation is then described. The duration of the study is sufficient to evaluate fully the dermal irritation.

Description of the Test Procedure

Healthy adult animals at least 3 in number are used in both experimental and control groups. Animals are kept in the experimental animal room having temperature (75 ± 2°F), humidity (30-70%), and 12 : 12 hrs light: dark condition. Animals are fed conventional laboratory diet and water *ad libitum.*

A dose of 0.1 ml of liquid or 0.1 gm of solid or semisolid is applied to the upper vault of the vagina. Exposure duration is 4 hrs. Longer exposure may be indicated under certain conditions. At the end of the exposure period residual substance is removed where practicable using water or appropriate solvent without disturbing the epidermis. The treatment schedule is given below :

Group 1	-	Control
Group 2	-	Non transgenic seed
Group 3	-	Transgenic seed

Observation

Observation period is not fixed but is sufficient to evaluate fully the effects of the test substance. Normally it need not exceed 14 days after application. Animals are examined for signs of erythema and oedema and responses scored at 30-60 minutes, 24, 48, 72 hrs and then at 7 and 14 days. Mucous memberane irritation is scored and recorded as per the grades given in table below:

References

Draise, J.H. The approval of chemical in Food, Drug and Cosmetics pp. 46-48. Association of Food and Drug Officials of United States, Austin Texas 1959.

Draise, J.H. Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics; pp. 46-59. Association of Food and Drugs Official of the United States, Topeka, Kanasas 1965.

Evaluation of Skin Reaction

Erythema and Eschar Formation	***Value***
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Maximum possible - 4

Oedema Formation	
No Oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre) and extending beyond area of exposure)	4

Maximum Possible - 4

Title : SKIN SENSITIZATION TEST OF TRANSGENIC SEED IN GUINEA PIGS

Adoption : OECD 406

Application and Limitation of Test

In the assessment and evaluation of the toxic characteristics of a substance, determination of its potential to provoke skin sensitization reaction (allergic dermatitis) is important. Information derived from skin sensitization serves to identify the possible hazards to a population exposed to the substance.

Principle

After initial exposure to a test substance the animals are subsequently subjected for 9 injections, then a challenge exposure to establish a hypersensitive state. Sensitization is determined by examining the reaction to the challenge exposure.

Desicription of Test Procedure

The guinea pigs are the generally recommended species. A sufficient number of animals are used. Animals are kept in experimental animal room having temperature (75 ± 2°F), humidity 30-70% and 12 : 12 light: dark condition. Animals are fed on conventional laboratory diet and water *ad libitum*. It is essential that guinea pigs receive an adequate amount of ascorbic acid. A treatment and a control groups are simultaneously taken. Animals are clipped off at dorsal side for the area 6 x 6 cm. The test substance 0.5ml is administered intradermally as a initial dose. There after nine subsequent injections are given intradermally on every alternate days. After giving a rest period of 15 days a booster dose of 0.05 ml is injected. The treatment schedule is given below :

Group 1 - Control
Group 2 - Non transgenic seed
Group 3 - Transgenic seed

Observation

Scoring of Skin reaction was performed on day 2 and then 24 hours after each injection. On day 36 and 37 animals are shaved again to check the intensity of erythema or edaema. With administration of booster dose, skin sensitization reaction was observed. The subsquently spreaded to longer area of the skin and resulted in necrosis at site of injection. Scored reaction are recorded in form of table.

Refereces

Draize, J.H., Food Drug Cosmets. Law J. 10, 722, 1955.

Evaluation of Skin Reaction

Erythema and Eschar Formation	***Value***
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Maximum possible - 4

Oedema Formation	
No Oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre) and extending beyond area of exposure)	4

Maximum Possible - 4

SUBCHRONIC ORAL TOXICITY – GOATS – 90 DAYS STUDY FOR GENETICALLY ENGINEERED SEEDS

OBJECTIVE

The objective of this study is to compare the whole someness of engineered seeds with control seeds and control seeds lines will be administered to the goats through the diet for 90 days.

MATERIAL AND METHODS

The methods, species of animals and the route of administration described in this protocol are based up on the standard OECD guidelines No. 408 (1993). This procedure deals with handling, maintaining and other procedures to be followed while dealing with feeding studies with goats. In order to maintain even distribution, the goats will be provided a number, based on random selection.

The test material will be administered in the diet. This route of administration was selected because it represents the most likely route of exposure of goat species in their natural habitat.

The test substance will be properly identified as per the detailed specification provided by the sponsor.

Treatment Groups

A group of 12 goats (6 males and 6 females) will be assigned to each group by the indiscriminate draw to each of the treatment and control group. All goats will be uniquely identifiable with an identification mark on the body and/or with a number plate around their neck.

The test will comprise feeding of the goats for 90 days regularly with concentrate of which 12.5% will be test seed and the concentrate itself will be 10% of the total feed i.e. concentrate and green grass. The consumption range of the feed will be pre-determined.

Each group is fed for 90 days and observed. An additional control group will be fed normal diet which will not contain cotton seeds throughout the test period.

Duration of the study.

All animals in the treatment groups will get Indian hybrid control cotton seeds in diet during acclimation.

Analysis will be initiated during this period itself viz., feed consumption, weight gain etc. This will facilitate statistical analysis.

Pilot study will be done before acclimation to assess the consumption of cotton seeds. Parameters like feed consumption, weight gain etc. will also be assessed for this group.

This study will be divided as under.

1. Acclimation: From receipt of the animals till the initiation of the study (a minimum duration of 15 days)

2. Exposure : 90 day

Test animals

Goat husbandry is generally associated with agriculture in Indian rural set ups. The availability of standard genetically defined goats and dietary and husbandry conditions, also make goats ideal in the Indian context and safety data on this ruminant model will be appropriate.

All goats will be 12 months old and healthy at the initiation of the study. The body weight will range between 15 and 18kg. Each treatment and control group will have 12 animals. The Barbari goats will be obtained from the State Animals Husbandary Departments. All the animals will be acclimated to their pens and facilities from the time of receipt until the initiation of the study.

ANIMAL CARE AND FACILITY

Animal Species

Goat - The Indian Barberi breed

Source

State Animal Husbandary Departments.

Number of animals

Twelve animal (6 males and 6 females) per group

Age and weight

Age of the animals will be 12 months and the weight between 15 and 18 kg.

Acclimation

The animals will undergo an acclimation for a period of not less than 15 days prior to the actual studies. The goats will be given anti-healminth drugs and also drugs for treatments for ectoparasites before the initiation of the study. All animals in the treatment groups will get Indian hybrid control cotton seeds control in diet during acclimation and group will not be given any cotton but will have groundnut cake instead in its diet.

Animals identification

Each animal will be numbered accordingly with the help of a tag around the neck.

Housing the animal care

Goats will be housed individually in a well constructed, cemented pens and maintained under strict hygienic conditions of veterinary care.

