

Report on Environmental release of Genetically Engineered Mustard (*Brassica juncea*) hybrid DMH-11 and use of parental events (Varuna bn3.6 and EH2 modbs2.99) for development of new generation hybrids*

- Application submitted by Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus, New Delhi
- CGMCP through extensive R&D work, financially supported by the Department of Biotechnology (DBT) and the National Dairy Development Board (NDDB), has developed male sterile and restorer lines using three transgenes- *barnase*, *barstar* and *bar* for hybrid seed production in *B.juncea*, a major oilseed crop of India.
- The summary of the application is detailed below:

Project Title:	Environmental release of Genetically Engineered Mustard (<i>Brassica juncea</i>) hybrid DMH-11 and use of parental events (Varuna bn3.6 and EH2 modbs2.99) for development of new generation hybrids
Common name of the plant:	Indian mustard
Scientific name of the plant:	<i>Brassica juncea</i> (L.)
Introduced genes:	Male sterility, MS (barnase line), and restoration of fertility, RF (barstar line) Selection marker (<i>bar</i>), required only for hybrid seed production stage.
Field studies (BRL I and BRL II)	Conducted under the overall supervision of the Directorate of Rapeseed Mustard Research (DRMR), Indian Council of Agriculture Research (ICAR).
Biosafety Research Level I (BRL I) field trials 3 locations, for two years	Conducted at 3 locations during 2010-11 and 2011-12. 1. Krishi Vigyan Kendra (KVK), Kumher, Bharatpur, Rajasthan. 2. Agricultural Research Station, Navgaon, Alwar, Rajasthan. 3. Agricultural Research Station, Sriganganagar, Rajasthan.
Biosafety Research Level II (BRL II) field trial 3 locations, for one year	Conducted at 3 locations during 2014-15. 1. Indian Agricultural Research Institute (IARI), New Delhi 2. Punjab Agricultural University (PAU), Ludhiana, Punjab 3. Regional Research Station (RRS), PAU, Bathinda, Punjab
Cloning, expression, purification and production of recombinant pure protein	Experiments carried out at M/s. Premas Biotech Pvt Ltd, Manesar. DSIR recognized, ISO 9001:2008 certified research and manufacturing facility located near New Delhi, India.
Compositional analysis	Conducted at Food and Drug Toxicology Research Centre (FDTRC) of the National Institute of Nutrition (NIN), Hyderabad. It is a Research Institute working under the aegis of Indian Council of Medical Research (ICMR), Ministry of Health and Family Welfare, Government of India.

ELISA kit development	Developed by M/s Amar Immunodiagnostics, Hyderabad. A research and development based Indian enterprise and a leading exporter of top quality GMO testing kits in India and across the world.
Allergenicity and Toxicity Assessment	Conducted at FDTRC of NIN, Hyderabad, an ICMR institute
Soil Microflora Assessment	Conducted at CSIR-IMTECH, Chandigarh, a constituent establishment of the Council of Scientific & Industrial Research (CSIR), Ministry of Science and Technology, Government of India. The institute houses International Depository Authority (IDA) and Microbial Type Culture Collection and Gene Bank (MTCC), a depository for microbial cultures.
Environmental safety studies (weediness and crossability)	Conducted by CGMCP, University of Delhi, Delhi.

Tests done by applicant as per various Regulatory Guidelines stipulated by Regulatory Agencies

Category	Tests Done	Relevant Guidelines
Molecular characterization	<ul style="list-style-type: none"> Gene sequences, constructs and molecular characterization Expression studies of the three inserted genes – <i>bar</i>, <i>barnase</i> and <i>barstar</i> Cloning, expression, purification and production of three expressed proteins 	<ul style="list-style-type: none"> Recombinant DNA Safety Guidelines, 1990 Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008 prepared by Indian Council of Medical Research (ICMR), Ministry of Health and Family Welfare, Government of India
Food safety Studies	<ul style="list-style-type: none"> Equivalence of the Bar, Barnase and Barstar recombinant proteins produced in bacteria with that expressed in GE plants Bioinformatics analysis of the three proteins Pepsin digestibility of the three proteins Heat stability of the three proteins Acute oral toxicity of the three proteins in mice Sub-chronic toxicity of leaves and seeds containing the three proteins in rats Compositional analysis 	<ul style="list-style-type: none"> Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008 prepared by Indian Council of Medical Research (ICMR), Ministry of Health and Family Welfare, Government of India