Food and water

Each animal will be allowed access to food for the whole day. Clean drinking water will be provided *ad libitum*. Feed consisting of wheat bran, gram, salt, minerals, cotton seeds and grass will form the daily diet of the goats.

The test will comprise feeding of the goats for 90 days regularly with concentrate of which 12.5% will be cotton seed and the concentrate itself will be 10% of the total feed i.e. concentrate and green grass. The consumption range of the feed will be predetermined.

Bedding

No bedding will be used; instead the floor will be made of rough cement/concrete to avoid slipping of goats while walking or standing.

Exercise

Though the goats do not need any strenuous exercise, they will however, be allowed to go out of their pens in an open field for about 2-3 hours each day but ensuring that they do not eat any other foliage. The area of their movement would be devoid of any vegetation but water will be provided during this period of their routine.

Animal diet

The test diet will be prepared by blending the test substance directly with the ration. Blending is normally done with a blender. Unless otherwise specified, the diets will be prepared every day. The diets will be provided to the goats from day 0 of the 90 day exposure period. Every batch of concentrate will be analysed and relevant record will be maintained. Cotton seed will be added to the concentrate everyday to avoid the concentrate going rancid because of the presence of cotton oil, if the concentrate is blended with cotton seed and stored. The ingredients will be purchased in bulk and made available for mixing; but the mixing and blending of the constituents will be done daily. The feed ingredients will be maintained in a dry and clean room to avoid attack by fungus. The test material will be crushed and mixed with the feed. The analysis of the feed will be for the following parameters: Crude protein, fat, acid detergent fiber, neutral detergent fiber, Calcium, phosphrus, Magnesium, Sodium, Potassium, Copper, Zinc, Manganese, Iron, Vitamin A, Vitamin D, Vitamin E. The analysis will be done on the mix and the raw ingredients. Also the mix will be randomly analysed once a week.

Housing and environmental conditions

Goals will be housed in properly constructed pens. Each pen measuring 1.5 sq. mt. per goat, allowing proper movement to the animals. The floor of the pen would be constructed of concrete and the walls of bricks. The roof will be made of corrugated sheet. At initiation of the study, each pen will hold a single goat and goat will be identifiable by a number. During the test, the temperature in the housing will be 25-30°C approximately. If necessary, air cooler will be provided to maintain the specified temperature. Relative humidity will be recorded at 24 hour interval. The goats will be provided a 16 hour light and 8 hour dark photoperiods during the test. Housing and animal husbandry practices will be followed as mentioned by Devendra and McLeroy 1982.

EXPERIMENTAL DESIGNS

Design

The study will be conducted as a randomised block design in which goats wil be distributed randomly in different treatment groups evenly consisting of a single goat as a replcate.

The study would have at least three following groups

1. Geneticaly engineered cotton line
2. Indian hybrid cotton line
3. Control group-Normal diet without cotton seeds but ground nut, instead.

Observations

All the animals will be observed daily for morbidity, mortality and clinical signs.

Daily observations

The general health of all the animals will be monitored daily and relevant records will be maintained. Any adverse observation will be documented. Animals found moribund or dead during the study period will be necropsied to the extent necessary to determine the probable cause.

Body weight and temperature

Body weights will be measured weekly at a predetermined time along with their health status. A chart of weekly temperature will also be maintained.

Body weight/feed consumption

Individual body weights will be taken at the initiation of the experiment, during the exposure period and at the end of the exposure period. Average feed consumption for individual animal will be maintained for the entire period. Determination of feed consumption and body weight will continue, if the study period is extended. Daily feed offered and refused will be measure for the concentrate and grass.

Feed intake

Goats will have access to the experimental feed (concentrate) from 9 a.m. to 12 p.m. each day.

Necropsy and Pathological examinations

Goats found moribund or dead during the study period will be necropsied to the extent necessary to determine the probable reason. Any gross lesions observed at necropsy will be processed for histopathological examinations.

Hematological observations

Following parameters would be assessed:

- Total RBC count
- Total WBC count
- Differential leucocytic count
- Haemoglobin concentration
- Clotting time
- ESR immunoglobulin profile

Clinical biochemistry

The following parameters will be analysed.

- Total Serum protein
- Glucose
- Blood urea
- Nitrogen
- Non-protein
- Nitorgen
- Bilirubin
- Histamine
- Got
- Gpt
- Alkaline phosphatase
- LDh

Necropsy

All the animals are sacrified on day 91. Goats are sacrified by administration of a saturated solution of magnesium sulphate intravenously and the autopsy is carried out as the standard procedure by the venterinary pathologist of the study.

Organ weights

The gross lesions in the organ are noted and weights of the following organs are recorded:

- Adrenals,
- Heart,
- Liver,
- Gonads (testes and ovaries),
- Brain,
- Kidneys,
- Spleen

Histopathological examinations

Following organs are preserved in 10% buffered formalin:

- Adrenals
- Kidneys
- Testes
- Liver
- Thymus
- Lungs
- Colon
- Spleen
- Ovaries
- Stomach (all 4 compartments)
- Heart
- Small intestine

Histopathological examinations of these organs will only be conducted if gross lesions are noted.

The tissues are subjected to dehydration procedure and processed in a tissue processor through different grades of alcohol and cleared in chloroform. They are embedded in paraffin wax, sectioned at 7 to 10 microns and stained with Haematoxylin-Eosin.

Disposal

The carcass will be mutilated by using Calcium hydroxide and buried deep ensuring that these are not removed by men or other animals like dogs and jackals.

References

1. OECD (1982). Guidelines for testing of chemicals Section 4, Health effects (No. 407-409) Organisation of European Cooperation and Development, Paris.
2. Schalm, O.W. (1969). Veterinary Hematology, Lea and Febiger, Philadalphia.
3. Devendra C. and McLeroy, G.B. (1982). Goat and Sheep Production in the tropics. Intermediate Tropical Agricultural Series, Longman, London.

Title : ACUTE ORAL TOXICITY TEST OF TRANSGENIC VEGETABLES IN RAT

Adoption: **OECD 401**

Application and limitation of tests

Acute oral toxicity is the adverse effects occuring within a short time of oral administration of a single dose of a test chemical or multiple doses given within 24 hrs. It is the initial step to find out median lethal dose (LD50) value which serve as basis for classification and labelling of the compound. It also forms a basis for selection of dose for subchronic studies. It will provide information on target organ toxicity after single exposure.

Principle

The test compound is administered orally by gavage in numerical doses to grous of animals, one dose per group. Signs of toxicity and death of animals are observed during 14 days observation period. The dead animals are necropsied during and the surviving animals are sacrificed and necropsied after the 14 days observation period for gross and histopathological studies, clinical biochemistry and haematological examiniation.

Description of the test Procedure

Animals

Healthy rats kept under standard animal husbandry conditions are used. At least 10 animals (male/female) are dosed. The weight variation of animals does not exceed 5-10g.

Animal maintenance

Animals are acclimatized to the experimental animal room having temperature 75 ± 2°F, humidity 30-70% and 12:12 hrs light dark condition. Animals are caged with maximum of 2 animlas in each polypropylene cages. Standard animal diet and water *ad libitum* is given to animals.

Preparation of dose

Test sample i.e. concentrated paste or cryogenic dehydrated powder of transgenic vegetables dissolved/suspended in groundnut oil is administrated to rat fasted overnight. The volume does not exceed 1 ml/100 g body weight. At least four doses of the test sample spaced in geometrical factor are selected. The treatment schedule of short term toxicity is given below:

Group 1 - Control (normal diet)

Group 2 - Non transgenic vegetables

Group 3 - Transgenic vegetables

Limit test dose

If a test sample at 5000 mg/kg body weight produces no mortality, then other doses are not essential.

Observations

The dosed animals are observed twice daily for 14 days to record the signs of poisoning and death of animals. The signs of poisoning include tremor, convulsion, salivation, diarrhoea, lethargy, sleep, coma, dyspnea, nasal bleeding etc. The time of death of animals is recorded. The body weight, food and water intake is recorded daily and monitored weekly. All the animals (moribund/live) are sacrificed after 14 days and examined for gross and histopathological changes, clinical biochemistry and haematological examination.

Pathology

The liver, kidney, gonads, adrenals, spleen and brain are weighed immediately after autopsy. All animals are subjected for gross pathological changes. The vital organs like liver, kidney, brain, gonads, spleen, adrenal, thyroid, lungs, heart, stomach, duodnum, jejunum, colon, uterus, prostate are fixed in formal saline solution and tissues embeded in parafin wax and section cut at 6 um on rotary microtome. The prepared slides are then stained in haematoxylin eosin for microscopic examinations.

Haematology

Haematology is carried out in oxalated blood using standard methods of Wintrobe and Landsberg 1935 and Kolmer et. al. 1951. Blood is analysed for WBC, RBC, Hb differential leucocytes, clotting and prothrombin time and ESR.

Clinical Enzymes

Serum and blood are analysed for:

(i) Glutamic oxaloacetic transaminase (GOT), (ii) Glutamic pyruvic transaminase (GPT), (iii) Alakline phosphatase (Orthophosphoric monoester hydroxylase ALP), (iv) Bilirubin (v) Blood glucose (vi) Blood urea nitrogen, (BUN) (vii) Non Protein nitrogen, (NPN) by the method of Wootton (1982), (viii) Acetylcholinesterase (AchE) by the method of Hestrin 1949 and (ix) Protein by the method of Lowry et. al. 1951.

Calculations

LD50 values and its range are calculated by the procedure of Weil 1952 and toxicity rating is done by Gleasons et al. 1969. All observed data are recorded and calculated by appropriate methods. The statistical evaluation is done by Fisher's student t' test. The results are summerised in tabular form.

References

Weil, C.S., tables for convenient calculation of median effective dose (LD or ED) and instruction in their use. Biometrics, 8, 249, 1952.

Gleason, M.N., Gosselin, R.E., Hodge, H.C. and Smith, R.P. Clinical toxicology of commerical products. Acute posioning 3rd ed. Williams and williams, Baltimore, Maryland.

Report on Acute Oral Toxicity

Test Animal.....

Rats..... Sex : Male/Female.....

Test Sample
Solid, Liquid, any other

Nature of vehicle
dist. water, peanut oil, corn oil, any other

Date of experiment started .....

Date of experiment terminated .....

LD50.....mg/kg; Range..... to .....mg/kg

Dosage (mg/kg)	Animals Died/Dosed	Death	Symptoms of toxicity
1. Control			
2.			
3.			
4.			

Statistical Method

Gross Pathology

Observations

Conclusions

Toxicity Rating

Report on Acute Oral Toxicity

Test animal : Rat.

Test Chemical : Solid, Liquid, any other

Nature of vehicle: dist. water, peanut oil, corn oil, any other

Date of expt. started....., date of expt. terminated.....

FOOD (G) WATER (ML) INTAKE OF MALE OR FEMALE ANIMALS EXPOSED TO.....FOR 14 DAYS

Dosage (mg/kg/day)	**Days**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MALE														
1. Control														
2.														
3.														
4.														
FEMALE														
1.														
2.														
3.														
4.														

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated : :.....

RELATIVE ORGAN WEIGHT OF MALE OR FEMALE ANIMALS EXOSED TO ..... FOR 14 DAYS

Dosage (mg/kg/day)	Liver	Kidney	Adrenalm	Heart	Spleen	Brain	Pituitary	Testes Ovary	Epididymis Uterus	Cervix	Vagina
MALE											
1. Control											
2.											
3.											
4.											
FEMALE											
1.											
2.											
3.											
4.											

*Organ weight / Body weight x 100

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated :.....

BLOOD PICTURE OF MALE OR FEMALE ANIMALS EXPOSED TO:..... FOR 14 DAYS

Dosage (mg/kg/day)	RBC (x 10 mm)	WBC (x 10 mm)	Hb	PVC	platelet	Differential Leucocyte count (%)			
						Neutrophils	Lymphocytes	Monocytes	Eosinophils
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1.									
2.									
3.									
4.									

Report on Acute Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated:.....

BIOCHEMICAL CHANGES IN MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 14 DAYS

Dosage (mg/kg/day)	Blood Sugar	Alk. Phos.		Protein		GOT		GPT	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1. Control									
2.									
3.									
4									

SUBCHRONIC (90 DAYS) ORAL TOXICITY TEST OF TRANSGENIC VEGETABLES IN RAT

Adoption: OECD 408

Application and limitation of test

Subchronic oral toxicity is the advsese effect occuring as a result of repeated daily oral dosing of a chemical to the animals. In the evaluation of toxic characteristics of a chemical the subchronic oral toxicity provides information on possible health hazards due to repeated exposure over a limited period of time. It will provide the information on target organ and the possibility of cumulation and for the selection of dose level for chronic studies.

Principle

The test compound is orally administered in three doses to animals for a period of 90 days. The animals are observed for any signs of toxicity and death during the period of exposure. Vital tissues of moribund and sacrificed animals are put for histopathological studies. Clinical biochemistry and haematological examinations are also made.

Description of the test Procedure

Rat is the preferred rodent model for subchronic oral toxicity studies. Healthy animals kept under standard animal husbandry conditions are used. At least 20 animals of 6-8 weeks old are used per group for three dose levels. The weight of the animals does not vary +20 g.

Animal maintenance

Animals are acclimatized to the experimental animal room having temperature (75+2 F), humidity (30-70%) and 12:12 hr light: dark conditions. Animals are given commercial feed and water *ad libitum*.