Environmental safety studies	<ul style="list-style-type: none"> • Research & Development Phase including primary field trials 2004-2007. • BRLI trials for two growing seasons (2010-11, 2011-12) • BRLII trials for one growing season (2014-15) • Weediness potential and aggressiveness parameters • Impact on soil microflora during BRLI and BRLII trials • Crossability and pollen flow studies • Pollination behaviour, pollen morphology and physiology 	<ul style="list-style-type: none"> • Revised Guidelines for Research in Transgenic Plants, 1998 (DBT) • Guidelines for the monitoring of Confined Field Trials of Regulated, Genetically Engineered (GE) Plants, 2008 (DBT & MoEF&CC)
Detection Protocols	<ul style="list-style-type: none"> • Protocol for testing at a level of detection (LOD) of 0.01% • Development of ELISA kits for Bar, Barnase and Barstar 	<ul style="list-style-type: none"> • Tests and Detection methods developed as part of previous Hon'ble Supreme Court of India directives

Summary:

- The Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus, New Delhi sought approval from GEAC, MoEF&CC, Government of India for environmental release of GE mustard (*B. juncea*) hybrid DMH-11 and use of parental events (Varuna bn 3.6 and EH-2 modbs 2.99) for development of new generation hybrids.
- The biosafety studies were carried out in various national institutes like National Institute of Nutrition (NIN) Hyderabad an ICMR institute; Institute of Microbial Technology (IMTECH) Chandigarh, a CSIR institute; Directorate of Rapeseed Mustard Research (DRMR) Bharatpur, an ICAR institute.

Results of Food Safety Assessment Studies

- The molecular characterization data reveals that the two GE parental events each have a single copy of the transgene integrated in the genome. The insertion of these genes does not lead to disruption of any known endogeneous genes. These genes are stably integrated and their stable expression and inheritance has been shown across several generations.
- The Food and Feed Safety Studies have led to the following conclusions: from the compositional analysis studies it is clearly evident that GE parents are substantially equivalent to their non-GE comparators in terms of key parameters such as oil, protein, carbohydrate, glucosinolate, erucic acid, fatty acids, allylisoithiocyanate, peroxide value, sodium, calcium, magnesium, potassium and minerals when analysed in leaf and seeds. Also, hybrid DMH-11 is very similar in its composition to the commercially cultivated

segment

- From the toxicity and allergenicity studies, it was concluded that the GE Mustard, the parental lines and hybrid DMH-11, does not raise any public health or safety concerns for human beings and animals with respect to overall nutritional characteristics.
- The introduced proteins i.e. Barnase and Barstar are expressed at negligible to non detectable levels in the edible parts and have been derived from commonly occurring non-pathogenic bacteria. None of the three proteins has been shown to be toxic or allergenic through bioinformatics and acute toxicity studies in experimental animals. All the three proteins are rapidly degraded in simulated gastric conditions of the mammalian digestive systems.
- Sub-chronic toxicity studies in experimental animals using edible plant parts i.e. leaves and seeds also did not show any adverse effects. Mustard oil does not contain any proteins. Therefore, the probability of oil extracted from DMH-11 or any other future hybrids to have any of the three proteins is nil/ negligible. There is more than 20 years of history of safe use of proteins from the three genes – *bar*, *barnase* and *barstar* in rapeseed (*B. napus*), as oil and meal extracted from GE rapeseed is being consumed in a large number of developed and developing countries and so far no toxicity or allergenicity have been reported.
- In conclusion, based on the history of safe use of the host and the donor organisms, transgene expression analysis, composition analysis, acute and sub-chronic toxicity assays of purified proteins and whole leaf and seeds, respectively, it can be concluded that the use of leaves, seed and oil derived from GE mustard lines is not likely to pose any risk to humans and animals.