Preparation of dose

Test sample i.e. concentrated paste or cyyogenic dehydrated powder of transgenic vegetables dissolved/suspended in peanut oil is orally administered by gavage to animals consequantly (5 days/week) for 90 days. The selection of the dose is made on the basis of acute toxicity studies of the test chemical. At least three dose level, one maximum, one minimum and one intermediate are used. Consideration is given that the highest dose may result toxic effects without causing excessive lethality and lowest dose may not produce any toxic effects. A group of vechicle control is also used.

Limit test dose

If a test at one dose level of at least 1000 mg/kg/body weight (but expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects, then a full study using three dose levels may not be considered necessary.

The treatment schedule is given below:

Group 1 - Control

Group 2 - Non transgenic vegetables

Group 3 - Transgenic vegetables

Observations

Animals are observed once daily to record the signs of posioning, like tremor, convulsion, diarrhoea, lethargy, dyspnea and nasal bleeding etc. The time of death is also recorded. The body weight, food and water intake is recorded daily and monitored weekly. At the end of 90 days animals are weighed and sacrificed.

Pathology

The liver, kidney, gonads, adrenals, spleen and brain are weighed immediately after autopsy. All animals are subjected for gross pathological changes. The vital organs like liver, kidney, brain, gonads, spleen, adrenal, thyroid, lungs, heart, stomach, duodenum, jejunum, colon, uterus, prostate are fixed in formal saline solution and tissues embeded in parafin wax and section cut at 6 um on rotary microtome. The prepared slides are then stained in haematoxylin eosin for microscopic examinations.

Haematology

Haematology is carried out in oxalated blood using standard methods of Wintrobe and Landsberg 1935 and Zolmer et. al. 1951. Blood is analysed for WBC, RBC, Hb differential leucocytes, clotting and prothrombin time and ESR. Immunoglobulin profile (IGM, IGA, IGE).

Clinical Enzymes

Serum and blood are analysed for

(i) Glutamic oxaloacetic transaminase (GOT), (ii) Glutamic pyruvic transaminase (GPT), (iii) Alakline phosphatase (Orthophosphoric monoester hydroxylase ALP), (iv) Bilirubin (v) Blood glucose (vi) Blood urea nitrogen, (BUN) (vii) Non protein nitrogen, (NPN) by the method of Wootton (1982), (viii) Acetylcholinesterase (AchE) by the method of Hestrin 1949 (ix) Protein by the method of Lowry et. al. 1951. (x) Serum histamine level.

Calculation and evaluation of data

All observed data are recorded and calculated by appropriate methods. The statistical evaluation is done by Fisher's student 't' test 1950. The results are summerised in tabular form.

References

Wintrobe, M. and Landsberg, J.W. A standard technique for blood sedimentation test. American J. Med. Sci. 189, 102, 1935.

Kolmer, K.A. Spaulding, E.H. and Robinson, H.W. Approved laboratory techniques Ved Scientifc Book Agency Calcutta, India, 1951.

Wootton, I.D.P. Microanalysis in Medical Biochemistry Sixth Edition, Churchill Ltd., London, 1982.

Hestrin, S.H. The reaction of Acetylcholine and other carboxylic acid derivatives with hydroxyl amine and its analytical applications J. Biol. Chem. 180, 249, 1949.

Lowry, O.H. Rosenburgh, N.J. Farr, A.L. and Randall, R.J. Protein measurement with the Folin Phenol reagent J. Biol. Chem. 193, 265, 1951.

Fisher, R.A. Statistical methods for research workers 11th edition Edinburgh Oliver and Boyd, 1950.

Report on Subchronic Oral Toxicity

Test Animal : Rat.

Test Chemical : Solid, Liquid, any other

Nature of vehicle : dist. water, peanut oil, corn oil, any other

Date of expt. started :..... , Date of expt. terminated :.....

FOOD (G) WATER (ML) INTAKE OF MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Weeks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MALE													
1. Control													
2.													
3.													
4.													
FEMALE													
1.													
2.													
3.													
4.													

Report on Subchronic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated : :.....

RELATIVE ORGAN WEIGHT OF MALE OR FEMALE ANIMALS EXOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Liver	Kidney	Adrenalm	Heart	Spleen	Brain	Pituitary	Testes Ovary	Epididymis Uterus	Cervix	Vagina
MALE											
1. Control											
2.											
3.											
4.											
FEMALE											
1.											
2.											
3.											
4.											

*$\frac{\text{Organ weight}}{\text{Body weight}} \times 100$

Report on Subchronic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... date of expt. terminated :.....

BLOOD PICTURE OF MALE OR FEMALE ANIMALS EXPOSED TO:..... FOR 13 WEEKS

Dosage (mg/kg/day)	RBC (x 10 mm)	WBC (x 10 mm)	Hb	PVC	platelet	Differential Leucocyte count (%)			
						Neutrophils	Lymphocytes	Monocytes	Eosinophils
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1.									
2.									
3.									
4.									

Report on Subchrpnic Oral Toxicity

Test Animal : Rat

Test Chemical : Solid, Liquid, any other

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated:.....

BIOCHEMICAL CHANGES IN MALE OR FEMALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Blood Sugar	Alk. Phos.		Protein		GOT		GPT	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
MALE									
1. Control									
2.									
3.									
4.									
FEMALE									
1. Control									
2.									
3.									
4									

PRIMARY SKIN IRRITATION TEST OF TRANSGENIC VEGETABLES IN RABBIT

Adoption: OECD 404

Application and limitation of test:

The assessment and evaluation of the toxic characterestics of a substances, determination of the irritant effects on the skin of mammals is an important initial step. Information derived from the test serves to indicate the existence of possible hazard likly to arise from exposure of the skin to the test substance.

Principle

The substances to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed.

Description of the test procedure

Animals

At least three adult rabbit should be used. Additional animals may be required to clarify equivocal responses.

Animal maintenance

Animals are acclimatized to the experimental animal room having temperature 75+2F, humidity 30-70% and 12:12 hrs light dark cycle. Animals are caged with maximum of two animals in each cage. Standard animal diet and water *at libitum*.

Prepration of dose and limit test dose

Test sample i.e. transgenic vegetable at a dose of 0.5ml of liquid or 0.5g of solid is applied to the test side. The treatment schedule is given below:

Group 1 - Control

Group 2 - Non transgenic vegetables

Group 3 - Transgenic vegetables

Observations

Animals are observed for signs of erythema and oedema and the responses scored

at 30-60 minutes, and then at 24, 48, 72 hours and 7 and 14 days after patch removal. Dermal irriation is scored and recorded as per the grades given in the table below.

References

Draise, J.H. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics pp, 46-48. Association of Food and Drug officials of united States, Austin, Texas 1959.

Draise, J.H. Appraisal of the Safety of chemicals in Foods, Drugs and Cosmetics; pp 46-59. Association of Food and Drugs official of the United States, Topeka, Kanasas 1965.

Evaluation of Skin Reaction

Erythema and Eschar Formation	***Value***
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Maximum possible - 4

Oedema Formation	
No Oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre) and extending beyond area of exposure)	4

Maximum Possible - 4

Title : IRRITATION TO MUCOUS MEMBRANE TEST OF TRANSGENIC SEED IN FEMALE RABBIT

Adoption : OECD 405

Application and Limitation of Test

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant effects on the mucous membrane of the rabbit is an important step. Information derived from this study serves to indicate the existence of possible hazards likely to arise from exposure on the mucous membrane to the test substance.

Principle:

The substance tested is applied in a single dose to the mucous membrane of the experimental animals. Simultaneous animals in the control group are also taken. The degree of irrritation is read and scored at specific interval. The complete evaluation is then described. The duration of the study is sufficient to evaluate fully the dermal irritation.

Description of the test procedure

Health adult animals at least 3 in number are used in both experimental and control groups. Animals are kept in the experimental animal room having temperature (75 ± 2°F), humidity (30-70%), and 12:12 light: dark condition. Animals are fed conventional laboratory diet and water *ad libitum*.

A dose of 0.1 ml of liquid or 0.1 gm of solid or semisolid is applied to the upper vault of the vagina. Exposure duration is 4 hrs. Longer exposure may be indicated under certain conditions. At the end of the exposure period residual substance is removed where practicable using water or appropriate solvent without disturbing the epidermis. The treatment schedule is given below:

Group 1 - Control -
Group 2 - Non transgenic seed
Group 3 - Transgenic seed

Observation

Observation period is not fixed but is sufficient to evaluate fully the effects of the test substance. Normally it need not exceed 14 days after application. Animals are examined for signs of erythema and oedema and the responses scored at 30-60 minutes, 24, 48, 72 hrs and then at 7 and 14 days. Mucous membrane irritation is scored and recorded as per the grades given in table below:

References

Draise, J.H. The approaval of chemical in Food, Drug and cosmetics pp, 46-48. Association of Food and Drug Officials of United States, Austin Texas 1959.

Draise, J.H. Appraisal of the Safety of chemicals in Foods, Drugs and Cosmetics; pp 46-59. Association of Food and Drugs official of the United States, Topeka, kanasas 1965.

Evaluation of Skin Reaction

Erythema and Eschar Formation	***Value***
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Maximum possible - 4

Oedema Formation	
No Oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre) and extending beyond area of exposure)	4

Maximum Possible - 4

SUBCHRONIC (90 DAYS) ORAL TOXCITY OF LEAVES OF TRANSGENIC PLANTS IN MALE RABBIT

Adoption: **OECD guideline No. 408**

2. METHOD

A. Application and limitation of test

Subchronic oral toxicity

Subchronic oral toxicity is the adverse effects occuring as a result of the repeated daily oral dosing of a transgenic material/product to experimental animals for part (not exceeding 10 per cent) of the life span. In the assessment and evaluation of the toxic characteristics of a transgenic material/product, the determination of subchronic toxicity provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information in target organs, the possibilities of cumulation and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

Dose is the amount of test substance administered. Dose is expressed as weight of test substance per unit weight of food (percent).

Principle of the test method

The transgenic and nontransgenic leaves are administered orally as part of the green vegetable diet (in addition to the standard rabbit pellet diet), one dose per group, for a period of 90 days. During the period of administration the animals are observed daily to detect sign of toxicity. Animals which die during the period of administration are necropsied and at the conclusion of the test all surviving animals are sacrificed and necropsied and histopathological examinations carried out. Clinical biochemistry and haematological examinations are also made.

B. Description of the Test Produre

Preparations

Healthy adult male rabbits are acclimatised to the laboratory conditions for atleast 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. The transgenic and nontransgenic leaves will be administered in the diet. All animals shall be dosed by the same method during the entire experimental period.

Species and number

Young adult male rabbits shall be used for the study. At the commencement of the study, the body weight variation of animals used should not exceed by ± 20 per cent of the mean weight. 10 animals per group shall be used.

Housing and feeding conditions

The temperature in the experimental animal room is maintained at 75+5°F (22 + 3° C) and the relative humidity at 30-70 per cent. Artificial lighting at a light:dark ratio of 12:12 is used. The animals are housed individually in standard rabbit cages.

For feeding, the leaves and conventional laboratory rabbit pallet diet are placed in separate enamel trays. Drinking water is provided ad libitum in another separate enamel bowl. Standard hygiene procedures will be implemented.

Animal model

Healthy adult male rabbits weighing 1.5-2 kg will be used for the study.

Sample administration

The transgenic and non-transgenic leaves will be administered as part of the diet. Leaves from both the transgenic and non-transgenic lines will be delivered each week. The fresh leaves are conserved in plastic bags in a refrigerator and the rabbits are fed fresh leaves every day. The leaves and the pellets are placed in separate enamel trays and the remainder collected after 24 hours, weighed and removed.

Dosage

The daily quantities of the leaves and pellets are fed for 90 days as per the following schedule:

Group	Leaves	Pellets
T1	No leaves	Ad libitum
T2	10% TL	Ad libitum
T3	ad libitum Tl	Ad libitum
T4	10% NL	Ad libitum
T5	Ad libitum NL	Ad libitum

TL: *Transgenic leaves*
NL: *Non-transgenic leves*

If pair feeding (PF) is required, two more groups i.e. one PF TL and one PF NL will be added.

Laboratory Investigations

Animals will be observed daily for the changes in skin and fur, detail untoward

CNS, respiratory, ocular and gastrointestinal symptoms, for haematuria from day 1 through day 90. The consumption of diet (leaves and pellet), water as well as body weight and feed efficiency ofthe animals will be recorded daily.

The death, if any, will also recorded daily in the morning and the dead animal is examined for pathological changes. At the end of 90 days, all animals are weighed and sacrificed.

Clinial examination

The following examination are made at the end of 90 days of exposure:

(a) ***Haematology***: Haematocrit, haemoglobin concentration erythrocyte count, total and differential leucocyte counts, ESR, and a measure of clotting potential i.e. clotting time, prothrombin time and immunoglobulin profile are evaluated.

(b) ***Biochemistry:*** Clinial diagnostic enzymes such as liver and serum GOT, GPT, alkaline phosphatase and LDH are assayed. The levels of protein, glucose, serum bilirubin, blood urea nitrogen, non protein nitrogen and serum histamine are also evaluated.

(c) ***Pthology*** : *Gross necropsy:* All animals will be subjected to a full gross necropsy which included examination of the external surface of body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Microscopic examination will be conducted by closely observing the various organs, viz. stomach, jejunum, ileum, colon, spleen, pancreas, heart, brain, liver, kidney, adrenals, thymus, thyroid, prostate and testes. The above organs will be weighed wet as soon as possible after dissection to avoid drying.

Histopathology

Full histopathology will be carried out on above organs of all animals in the control and dosed groups. All gross lesions will be examined.