Results from Environmental Risk Assessment studies

- The weediness Potential of GE mustard hybrid DMH-11 is similar to that of the varieties commonly grown in India. There is no risk of any aggressiveness or any weediness potential in the hybrid DMH-11. Therefore, potential of GE hybrid DMH-11 becoming a weed in mustard fields or in natural ecosystem is highly unlikely.
- Crossability and gene flow study lead to the conclusion that intraspecies gene flow could occur between DMH-11 and varieties of *B. juncea* grown in close proximity to GE hybrid DMH-11. However, the cross between adjoining crops has been found to occur upto a distance of 20 m. Moreover, the progeny of such crosses will not have any survival advantages in the absence of selection pressure. In cases where barnase gene is transferred, there will be co-expression of barstar thus, the resultant plant would be normal. The data on interspecies crossability of GE mustard hybrid DMH-11 shows no crossability with related *Brassica species*. Thus the probability of persistence of progeny of such crosses in the environment is negligible.
- As for the effect on Soil microbial community it can be concluded that the transgenes present in GE mustard lines have been cloned from highly abundant non-pathogenic soil microorganisms. Therefore, the proteins expressed by the GE mustard are expected to be already widely present in nature and their presence in the GE mustard is not

expected to present any new toxicity risks to soil microorganisms in these environments. In addition, the introduced proteins are expressed at very low levels as intracellular proteins and not found in the root exudates of GE mustard. Hence, considering the remote possibility of exposure of soil micro-organisms to barnase/barstar or bar proteins, such an exposure will not create any harm to microbes or will not disturb microbial gene pool in terms of microbial abundance and diversity in the soil. Further prevalence of predominant bacterial genera remained same in both GE and non GE mustard. Therefore, GE mustard is expected to exert an effect on rhizospheric microbial population that is similar to the effect of non-GE conventional parents and local checks.

- Effect on pests, diseases and beneficial organisms, it was observed that natural occurrence of all the major pests and diseases and predators of mustard were similar in GE hybrid DMH-11 to their non-GE comparators and conventional zonal check variety. Honey bees foraging were also observed to be similar on GE mustard as it was on conventionally grown mustard varieties. The transgenes have been found to have no unintended effect on the pest and disease occurrence and on beneficial organisms.
- Agronomic and phenotypic data demonstrates that presence of transgenes in the hybrid does not lead to any unintended effect on the agronomic parameters. The efficacy evaluation has proven the presence of hybrid vigour in the hybrid. The GE hybrid DMH-11 is superior as compared with zonal and national check (Varuna) in terms of yield.
- These results collectively reveal that the introduction of the *barnase*, *barstar* and *bar* genes to mustard did not lead to any unintended effects on the food/feed and environmental safety of the GE plants on either the parental lines or the hybrid DMH-11.

Final Recommendations

- Sub-committee prepared this document on "Assessment of food and environmental safety (AFES)" and critically examined the data requirement particularly as the resultant hybrid is based on stacked events. The evaluation of such stacked events depends primarily on the two criteria: The process used for generating the stacked event and prior approval status of the individual event/s. Since in this case none of the individual events are approved and are to be used only for hybrid seed production, the applicant was required to submit a dossier containing detailed characterization report of the individual parent event (s), food and environmental safety studies data for hybrid as well as parental events. It was noted that the data generated and submitted in final dossier by the applicant to GEAC is comprehensive and in compliance with all the existing guidelines published, protocols and measurable end points prescribed by RCGM and GEAC as well as the international best practices.
- The AFES report presented by the sub-committee herein contains thorough assessment of biosafety data generated by the applicant, its comparison with other such international assessment by well known regulatory agencies such as EFSA, OGTR and Canadian regulatory authorities and existing scientific literature on the subject in peer reviewed journals yet addressing the specific uses of mustard in Indian context. Therefore, sub-committee is of the opinion that both the genetically engineered parents

equivalent to non-GE parents and conventional mustard, and its consumption is safe for human and animal health. With regard to the environment, the sub-committee concluded that environmental release of parental lines for hybrid production DMH-11 may not pose any risk to biodiversity and the agro-ecosystem as the GE material under review have been demonstrated to have no/ negligible effect on non-target organisms including honey bees, soil microbes and other non-target beneficial organisms.

- In summary, in the opinion of sub-committee, the environmental release of the parental lines and the hybrid assessed here is safe and effective following good stewardship practices in handling production of hybrids and their cultivation.
- However, in order to have wider inputs from various stakeholders the sub-committee recommends that the AFES document be made public before the final decision by the GEAC is arrived at.