The tissues are fixed in formalin, embedded in paraffin wax, sectioned at 6-8 microns and stained with Haematoxilin-Eosin for microscopic examinations

Data and Reporting

All observed results, quantitative and incidental, will be evaluated by appropriate, geneally accepted statistical methods. The data are summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions the types of lesions and the percentage of animals displaying each type of lesions.

Evaluation of results

The findings of the subchronic oral toxicity study should be considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will

include the presence or absence, the incidence and severity, of abnormalities, including behavioural and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic, effects.

Test report

- The test report will include the following informations:
- species/strain used;
- toxic response date by sex and dose;
- time of death during the study or whether animals survived to termination;
- toxic or other effects;
- the time of observation of each abnormal sign and its subsequent course;
- food and body weight data;
- ophtholmological effects;
- haematological tests employed and results with relevant baseline data;
- clinical biochemistry tests employed and results with relevant baseline data;
- necropsy findings;
- detailed description of all histopathological findings; and
- statistical treatment of results where appropriate.

Report on Subchronic Oral Toxicity

Test Animal : Rabbit (Male)

Test Chemical : Leaves, administered is part of diet.

Nature of vehicle : dist. water, peanut oil, corn oil, any other

Date of expt. started :.....

Date of expt. terminated :.....

FOOD (G) WATER (ML) INTAKE OF MALE ANIMALS EXPOSED

TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	**Weeks**												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MALE													
1. Control													
2.													
3.													
4.													

Report on Subchronic Oral Toxicity

Test Animal : Rabbit

Test Chemical :Leaves administered as part of diet.

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

RELATIVE ORGAN WEIGHT OF MALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Liver	Kidney	Adrenalm	Heart	Spleen	Brain	Pituitary	Testes	Epididymis	Cervix	Vagina
MALE											
1. Control											
2.											
3.											
4.											

*Organ weight / Body weight x 100

Report on Subchronic Oral Toxicity

Test Animal : Rabbit, Male

Test Chemical : Leaves administered as part of diet.

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

Blood Picture of Male Animals Exposed to..... for 13 Weeks

Dosage (mg/kg/day)	RBC (x 10 mm)	WBC (x 10 mm)	Hb	PVC	platelet	Differential Leucocyte count (%)			
						Neutrophils	Lymphocytes	Monocytes	Eosinophils
MALE									
1. Control									
2.									
3.									
4.									

Report on Subchronic Oral Toxicity

Test Animal : Rabbit, Male

Test Chemical : Leaves administered as part of diet.

Nature of vehicle : Dist, water, peanut oil, corn oil, any other

Date of expt. started :..... Date of expt. terminated :.....

BIOCHEMICAL CHANGES IN MALE ANIMALS EXPOSED TO ..... FOR 13 WEEKS

Dosage (mg/kg/day)	Blood Sugar	Alk. Phos.		Protein		GOT		GPT	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
MALE									
1. Control									
2.									
3.									
4.									

PROTOCOL FOR ALLERGENICITY TESTING OF GENETICALLY TRANSFORMED PRODUCTS IN ANIMAL MODEL

INTRODUCTION

India is the 7th largest country in the world. Its population was 885 million in mid 1993 (16% of global population) which is expected to rise to 1015 million by 2000 AD and 2530 million by 2050 AD. It is absolutely necessary to increase the food production to feed the growing population. Although the food production has increased but it was almost proportional to the rise in the population with the result the per capita consumption remained almost static during the last one decade. Therefore, in order to further improve the quality and quantity of agricultural crops, various means are to be adopted. India has realised that there is no escape from the use of modern biotechnology involving the application of genetically modified plants to accomplish increased food production. Modern biotechnology includes genetically modified agricultural crops or foods, modified through human intervention by recombinant DNA technology. However, the unfamiliarity with these new inventions had aroused great concern among regulatory agencies for the right assessment of risk from the use of genetically modified plants to the habitat including human and animals. This situation is true, not only in India but also in all the developing countries as well as in the developed nations (Ghosh, 1997).

It is known that almost any food may be responsible for allergic disorders. Therefore, it is important and also would be appropriate not to overlook the allergenic potentials of genetically derived foodstuffs. For foods or food components produced by biotechnology, the safety assessment is particularly important (FAO/WHO, 1996). Unfortunately, model experimental protocols have not yet been designed to assess the allergenicity of new genetically modified foods. The tests to assess allergenicity are required in the case of transgenic crops, since exogenous proteins are engineered (Dean, et.al., 1996; McClintock, et.al., 1995; Niestiji, et.al., 1994; Taylor, 1985). To minimise the risk of allergenicity in exposed population, it is essentially required to identify whether the allergenic proteins are present or not, in the genetically engineered crop (Taylor, 1997). The assessment of allergenic potentials must be conducted in a careful step wise manner by using the in-vivo and in-vitro immunological tests.

A protocol is therefore, proposed by designing appropriate experiments to gather data on allergenicity testing in laboratory animals, which may help to evaluate the allergic potentials of new foodstuffs and thereby minimising the risk of allergenicity in exposed human population.

Brief Background of Allergy

Food allergy appears to be relatively common in the community. In taking the past histories of atopic patients, acute episodes of urticaria, angioedema, itching or

gastrointestinal disorders following the ingestion of certain foods have been reported by substantial number of patients. These manifestations of hypersensitivity to foods are well known.

Allergic reactions occur when an already sensitised indiviual is re-exposed to the same foreign substance or allergen. Allergic responses range from the familiar runing nose and sneezing to systemic anaphylaxis and death. These responses do not occur when a naive individual is first exposed to an allergen. The initial response takes time and usually does not cause any symptoms. once an individual is sensitised, the allergic reactions often become worse with each exposure, as each re-exposure not only produces allergic symptoms but also increases the level of antibody present.

There are two distinct types of allergic reactions to foods, one is characterised by the rapid appearance of symptoms, often within a few minutes after the offending food is eaten and the second is delayed type in which a number of hours or even a day or more may elapse between the ingestion of the allergenic food and the appearance of symptoms. It has been suggested that the actual allergen in the case of the immediate type of reactions to foods are the whole proteins. With delayed reactions, the allergen may be some protein breakdown product formed during the process of digestion of the protein (Goldman, et.al., 1963).

The first description of the mechanism of allergic reaction was presented by Prausnitz and Kustner in 1921, which is similar to the PCA test used for the assay of IgE production in experimental animals. Prausnitz and Kustner proposed the existence of an ``atopic reagin'' in the serum of allergic subjects. Some 45 yrs. later, Ishizaka and colleagues isolated this atopic reagin and showed that it was a new class of immunoglobulin IgE. It is the presence of such highly specific antibodies which provide the basis for detecting the specific allergen to which the patient reacts. The level of specific IgG has little correlation to symptoms of food allergy. Other immunoglobulins (IgA, IgM & IgD) play no known role in allergic disorders.

Animal Model

Unfortunately, no established animal model is available in literature for assessing the allergenicity of genetically modified foods, however, rabbit or guinea pig could be used to detect allergenicity. Brown Norway rats (Atkinson and Miller, 1994; Atkinson, et.al. 1996) have been used in experimental studies but it is yet to be accepted as an animal model by the regulatory agencies. However, this could be used to generate additional data.

Treatment Schedule

Normal adult healthy animals are kept under proper husbandry conditions with 12 hour light and 12 hour dark period. Before the start of experiment, animals are kept one week for acclimation. Animals are randomly divided into two groups, based on diet fed. Experimental group consists of ten animals, sensitised to the test protein(s) by incorporating into the feeding pellets such that 10% of the total diet is from the test compound. The control group of ten animals are fed 10% of the total diet of non-transgenic foodstuff. Animals are fed for sixty days. Water is provided *ad libitum*. Sera from the treated animals are used to assess allergenicity.

Preparation of Antigen/Allergen

Collect the test material in as pure form as possible, grind into fine powder form and defat with ether. Extract the defvatted material with buffered saline, 2% wt./vol/., however, this proportion can be varied depending upon the type of test compound. Allow the mixture to stand at 20*c for 72 hr., shake during this period 8-10 times for 30 minute each. Filter through Whatman No. 1 filter paper. Dialyse through dialysis sac, sterilise by passing through memberane filter, transfer to sterile vials and lyophilise and seal the vials.

Experimental Protocol

The following *in-vivo* and *in-vitro* immunological assays could be used for the detection of reactogenic antibodies in the test sera:

IN-VIVO ASSAYS

1. Passive Cutaneous Anaphylaxis (PCA)

Application and Limitations of Test

PCA is an *in-vivo* method usually employed to assay the specific IgE present in serum. It is a useful immunological tool to detect as little as 0.1 ug antibody protein. In this test the anaphylactic reaction is visualised as a local skin reaction.

Sex of Animals

Male and/or female healthy young adult animals can be used. if females are used they should be nulliparous and non-pregnant.

Housing and feeding Conditions

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. If guinea pigs are used, it is essential that animals receive an adequate amount of ascorbic acid.

Preparation of the Animals

Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by close clipping, shaving or by chemical depilation. Care should be taken to avoid adbarding the skin.

Principle

PCA could be produced with the sera of allergic host by challenging intradermally sensities sites with intravenously injected antigen/allergen plus dye. Well-defined blue areas appear, indicating the sites of antigen-induced extravasation of fluid due to interaction with tissue fixed antibody.

Description of Test Procedure

Naive animals are shaved on the back and flanks, avoiding abrasion of skin. Unblemished skin sites are selected and cleaned with 70% alcohol. Injections are spaced approximately 3-4 cm apart.

0.1 ml of test serum is injected intradermally. Doses are usually in the ratio of 1 : 2 : 4 or 1 : 3 : 9. The doses are injected using tuberculin syringe(s). The syringe used for intradermal injection must not leak even under heavy injection pressure.

Plastic disposable sterilised tuberculin syringes are satisfactory in this respect. Glass tuberculin syringes must be checked for leaks both at the needle butt and past the piston. Needles with `short bevel' points, usually 1/2 inch x 26 gauge are used 24 to 48 hour later, 0.6 ml of antigen/allergen (1 mg/ml) is injected intravenously together with 0.4 ml of Evans blue (2% in physiological saline). For the intravenous injection the piston of the syringe must be easy moving so that there is no doubt that the needle is in the vein when the smallest pressure is applied. The needle must be very sharp. For intravenous administration in guinea pigs, the vein which runs on the dorsal surface of the hind foot between the metatarsals of the outer and middle toes or ear vein or intracardiac route can be used. In rabbits, the vein running the margin of ear is the most useful site but other veins which are easily accessible can also be used.

Observation

30-45 min. later, animals are killed. The skin is opened and reflected so that the lesions can be evaluated. Measurements of diameter and the assessment of intensity are usually made. This can be postponed until all the animals have been killed but the delay is not advised and should not exceed 2 hours. The skin must not be allowed to dry. Intensity of bluing is often expressed arbitrarily as + to +++. Since the relationship between area of response and the dose is roughly linear, the plot of mean diameter upon log dose will also be linear. A table showing both would usually be preferred.

When the potency of sensitising antibody is unknown, a wider range of doses may be useful . The highest dose should give lesions of about 15 to 20 mm. diameter and the smallest about 5 mm.

Report on PCA

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

Report of PCA :

Group		Animal No.	Area of Dye extravasion		
			Undiluted	1:2 diluted	1:4 diluted
			Sera	Sera	Sera
Experimental		1			
		to			
		10			
Control		11			
		to			
		20			

2. Prausnitz - Kustner (PK) Test

Application and Limitations of Test

PK test is a reasonably accurate biological test when determining reaginic potency by wheal size. It's coefficient of variation is 20-30 per cent. The minimum dose of IgE antibody to give PK reaction has been estimated as 1 - 100 pg. Before testing the sera in a naive animal, it should be ascertained that the naive animal should not have any reactivity to the test antigen/allergen.

Principle

When normal skin is injected with reaginic serum, the reaginic antibodies become

attached to the skin mast cells and the injected area of skin acquires the specific skin reactivity towards antigen/allergen challenged.

Sex of Animals

Male and/or female healthy young adult animals can be used. If females are used they should be nulliparous and non-pregnant.

Housing and Feeding Conditions

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. If guinea pigs are used, it is essential that animals receive an adequate amount of ascorbic acid.

Preparation of the Animals

Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by close clipping, shaving or by chemical depilation. Care should be taken to avoid avrading the skin.

Description of Test Procedure

Intradermal injections are spaced approximately 3 - 4 cm apart. Unblemished skin sites are selected and cleaned with 70 % alcohol. The syrings used for the intradermal injections must not leak under injection pressure. Plastic disposable sterilised tuberculin syrings are satisfactory in this respect but the calibration at 0.05 ml must be checked.

If glass tuberculin syringes are used, they must be checked for leaks both at the needle butt and past the piston.

0.05 ml of serum (or a dilution of it) is then injected intradermally in the indicated position. Control site is injected with 0.05 ml of physiological saline. Care is taken to inject as far as possible always to the same depth of skin. The resulting injection wheals are then carefully outlined with a black or blue felt pen or ball-point per or otherwise marked so as not to rub off easily. 24 to 48 hour later, the experimental and control sites are challenged with 0.5 ml antigen/allergen.

Observation

A wheal and flare formation (> 3 mm) in the skin within 20 - 30 min. indicate positive reaction. The reaction wheals are outlined with a ball-point pen. Control site should not give wheal and flare response (Prausnitz and Kustner, 1921).

Report on PK Test

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

Report on PK Reaction

Animal Number	Group	Wheal & Flare response
	Experimental	
1		
to		
10		
	Control	
11		
to		
20		

IN-VITRO ASSAYS

3. Radioallergosorbent (RAST)/RAST Inhibition Test

Application and Limitations of Test

It is considered to be a highly sensitive and allergen specific of all the currently available laboratory tests. RAST is a direct assay method where only the radioactivity is measured. In this test a smll increase of radioactivity above background becomes significant and it is this that gives the method its great sensitivity. 10 pg of IgE being quoted as the lower limit of sensitivity.

Principle

The antigen/allergen coupled to an insoluble polymer is added to the serum to be investigated, if the antibodies to the antigen/allergen are present, they react with the conjugate. After the removal of all unbound serum components, 125-I labelled anti-IgE antibodies are added. They will bind to the antibodies of the IgE class which have reacted with the polymer-coupled antigen/allergen. The uptake of labelled antibodies measured in terms of radioactivity is proportional to the amount of IgE antibodies (Wide, et.al., 1967).

Description of Test Procedure

Insoluble Polumer-allergen conjugate : The allergen in 1 ml solution is coulpled to 100 mg CNBr-activated Sephadex G 25 or paper discs. The particles/discs are then suspended in a concentration of 1 mg/ml of 0.1 M tris buffered saline solution of pH 7.4 with 1% Tween 20 and 0.2% bovine serum albumin. The conjugates are stable for atleast 3 months at + 4°C.

125-I labelled anti-IgE antibodies : The purified antibodies labelled with 125-I can be purchased from the market or may be labelled with 125-I using the chloramine - T.

5-50 ul of serum and 0.5 ml of suspension of polymer-allergen conjugate are mixed in a test tube and incubated for 6-24 hour with low vertical rotation. The suspension is centrifuged at 3000 rpm and washed three times with tris buffer saline solution of pH 7.4 containing 1% tween-20. 100 ul of labelled anti-IgF antibodies in a concentration corresponding to about 40,000 cpm is added to the tubes. The mixture is incubated, centrifuged and washed. The bound radioactivity is measured in Scintillation counter. The results obtained with unknown sera were compared with known non-allergic sera and with diluent.

In Rast inhibition, allergen is added to serum from a host sensitive to this allergen. An inhibition of reaction takes place in RAST system when tested for antibodies to this particular allergen.

Standard curve

The standard curve or dose response curve is obtained by using various concentrations of standard. It is preferable to use as many standard concentrations as possible (10-12 concentrations) but they should not be less than five. It is better to include serial double dilutions of standards but where the working range of standard curve is small, other intermediate dilutions can also be included to increase the number of standards in the assay.

Observations

Ths results are regarded as positive (+) when the radioactivity uptake is 2 - 5 times that of the control and strongly positive (++) when the radioactivity is higher. All tests are conducted in duplicate.

RAST Score = cpm in test sera (mean of duplicate)/cpm in known negative (mean of duplicate)

Score of two or greater indicates allergen specific IgE.

Report on RAST

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

Animal Number	Group	Counts per minute (cpm)
	Experimental	
1		
to		
10		
	Control	
11		
to		
20		

4. Enzyme-Linked Immunosorbent Assay (ELISA)

Application and Limitations of Test

ELISA is an *in-vitro* assay of high sensitivity, close to solid phase radioimmuno assay. It is a relatively simple test which does not require special equipments and uses antibodies which remain stable for long periods under frozen conditions. IgE levels ranging from 24 - 24000 ng/ml are found in human sera and correlate well with RAST results. Reproducibility is said to be as high as ±3% and it is claimed that as little as 10 ng IgE/ml can be detected and that the test may be completed within 24 hour. The importance

of high quality anti-IgE antibodies is stressed and it is also pointed out that less potent antibodies may require higher temperature and longer incubation periods to obtain maximum accuracy.

Principle

The IgE under test is made to react with solid phase anti-IgE. In ELISA, an enzyme is used in place of 125-I. The label taken up by the washed solid phase is proportional to the IgE content of the sample under test and is measured spectrophotometrically.

Description of Test Procedure

The test is conducted by adsorption or coating of wells of microtitre plates with the test proteins. Unadsorbed proteins are removed by three washings. Test sera is incubated in the washed plates. The plates are again washed followed by incubation with enzyme labelled anti-IgE antibodies. Substrate is added in the washed plates, to produce colour. Optical density (OD) of colour is measured in automatic scanner or ELISA plate reader, which is directly proportional to the concentration of IgE antibodies (Vos, et.al., 1979). (If protein extract of the sample is unable to coat the wells, in that case the assay may be conducted for the estimation of total IgE, yet the estimation of specific IgE is essential as total IgE will not reflect allergenicity).

Observations

Blank values, i.e. those obtained from the wells which were not coated with antigen/ allergen are subtracted from the test values. Test is considered positive when the values are two fold or more higher than the controls. Each plate must contain positive and negative controls. Each sera must be tested in duplicate or triplicate wells.

Report on ELISA

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

Group		Animal No.		Optical Density				
				Dilutions of Sera				
				2 $\log^2$	2 $\log^3$	2 $\log^4$	2 $\log^5$	to 2 log
Experimental		1						
		to						
		10						
Control		11						
		to						
		20						

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ADDENDUM TO THE "REVISED GUIDELINES" - AUGUST, 1998

Dated: 24th September, 1999.

1. Please add the following **'sub-para xi'** after the 'sub-para x' of para **B. RCGM (Review Committee on Genetic manipulation)** at page 4 of the "Revised guidelines", which reads as under:

Sub-para xi : RCGM can approve applications for generating research information on transgenic plants. Such information may be generated in contained green house as well as in very small plots, as research needs to be conducted in such environment for seeking answers to specific environmental safety issues emanating from the use of transgenic plants. The small experimental field trials should be limited to a total area of 20 acres in multi-locations in one crop season. In one location where the experiment is conducted with transgenic plants, the land used should not be more than one acre. Any experiment beyond the above limits in one crop season would require the approval of the Genetic Engineering Approval Committee (GEAC).

2. Instead of the following caption of "Appendix III" at page 18 of the "Revised Guidelines....."

"Application seeking Non-Regulated Status in India under the Environment (Protection) Act-1986 for Transgenic Plants".

please read as:

"Application seeking Approval under Rules 8,9,10 & 11 of the Notification No, GSR 1037(E) dated 05.12.1989 issued by the Ministry of Environment & Forests under the Environment (Protection) Act, 1986, for transgenic plants".

3. Instead of the following caption on page 21 of the "Revised Guidelines....."

"Particulars of the application seeking Non-Regulated status under Environment (Protection) Act-1986".

please read as:

"Particulars of the application seeking Approval under Rules 8,9,10 & 11 of the Notification No, GSR 1037(E) dated 05.12.1989 issued by the Ministry of Environment & Forests under the Environment (Protection) Act, 1986, for transgenic plants".